Organic & Biomolecular Chemistry

Dynamic Article Links

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 2357

www.rsc.org/obc

**PAPER**

# Synthesis and luminescence properties of new red-shifted absorption lanthanide(III) chelates suitable for peptide and protein labelling†

**Nicolas Maindron,[a] Séverine Poupart,[a] Maxime Hamon,[a] Jean-Baptiste Langlois,[a] Nelly Plé,[b,c] Ludovic Jean,[a,b] Anthony Romieu*[a,b] and Pierre-Yves Renard*[a,b,d]**

***Received 5th October 2010, Accepted 23rd December 2010***
**DOI: 10.1039/c0ob00832j**



The synthesis and photo-physical properties of an original bis-pyridinylpyrazine chromophore efficiently sensitising europium(III) and samarium(III) are described. The corresponding lanthanide(III) complexes display in aqueous solutions a maximum excitation wavelength which is significantly red-shifted compared to the usual terpyridine-based chelates, and a valuable luminescence brightness above 2 000 $dm^3\ mol^{-1}\ cm^{-1}$ at 345 nm was obtained with a europium(III) derivative. Further functionalisation with three different bioconjugatable handles was also investigated and their ability to efficiently label a model hexapeptide was evaluated and compared. Finally, the best bioconjugatable europium(III) chelate was used in representative labelling experiments involving monoclonal antibodies and the luminescence features of the corresponding bioconjugates remained satisfactory.

## Introduction

A wide range of bio-analytical and biomedical applications involved in various fields such as diagnostic, drug discovery and molecular imaging (both *in cellulo* and *in vivo* contexts),[1] require accurate investigations of biological events and/or materials through the use of optical bioprobes. Since the targeted analytes are often present in minute concentrations in complex biological media, these biomolecular tools must be as sensitive as possible. Besides, they have to give rise to a specific and easy-to-measure signal. For more than 25 years,[2,3] luminescence bioprobes based on lanthanide complexes, have turned out to be well-suited tools for such challenging applications.[4,5] Indeed, these luminescent chelates possess remarkable photophysical properties.[6] For instance, long-lived emission of lanthanide(III) cations can be temporally resolved from scattered light and background fluorescence (*e.g.*, from biological matrix) to dramatically enhance measurement sensitivity.

The poor absorption of a lanthanide ion can be overcome by its insertion inside an adequate organic ligand which is able to absorb UV–visible light and subsequently transfer it onto the metal ion *via* its triplet state (a process called luminescence sensitisation or antenna effect).[6] This transfer leads also to an important apparent Stokes' shift of up to about 250 nm (compared to 30–50 nm for most organic fluorescent dyes).[2] These unique spectroscopic features combined with lanthanide(III) cation's narrow emission bands in the red-light region explain why lanthanide luminescent chelates are valuable tools in a large panel of highly sensitive bioaffinity assays.[7] Using these lanthanide(III) chelates, a sub-picomolar detection threshold is readily achieved,[8] whereas with other fluorescent probes the highest limit of detection reached is in the picomolar range.[2]

A lanthanide luminescent bioprobe must fulfil several requirements to be efficient: high thermodynamic stability as well as kinetic inertness in various biological matrices and at physiological pH, good absorption and emission properties, photostability and water solubility (for *in cellulo* applications, non-cytotoxicity is also needed).[4] Finally, one of the challenges in the design of luminescent lanthanide probes dedicated to bioanalyses and bioimaging applications, is to be able to increase the excitation wavelength as high as possible. The long-wave UV region or visible spectrum are preferred and targeted (especially wavelengths around or above 350 nm) for two intrinsic reasons: biomolecules/biopolymers (*e.g.*, DNA) are usually damaged by short- or medium-wave UV light, and tissues' autofluorescence excitation wavelengths are usually below 320 nm. Three strategies are generally adopted to extend the excitation window of Ln(III) complexes (mainly applied to Eu(III) complexes) to a long-wavelength region.[9] Diminishing the energy gap between the lowest $S_1$ state and the $T_1$ state of the antenna ligand is considered as an effective way to extend the

[a]*Equipe de Chimie Bio-Organique, COBRA-CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France. E-mail: pierre-yves.renard@univ-rouen.fr, anthony.romieu@univ-rouen.fr; Fax: +33 2-35-52-29-71; Tel: +33 2-35-52-24-14 (or 24-15); Web: http://ircof.crihan.fr*
[b]*Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France*
[c]*Equipe de Chimie Hétérocyclique, COBRA-CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France*
[d]*Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France*

† Electronic supplementary information (ESI) available: Detailed synthetic procedures for compounds **16** *and* **25**, characterisation data for compounds **6**, **8**, **10**, **17** and **18**, ESI mass spectra of Ln(III) chelates, bioconjugatable derivatives and luminescent labelled peptides. See DOI: 10.1039/c0ob00832j

excitation wavelength for the Eu(III) complex into the visible-light region through the usual triplet energy-transfer pathway.[10,11] Another promising means of longer-wavelength sensitisation of Eu(III) emission is through the singlet pathway, in which the excited-state energy of a chromophore is directly transferred from its $S_1$ state to the luminescent states of the Eu(III) center (*i.e.*, charge transfer transitions).[12] Two- (or multi-) photon excitation, that is, simultaneous absorption of two photons of half energy is the third way to circumvent the use of UV light and has recently been successfully applied to Eu(III) complexes and nanoprobes for live cell imaging applications.[13]

With the goal in mind to develop a new structurally simple Ln(III) chelate bio-labelling reagent that displays UV-A or visible absorption maximum, we thought to implement the first strategy to Ln(III) chelates comprising a terpyridine unit as antenna, by replacing its central pyridine ring by a pyrazine moiety. Indeed, the commercially available Ln(III) terpyridine-based chelates currently used for diagnostics or drug discovery purposes, generally possess a maximum excitation wavelength below 340 nm. These stable luminescent complexes are generally composed of a 4′-aryl-substituted 2,2′:6′,2″-terpyridine unit as the light-absorbing moiety, methylenenitrilo diacetic acids as the chelating arms, and a bioconjugatable group for coupling to biomolecules/biopolymers (Fig. 1).[14] To date, to our knowledge, there are only two Ln(III) chelate labels bearing a diazine ring within their chromophoric moiety (Fig. 2).[15,16] The first one is the bis-pyrazolylpyrazine Ln(III) chelate **4** that displays in borate buffer (pH 8.5) a moderate luminescence brightness ($\varepsilon \times \Phi$) 250 $dm^3\ mol^{-1}\ cm^{-1}$ at 336 nm for Eu(III) ion,[17] and a good value of 6 380 $dm^3\ mol^{-1}\ cm^{-1}$ for Tb(III) ion thanks to the sensitiser triplet-state energy level. Interestingly, the introduction of this pyrazine unit instead of the central pyridine ring of the original bis-pyrazolepyridine Ln(III) chelate, leads to a significant bathochromic shift of 15 nm of the excitation wavelength.[18,19] The second one **5**, based on a

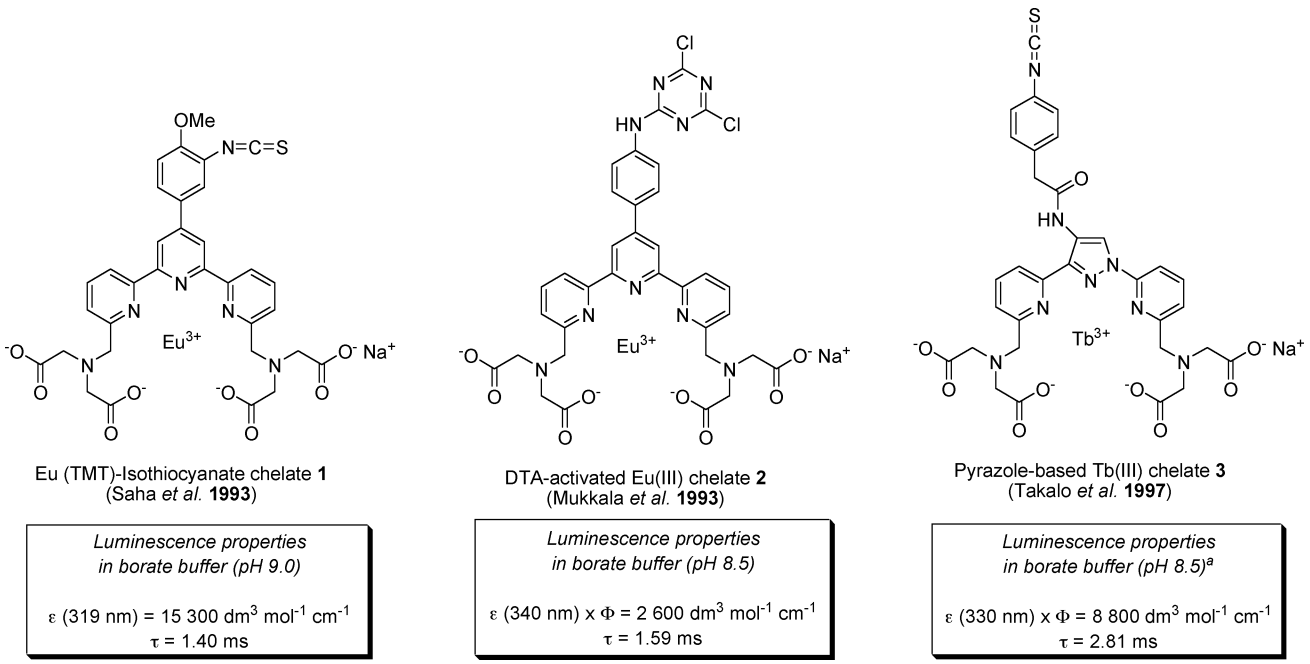



**Fig. 1** Examples of Ln(III) chelates based on a terpyridine or a terpyridine-like pyrazole ligand. [a]Determined only for the chelate without the bioconjugatable arm.

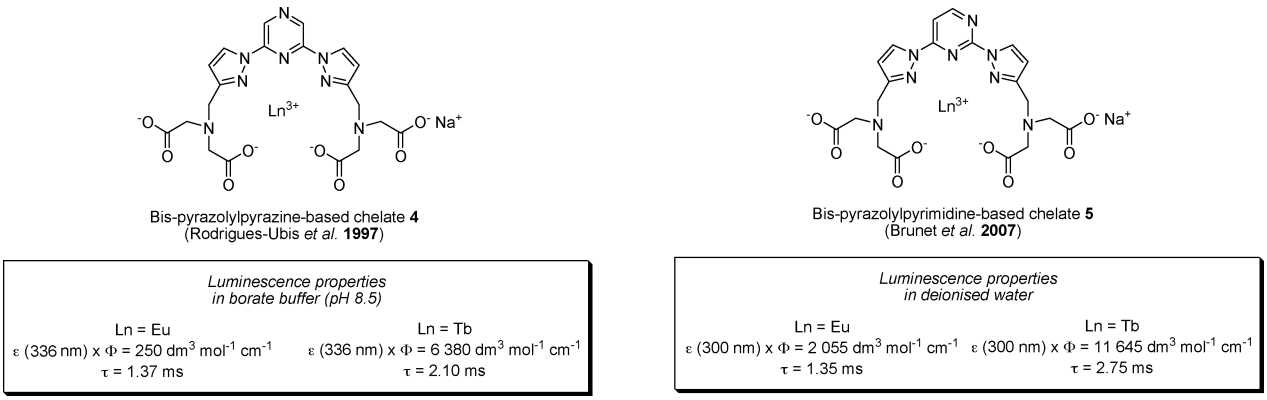



**Fig. 2** Structure and luminescence properties of lanthanide(III) chelates based on a tris-azaheterocyle ligand comprising a diazine unit, and already reported in the literature.

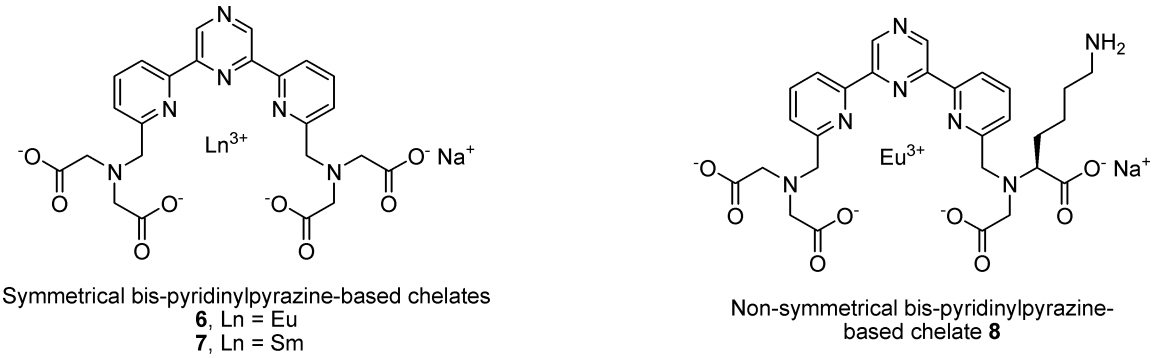



**Fig. 3** Structures of lanthanide(III) chelates composed of a bis-pyridinylpyrazine unit as the energy-absorbing and donating ligand, studied in this work.

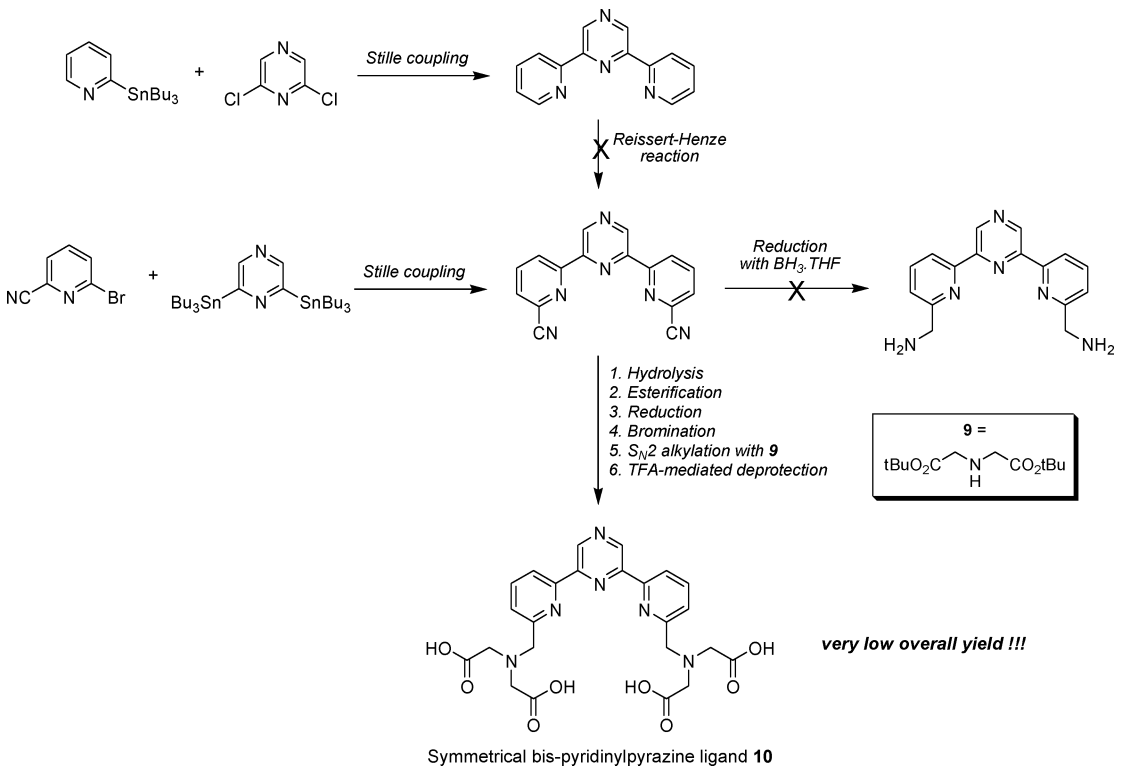



**Fig. 4** First synthetic pathways explored for the preparation of symmetrical bis-pyridinylpyrazine ligand **10**.

bis-pyrazolylpyrimidine ligand, shows better luminescence brightness in water for both Eu(III) and Tb(III) ions: 2 055 $dm^3$ $mol^{-1}$ $cm^{-1}$ and 11 645 $dm^3$ $mol^{-1}$ $cm^{-1}$ respectively. However, the main drawback of such chelates remains an excitation maximum at the more energetic wavelength of 300 nm. Thus, it seems relevant to examine the chemistry of 2,6-di(2-pyridinyl)pyrazine ligand[20] whose functionalisation with chelating arms and its subsequent complexation with Ln(III) ions have never been explored.

Here we report the first synthesis along with the photophysical properties of Eu(III) chelate **6** based on a bis-pyridinylpyrazine chromophoric moiety functionalised with two methylenenitrilo diacetic acids moieties (Fig. 3). The preparation of the non-symmetrical derivative **8** bearing a lysine residue within one of the two chelating arms is also described. Its potential utility in the context of luminescent bio-labelling is illustrated through its conjugation to a model hexapeptide and monoclonal antibodies.

## Results and discussion

### Synthesis of the symmetrical functionalised bis-pyridinylpyrazine ligand

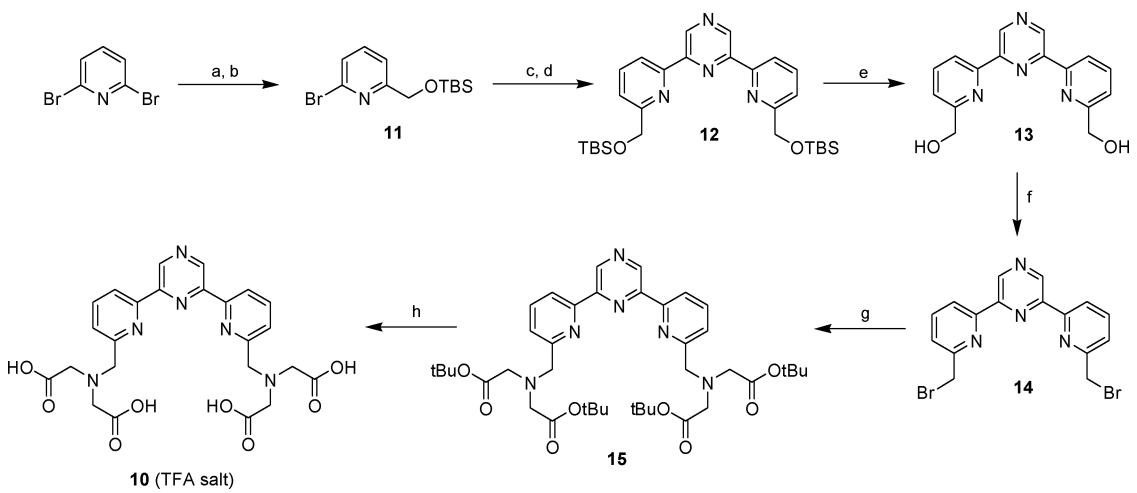
The bis-pyridinylpyrazine core structure was obtained *via* a tricky to optimise and efficient Stille cross-coupling reaction between 2,6-dichloropyrazine and 2-(tributylstannyl)pyridine (Fig. 4). The most frequently used synthetic method to introduce the chelating arms onto the 6,6″-positions of a terpyridine scaffold, involves a key di-cyano derivative which is obtained through a modified Reissert–Henze reaction.[21] Thus, it was theoretically possible to get the targeted 6,6″-functionalised bis-pyridinylpyrazine ligand **10** by using one of the two following synthetic strategies: (1) the nitrile functions could be reduced with borane–tetrahydrofuran complex to afford the corresponding primary bis-amine which would then be twice carboxymethylated with an alkyl bromoacetate followed

by hydrolysis of the esters to give the free methylenenitrilo diacetic acids, or (2) the nitrile functions could be hydrolysed to carboxylic acids, which would be esterified and subsequently reduced to alcohols. Thereafter, the primary bis-alcohol would be converted into the corresponding bis-bromide by treatment with $PBr_3$ which could undergo $S_N2$ reaction with an iminoacetic acid ester followed by hydrolysis to give the desired ligand.[22] However, introduction of nitrile functions onto the bis-pyridinylpyrazine scaffold by means of the Reissert–Henze reaction gave poor yields, thus we decided to perform their incorporation prior to the Stille cross-coupling reaction. The optimised Stille reaction between 2,6-(tributylstannyl)pyrazine and 2-bromo-6-cyanopyridine efficiently afforded the bis-nitrile derivative.[23] Reduction with borane–tetrahydrofuran complex failed but nitriles hydrolysis followed by esterification was successful. The main drawback of this synthetic scheme is that the hydride source ($NaBH_4$ ou DIBAL-H), used for reduction of esters, led to an unresolved mixture of desired bis-alcohol and boron (or aluminium) complex salts. Consequently, the $PBr_3$-mediated bromination reaction was performed on the crude reduction mixture to give the bis-bromide derivative with an unsatisfactory 9% overall yield for the two steps. The substitution of the bromine atoms by di-*tert*-butyl iminodiacetate **9** followed by *tert*-butyl deprotection gave ligand **10** with a low 13% overall yield for this two-step reaction sequence. The insignificant overall yield for this 8-step synthetic procedure, which amounted to 0.2%, led us to discard this approach and develop another synthetic route able to circumvent the tedious ester reduction step.

We decided then to investigate a synthetic strategy involving the introduction of a protected 2-(hydroxymethyl) pyridine moiety on the 2,6-dichloropyrazine *via* the same Stille cross-coupling reaction (Scheme 1). The required trimethyltin derivative was easily obtained from the commercially available 2,6-dibromopyridine in three steps and with a good 72% yield: the first step allowed to replace one of the two bromine atoms by the hydroxymethyl group in almost quantitative yield as described by Cai *et al.*[24] Thereafter, the primary alcohol was protected as a *tert*-butyldimethylsilyl (TBS) ether to afford compound **11**. The key trimethyltin derivative was efficiently generated by trapping the lithiated intermediate with trimethyltin chloride in dry THF from −78 °C to room temperature for 4 h (89% yield for the crude product). The Stille cross-coupling reaction between this stannane intermediate and 2,6-dichloropyrazine was performed with $Pd(PPh_3)_4$ as a Pd(0) catalyst in an argon deaerated xylene solution at 110 °C overnight to give the desired bis-TBS ether **12** of bis-pyridinylpyrazine in a moderate 50% yield. The removal of TBS ethers was performed with a 1.0 M solution of TBAF in THF and afforded the desired bis-alcohol **13**. This bis-pyridinylpyrazine derivative was isolated in a pure form by simple precipitation in a 0.1% aq. trifluoroacetic acid (TFA) and MeOH (1 : 1) mixture (79% yield). Next, bis-bromination reaction of **13** was carried out in dry DMF with $PBr_3$ to give the bis-halide compound **14** in a quantitative yield. This crude product was then directly engaged in the substitution step with *tert*-butyliminoacetate **9**. This latter reaction was performed with DIEA as base, NaI as catalyst and in dry DMF. Finally, the removal of *tert*-butyl esters was achieved in a mixture of TFA and $CH_2Cl_2$ (1 : 1) containing 2.5% of water. The symmetrical tetrakis(acetic acid) ligand **10** was purified by flash-chromatography on a RP-$C_{18}$ silica gel column. All spectroscopic data, in particular NMR and mass spectrometry, were in agreement with the structure assigned (see ESI†). By comparison with the synthetic route initially explored (Fig. 1), this alternative pathway led to the desired ligand **10** with an improved overall yield of 7.6%.

### Synthesis of the non-symmetrical functionalised bis-pyridinylpyrazine ligand suitable for bioconjugation studies

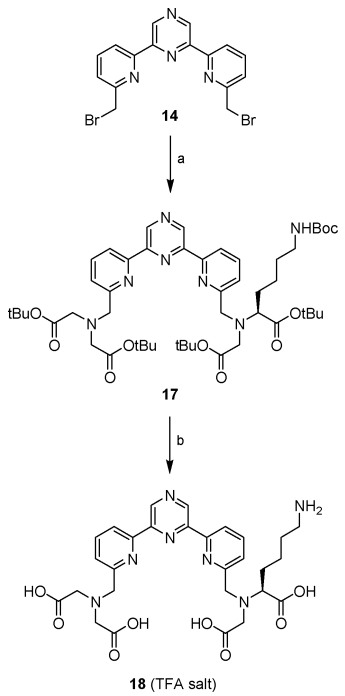
In order to have a suitable chemically reactive group for the bioconjugation of the corresponding Ln(III) chelates derived from bis-pyridinylpyrazine ligand to various biomolecules/biopolymers, we also explored the synthesis of the non-symmetrical ligand **18** bearing a lysine residue from the dibromide derivative **14**



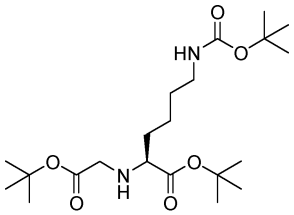

**Scheme 1** *Reagents and conditions*: a) *n*BuLi (1.0 equiv.), THF, −70 °C, 15 min then DMF (1.5 equiv.), −78 °C, 15 min then MeOH, $CH_3CO_2H$, −78 °C to rt then $NaBH_4$ (1.0 equiv.), rt, 92%; b) TBS-Cl (1.2 equiv.), imidazole (4.0 equiv.), DMF, rt, 25 min, 88%; c) *n*BuLi (1.1 equiv.), THF,−78 °C, 90 min then $Me_3SnCl$ (1.05 equiv.), rt, 4 h, 89%; d) 2,6-dichloropyrazine (0.75 equiv.), $Pd(PPh_3)_4$ (0.05 equiv.), xylene, 110 °C, 6 h, 50%; e) 1 M TBAF in THF (2.3 equiv.), THF, rt, 2 h 30, 79%; f) $PBr_3$ (3.0 equiv.), DMF, 0 °C to rt, 4 h, 95%; g) secondary amine **9** (2.2 equiv.), NaI (1.1 equiv.), DIEA (10 equiv.), DMF, rt, 6 h 30, 64%; h) TFA, $H_2O$ (50 equiv.), $CH_2Cl_2$, 0 °C to rt, 5 h, 44%.

(Scheme 2). Indeed, the post-synthetic derivatisation of the ε-amino group of this original chelating arm with an homobifunctional (or trifunctional) cross-linker should enable the introduction of a bioconjugatable moiety reactive towards the primary amines of proteins (*e.g.*, *N*-hydrosuccinimidyl ester, NHS ester). Firstly, the required fully-protected chelating arm **16** derived from lysine was readily prepared from commercially available Fmoc-L-Lys(Boc)-OH using a three-step method (see ESI†). The one-pot synthesis leading to fully-protected non-symmetrical intermediate **17** was a tricky step. Indeed, dibromide derivative **14** was flanked by two equivalent reactive sites, whereas the two amines involved in the alkylation process did not exhibit the same reactivity (presumably due to steric considerations, secondary amine **9** was found to react faster than the lysine derivative **16**). Consequently, a slight excess (1.1 equiv.) of less reactive secondary amine **16** was first added to a dry DMF solution containing **14**. The mixture was stirred at room temperature for 9 h prior to the addition of secondary amine **9** in several portions over a period of 48 h. Eventually, the desired compound **17** was isolated from the complex reaction mixture (containing **17** and the two possible symmetrical compounds) with a modest 18% yield after two successive purifications: a normal phase flash-chromatography followed by a semi-preparative RP-HPLC purification. The removal of acid-labile protecting groups Boc and *tert*-butyl was carried out with a mixture of TFA–$CH_2Cl_2$ (1 : 1) containing 2.5% of water. The non-symmetrical ligand **18** was isolated in a pure form by semi-preparative RP-HPLC. Its structure was confirmed by NMR and ESI mass spectrometry measurements (see ESI†). This desymmetrisation process involving a sequential alkylation of two different secondary amines led logically to the desired ligand **18** with a lower overall yield (2.6%) than the one obtained for ligand **10**.




**Scheme 2** *Reagents and conditions*: a) secondary amine **16** (1.1 equiv.), NaI (0.6 equiv.), $K_2CO_3$ (15 equiv.), DMF, rt, 9 h then secondary amine **9** (1.05 equiv.), rt, 72 h, 18%; b) TFA, $H_2O$ (40 equiv.), $CH_2Cl_2$, 0 °C to rt, 20 h, 54%.



**16**

### Synthesis and characterisation of Ln(III) chelates

Bis-pyridinylpyrazine ligands **10** and **18** were converted to the corresponding Eu(III) chelates by treatment with aq. europium(III) chloride. The completion of these latter reactions was checked by UV–visible spectrophotometric measurements and purification was achieved by flash-chromatography on a RP-$C_{18}$ silica gel column. The stoichiometry [1 : 1] and structures of these Eu(III) chelates were confirmed by ESI mass spectrometry analyses (see ESI†). Interestingly, the Tb(III) and Sm(III) complexes of symmetrical ligand **10** were also prepared from their respective hexahydrate trichloride salts (for ESI mass spectra, see ESI†). The Tb(III) chelate was found to be non luminescent whereas Sm(III) chelate **7** exhibited a modest quantum yield of 0.5%.[25] Thus, we decided to focus our next comprehensive spectral and bioconjugation studies only on the Eu(III) chelates.

### Photo-physical properties of the bis-pyridinylpyrazine ligands and the corresponding Eu(III) chelates

The photo-physical features for Eu(III) chelates **6** and **8** are presented in Table 1. The ligand triplet-state level was not measured; nevertheless, since the Tb(III) chelate was not luminescent contrary to the Sm(III) derivative, we can reasonably postulate an energy level of around 20 000 $cm^{-1}$. The UV–vis absorption spectra in deionised water (see Fig. 5 for ligand **18**) displayed for both ligands **10** and **18** a broad band between 260 and 350 nm with two maxima located at 293 ($\varepsilon$ = 13 600 $dm^3\ mol^{-1}\ cm^{-1}$) and 310 nm ($\varepsilon$ = 14 000 $dm^3\ mol^{-1}\ cm^{-1}$). The molar extinction coefficients are virtually the same for each ligand. Upon complexation with Eu(III) cation, the broad absorption band splits into two narrower bands with a large bathochromic shift from 310 to 344 nm due to conformational changes leading to an improvement of the flatness of the structure which favours the π-electron delocalisation (Fig. 5).[26] In this case, the red-shift was accompanied with a slight hyperchromic effect ($\varepsilon$ = 15 500 $dm^3\ mol^{-1}\ cm^{-1}$). The higher energy absorption band of the free ligand underwent no significant shift ($\lambda$ = 296 nm), but its absorption coefficient decreased ($\varepsilon$ = 11 200 $dm^3\ mol^{-1}\ cm^{-1}$). The corresponding emission spectra of Eu(III) chelates **6** and **8** present the usual $^5D_0 \rightarrow {}^7F_J$ ($J$ = 1–4) transitions typical of the Eu(III) ion. The luminescence brightness of Eu(III) chelates **6** and **8** were measured in deionised water by using a relative method[27] using rhodamine 6G[28] and the Eu(TMT)$AP_3$ chelate **19** recently reported by us,[29] as standards.

**Table 1** Photophysical properties of Eu(III) chelates in borate buffer pH 8.5 (chelates **4** and **20**) or in deionised water (chelates **6** and **8**) at 25 °C

| Chelate | $\lambda_{abs,\,max}$ (nm) | $\varepsilon$ (dm$^3$ mol$^{-1}$ cm$^{-1}$) | $\lambda_{em,\,max}$ (nm)[c] | $\Phi$ (%) | $\tau_{H2O}$ (ms) | $\tau_{D2O}$ (ms) | $q_{H2O}$[g] |
|---|---|---|---|---|---|---|---|
| **4**[a] | 335 (336) | 8 400 (10 900) | 621 | 3.0 (13.0) | 1.37 (1.30) | — | 0.5 |
| **20**[b] | 333 | 12 100 | — | 17.6 | 1.31 | — | — |
| **6** | 344 | 15 500 | 615 | 15.7 (14.2)[d] | 1.35[e] | 2.35[f] | 0.3 (0) |
| **8** | 345 | 15 500 | 616 | 14.5 (13.2)[d] | 1.30[e] | 2.30[f] | 0.35 (0) |

[a] Data reported by Rodrigues-Ubis *et al.*[16] and values inside brackets reported by Latva *et al.*[19] [b] Data reported by Mukkala *et al.*[38] and Latva *et al.*[19] [c] Only the most intense emission band corresponding to the $^5D_0 \rightarrow {}^7F_2$ transition is reported. [d] Determined by using Eu(TMT)-AP$_3$ **19** ($\Phi$ = 16.9% in deionised water)[29] or R6G ($\Phi$ = 88% in ethanol, values inside brackets)[28] as standard (excitation at 345 nm). [e] A solution of chelate in deionised water at 10 µM was used. [f] A solution of chelate in heavy water at 10 µM was used. [g] Calculated according to the following equations: $q_{H2O} = 1.05[(\tau_{H2O})^{-1} - (\tau_{D2O})^{-1}]$[32] or $q_{H2O} = 1.11[(\tau_{H2O})^{-1} - (\tau_{D2O})^{-1} - 0.31]$[31] (inside brackets).

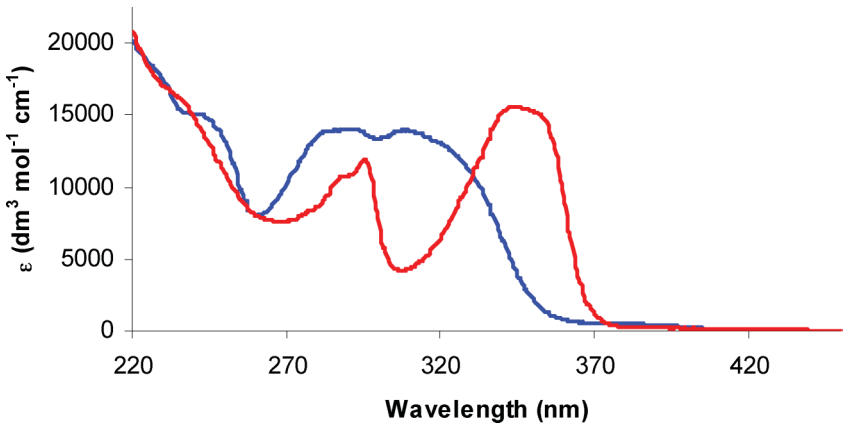
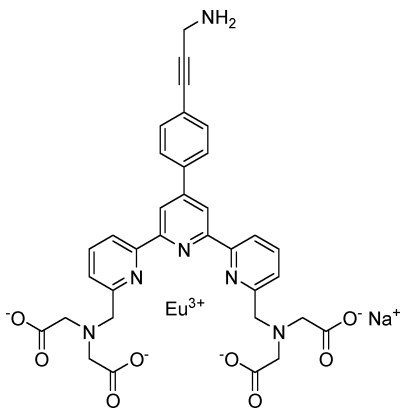
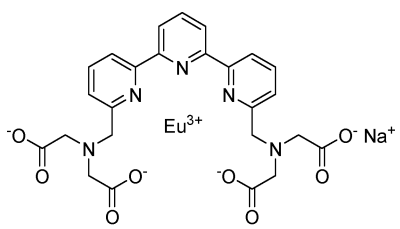



**Fig. 5** Absorption spectra of non-symmetrical bis-pyridinylpyrazine ligand **18** (—) and corresponding Eu(III) chelate **8** (—) in deionised water at 25 °C (concentration: 9.0 µM).

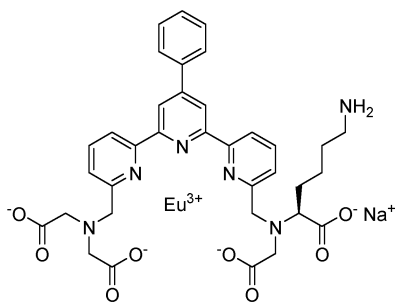



Eu(TMT)-AP$_3$ chelate **19**
(Poupart *et al.* **2006**)

Under excitation at 345 nm, the luminescence brightness varied from 2 200 to 2 500 for **6** and from 2 000 to 2 250 for **8** according to the reference used. Interestingly, the presence of a free primary amine within the structure of chelate **8** does not lead to a substantial quantum yield loss as previously observed for the terpyridine analogue **21**.[19] As compared with chelate **4**, the replacement of the two pyrazole rings by pyridine moieties, in spite of a lack of luminescence in the case of Tb(III), leads to better spectroscopic properties for Eu(III) derivative. Moreover, compared to terpyridine analogue **20**[30], the pyrazine introduction has been revealed to be beneficial because the excitation maximum was red-shifted (10 nm), and a slight improvement of luminescence yield was also observed (Table 1, see entries 2–4). Interestingly, the small size of this novel chromophoric unit also has a beneficial effect on the water-solubility of the resulting lanthanide(III) chelates. Indeed, **6** and **8** were found to be perfectly soluble in water and related aq. buffers in the concentration range of 1 µM to




Terpyridine-based chelate **20**
(Mukkala *et al.* **1992**)




4'-Aryl-terpyridine-based chelate **21**
(Latva *et al.* **1997**)

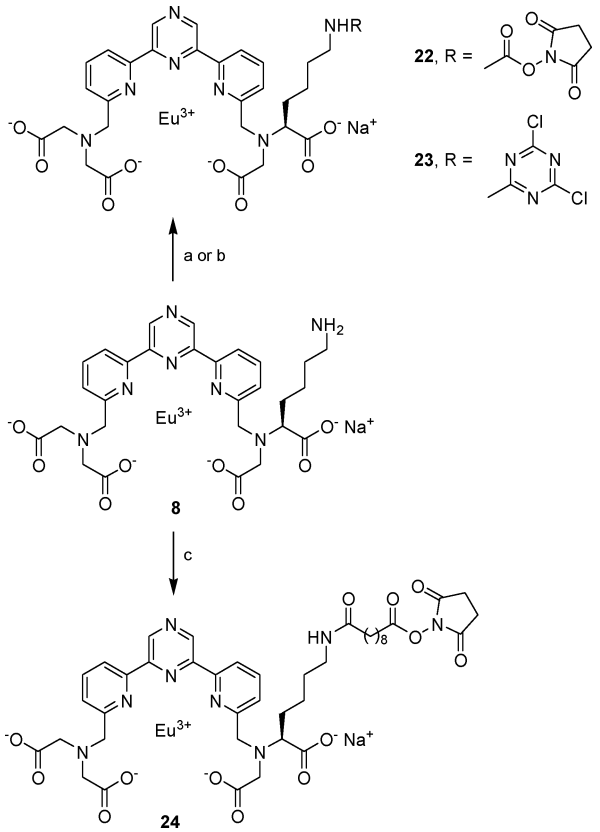
 

1 mM, contrary to 4′-aryl-substituted 2,2′:6′,2″-terpyridine-based chelates such as **19**.

Finally, luminescence lifetime measurements in water and heavy water were performed to calculate, according to the two equations reported in the literature,[31,32] the average number ($q$) of water molecules in the first coordination sphere of Eu(III), which was found to be close to 0. It clearly indicates that the complexation provided by the nine binding sites efficiently expels water molecules from the inner coordination sphere of the metal ion thus avoiding strong luminescence quenching through OH oscillators.

### Selection of the best bioconjugatable group for protein labelling with luminescent Eu(III) chelate 8

To demonstrate the potential utility of Eu(III) chelate **8** as a reagent for biopolymer labelling (especially proteins), it was necessary to derivatise its primary amino group under mild conditions in order to introduce a bioconjugatable moiety reactive towards the $NH_2$ groups of ε-lysine residues present in selected proteins. Thus, we investigated the conversion of **8** into three different derivatives **22–24** by reaction with *N*,*N*′-disuccinimidyl carbonate (DSC), cyanuric chloride (*i.e.*, 2,4,6-trichloro-1,3,5-triazine) and disuccinimidyl sebacate (DSSEB) respectively (Scheme 3). The identity of the reaction products was confirmed by ESI mass analyses (see ESI†). Firstly, the reaction of **8** with DSC in dry DMSO containing TEA was found to be complete only by using an excess of this reagent, probably due to the steric



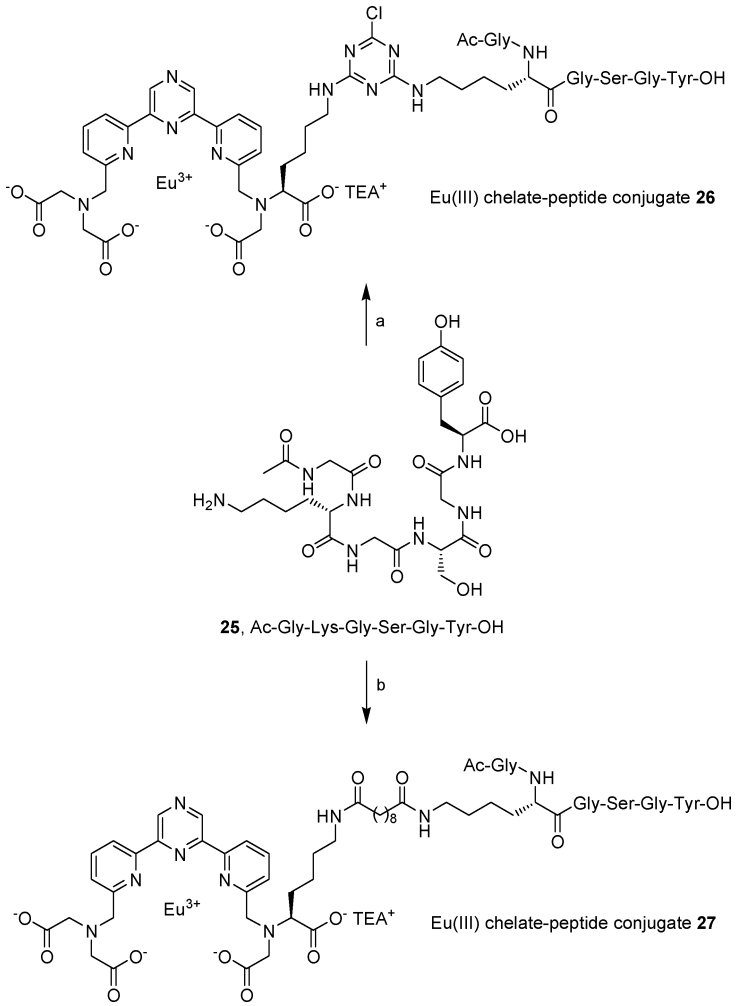

**Scheme 3** *Reagents and conditions*: a) DSC (4 equiv.), DMSO, TEA, rt, 24 h; b) cyanuric chloride (1.0 equiv.), acetone, aq. $NaHCO_3$ (pH 8.7), 0 °C, 4 h; c) DSSEB (1 equiv.), TEA, DMSO, rt, 6 h.

hindrance. Thus, to remove the unreacted reagent, the activated-carbonate chelate **22** was purified by RP-HPLC but premature hydrolysis of its active carbamate moiety occurred despite the use of a non-basic aq. mobile phase (*i.e.*, deionised water). Conversely, the complete amidification of the amino group of **8** with DSSEB was successfully achieved with only one equivalent of this homobifunctional cross-linking reagent. Interestingly, RP-HPLC analysis of the crude reaction mixture has shown that roughly one third of the newly formed NHS ester chelate **24** underwent a further reaction with a second chelate unit to give the corresponding dimer, chemically unreactive towards the proteins. Thus, no chromatographic purification was required and it was possible to use directly the crude activated chelate **24** in peptide and protein labelling (*vide infra*). Finally, we have also studied the $S_NAr$ reaction between **8** and cyanuric chloride, aimed at getting the corresponding dichlorotriazinyl activated chelate **23**, even if solely the mono-substitution of chlorine atoms by primary amines is quite difficult to obtain.[33] This reaction was performed in a mixture of aq. $NaHCO_3$ buffer (0.1 M, pH 8.7) and acetone at 0 °C for 4 h and provided a mixture of target activated chelate **23**, a dimer (resulting from two chlorine substitutions) and starting free amino chelate **8** in a ratio of 60 : 4 : 36. This crude mixture was also used in labelling reactions without purification.

In order to select the best bioconjugatable group between the succinimidyl sebacate and the dichlorotriazinyl moiety from the point of view of chemoselectivity (towards $NH_2$ groups) and efficiency of labelling, we studied the reaction of activated chelates **23** and **24** with model hexapeptide Ac-Gly-Lys-Gly-Ser-Gly-Tyr-OH **25** under mild aq. conditions (Scheme 4). This short sequence was chosen to get within the same peptidyl architecture the three representative nucleophilic amino acid side-chains able to react with the studied bioconjugatable moieties. (*i.e.*, primary amine, primary alcohol and phenol). These labelling reactions were carried out in aq. $NaHCO_3$ buffer (0.1 M, pH 8.5) with three equivalents of activated chelates **23** or **24**. As expected, the lysine residue of **25** readily reacted on each one of the two activated chelates, contrary to its serine. The resulting Eu(III) chelate–peptide conjugates **26** and **27** were isolated by RP-HPLC and their structures confirmed by ESI mass spectrometry (see ESI†). Interestingly, LC-MS analysis of the crude labelling mixture with activated chelate **23** (see ESI†) revealed the significant formation of two other peptides derivatives which were identified as the mono-labelled cyclo-peptide **28** ($t_R$ = 13.1 min, 1428.37 [M − H]$^-$) and bis-labelled derivative **29** ($t_R$ = 15.1 min, 1159.11 [M − 2H]$^{2-}$). The formation of these non-desired side-products can be inferred from the chemical modification of tyrosine residue.[34] It led to a decrease in the mono-labelling efficiency (5.3% isolated yield for **23** against 16.6% for **24**). Thus, these preliminary experiments have clearly shown the superior ability of the NHS activated Eu(III) chelate **24** to label selectively the lysine residues within peptides. This latter bio-labelling reagent was chosen for the further bioconjugation experiments involving monoclonal antibodies (mAb).

### Antibody labelling using activated chelate 24

The commonly used mAb 9E10 and 12CA5 that recognise the c-myc epitope tag[35] and infleunza hemagglutinin (HA) epitope tag[36]




**Scheme 4** *Reagents and conditions*: a) activated Eu(III) chelate **23** (3.0 equiv.), 0.1 M aq. $NaHCO_3$ (pH 8.5), rt, overnight; b) activated Eu(III) chelate **24** (3.0 equiv.) under the same conditions. $TEA^+$ = triethylammonium counter ion.

respectively, were chosen as the representative proteins. Anti-c-myc and anti-HA mAb were labelled through incubation with a 70-fold molar excess of activated Eu(III) chelate **24** in phosphate buffer (pH 6.5). The resulting protein luminescent conjugates were initially purified by size-exclusion chromatography over a Sephadex® G-25 column but a very poor resolution between unreacted labelling reagent and labelled proteins was obtained. Thus, the luminescent mAb had to be isolated by affinity chromatography using a gel-immobilised protein A. Table 2 reports quantum yields of luminescent proteins in Tris-HCl/glycine buffer, together with the attached chelate to protein molar ratios (F/P), estimated from the relative intensities of protein and chelate absorptions. As an illustrative example, the absorption/excitation/emission spectra of chelate–12CA5 conjugate are also presented in Fig. 6. Monoclonal antibody 12CA5 was found to be labelled more efficiently as inferred from an F/P ratio twice as high as the one determined for mAb 9E10.[37] Even though the difference of reactive lysine residues between these two mAb is unknown, the significant ratio improvement could be partly ascribed to the lower concentration of the 9E10 mAb aliquots used in these bioconjugation experiments (*i.e.*, 0.7 against 1.8 mg $cm^{-3}$). When compared to the non-covalently bound Eu(III) chelate **8**, the excitation/emission maxima of the luminescent protein conjugates remain unchanged (Fig. 6). Furthermore, the quantum yield of grafted Eu(III) chelate was slightly reduced by the same extent with mAb 9E10 and 12CA5 (Table 2) as compared to the value of free luminescent label (Table 1, see entries 3–4). However, the luminescence brightness remains satisfactory and proved that the activated Eu(III) chelate **24** is a suitable luminescent tag for various bio-analytical applications.

**Table 2** Quantum yields (excitation at 345 nm) and chelate to protein molar ratios of luminescent bioconjugates determined at 25 °C

| Luminescent bioconjugate | $\Phi$ (%) (Eu(TMT)-$AP_3$) | $\Phi$ (%) (R6G) | F/P |
|---|---|---|---|
| Anti c-myc 9E10 | 10.9 | 9.3 | 3.6 |
| Anti-HA 12CA5 | 10.0 | 8.5 | 7.7 |
| Hexapeptide **27** | 13.3 | 10.3 | 1.0 |

## Conclusion and future work

In summary, we have achieved the synthesis of new luminescent water-soluble Ln(III) chelates based on an original

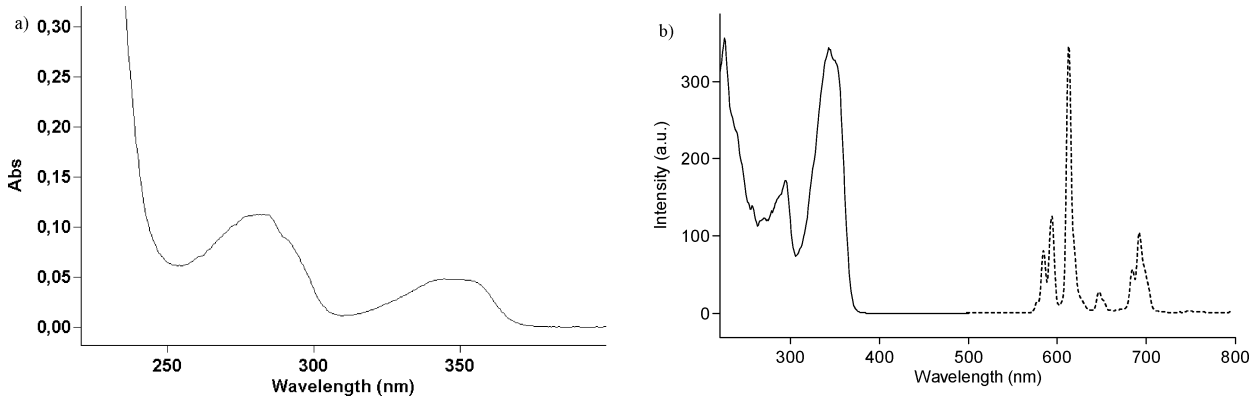
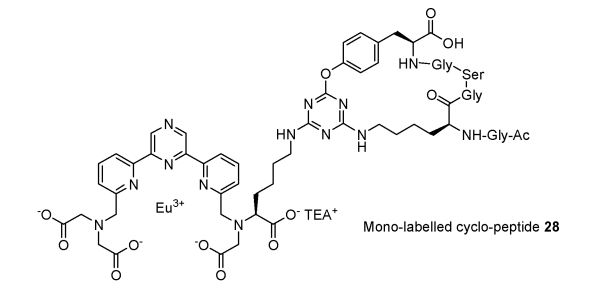
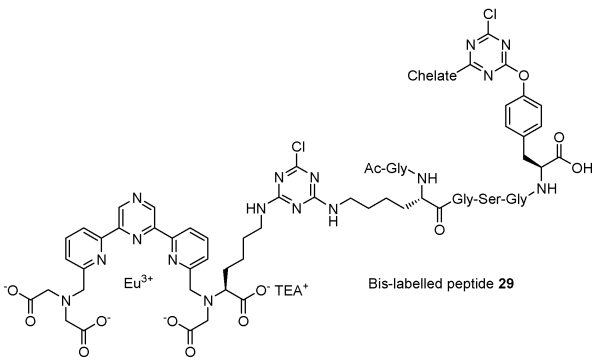



**Fig. 6** (a) Absorption spectrum of luminescent labelled mAb anti-HA 12CA5 in Tris·HCl/glycine buffer at 25 °C; (b) Excitation (—) (Em. 615 nm) and emission (---) (Ex. 345 nm) spectra of luminescent labelled mAb anti-HA 12CA5 in Tris·HCl/glycine buffer at 25 °C.






Mono-labelled cyclo-peptide **28**




Bis-labelled peptide **29**

bis-pyridinylpyrazine ligand which was functionalised at the 6,6″-positions through an original synthetic pathway aimed at introducing the chelating arms. The ability of these tri-azaheterocyclic ligands to efficiently sensitise europium(III) was also proved. Indeed, the corresponding chelates **6** and **8** have shown good luminescence properties in water: luminescence yield above 2000 $dm^3\ mol^{-1}\ cm^{-1}$ and a decay time around 1.30 ms. The most interesting feature is the red-shift of the maximum absorption wavelength compared to the usual terpyridine-based Ln(III) chelates. Indeed, the absorption maximum is located at 344 nm and stays barely unaltered until 354 nm. Furthermore, a comprehensive bioconjugation study conducted with the non-symmetrical Eu(III) chelate **8** and a model hexapeptide, has enabled us to select the most appropriate bioconjugatable group (*i.e.*, succinimidyl sebacate) for an efficient labelling of lysine residues of proteins. Thus, Eu(III) chelates based on this new scaffold could be potential candidates as luminescent markers for bioaffinity assays. However, a more straightforward synthetic pathway for the introduction of the bioconjugation arm within the ligand scaffold would improve the global process. As a general rule, location of the bioconjugatable moiety on the pyrazine-chromophoric moiety would be beneficial. With this goal in mind, a new synthetic strategy involving an aminopyrazine derivative is currently under investigation.

## Experimental Section

See ESI† for general remarks and details on experimental procedures for peptide synthesis.

### High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps. **System A**: HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 4.6 × 100 mm) with $CH_3CN$ and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 80% (40 min) of $CH_3CN$] at a flow rate of 1.0 mL $min^{-1}$. Dual UV detection was achieved at 220 and 254 nm. **System B**: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 4.6 × 100 mm) with MeOH and 0.1% aq. TFA, 0.1% as eluents [90% TFA (5 min), followed by linear gradient from 10 to 90% (40 min) of MeOH] at a flow rate of 1.0 mL $min^{-1}$. Triple UV detection was achieved at 220, 290 and 320 nm. **System C**: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 4.6 × 100 mm) with MeOH and triethylammonium acetate buffer (TEAA 25 mM, pH 7.0) as eluents [100% TEAA (10 min), followed by linear gradient from 50 to 100% (25 min) of MeOH] at a flow rate of 1.0 mL $min^{-1}$. Triple UV detection was achieved at 220, 290 and 345 nm. **System D**: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 4.6 × 100 mm) with $CH_3CN$ and aq. TFA, 0.1% as eluents [80% TFA (5 min), followed by linear gradient from 20 to 100% (32 min) of $CH_3CN$] at a flow rate of 1.0 mL $min^{-1}$. Triple UV detection was achieved at 220, 260 and 285 nm. **System E**: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 4.6 × 100 mm) with $CH_3CN$ and TEAA (25 mM, pH 7.0) as eluents [100% TEAA (2 min), followed by linear gradient from 0 to 80% (40 min) of $CH_3CN$] at a flow rate of 1.0 mL $min^{-1}$. Dual UV detection was achieved at 254 and 345 nm. **System F**: semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 21.2 × 250 mm) with $CH_3CN$ and aq. TFA, 0.1% as eluents [80% TFA (5 min), followed by linear gradient from 20 to 60% (13 min) then from 60 to 100% (32 min) of $CH_3CN$] at a

flow rate of 16.0 mL $min^{-1}$. Dual UV detection was achieved at 260 and 285 nm. **System G**: semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 21.2 × 250 mm) with MeOH and aq. TFA, 0.1% as eluents [100% TFA (5 min) followed by a linear gradient from 0 to 10% (3 min) then from 10 to 90% (40 min) of MeOH] at a flow rate of 14.0 mL $min^{-1}$. Dual UV detection was achieved at 260 and 285 nm. **System H**: semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 10 × 100 mm) with $CH_3CN$ and triethylammonium bicarbonate buffer (TEAB 50 mM, pH 7.5) as eluents [100% TEAB (5 min) followed by linear gradient from 0 to 80% (80 min) of $CH_3CN$] at a flow rate of 4.0 mL $min^{-1}$. Dual UV detection was achieved at 254 and 345 nm. **System I**: LC-MS with RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 µm, 2.1 × 150 mm) with $CH_3CN$ and TEAB (50 mM, pH 7.5) as eluents [95% TEAB (2 min) followed by linear gradient from 5 to 85% (40 min) of $CH_3CN$] at a flow rate of 0.25 mL $min^{-1}$. Dual UV detection was achieved at 220 and 345 nm. ESI-MS detection in the negative mode (full scan, 150–1500 a.m.u., data type: centroid, sheat gas flow rate: 60 arb unit, aux/sweep gas flow rate: 20, spray voltage: 4.5 kV, capillary temp: 270 °C, capillary voltage: −10 V, tube lens offset: −50 V).

### 2-Bromo-6-[(*tert*-butyldimethylsilyloxy)methyl]pyridine (11)

(*a*) *2-Bromo-6-(hydroxymethyl)pyridine*: To a cooled mixture of dry THF (8.0 mL) and *n*-BuLi in hexane (1.35 M, 9.4 mL, 12.7 mmol), a solution of 2,6-dibromopyridine (3.0 g, 12.7 mmol) in dry THF (16.0 mL) was added dropwise under an argon atmosphere, in order to keep the mixture below −70 °C. The resulting reaction mixture was left stirring for 15 min. and dry DMF (1.5 mL, 19.7 mmol) was added. The reaction solution was further stirred at −78 °C for 15 min, followed by addition of acetic acid (810 µL) in methanol (12.0 mL). Afterwards, $NaBH_4$ (0.48 g, 12.7 mmol) was introduced in several portions. The cooling bath was removed and the reaction mixture was warmed up to room temperature overnight. Thereafter, the reaction mixture was carefully quenched with sat. $NH_4Cl$ (40 mL), then extracted with AcOEt (2 × 40 mL). The organic phase was washed with brine (40 mL), dried over $MgSO_4$, filtered and concentrated under vacuum. The resulting yellow oil was purified by flash-chromatography on a silica gel column with a step gradient of AcOEt (20–30%) in cyclohexane to afford the primary alcohol as a pale yellow oil (2.20 g, yield 92%). *R*f (cyclohexane–AcOEt, 7: 3, v/v) 0.29; $\delta_H$ (300 MHz, $CDCl_3$) 7.55 (1 H, t, *J* 7.7), 7.39 (1H, d, *J* 7.7), 7.28 (1H, d, *J* 7.6), 4.74 (2H, s), 3.20 (1H, bs); $\delta_C$ (75.4 MHz, $CDCl_3$) 161.6, 141.3, 139.3, 126.6, 119.5, 64.2; MS (ESI+): *m/z* 188.07 and 190.07 $[M + H]^+$, calcd for $C_6H_6BrNO$: 188.02.

(*b*) *Silylation*: The alcohol (2.12 g, 11.3 mmol) was dissolved in dry DMF (10.0 mL). Imidazole (3.08 g, 45.2 mmol) and TBS-Cl (2.04 g, 13.5 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 25 min. Thereafter, the reaction mixture was extracted with $Et_2O$ (2 × 30 mL). The combined ethereal phases were washed with deionised water (3 × 5 mL), dried over $MgSO_4$, filtered and concentrated. The resulting yellow oily residue was purified by flash-chromatography on a silica gel column with a step gradient of AcOEt (1–3%) in cyclohexane. The desired TBS ether **11** was obtained as a viscous turbid liquid (3.01 g, yield 88%). *R*f (cyclohexane–AcOEt, 99: 1, v/v) 0.15; $\delta_H$ (300 MHz, $CDCl_3$) 7.57 (1H, t, *J* 7.7), 7.47 (1H, dd, *J* 7.7 and 0.9), 7.33 (1H, dd, *J* 7.7 and 0.8), 4.80 (2H, s), 0.95 (9H, s), 0.11 (6H, s); $\delta_C$ (75.4 MHz, $CDCl_3$) 163.3, 141.0, 139.1, 126.1, 118.7, 65.5, 26.0, 18.4, −5.3; MS (ESI+): *m/z* 302.07 and 304.00 $[M + H]^+$, calcd for $C_{12}H_{20}BrNOSi$: 302.29.

### 2,6-Bis[6-((*tert*-butyldimethylsilyloxy)methyl)pyridin-2-yl]pyrazine (12)

(*a*) *2-Trimethyltin-6-*[(*tert-butyldimethylsilyloxy*)*methyl*] *pyridine*: To a solution of silyl ether **11** (2.0 g, 6.62 mmol) in dry THF (14.0 mL) was added dropwise, under an argon atmosphere at −78 °C, a solution of *n*-BuLi in hexane (1.4 M, 5.2 mL, 7.3 mmol). The dark red solution was stirred during 90 min at −78 °C before adding a solution of trimethyltin chloride (1.38 g, 6.95 mmol) in dry THF (14.0 mL). The reaction mixture was left running at room temperature for 4 h under an argon atmosphere. Thereafter, the mixture was evaporated to dryness and the resulting yellow-brown paste was triturated twice with $Et_2O$ and filtered. The filtrate was evaporated and the stannane derivative was obtained as an orange oil and was used in the next step without further purification (2.40 g, yield 89%). $\delta_H$ (300 MHz, $CDCl_3$) 7.53 (1H, t, *J* 7.6), 7.35 (1H, d, *J* 7.3), 7.29 (1H, d, *J* 7.2), 4.85 (2H, s), 0.96 (9H, s), 0.32 (9H, s), 0.12 (6H, s).

(*b*) *Stille-coupling reaction*: To a xylene solution (6 mL) deaerated with argon were added $Pd(PPh_3)_4$ (87 mg, 0.08 mmol), 2,6-dichloropyrazine (224 mg, 1.50 mmol) and trimethyltin derivative (1.27 g, 3.30 mmol). The resulting reaction mixture was heated to 110 °C overnight. The desired compound as well as the monocoupling derivative were detected by NMR analyses. Further amounts of stannane (270 mg, 0.70 mmol) and $Pd(PPh_3)_4$ (12 mg, 0.01 mmol) were therefore added and the mixture was left running 24 h at 110 °C. The reaction mixture was filtrated on a Celite® pad. After solvent removal, a brown solid was recovered. Purification of this crude product was achieved by flash-chromatography on a silica gel column with a step gradient of AcOEt (2–4%) in cyclohexane as the mobile phase to give the bis-pyridylpyrazine derivative **12** as a white foam (520 mg, yield 50%). *R*f (cyclohexane–AcOEt, 96: 4, v/v) 0.24; $\delta_H$ (300 MHz, $CDCl_3$) 9.62 (2H, s), 8.39 (2H, d, *J* 7.8), 7.89 (2H, t, *J* 7.8), 7.59 (2H, d, *J* 7.7), 4.95 (4H, s), 0.99 (18H, s), 0.17 (12H, s); $\delta_C$ (75.4 MHz, $CDCl_3$) 161.4, 153.4, 149.7, 142.7, 137.7, 120.9, 119.7, 66.3, 26.1, 18.5, −5.2; MS (ESI+): *m/z* 523.33 $[M + H]^+$, calcd for $C_{28}H_{42}N_4O_2Si_2$: 522.83.

### 2,6-Bis[6-(hydroxymethyl)pyridin-2-yl]pyrazine (13)

To a solution of bis-silyl ether **12** (547 mg, 1.05 mmol) in THF (10 mL) was added a 1.0 M solution of TBAF in THF (2.4 mL, 2.41 mmol). The resulting reaction mixture was stirred at room temperature for 3 h and concentrated under reduced pressure to give an orange-brown viscous oily residue. This crude product was first triturated in cyclohexane and then in a mixture of aq. TFA 0.1% and methanol (1: 1, v/v) to give a the bis-alcohol **13** as a white solid which was dried by lyophilisation (244 mg, yield 79%). $\delta_H$ (300 MHz, DMSO-$d_6$) 9.57 (2H, s), 8.45 (2H, d, *J* 7.7), 8.06 (2H, t, *J* 7.8), 7.63 (2H, d, *J* 7.6), 5.58 (2H, t, *J* 5.8), 4.71 (4H, d, *J* 5.2); $\delta_C$ (75.4 MHz, DMSO-$d_6$) 162.2, 152.3, 149.1, 142.1, 138.2, 121.6, 119.5, 64.3; MS (ESI+): *m/z* 292.50 $[M + H]^+$, calcd for $C_{16}H_{14}N_4O_2$: 294.31.

**2,6-Bis[6-(bromomethyl)pyridin-2-yl]pyrazine (14)**

$PBr_3$ (0.29 ml, 3.1 mmol) was added to dry DMF (10 mL) under an argon atmosphere and the resulting solution was cooled to 0 °C. Bis-alcohol **13** (303 mg, 1.03 mmol) previously dissolved in dry DMF (10 mL) was added to the $PBr_3$ solution. The resulting reaction mixture was stirred at room temperature for h. After DMF removal under high vacuum, the resulting residue was diluted in $CH_2Cl_2$ (50 mL) and washed with aq. sat. $NaHCO_3$ (2 × 30 mL). The aq. phase was then re-extracted with $CH_2Cl_2$ (30 mL). The organic phases were collected and washed with brine (40 mL), dried over $MgSO_4$, filtered and concentrated. A clear brown solid was obtained. The compound was used in the next step without further purification (410 mg, yield 95%). *R*f (cyclohexane–AcOEt, 9: 1, v/v) 0.20; $\delta_H$ (300 MHz, $CDCl_3$) 9.71 (2H, s), 8.45 (2H, dd, *J* 7.7 and 0.8), 7.90 (2H, t, *J* 7.9), 7.55 (2H, dd, *J* 7.7 and 0.8), 4.67 (4H, s); $\delta_C$ (75.4 MHz, $CDCl_3$) 156.9, 154.2, 149.1, 143.3, 138.2, 124.3, 120.7, 33.9.

**Fully-protected symmetrical bis-pyridinylpyrazine ligand (15)**

To a solution of di-brominated derivative **14** (157 mg, 0.37 mmol) and NaI (61 mg, 0.41 mmol) in dry DMF (8 mL) were added DIEA (0.65 mL, 3.7 mmol) and secondary amine **9** (200 mg, 0.81 mmol). The reaction mixture was stirred at room temperature for 6 h and DMF was removed under high vacuum. The resulting residue was dissolved in $CH_2Cl_2$ (50 mL) and the organic phase was washed with an aq. sat. $NaHCO_3$ solution (3 × 20 mL). The aq. phase was re-extracted with $CH_2Cl_2$. The combined organic phases were washed with brine, dried over $MgSO_4$, filtered and evaporated to dryness. The resulting brown viscous oily residue was purified by flash-chromatography on a silica gel column with a step gradient of MeOH (0–5%) in $CH_2Cl_2$ to afford the *tert*-butyl ester **15** as a yellow oil (199 mg, yield 64%). *R*f ($CH_2Cl_2$–MeOH, 95: 5, v/v) 0.30; $\delta_H$ (300 MHz, $CDCl_3$) 9.66 (2H, s), 8.42 (2H, d, *J* 7.8), 7.87 (2H, t, *J* 7.8), 7.73 (2H, d, *J* 7.7), 4.15 (4H, s), 3.56 (8H, s), 1.48 (36H, s); $\delta_C$ (75.4 MHz, $CDCl_3$) 170.7, 159.3, 153.6, 149.7, 142.8, 137.7, 124.1, 119.9, 81.2, 59.7, 55.9, 28.3; MS (ESI+): *m/z* 749.20 $[M + H]^+$, 771.27 $[M + Na]^+$ and 787.33 $[M + K]^+$, calcd for $C_{40}H_{56}N_6O_8$: 748.91.

**Bis-pyridinylpyrazine ligand (10)**

Tetrakis-*tert*-butyl ester **15** (200 mg, 0.27 mmol) was dissolved in $CH_2Cl_2$ (10 mL) and deionised water (0.5 mL) and cooled to 0 °C. TFA (10 mL) was added and the reaction mixture was stirred at room temperature for 5 h. Thereafter, the mixture was evaporated to dryness and the residue was co-evaporated thrice with cyclohexane and finally triturated with $Et_2O$ to give a pale brown powder. This solid was diluted in deionised water and lyophilised. A white amorphous powder was obtained (170 mg). A batch of 100 mg was purified by flash-chromatography on a RP-$C_{18}$ silica gel column with a step gradient of MeOH (0–30%) in aq. TFA 0.1%. The product-containing fractions were lyophilised to afford the TFA salt of ligand **10** as a pale yellow solid (40 mg, yield 44%). $\delta_H$ (300 MHz, DMSO-$d_6$) 9.56 (2H, s), 8.48 (2H, d, *J* 7.5), 8.06 (2H, t, *J* 7.8), 7.69 (2H, d, *J* 7.3), 4.17 (4H, s), 3.64 (8H, s); $\delta_C$ (75.4 MHz, DMSO-$d_6$) 172.0, 152.6, 149.0, 142.2, 138.4, 124.3, 120.0, 59.0, 54.4; MS (ESI+): *m/z* 525.27 $[M + H]^+$, calcd for $C_{24}H_{24}N_6O_8$: 524.48; HPLC (system B): $t_R$ = 13.8 min, purity 96% (max plot 220–450 nm); $\lambda_{max}$(recorded during the HPLC analysis)/nm 229, 243, 283, and 310. The elemental composition was accurately determined by UV-titration with an aq. solution of known concentration of $EuCl_3$ and by assuming the expected stoichiometry [1 : 1] for the complex. An average value of 0.5 molecule of TFA per molecule of ligand was found.

**Eu(III) chelate (6)**

Bis-pyridinylpyrazine ligand **10** (TFA salt, 70 mg, 48 μmol) was dissolved in deionised water (1 mL) and $NaHCO_3$ was added to adjust the pH of the medium to approximately 7.5. Then, $EuCl_3 \cdot 6H_2O$ (43 mg, 120 μmol) was added and the resulting reaction mixture was stirred at room temperature for 1 h. Thereafter, the crude Eu(III) complex was purified by flash-chromatography on a RP-$C_{18}$ silica gel column with deionised water. The product-containing fractions were lyophilised to afford the desired Eu(III) chelate **6** in a pure form as a white solid (Na salt, 34.3 mg, 49.3 μmol, quantitative yield). MS (ESI–): *m/z* 671.20 and 673.20 $[M - H]^-$, calcd for $C_{24}H_{21}EuN_6O_8$: 673.42; HRMS (ESI–): *m/z* 671.0541 and 673.0555 for $C_{24}H_{20}EuN_6O_8$. HPLC (system C): $t_R$ = 8.4 min, purity 100% (345 nm); $\lambda_{max}$(recorded during the HPLC analysis)/nm 229, 295 and 344.

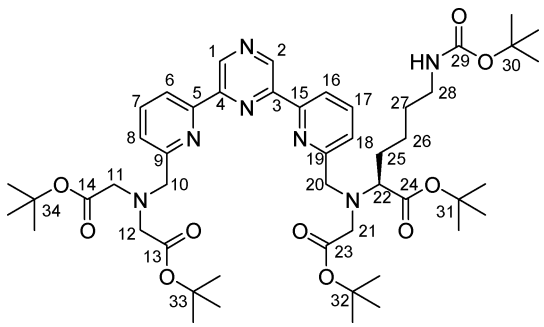
**Sm(III) chelate (7)**

Bis-pyridinylpyrazine **10** (TFA salt, 3.4 mg, 5.8 μmol) was dissolved in 0.1 M aq. $NaHCO_3$ (200 μL, pH 8.7). Thereafter, a 41 mM $SmCl_3 \cdot 6H_2O$ solution in deionised water (143 μL, 6.0 μmol) was added and the resulting reaction mixture was stirred at room temperature for 1 h. Thereafter, the reaction mixture was purified by flash-chromatography on a RP-$C_{18}$ silica gel column with deionised water. The product-containing fractions were lyophilised to afford the desired Sm(III) chelate **7** in a pure form as a white powder (Na salt, 3.7 mg, 5.3 μmol, yield 92%). MS (ESI–): *m/z* 667.33, 669.33, 672.40, 674.20 $[M - H]^-$, calcd for $C_{24}H_{21}SmN_6O_8$: 671.82; HPLC (system C): $t_R$ = 11.3 min; $\lambda_{max}$(recorded during the HPLC analysis)/nm 233, 294 and 343.

**Protected non-symmetrical bis-pyridinylpyrazine ligand (17)**

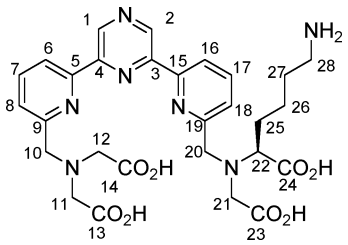
To a solution of di-brominated derivative **14** (122 mg, 0.29 mmol) in dry DMF (5 mL) were introduced NaI (25 mg, 0.17 mmol), anhydrous $K_2CO_3$ (601 mg, 4.35 mmol) and secondary amine **16** (133 mg, 0.32 mmol). The resulting reaction mixture was stirred at room temperature for 9 h. Thereafter symmetrical secondary amine **9** was added in several portions: 21.0 mg (0.09 mmol), 21.0 mg (0.09 mmol) after 27 h and 31.0 mg (0.12 mmol) after two days. The reaction mixture was finally stirred at room temperature for additional 24 h after the addition of the third aliquot of **9**. Thereafter DMF was evaporated and the residue was diluted in a mixture of cyclohexane/AcOEt (1: 1, v/v) which was filtered. The filtrate was evporated to dryness and the resulting orange viscous oily residue was firstly purified by flash-chromatography on a silica gel column cyclohexane/AcOEt (6: 4, v/v). Thereafter, a second purification was achieved by semi-preparative RP-HPLC (system F). The product-containing fractions were lyophilised to give the desired ligand as a brown foam (47 mg, yield 18%). $\delta_H$ (300 MHz, $CDCl_3$) 9.67 (1H, s, $H_1$ or

$H_2$), 9.64 (1H, s, $H_2$ or $H_1$), 8.49 (1H, d, *J* 7.7, $H_6$ or $H_{16}$), 8.40 (1H, d, *J* 7.6, $H_{16}$ or $H_6$), 7.93 (1H, t, *J* 7.8, $H_7$ or $H_{17}$), 7.90 (1H, t, *J* 7.8, $H_{17}$ or $H_7$), 7.81 (1H, d, *J* 7.6, $H_8$ or $H_{18}$), 7.70 (1H, d, *J* 7.5, $H_{18}$ or $H_8$), 4.63 (1H, bs, NH), 4.23 (2H, s, $H_{10}$), 4.20 (1H, d, *J* 15.2, $H_{20}$), 4.10 (1H, d, *J* 15.3, $H_{20}$), 3.83 (4H, s, $H_{11}$, $H_{12}$), 3.54 (2H, s, $H_{21}$), 3.38 (1H, t, *J* 7.4, $H_{22}$), 3.07 (2H, bs, $H_{28}$), 1.77–1.65 (2H, m, $H_{25}$), 1.58–1.30 (49 H, m, H, $H_{26}$, $H_{27}$, $H_{t\text{-}Bu}$); $\delta_C$ (75.4 MHz, $CDCl_3$) 172.3 ($C_{23}$), 170.9 ($C_{24}$), 168.6 ($C_{14}$, $C_{13}$), 159.5 ($C_{29}$), 156.1 ($C_9$ or $C_{19}$), 155.7 ($C_{19}$ or $C_9$), 153.7 ($C_5$ or $C_{15}$), 153.0 ($C_{15}$ or $C_5$), 149.8 ($C_3$ or $C_4$), 149.4 ($C_4$ or $C_3$), 142.7 ($C_1$ or $C_2$), 142.3 ($C_2$ or $C_1$), 138.5 ($C_7$ or $C_{17}$), 138.0 ($C_{17}$ or $C_7$), 125.1 ($C_8$ or $C_{18}$), 124.3 ($C_{18}$ or $C_8$), 120.9 ($C_6$ or $C_{16}$), 120.1 ($C_{16}$ or $C_6$), 82.8 ($C_{33}$, $C_{34}$), 81.7 ($C_{31}$ or $C_{32}$), 81.3 ($C_{32}$ or $C_{31}$), 79.1 ($C_{30}$), 64.4 ($C_{22}$), 58.8 ($C_{10}$), 58.2 ($C_{20}$), 55.1 ($C_{11}$, $C_{12}$), 53.5 ($C_{21}$), 40.4 ($C_{28}$), 29.7 ($C_{25}$ or $C_{27}$), 29.6 ($C_{27}$ or $C_{25}$), 28.5 ($C_{t\text{-}Bu}$), 28.4 ($C_{t\text{-}Bu}$), 28.2 ($C_{t\text{-}Bu}$), 23.5 ($C_{26}$); MS (ESI+): *m/z* 920.33 $[M + H]^+$, 942.27 $[M + Na]^+$ and 958.33 $[M + K]^+$ calcd for $C_{49}H_{73}N_7O_{10}$: 920.14; HPLC (system C): $t_R$ = 26.7 min; $\lambda_{max}$(recorded during the HPLC analysis)/nm 230, 243, 283 and 312.



**Non-symmetrical bis-pyridinylpyrazine ligand (18)**

Fully-protected ligand **17** (60 mg, 66 µmol) was dissolved in a mixture of $CH_2Cl_2$ (1.9 mL) and deionised water (90 µL) and cooled to 0 °C. Then, TFA (1.9 mL) was added and the resulting reaction mixture was stirred at room temperature for 7 h. The reaction was checked for completion by RP-HPLC (system B). Further amount of TFA (0.3 mL) was added and the reaction mixture was stirred overnight. Thereafter, the reaction mixture was evaporated to dryness and the resulting brownish paste was purified by semi-preparative RP-HPLC (system G). The product-containing fractions were lyophilised to afford the TFA salt of non-symmetrical bis-pyridinylpyrazine ligand **18** as a brown powder (26.3 mg, yield 54%). $\delta_H$ (300 MHz, DMSO-$d_6$+$D_2O$) 9.55 (1H, s, $H_1$ or $H_2$), 9.53 (1H, s, $H_2$ or $H_1$), 8.47 (1H, d, *J* 7.2, $H_6$ or $H_{16}$), 8.44 (1H, d, *J* 7.2, $H_{16}$ or $H_6$), 8.05 (1H, t, *J* 7.7, $H_7$ or $H_{17}$), 8.03 (1H, t, *J* 7.7, $H_{17}$ or $H_7$), 7.73 (1H, d, *J* 7.7, $H_8$ or $H_{18}$), 7.68 (1H, d, *J* 7.6, $H_{18}$ or $H_8$), 4.19 (2H, s, $H_{10}$), 4.13 (1H, d, *J* 15.5, $H_{20}$), 4.03 (1H, d, *J* 15.3, $H_{20}$), 3.67 (4H, s, $H_{11}$, $H_{12}$), 3.55 (2H, s, $H_{21}$), 3.39 (1H, t, *J* 7.3, $H_{22}$), 2.77–2.69 (2H, m, $H_{28}$), 1.74–1.64 (2H, m, $H_{25}$), 1.56–1.43 (4H, m, $H_{26}$, $H_{27}$); $\delta_C$ (75.4 MHz, DMSO-$d_6$+$D_2O$) 174.1 ($C_{24}$), 172.7 ($C_{23}$), 172.1 ($C_{13}$, $C_{14}$), 159.8 ($C_{19}$), 158.5 ($C_9$), 152.6 ($C_5$ or $C_{15}$), 152.5 ($C_{15}$ or $C_5$), 149.1 ($C_3$ or $C_4$), 149.0 ($C_4$ or $C_3$), 142.1 ($C_1$, $C_2$), 138.3 ($C_7$ or $C_{17}$), 138.2 ($C_{17}$ or $C_7$), 124.3 ($C_8$), 123.9 ($C_{18}$), 119.9 ($C_6$ or $C_{16}$), 119.8 ($C_{16}$ or $C_6$), 63.2 ($C_{22}$), 59.0 ($C_{10}$), 57.4 ($C_{20}$), 54.4 ($C_{11}$, $C_{12}$), 52.6 ($C_{21}$), 38.7 ($C_{28}$), 29.0 ($C_{25}$), 26.8 ($C_{27}$), 22.8 ($C_{26}$); MS (ESI+): *m/z* 596.27 $[M + H]^+$, calcd for $C_{28}H_{33}N_7O_8$: 595.60; HPLC (system B): $t_R$ = 13.7 min, purity 93% (max plot 220–450 nm); $\lambda_{max}$(recorded during the HPLC analysis)/nm 229, 243, 282 and 315. The elemental composition was accurately determined by UV-titration with an aq. solution of known concentration of $EuCl_3$ and by assuming the expected stoichiometry [1 : 1] for the complex. An average value of 1.3 molecules of TFA per molecule of ligand was found.



**Non-symmetrical Eu(III) chelate (8)**

This complex was prepared and purified by the same procedure used for **6**, and obtained as a pale brown powder (Na salt, 6.3 mg, 8.2 µmol, yield 78%). MS (ESI+): *m/z* 744.07 and 746.13 $[M + H]^+$; MS (ESI–): *m/z* 742.20 and 744.13 $[M - H]^-$ and 855.87 and 857.87 $[M + TFA - H]^-$, calcd for $C_{28}H_{30}EuN_7O_8$: 744.55; HRMS (ESI–): *m/z* 742.1276 and 744.1290 for $C_{28}H_{29}EuN_7O_8$; HPLC (system C): $t_R$ = 5.2 min (broad peak), purity 98% (345 nm); $\lambda_{max}$(recorded during the HPLC analysis)/nm 224, 294 and 344.

**Dichlorotriazine derivative (23)**

Amino Eu(III) chelate **8** (1.0 mg, 1.3 µmol) was dissolved in 0.1 M aq. $NaHCO_3$ (200 µL, pH 8.7). Thereafter, a 13 mM cyanuric chloride solution in HPLC-grade acetone (100 µL, 1.3 µmol) was added at 0 °C. The resulting reaction mixture was stirred at 0 °C for 4 h. The reaction was checked for completion by ESI-MS. The crude activated Eu(III) chelate **23** was used in the next bioconjugation step without further purification. MS (ESI–): *m/z* 891.07 $[M - H]^-$, calcd for $C_{31}H_{29}Cl_2EuN_{10}O_8$: 892.50; HPLC (system E): $t_R$ = 13.5 min; $\lambda_{max}$(recorded during the HPLC analysis)/nm 234, 295 and 344.

**Succinimidyl sebacate derivative (24)**

To a solution of DSSEB reagent (0.55 mg, 1.4 µmol) in DMSO (50 µL) was added at room temperature, every 30 min, 10 µL of a mixture containing amino Eu(III) chelate **8** (1.0 mg, 1.3 µmol) and TEA (2 µL) in DMSO (98 µL). After each addition, the reaction mixture was vortexed and finally left at room temperature for 45 min. The reaction was checked for completion by LC-MS (system I). The crude NHS ester **24** was stored at 4 °C and used in the next bioconjugation step without further purification. MS (ESI–): *m/z* 1023.31 and 1025.29 $[M - H]^-$, calcd for $C_{42}H_{49}EuN_8O_{13}$: 1025.85. HPLC (system C): $t_R$ = 16.2 min; $\lambda_{max}$(recorded during the HPLC analysis)/nm 234, 294 and 343.

**Luminescent labelling of hexapeptide 25 with activated Eu(III) chelates 23 and 24**

(*a*) *Labelling with 23*: Hexapeptide **26** was dissolved in 0.1 M aqueous $NaHCO_3$ pH 8.7 (22.13 µM, 9.8 µL, 217 nmol) and mixed with the crude activated Eu(III) chelate **23** (150 µL, 650 nmol, the activation of amino chelate was assumed to be quantitative).

The resulting reaction mixture was stirred at room temperature overnight. The completion of the reaction was checked by HPLC (system E) and purified by semi-preparative RP-HPLC (system H). The fractions containing the luminescent labelled peptide were lyophilised. MS (ESI–): *m/z* 1464.40 [M – H]$^-$, calcd for $C_{57}H_{67}ClEuN_{17}O_{18}$: 1465.67; HPLC (system E): $t_R$ = 12.4 min, purity 95%; $\lambda_{max}$(recorded during the HPLC analysis)/nm 229, 295, 343 nm.

(*b*) *Labelling with* ***24***: The same procedure as described above. MS (ESI–): *m/z* 1519.67 [M – H]$^-$, calcd for $C_{64}H_{83}EuN_{14}O_{20}$: 1520.41; HPLC (system E): $t_R$ = 12.2 min, purity 97%; $\lambda_{max}$(recorded during the HPLC analysis)/nm 227, 295 and 343.

For both labelling reactions, the bioconjugation yield was determined through quantification of the labelled peptide by UV–vis spectroscopy at the $\lambda_{max}$ of the chelate (*i.e.*, 345 nm)

### Labelling of mAb with activated Eu(III) chelate 24

(*a*) *Labelling of anti c-myc 9E10 mAb*: An aq. solution of anti c-myc 9E10 mAb (0.7 mg cm$^{-3}$, 500 µL, 2.33 nmol) in phosphate buffer (0.1 M, pH 6.5) was mixed with the crude activated Eu(III) chelate **24** (72 equiv., the activation of amino chelate was assumed to be quantitative). The resulting reaction mixture was left at room temperature overnight without stirring. Thereafter, the crude mixture was purified by affinity chromatography (*vide infra*).

(*b*) *Labelling of anti HA 12CA5 mAb*: The same procedure as described above was applied to an aq. solution of anti HA 12CA5 mAb (1.8 mg cm$^{-3}$, 500 µL, 6.00 nmol) in phosphate buffer pH 6.5.

(*c*) *Purification by affinity chromatography*: ~ 1 mL of a gel-immobilised protein A was used. First, the gel was rinsed with 0.1 M borate buffer (containing 0.15 M NaCl, pH 8.5, 20 mL). The binding step was carried out with the same buffer and the elution of the labelled antibodies was performed with a glycine buffer (0.1 M, pH 3.5). Each acidic fraction (~750 µL) was neutralised by adding 50 µL of Tris-HCl buffer (0.5 M, pH 8.5). The elution of the desired labelled antibodies was checked by UV absorbance measurements. The luminescence features of labelled mAb were directly determined in this mixture of buffers. After using, the column was washed with a solution of aq. HCl (pH 1.5), neutralised with borate buffer and stored in PBS buffer containing $NaN_3$ 0.01% at 4 °C.

(*d*) *Labelling molar ratio F/P*: They were measured by recording the UV absorbance at 280 and 345 nm respectively. The absorbance value at 345 nm enabled us to determine the concentration of Eu(III) chelate grafted to the protein. At this wavelength, the Eu(III) chelates were the unique absorbing-species. The mAb concentration could be measured by subtracting from the UV absorbance value at $\lambda$ = 280 nm the residual absorbance at the same wavelength coming from grafted Eu(III) chelates (the chelate absorbance value at 345 nm was found to be twice higher than the one at 280 nm). The measured values were inserted into the following equation:

$$[\text{mAb}]\ (\mu\text{M}) = (A_{280} - 0.5 \times A_{345})/(1.48 \times 0.15)$$

The average molecular weights of the two mAb are 150 000 Da (hence 0.15 in the former formula).

### Luminescent measurements

Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer using either a semi-micro quartz fluorescence cell (Hellma, 104F-QS, light path: 10 × 4 mm, 1400 µL) for ligands and Eu(III) chelates, or an ultra-micro fluorescence cell (Hellma®, 105.51-QS, light patch: 3 × 3 mm, 45 µL) for labelled mAb. Emission spectra were recorded in "phosphorescence mode" with the following parameters: delay time: 0.1 ms, gate time: 3 ms and total decay time: 10 ms, and after excitation at 345 nm. Lifetime measurements were achieved by using the following parameters: delay time: 0 ms, flash number: 1, excitation slit: 5 nm, emission slit: 10 nm, total decay time: 10 ms, cycle number: 20 and PMT (sensitivity): medium, $\lambda_{exc}$: 345 nm, $\lambda_{em}$: 615 nm. The luminescence quantum yields were measured in deionised water (except for labelled mAb which were performed in a mixture of Tris-HCl and glycine buffers, *vide supra*) at 25 °C by a relative method[27] using two standards: rhodamine 6G ($\Phi$ = 88% in ethanol)[28] and Eu(TMT)-AP$_3$ ($\Phi$ = 16.9% in deionised water)[29] All $\Phi$ are corrected for changes in refractive index. Excitation was achieved at 345 nm. To minimise the inner filter effect, samples with an absorbance below 0.1 unit were used.

### UV–visible titrations

A solution of bis-pyridinylpyrazine ligand (estimated concentration: 10 µM) was prepared in deionised water and transferred into a quartz UV-cell (Varian, 6610000800, cell Open Top, 10 × 10 mm, 3.5 mL). Titration was performed by adding periodically aliquots (usually 10 µL then 2 µL when the ratio between the ligand and the metal was closed to 1 : 1) of an exactly known concentration aq. $EuCl_3$ in deionised water ([$EuCl_3$] = 0.48 or 0.24 mM). After each addition, the solution was stirred for 5 min before recording the corresponding UV-visible spectrum (220–450 nm) at 25 °C. For drawing the titration curves, changes of absorbance at 354 nm was used.

## Acknowledgements

This work was supported by the "Ministère de l'enseignement supérieur et de la recherche" through a young investigator grant (ACI-JC 4064), and a PhD grant for Séverine Poupart. La Région Haute-Normandie *via* the CRUNCh program (CPER 2007–2013), and *via* a shared CNRS - Région Haute-Normandie grant for Nicolas Maindron, and Institut Universitaire de France (IUF) are also greatly acknowledged for their financial support. We thank Albert Marcual (COBRA-CNRS UMR 6014) for high-resolution mass analyses and Dr Hervé Volland (iBiTecS, LERI, CEA-Saclay) for kind gift of monoclonal antibodies and gel-immobilised protein A.

## Notes and references

1 (*a*) H. Bazin, E. Trinquet and G. Mathis, *Rev. Mol. Biotechnol.*, 2002, **82**, 233; (*b*) G. Mathis, *Clin. Chem.*, 1995, **41**, 1391; (*c*) C. P. Montgomery, B. S. Murray, E. J. New, R. Pal and D. Parker, *Acc. Chem. Res.*, 2009, **42**, 925; (*d*) C. P. Montgomery, E. J. New, L. O. Palsson, D. Parker, A. S. Batsanov and L. Lamarque, *Helv. Chim. Acta*, 2009, **92**, 2186.
2 I. Hemmilä, *Clin. Chem.*, 1985, **31**, 359.
3 E. Soini and I. Hemmilä, *Clin. Chem.*, 1979, **25**, 353.
4 J.-C. G. Bünzli, *Chem. Rev.*, 2010, **110**, 2729.

5 E. G. Moore, A. P. S. Samuel and K. N. Raymond, *Acc. Chem. Res.*, 2009, **42**, 542.
6 J.-C. G. Bünzli and C. Piguet, *Chem. Soc. Rev.*, 2005, **34**, 1048.
7 (*a*) J. C. G. Bünzli, *Springer Ser. Mater. Sci.*, 2005, **83**, 462; (*b*) I. Hemmilä and V.-M. Mukkala, *Crit. Rev. Clin. Lab. Sci.*, 2001, **38**, 441; (*c*) E. F. Dickson Gudgin, A. Pollak and E. P. Diamandis, *Pharmacol. Ther.*, 1995, **66**, 207.
8 P. R. Selvin, in *Top. Fluoresc. Spectrosc.*, ed. J. R. Lacowicz, Kluwer Academic/Plenum Publishers, New York, 2003, vol. 7, pp. 177.
9 For recent reviews describing these strategies, see: (*a*) Y. Ma and Y. Wang, *Coord. Chem. Rev.*, 2010, **254**, 972; (*b*) S. V. Eliseeva and J.-C. G. Bünzli, *Chem. Soc. Rev.*, 2010, **39**, 189.
10 For selected examples, see: (*a*) M. H. V. Werts, M. A. Duin, J. W. Hofstraat and J. W. Verhoeven, *Chem. Commun.*, 1999, 799; (*b*) A. Beeby, L. M. Bushby, D. Maffeo and J. A. G. Williams, *J. Chem. Soc., Perkin Trans. 2*, 2000, 1281; (*c*) A. Dadabhoy, S. Faulkner and P. G. Sammes, *J. Chem. Soc., Perkin Trans. 2*, 2002, 348; (*d*) R. Van Deun, P. Nockemann, P. Fias, K. Van Hecke, L. Van Meervelt and K. Binnemans, *Chem. Commun.*, 2005, 590.
11 For selected publications about sensitisation of lanthanide luminescence with azaxanthone-DOTA ligands, see: (*a*) P. Atkinson, K. S. Findlay, F. Kielar, R. Pal, D. Parker, R. A. Poole, H. Puschmann, S. L. Richardson, P. A. Stenson, A. L. Thompson and J. Yu, *Org. Biomol. Chem.*, 2006, **4**, 1707; corrigendum; , *Org. Biomol. Chem.*, 2006, 4, p. 3708; (*b*) R. A. Poole, G. Bobba, M. J. Cann, J.-C. Frias, D. Parker and R. D. Peacock, *Org. Biomol. Chem.*, 2005, **3**, 1013; (*c*) R. A. Poole, F. Kielar, S. L. Richardson, P. A. Stenson and D. Parker, *Chem. Commun.*, 2006, 4084; corrigendum; , *Chem. Commun.*, 2006, p. 4862; (*d*) R. A. Poole, C. P. Montgomery, E. J. New, A. Congreve, D. Parker and M. Botta, *Org. Biomol. Chem.*, 2007, **5**, 2055; (*e*) J. Yu, D. Parker, R. Pal, R. A. Poole and M. J. Cann, *J. Am. Chem. Soc.*, 2006, **128**, 2294.
12 For selected examples, see: (*a*) C. Yang, L.-M. Fu, Y. Wang, J.-P. Zhang, W.-T. Wong, X.-C. Ai, Y.-F. Qiao, B.-S. Zou and L.-L. Gui, *Angew. Chem., Int. Ed.*, 2004, **43**, 5010; (*b*) A. D'Aleo, A. Picot, A. Beeby, J. A. Gareth Williams, B. Le Guennic, C. Andraud and O. Maury, *Inorg. Chem.*, 2008, **47**, 10258.
13 For selected examples, see: (*a*) L.-O. Palsson, R. Pal, B. S. Murray, D. Parker and A. Beeby, *Dalton Trans.*, 2007, 5726; (*b*) A. Picot, A. D'Aleo, P. L. Baldeck, A. Grichine, A. Duperray, C. Andraud and O. Maury, *J. Am. Chem. Soc.*, 2008, **130**, 1532; (*c*) G. Shao, R. Han, Y. Ma, M. Tang, F. Xue, Y. Sha and Y. Wang, *Chem.–Eur. J.*, 2010, **16**, 8647.
14 (*a*) V. M. Mukkala, M. Helenius, I. Hemmilä, J. Kankare and H. Takalo, *Helv. Chim. Acta*, 1993, **76**, 1361; (*b*) A. K. Saha, K. Kross, E. D. Kloszewski, D. A. Upson, J. L. Toner, R. A. Snow, C. D. V. Black and V. C. Desai, *J. Am. Chem. Soc.*, 1993, **115**, 11032; (*c*) H. Takalo, V. M. Mukkala, L. Merio, J. C. Rodriguez-Ubis, R. Sedano, O. Juanes and E. Brunet, *Helv. Chim. Acta*, 1997, **80**, 372.
15 E. Brunet, O. Juanes, R. Sedano and J. C. Rodriguez-Ubis, *Tetrahedron Lett.*, 2007, **48**, 1091.
16 J. C. Rodriguez-Ubis, R. Sedano, G. Barroso, O. Juanes and E. Brunet, *Helv. Chim. Acta*, 1997, **80**, 86.
17 A value of 1 400 $dm^3\ mol^{-1}cm^{-1}$ was reported by Latva *et al.* (ref. 19) but the nature and pH of aq. buffer for such measurement was not specified.
18 See photo-physical properties of compounds **9** and **12**, reported by Latva *et al.* (ref. 19).
19 M. Latva, H. Takalo, V.-M. Mukkala, C. Matachescu, J. C. Rodriguez-Ubis and J. Kankare, *J. Lumin.*, 1997, **75**, 149.
20 M. Darabantu, L. Boully, A. Turck and N. Plé, *Tetrahedron*, 2005, **61**, 2897.
21 W. K. Fife, *J. Org. Chem.*, 1983, **48**, 1375.
22 J. Hovinen and P. M. Guy, *Bioconjugate Chem.*, 2009, **20**, 404.
23 For the use of Stille cross-coupling reaction for the preparation of 4′-substituted 2,2′:6′,2″-terpyridines, see: R.-A. Fallahpour, *Synthesis*, 2003, 155.
24 D. Cai, D. L. Hughes and T. R. Verhoeven, *Tetrahedron Lett.*, 1996, **37**, 2537.
25 H. Hakala, P. Liitti, J. Peuralahti, J. Karvinen, V.-M. Mukkala and J. Hovinen, *J. Lumin.*, 2005, **113**, 17.
26 K. Nakamoto, *J. Phys. Chem.*, 1960, **64**, 1420.
27 A. T. R. Williams, S. A. Winfield and J. N. Miller, *Analyst*, 1983, **108**, 1067.
28 J. Olmsted, III, *J. Phys. Chem.*, 1979, **83**, 2581.
29 S. Poupart, C. Boudou, P. Peixoto, M. Massonneau, P.-Y. Renard and A. Romieu, *Org. Biomol. Chem.*, 2006, **4**, 4165; corrigendum; , *Org. Biomol. Chem.*, 2008, 6, p. 4669.
30 V. M. Mukkala, C. Sund, M. Kwiatkowski, P. Pasanen, M. Hogberg, J. Kankare and H. Takalo, *Helv. Chim. Acta*, 1992, **75**, 1621.
31 R. M. Supkowski and W. D. Horrocks, Jr., *Inorg. Chim. Acta*, 2002, **340**, 44.
32 W. D. Horrocks, Jr. and D. R. Sudnick, *J. Am. Chem. Soc.*, 1979, **101**, 334.
33 H. Karsilayan, I. Hemmilä, H. Takalo, A. Toivonen, K. Pettersson, T. Lövgren and V. M. Mukkala, *Bioconjugate Chem.*, 1997, **8**, 71.
34 Y. Gotoh, M. Tsukada and N. Minoura, *Bioconjugate Chem.*, 1993, **4**, 554.
35 G. I. Evan, G. K. Lewis, G. Ramsay and J. M. Bishop, *Mol. Cell. Biol.*, 1985, **5**, 3610.
36 (*a*) C. Chidley, K. Mosiewicz and K. Johnsson, *Bioconjugate Chem.*, 2008, **19**, 1753; (*b*) D. Maurel, J. Kniazeff, G. Mathis, E. Trinquet, J.-P. Pin and H. Ansanay, *Anal. Biochem.*, 2004, **329**, 253.
37 As reported by Perkin Elmer for LANCE® Eu-activated chelates (see http://las.perkinelmer.com/Content/manuals/man_ad0020.pdf), a F/P value of 4–10 chelates per protein (whose molecular weight is higher than 100 000) is a good labelling yield.
38 V. M. Mukkala, H. Takalo, P. Liitti and I. Hemmilä, *J. Alloys Compd.*, 1995, **225**, 507.