Aminopropargyl derivative of terpyridine-bis(methyl-enamine) tetraacetic acid chelate of europium (Eu (TMT)-AP_3): a new reagent for fluorescent labelling of proteins and peptides†

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The synthesis and photophysical properties of a new terpyridine-based europium(III) chelate (Eu (TMT)-AP_3) designed for peptide and protein labelling in aqueous solution phase is described. In order to obtain a stable, easy to handle, versatile and efficient labelling agent, a reactive aminopropargyl arm has been introduced onto the terpyridine moiety. As preliminary biochemical applications the chelate has been 1) efficiently covalently attached onto a representative biomolecule—monoclonal antibody—and 2) converted into iodoacetamido and aldehyde derivatives, and the photoluminescent Eu (TMT)-AP_3 was grafted onto cysteine and lysine amino acid residues respectively. These two different solution phase labelling methods yielded original fluorogenic FRET based probes suitable for "*in vitro*" detection of caspase-3 protease, a key mediator of apoptosis of mammalian cells.

Introduction

Lanthanide chelates, due to their unique luminescence properties (long decay-time, large Stokes' shift, narrow emission band and negligible concentration quenching), are gaining expanding applications in a wide variety of bioanalytical assays, in diagnostics, research, drug discovery, as sensing tools and in imaging.[1] These chelates contain an organic ligand that absorbs light (the "antenna"), a lanthanide ion that emits the light after energy transfer from the ligand to the metal ion and a reactive group for conjugation to target bioactive molecules (such as proteins, peptides, oligonucleotides,···) through selective reaction with their amino or mercapto groups. In addition to these features and with respect to energy-transfer processes and minimisation of non-radiative deactivation, the Ln(III) environment in a lanthanide-containing luminescent probe must also fulfil several other requirements: high thermodynamic stability, kinetic inertness, and a saturated coordination sphere.[2] Therefore, it is advantageous to resort to polydentate ligands for building a fitted coordination environment around Ln(III) ions. Among the numerous imaginative strategies developed to insert Ln(III) ions into functional edifices, those using functionalised terpyridine derivatives as polydentate ligands have received considerable attention[3] especially from the research groups of GE Healthcare (formerly Amersham-Biosciences) and Perkin Elmer Life and Analytical Sciences. Therefore, some chelates composed of a 4′-aryl-substituted 2,2′:6′,2″-terpyridine unit as the energy-absorbing and donating group, Eu(III), Sm(III) or Tb(III) as the emitting ions, methylenenitrilo (acetic acids) as the stable chelate-forming moieties, and iodoacetamido or isothiocyanato groups as the activated moieties for coupling to biomolecules are commercially available and routinely used in various types of time-resolved fluorometry (or quenching)-based assays (examples **1** and **2** in Scheme 1).[4–7] The synthetic strategies for the preparation of 2,2′:6′,2″-terpyridine ligands possessing functional groups directly attached to C-4′ are versatile and well established.[8] However, it is amazing to note that no general and efficient method for introducing an alkylamino group onto these functionalised terpyridine architectures has been reported to date. As the amino group is essential for activation and subsequent coupling of the Ln(III) chelate to biomolecules, several methods leading to the incorporation of a less reactive aminophenyl moiety have been developed.[6,9] They involve either the reduction of the aromatic nitro group of the corresponding 4′-aryl-substituted 2,2′:6′,2″-terpyridine or the cross-coupling of an (aminophenyl)acetylene derivative to the 4′-(4-bromophenyl)-2,2′:6′,2″-terpyridine ligand. As the aniline derivatives are often too unstable to be easily isolated in a pure form in good yields, these latter methods are not suitable for routine preparation of amino-terpyridines at a small scale (~1 mmol). Furthermore, the luminescence of the corresponding Eu(III) chelates is quite low because aromatic amines quench the excited state of the lanthanide ion by an electron transfer mechanism.[10] Their activation by acylation with a bifunctionnal reagent (iodoacetic anhydride or thiophosgene) leading to the thiol-specific iodoacetamido or amine-reactive isothiocyanato derivative, is essential to partially recover the luminescence.[6] Thus, especially, these chelates are not proper reagents for the labelling of proteins through traditional techniques involving the use of non-acylating bifunctionnal cross-linking reagents such as glutaraldehyde.[11]

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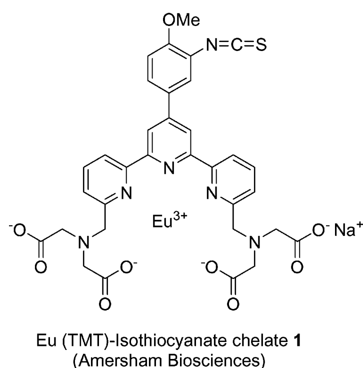
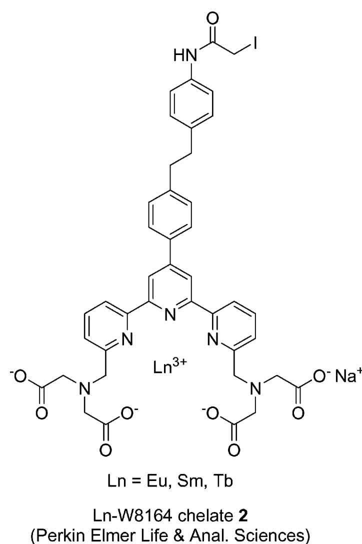
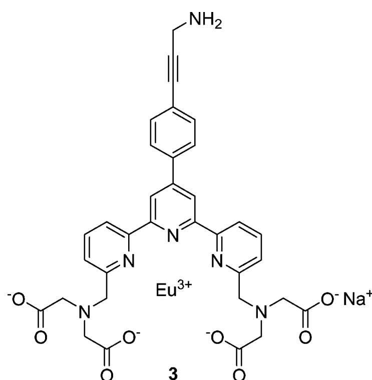




Scheme 1 Examples of commercially available Ln(III) chelates composed of a 4′-aryl-substituted 2,2′:6′,2″-terpyridine unit as the energy-absorbing and donating group.

With the goal in mind to develop a new label for biochemical applications, that displays good luminescence properties, is easy to prepare and to handle, and usable in a wide range of bioconjugate techniques involved in solution phase protein labelling, we have examined the chemical functionalisation of Ln(III) chelates derived from 4′-aryl-substituted 2,2′:6′,2″-terpyridine ligands, with an alkynylamino group (3-aminopropynyl or aminopropargyl, AP_3). Indeed, we thought that the structural features of this linker (small size, rigidity and presence of a reactive and stable primary alkylamino group) should be beneficial for the introduction of the luminescent label onto the target biopolymers without disrupting their original structure and properties. This AP_3 arm has been successfully used in the field of nucleic acids chemistry especially as a key component of fluorescent labelled nucleotides currently used in DNA sequencing.[12,13]

Here we report the first synthesis and luminescence properties of the aminopropargyl derivative of terpyridine-bis(methyl-enamine) tetraacetic acid europium chelate (Eu (TMT)-AP_3) **3**. The use of this chelate for solution phase labelling of peptides and proteins is illustrated by the preparation of two original FRET substrates suitable for a caspase-3 assay[14] and by its coupling to a monoclonal antibody.



Results and discussion

Synthesis of the AP_3-terpyridine ligand

Terpyridine ligand **9** was prepared in 3 steps from the already published 6,6″-bis(aminomethyl)-4′-(4-bromophenyl)-2,2′:6′,2″-terpyridine ligand **4** (Scheme 2).[15] The introduction of the four chelating carboxylic acid functions (by alkylation of the free aminomethyl groups) and the reactive AP_3 arm (by Pd(0)-catalyzed Sonogashira cross-coupling[16]) requires the use of reagents (bromoacetic acid and propargylamine) bearing a protected functional group (respectively CO_2H or NH_2). According to the nature of protecting groups used (acid- or base-labile), two different strategies have been tested.

We first examined the synthetic strategy which provides the full-protected ligand **6** whose reactive functions are masked with base-labile protecting groups (methyl ester for CO_2H and trifluoroacetyl for NH_2 respectively, path a in Scheme 2). Thus, bis(aminomethyl) terpyridine derivative **4** was carboxymethylated to the tetraester **5** by treatment with an excess of methyl bromoacetate in the presence of DIEA and potassium iodide as catalyst in dry DMF. Thereafter, reaction of **5** with *N*-propargyltrifluoroacetamide in the presence of Pd(II) and CuI in a mixture of triethylamine and DMF gave the desired ligand **6** in 49% yield. Finally, the removal of the protecting groups was achieved by treatment with 1 N sodium hydroxide in methanol. However, this treatment led to the complete decomposition of the ligand. Furthermore, HPLC analyses of the crude deprotection mixture have clearly shown the formation of numerous decomposing products which were not characterised. However, ^1H NMR analyses of the crude deprotected ligand have shown the loss of the signal (singlet around 3.70 ppm in D_2O) assigned to the methylene group of the AP_3 arm. This result suggests that the side-reaction(s) probably starts with proton abstraction from this carbon to give a carbanion which might decompose through different chemical pathways.

Consequently, we have explored another protection strategy (path b in Scheme 2). Indeed, we thought that the use of

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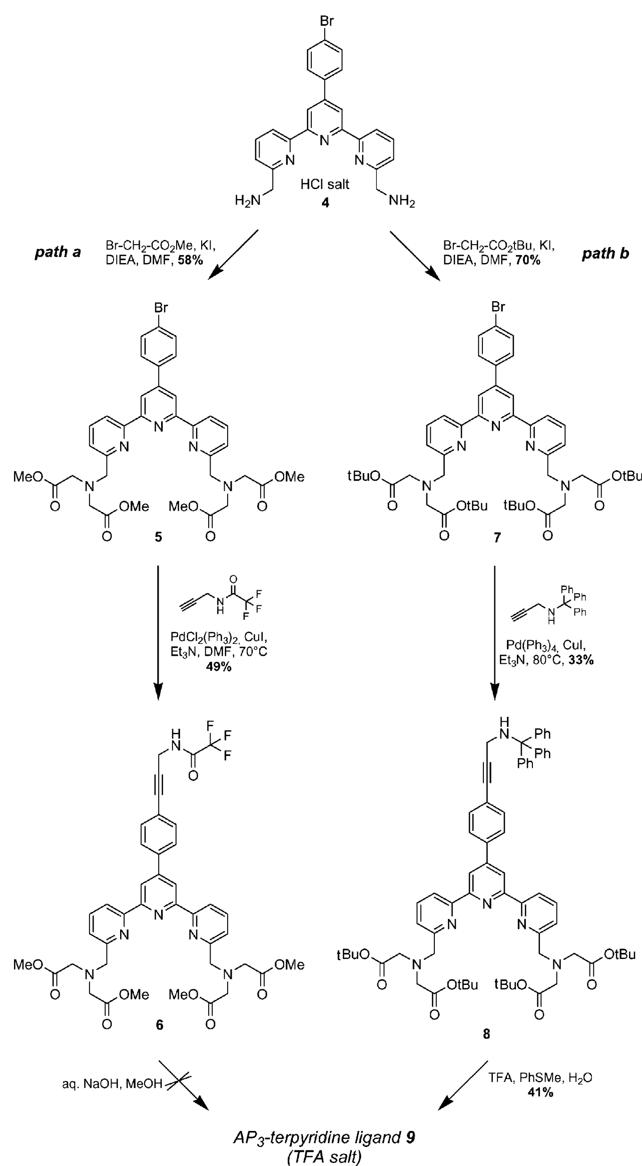
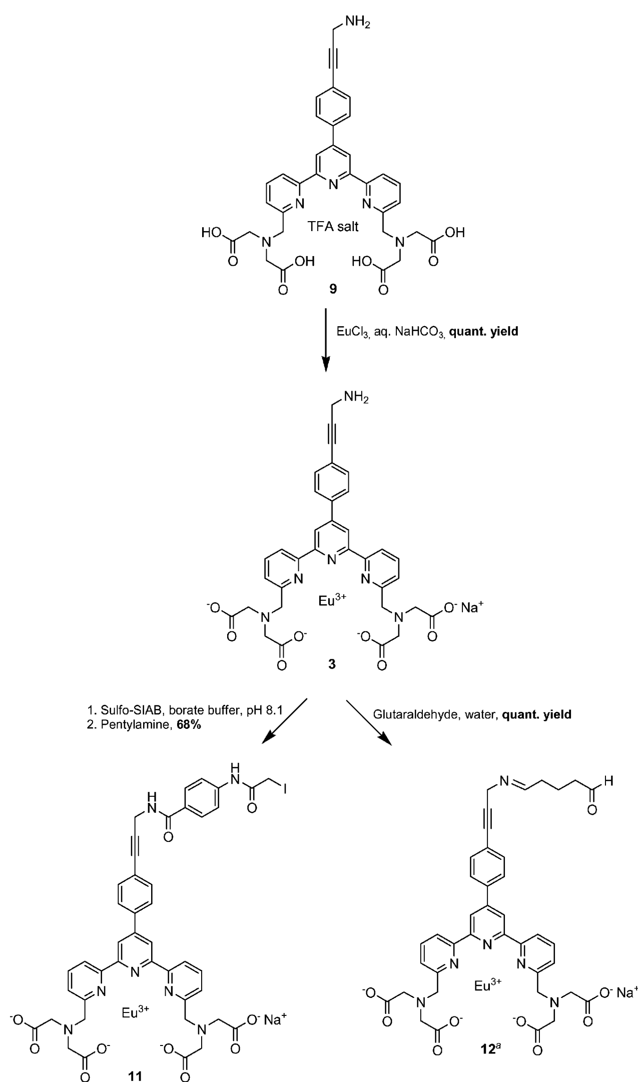


Scheme 2 Synthesis of the AP_3-terpyridine ligand **9**.

acid-labile protecting groups (*tert*-butyl ester for CO_2H and trityl for NH_2 respectively) which can be removed by treatment with trifluoroacetic acid and appropriate scavengers, was more suited to the stability of our AP_3-terpyridine ligand. With this goal in mind, the bis(aminomethyl) terpyridine derivative **4** was carboxymethylated to the tetraester **7** by treatment with an excess of *tert*-butyl bromoacetate as described above for the methyl ester. Thereafter, reaction of **7** with *N*-tritylpropargylamine in the presence of $Pd(Ph_3)_4$ and CuI in triethylamine gave the desired ligand in moderate yield. Unlike the Sonogashira cross-coupling reactions involving *N*-protected propargylamine and less sophisticated halogeno-aryl derivatives,[12] it is essential to use a significantly higher amount of Pd catalyst to get acceptable yields. That may be explained by partial inhibition of this catalyst through chelation of its metal centre within the terpyridine moiety. Furthermore, several attempts have clearly shown that more reproducible yields in **8** were obtained when $Pd(Ph_3)_4$ was used instead of $PdCl_2(Ph_3)_2$. Finally, the removal of the *tert*-butyl and trityl groups was achieved by treatment with a trifluoroacetic acid solution containing 5% of water and 5% of thioanisole. The crude AP_3-terpyridine ligand **9** was isolated by precipitation in Et_2O. However, further purification by flash-chromatography on a RP-C_{18} silica gel column was essential to remove hydrophobic side-products resulting from the reaction between thioanisole and *tert*-butyl or trityl cations. Thus, **9** was obtained in 41% yield and its structure was confirmed by detailed measurements, including ESI mass spectrometry and NMR analyses.

Synthesis and characterisation of the Eu (TMT)-AP_3 chelate

The AP_3-terpyridine ligand **9** was converted to the corresponding Eu(III) chelate **3** by treatment with aqueous europium(III) chloride (Scheme 3). Purification was achieved by flash-chromatography on a RP–C_{18} silica gel column and provided **3** in quantitative yield. The formation of this lanthanide chelate is clearly demonstrated by UV–visible spectrophotometric titration of **9** in water by following the variations in the UV–visible spectra of the ligand upon



Scheme 3 Synthesis of the Eu (TMT)-AP_3 chelate **3** and its corresponding iodoacetyl and aldehyde derivatives **11** and **12**.[a]mixture of reactive species (monomeric and polymeric forms).

addition of increasing amounts of $EuCl_3{\cdot}6H_2O$ salt. Addition of aliquots of the lanthanide solution immediately results in the displacement of the absorption maximum to lower energies, from 290 nm for the free ligand to 298 nm for 1 equiv. of added lanthanide salt. Upon further addition of europium the spectra remained unchanged. This result pointed to the formation of a single new absorbing species with a one metal to one ligand stoichiometry. In these conditions, the apparent association constant for the formation of the complex appears to be too high to be determined by a single titration curve. However, competitive binding experiments indicate that the TMT moiety readily displaces the Eu(III) ion from the EDTA–Eu complex[4] and so that the conditional stability constant of **3** could be estimated as superior to 10^{17}. ESI-MS spectra of **3** were recorded in either positive or negative modes, revealing in both cases the expected molecular peak (Fig. 1). Indeed, the positive integration mode displayed $[(L - 3H^+) + Na^+ + Eu^{3+}]^+$ as the major peaks with the expected isotopic pattern distribution (823.1 (85%) and 825.1 (100%)) and the spectrum recorded in the negative mode showed the presence of the $[(L - 4H^+) + Eu^{3+}]^-$ species at 799.2 and 801.2 m/z units (with 80% and 100% relative intensities, respectively).

Photophysical properties of the Eu (TMT)-AP$_3$ chelate

The excitation maxima, absorption coefficient (ε), emission maxima, mean metal luminescence lifetime (τ), quantum yield (Φ) and luminescence yield ($\varepsilon\Phi$) for the chelate **3** are presented in Table 1. Unlike Eu(III) chelates bearing a free aromatic NH_2 group,[6] **3** shows a strong metal luminescence in water solution upon excitation of the terpyridine ligand. As an example, the excitation and emission spectra of **3** are shown in Fig. 2. The emission spectrum presents the usual $^5D_0 \rightarrow {}^7F_J$ (J = 1–4) transitions typical of the Eu(III) ion. The luminescence quantum yield of this chelate was measured by a relative method using

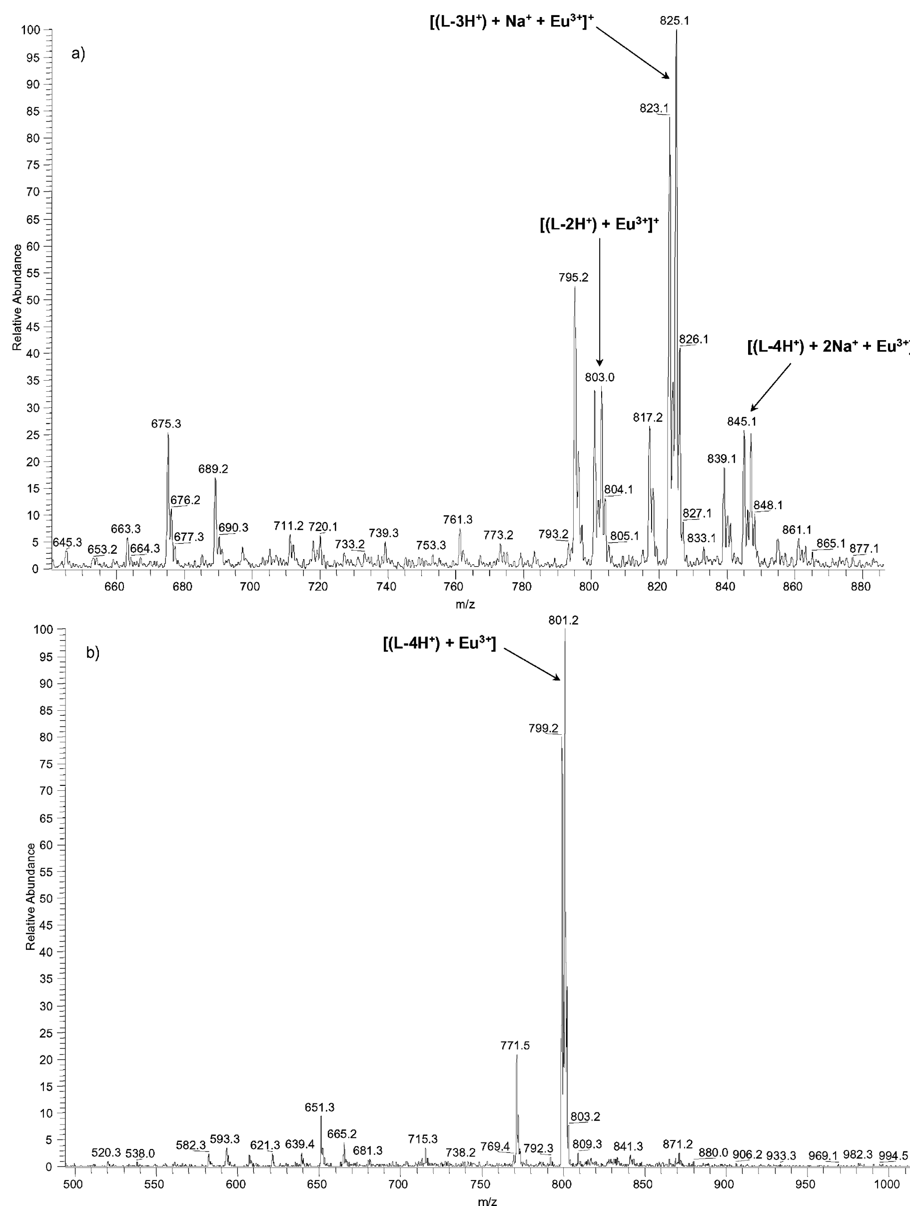


Fig. 1 ESI mass spectra of the Eu (TMT)-AP$_3$ chelate **3**. a) positive mode, b) negative mode, calcd mass for $C_{34}H_{28}N_6O_8Eu$ 800.60.

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Table 1 Photophysical properties of Eu (TMT)-AP_3 chelate **3** in deionised water at 25°C

λ_{max}, abs/nm	λ_{max}, em/nm[a]	ε/L mol^{-1} cm^{-1}	Φ[b]	$\varepsilon\Phi$[c]	τ/ms[d]
238, 298	616	25 000, 42 000	0.0064	210	1.41

[a] Only the most intense emission band corresponding to the $^5D_0 \rightarrow {}^7F_2$ transition is presented. [b] Determined by using $Ru(bpy)_3Cl_2$ as a standard ($\Phi = 0.028$, according to ref. 37). [c] ε value of **3** at $\lambda = 292$ nm (32 956 L mol^{-1} cm^{-1}) corresponding to the excitation wavelength selected for the quantum yield measurements was used for this calculation. [d] An aq. solution of **3** at a concentration of 11.0 10^{-6} mol L^{-1} was used. The measurement was also achieved in D_2O under the same conditions, $\tau(D_2O) = 2.28$ ms. As expected, the hydration number (q) obtained from the equation $q = A\,[\tau(H_2O)^{-1} - \tau(D_2O)^{-1} - 0.25]$ (according to ref. 39), where A = 5.0, was found to be close to zero ($q = 0.10$).

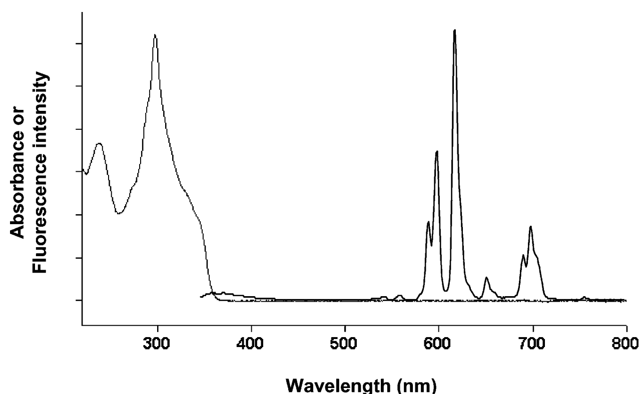
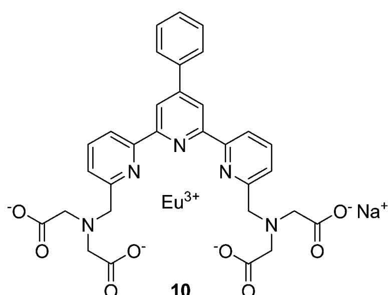


Fig. 2 Normalised absorption and emission spectra of **3** in deionised water at 25 °C.

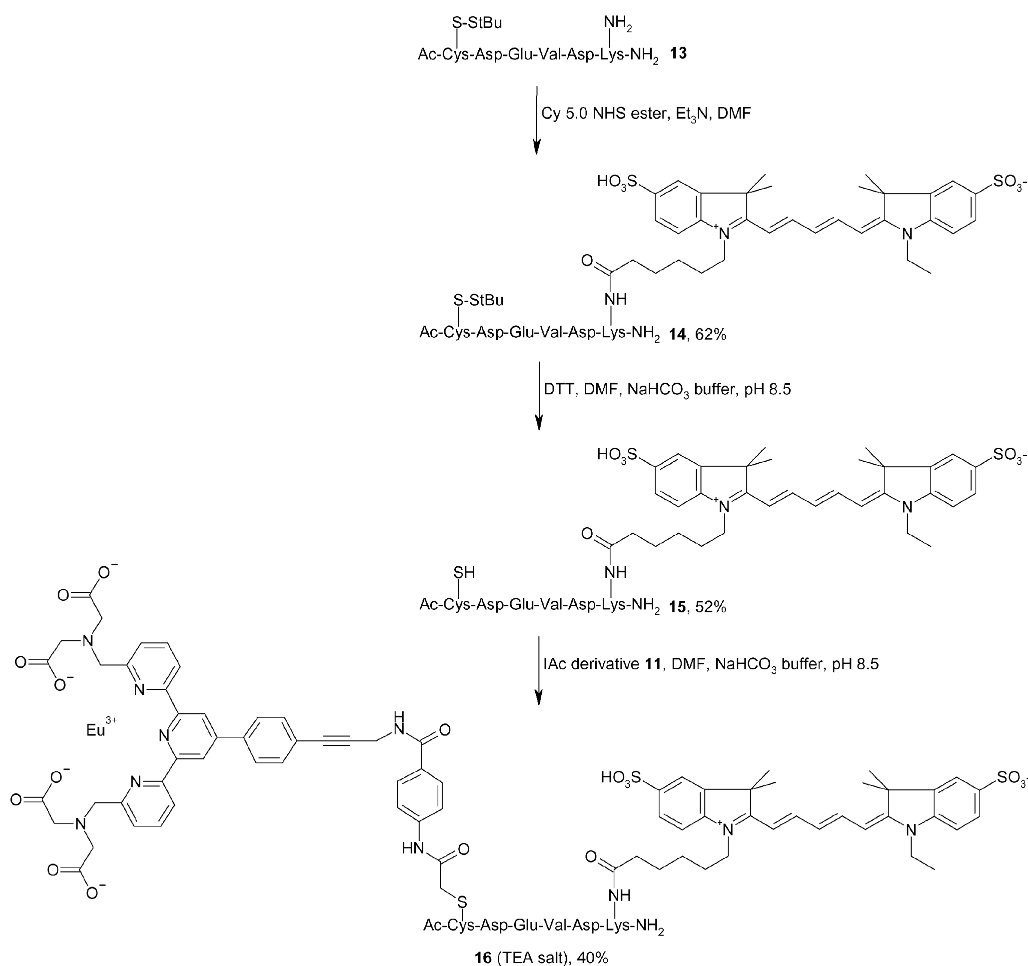
$Ru(bpy)_3Cl_2$ as a standard.[7] The luminescence yield of **3** was found to be comparable to values reported for some Ln(III) chelates derived from the parent compound **10** by substitution of its 4′-phenyl ring.[6] Therefore, these photophysical properties allowed us to consider some applications of **3** as labelling reagent of biopolymers.



Labelling peptides in solution with the Eu (TMT)-AP_3 chelate—application to the preparation of FRET substrates of caspase-3 protease

To demonstrate the utility of the Eu (TMT)-AP_3 chelate as a reagent for biopolymer derivatisation, **3** was converted to the corresponding iodoacetamido and aldehyde derivatives **11–12** (Scheme 3) and coupled to Cy 5.0 labelled hexapeptides bearing either a free cysteine or a lysine amino acid residue (Scheme 4 and 5). The use of non standard protecting groups removable under mild reducing conditions (S*t*-Bu for cysteine and Pydec[17] for lysine) has enabled the selective introduction of the two fluorescent labels at two different amino acid residues within the peptides, thus obtaining FRET substrates (Eu (TMT)-AP_3 chelate and Cy 5.0 dye act as donor and acceptor respectively) through original labelling methods. The target hexapeptides Ac-Cys(Eu (TMT)-AP_3)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 **16** and Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Eu (TMT)-AP_3)–NH_2 **21** contain the Asp-Glu-Val-Asp motif which is a specific substrate for caspase-3. The cleavage site is located after the aspartic acid residue at the C-terminal side. Caspases are Cysteine-ASpartic-acid-ProteASES that play a critical role as mediators for apoptotic cell death.[18] Caspase-3 has been specifically identified as being a key mediator of apoptosis of mammalian cells[19]: activation of caspase-3 indicates that the apoptotic pathway has progressed to an irreversible stage. There is thus a growing interest in identifying caspase inhibitors to minimise cell death in pathological conditions (neurodegenerative diseases for instance) but also for inducing caspase activation in cancer cells.[20] In addition, caspase-3 is widely used for monitoring apoptosis induction for general cytotoxicity screening. This interest for caspase-3 resulted in the development of several assays using a variety of formats and amenable to high throughput screening.[21] Peptides **13** and **17** have been chosen as suitable targets in order to get novel fluorogenic substrates useful for detecting apoptosis in whole cells and for cell-based high throughput screening assays for apoptosis inhibitors or inducers.[22]

Firstly, the iodoacetyl moiety was introduced on the AP_3 arm of **3** by acylation of its primary amino group with the commercially available heterobifunctional cross-linker sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (*i.e.*, Sulfo-SIAB) in borate buffer (pH 8.1). After completion of the reaction, a further derivatisation step of the unreacted Sulfo-SIAB reagent by treatment with a scavenging excess of pentylamine was achieved to facilitate the purification. Indeed, flash-chromatography on a RP-C_{18} silica gel column provided the thiol-reactive luminescent Ln(III) chelate **11** in a pure form (yield 68%). Its structure was confirmed by ESI mass spectrometry. Due to the poor stability of the couple of fluorophores (**3** and Cy 5.0) towards harsh acidic conditions currently used for the final deprotection of synthetic peptides, post-synthetic labelling of the peptidic backbone Ac-Cys-Asp-Glu-Val-Asp-Lys-NH_2 seems to be the best synthetic strategy to get the target caspase-3 substrate **16** in good yield and with a high degree of purity (Scheme 4). Thus, the crude peptide Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys-NH_2 **13** was firstly treated with the *N*-hydroxysuccinimidyl ester of Cy 5.0 dye in dry DMF in the presence of triethylamine. Purification by RP-HPLC provided the Cy 5.0 labelled peptide Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 **14** in 62% yield. It should be mentioned that trifluoroacetic acid was substituted by acetic acid (or formic acid) in the HPLC purification because prolonged exposure of Cy 5.0 dye to strong acid (such as 0.1% aq. trifluoroacetic acid solution) was detrimental for fluorescence (although little change in the absorption spectrum was observed).[23] The removal of the *tert*-butylthio protecting group from the cysteine residue was achieved by treatment with an excess of dithiothreitol (DTT) in a mixture of DMF and aq. sodium bicarbonate buffer (pH 8.5). Purification of the free sulfhydryl containing peptide–Cy 5.0 conjugate **15** was achieved by RP-HPLC. Finally, iodoacetamido activated Eu(III) chelate **11** was attached on the free cysteine of peptide **15** by using



Scheme 4 Preparation of the energy-transfer substrate of caspase-3 enzyme **16**.

a 2 : 1 molar excess (**11** : **15**) in sodium bicarbonate buffer (pH 8.5). The caspase-3 substrate **16** was purified by RP-HPLC by using a volatile aq. buffer (*i.e.*, triethylammonium bicarbonate (TEAB), 50 mM, pH 7.5) and acetonitrile as eluents to keep the integrity of the Eu(III) chelate. Due to the small differences in polarity between the peptides **15** and **16** and iodoacetamido derivative **11**, it is essential to use a slow linear gradient of increasing acetonitrile (0.4% min^{-1}) in aq. TEAB buffer to get peptide **16** in a pure form (yield 40%, purity >95%). Analysis of this fluorogenic substrate using MALDI-TOF mass spectrometry indicated the presence and integrity of Cy 5.0 dye and Eu(III) chelate within the peptide architecture (Fig. 3).

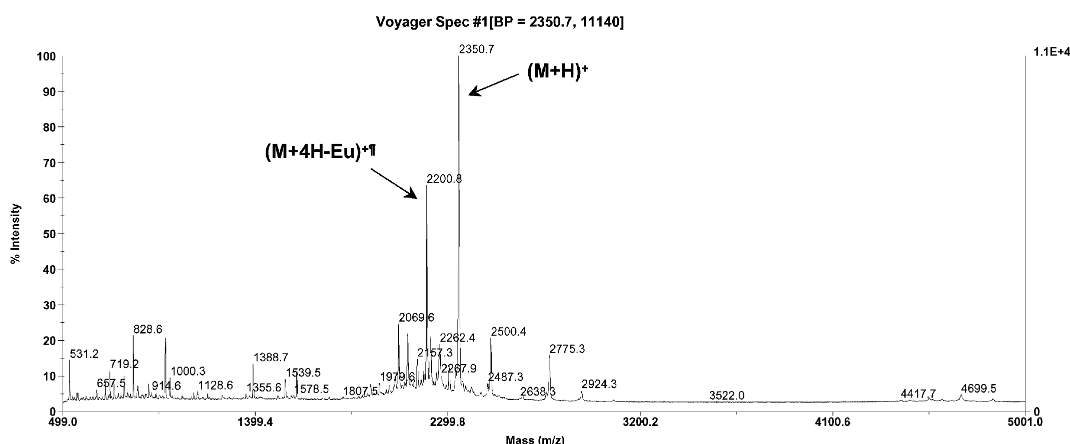


Fig. 3 MALDI-TOF mass spectrum of the fluorogenic substrate of caspase-3 protease **16**, in the positive mode, α-cyano-4-hydroxycinnamic acid was used as a matrix, $(M + H)^+$: m/z: calcd 2348.4, found 2350.7. ¶loss of Eu(III) occurred during the ionisation process.

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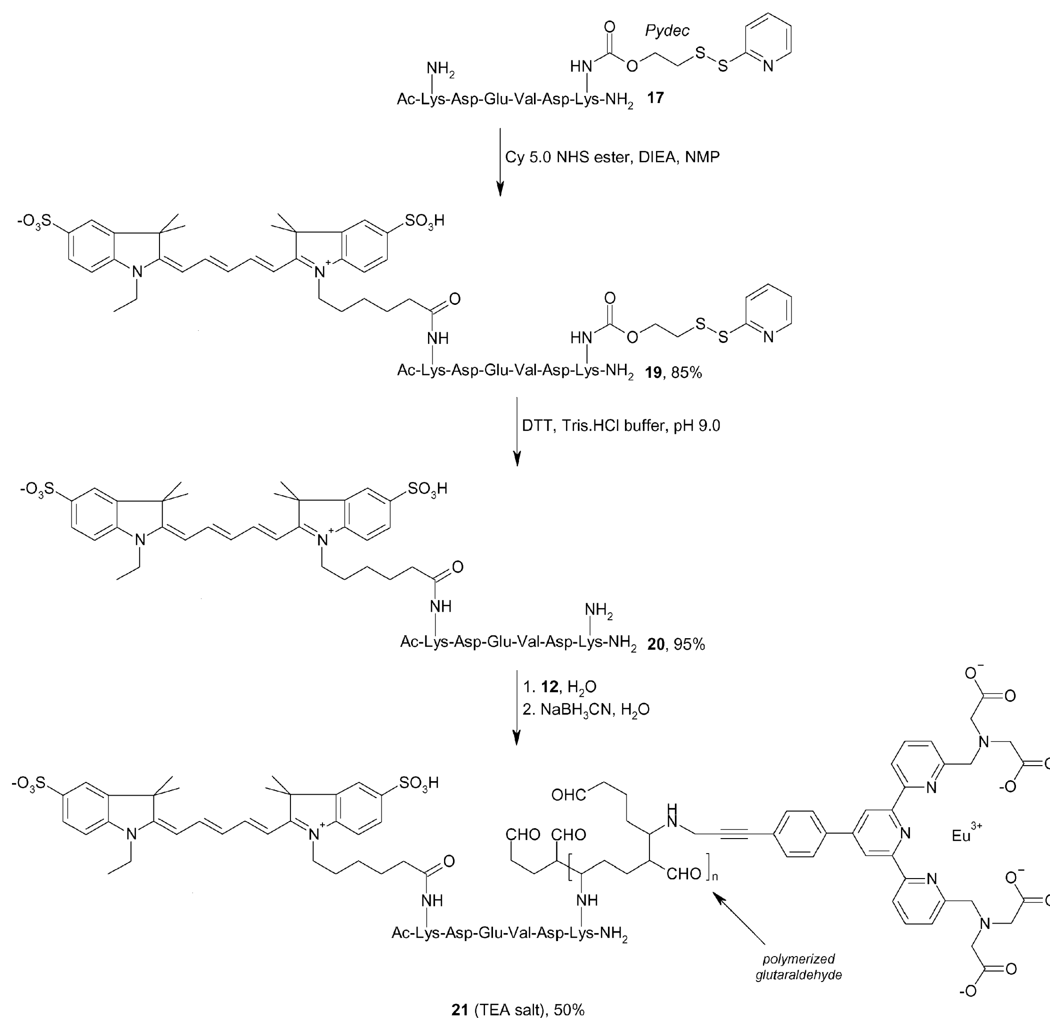
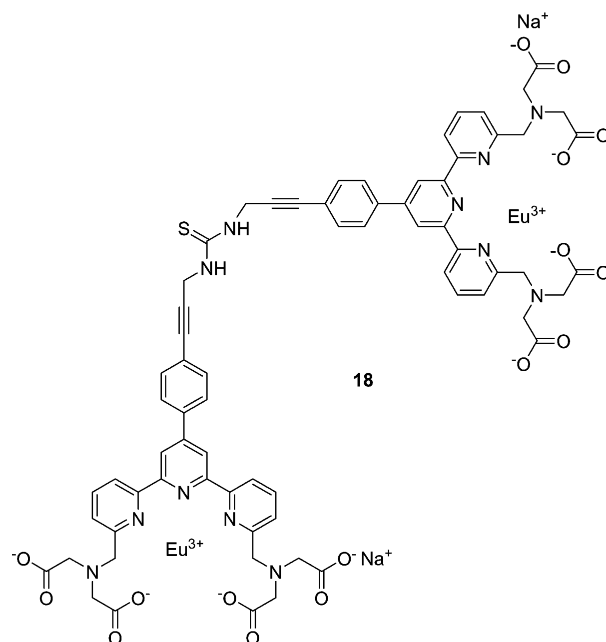


Scheme 5 Preparation of the energy-transfer substrate of caspase-3 enzyme **21**.

In addition to this first fluorescent labelling method, we have explored an alternative strategy for introducing the same FRET donor–quencher pair onto the two distinct lysine residues of hexapeptide **17** (Scheme 5). This original fluorescent labelling method involves the use of the Pydec moiety as protecting group of a lysine side chain. Indeed we recently designed this amine protecting group, fully compatible with the stability of biopolymers and fluorophores. It is of an easy synthetic access, and removable under mild reducing conditions. This protective group proved to be a powerful tool to efficiently differentiate two primary amino groups of otherwise similar reactivity.[17] The conversion of the Eu (TMT)-AP_3 chelate into an amine-reactive reagent is required for its coupling to a lysine residue. Chemical derivatisation of **3** with *N*,*N'*-disuccinimidyl carbonate (DSC) and thiophosgene was firstly explored. However, attempts to get the corresponding succinimidyl carbamate and isothiocyanato derivatives failed. Indeed, the stability of the DSC reagent was found to be poorly compatible with the neutral aq. conditions in which **3** is fairly soluble. As far as the reaction with thiophosgene is concerned, quantitative formation of the Eu(III) chelate dimer **18** bearing a thiourea linkage was observed (isolation by RP-HPLC and structure confirmed by ESI mass spectrometry), even under the experimental conditions currently used for the derivatisation



of aromatic amines with this reagent (slow addition of the aq. solution of **3** to a large excess of thiophosgene).

Since the dicarbonyl compounds such as glutaraldehyde are routinely used as protein cross-linking reagents,[24] we decided to modify the free amino group of **3** with this bis-aldehyde compound to form an activated derivative able to react with the free lysine residue (Scheme 3). The hexapeptide Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 **17**, synthesised by the solid-phase method as previously described,[17] was firstly treated with the *N*-hydroxysuccinimidyl ester of Cy 5.0 dye in dry NMP in the presence of DIEA. Purification by RP-HPLC provided the Cy 5.0 labelled peptide Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 **19** in 85% yield. The removal of the Pydec protecting group from the C-terminal side lysine residue was achieved by treatment with an excess of DTT (50 equiv.) in Tris HCl buffer (pH 9.0). Purification of the free amine containing peptide–Cy 5.0 conjugate **20** was achieved by RP-HPLC. Finally, the aldehyde derivative **12**, prepared immediately prior to use by treatment of Eu (TMT)-AP_3 chelate with a slight excess of glutaraldehyde (1.5 equiv.), was used to label the free amino group of the peptide **20** by using 2 : 1 molar excess (**12** : **20**) in deionised water. After reduction of the resulting Schiff base linkages with sodium cyanoborohydride, the caspase-3 substrate **21** was purified by RP-HPLC (yield 50%). On the RP-HPLC elution profile of this fluorogenic probe (see ESI†), a broad peak was observed, reflecting the presence of a mixture of dual-labelled peptides due to the propensity of glutaraldehyde to polymerise in aq. solution and to react with amines by several means such as aldol condensation or Michael-type addition.[25] Thus, it was not possible to get an easily interpretable ESI or MALDI-TOF mass spectrum of **21**. However, FRET measurements (see below) have confirmed the presence and integrity of the two fluorophores **3** and Cy 5.0 within the hexapeptide.

Photophysical characterisation and *in vitro* cleavage by caspase-3 of the FRET substrates

When we measured the fluorescence spectrum of the probe **16** (or **21**) after excitation of the donor Eu(III) chelate at 298 nm, a major emission at 672 nm corresponding to the Cy 5.0 fluorescence was observed (Fig. 4). The lack of the $^5D_0 \rightarrow {}^7F_2$ transition emission at 616 nm confirmed that the luminescence of the donor is fully transferred to the Cy 5.0 dye which shows a significant overlapping of its excitation spectrum with the emission spectrum of the used Eu (TMT)-AP_3 chelate. This Cy 5.0 emission occurred *via* the non-radiative transfer from the donor to the acceptor according to the theory of Förster.[26] The efficiency of the energy transfer obtained with this doubly labelled substrate is compatible with the use of this read-out for monitoring caspase-3 activity. Enzymatic action will result from de-quenching of donor luminescence upon separation of the two labels as a consequence of enzymatic peptide bond cleavage. As expected, incubation of peptide **16** with recombinant human caspase-3 has resulted in an increase of the fluorescence emission of Eu(III) chelate at 616 nm (Fig. 5). This de-quenching effect of the donor is concomitant to a decrease of Cy 5.0 acceptor emission at 672 nm due to the abolishment of FRET resulting from the protease cleavage. After a 1 h reaction time, the donor luminescence total recovery suggests that the peptide bond cleavage reaction induced by caspase-3 went to completion and is in agreement with an initial almost quantitative transfer efficiency. Furthermore, no non-specific cleavage of this probe was observed in a control reaction in which peptide **16** was incubated only with the caspase-3 buffer. Similar results were obtained when peptide **21** was submitted to the same enzymatic hydrolysis (see ESI†). These results confirmed that caspase-3 protease exhibits a high degree of substrate tolerance towards the amino derivative coupled to the C-terminal side of the Asp-Glu-Val-Asp motif. Indeed, a wide range of fluorophores such as coumarin,[27] phenoxazine[28] and rhodamine[29] derivatives have already been linked to this tetrapeptide through their aromatic amino groups to get flurorogenic probes suitable for cell-based apoptosis assays.

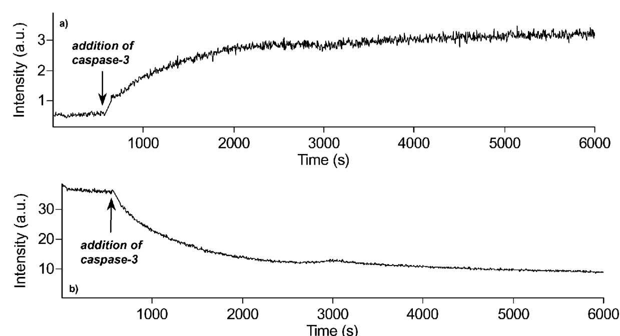


Fig. 5 Fluorescence emission time course (excitation at 298 nm) of peptide **16** (concentration 1 μM) with recombinant human caspase-3 (3.2 10^{-3} U, incubation time 3 h) in caspase assay buffer (100 mM NaCl, 40 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% (w/v) sucrose and 0.1% (w/v) CHAPS, pH 7.2, 37 °C) at 616 (a) and 672 nm (b).

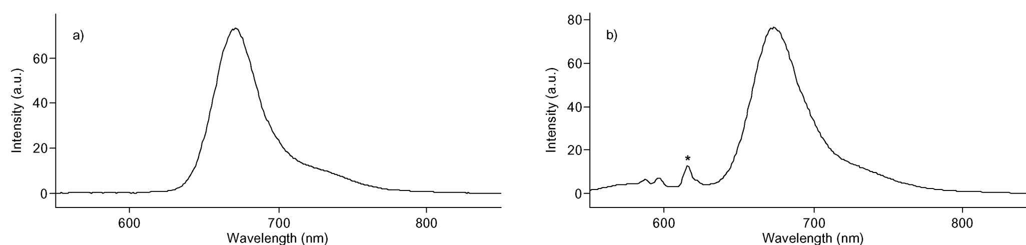


Fig. 4 Fluorescence emission spectra (excitation at 298 nm) of peptides **16** (a) and **21** (b) in caspase-3 buffer at 37 °C (concentration: 1 μM). *Residual emission at 616 nm is most likely due to an uncomplete energy transfer from the donor to the acceptor. As peptide **21** is a mixture of compounds with various content of polymerised glutaraldehyde, this results in Eu(III) labels which are more distant from the Cy 5.0 dye than the usual Förster distance.

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Antibody labelling using the Eu (TMT)-AP_3 chelate

As a further illustration of the applicability of the Eu (TMT)-AP_3 chelate synthesised for biomolecule derivatisation, **3** was used in the labelling of a monoclonal antibody (anti c-*myc* peptide monoclonal antibody, mAb), with the goal in mind to develop homogeneous TR-FRET titration assays (time-resolved fluorescent resonance energy transfer assay) for protein–protein interactions.[30] The reaction was easily performed under the same conditions described above for the introduction of **3** onto lysine residues within peptide architectures. This reaction proceeded smoothly and the protein conjugate was easily and completely purified by size-exclusion chromatography over a Sephadex® G-25 column. Protein quantification by UV spectroscopy (for IgG, 1 mg mL^{-1} has a mean absorbance at λ = 280 nm of 1.48) has enabled determination of a high labelling level of 5.8 Eu (TMT)-AP_3/mAb. The excitation and emission spectra of this protein conjugate are shown in Fig. 6. The main emission line of the chelate was observed as usual at around 616 nm. The average luminescence decay time was long (τ 1.03 ms) and comparable to those recently reported for protein conjugates of Eu(III) chelates derived from other 2,2′:6′,2″-terpyridine derivatives.[31] As far as the metal luminescence quantum yield of this protein conjugate is concerned, when measured for each lanthanide ion, it was found to be lower than that of the free Eu (TMT)-AP_3 chelate **3** (Φ 0.0038).

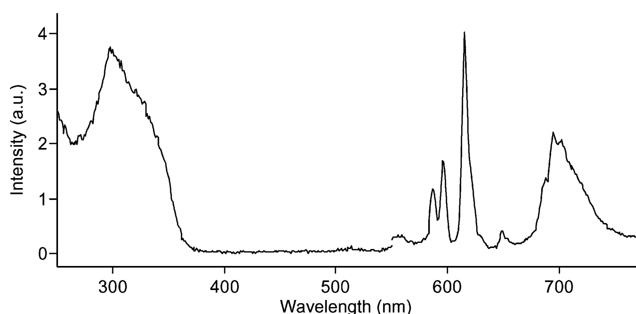

Fig. 6 Fluorescence emission and excitation spectra of the anti c-*myc* mAb labelled with the europium chelate **3** at 25 °C (concentration: 0.117 mg mL^{-1} in PBS buffer).

Conclusion

In this paper, we have described the first synthesis and photophysical properties of a luminescent Eu(III) chelate containing both the well-known nonadentate 6,6″-bis(aminomethyl)-4′-(phenyl)-2,2′:6′,2″-terpyridine ligand and an unusual aminopropargyl arm (AP_3 arm). The utility and the high reactivity of this latter linker are demonstrated through the preparation of iodoacetamido and aldehyde derivatives **11** and **12** which have been successfully used in solution-phase peptide labelling. Furthermore, the use of a non-standard thiol protecting group removable under mild reducing conditions for temporarily masking a lysine residue, has enabled the development of an efficient method for producing dual fluorescently-tagged peptides. We believe that this labelling strategy is complementary to the solid-phase methods[32] because the experimental conditions are compatible with a wide range of fluorophores showing poor or moderate chemical stability. In addition to the preparation of fluorogenic substrates of proteases, preliminary labelling experiments at the protein level have shown that it is possible to efficiently label antibodies with the Eu (TMT)-AP_3 chelate by means of dicarbonyl cross-linking reagents such as glutaraldehyde. As this latter protein conjugate does not completely maintain the photophysical properties (luminescence lifetime and quantum yield) of the free chelate, further efforts are in progress to improve the luminescence yield of **3** especially by introducing a further benzene ring between the AP_3 arm and the 4′-phenyl-2,2′:6′,2″-terpyridine moiety. Indeed, this approach has recently been used by Nishioka *et al.* to get efficient luminescent Eu(III) chelates for DNA labelling.[33]

Experimental

General

Column chromatographies were performed on silica gel (40–63 μm) from SdS. Reversed-phase column flash-chromatographies were performed on octadecyl-functionalised silica gel (mean pore size 60 Å) from Aldrich or Whatmann. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. Compounds were visualised by one of the two following methods (or both): (1) fluorescence quenching, (2) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol. MeOH was freshly distilled over $Mg(OMe)_2$ and stored over 4 Å molecular sieves. DMF was dried by distillation over BaO. DIEA and triethylamine were distilled from CaH_2 and stored over BaO. 6,6″-Bis(aminomethyl)-2,2′:6′,2″-terpyridine ligand **4** was prepared from 2-acetylpyridine and 4-bromobenzaldehyde by using the multi-steps synthetic procedure developed by Hovinen and Hakala.[15] *N*-Propargyltrifluoroacetamide, *N*-tritylpropargylamine and sulfo-cyanine dye Cy 5.0 were prepared by using literature procedures.[34] Hexapeptides Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys-NH_2 **13** and Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 **17** were synthesised on an automated peptide synthesizer (ABI 433A, Applied Biosystems) using the stepwise solid-phase synthesis method and Fmoc amino acids, as previously described.[17] $EuCl_3\cdot 6H_2O$ was purchased from Aldrich. *N*-Sulfosuccinimidyl[4-iodoacetyl]aminobenzoate (Sulfo-SIAB) was purchased from Pierce. Recombinant human caspase-3 enzyme (5.52 U mg^{-1}) was purchased from Sigma. The HPLC grade solvents (CH_3CN and MeOH) were obtained from Acros. Aqueous buffers for HPLC were prepared using water purified with a Milli-Q system. 1.0 M triethylammonium acetate (TEAA) and triethylammonium bicarbonate (TEAB) buffers were prepared from distilled triethylamine and glacial acetic acid or CO_2 gas. ^{1}H-, ^{13}C- and ^{19}F-NMR spectra were recorded on a Bruker DPX 300 (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from $CDCl_3$ (δ_H = 7.26, δ_C = 77.36) or D_2O (δ_H = 4.79).[35] J values are in Hz. ^{13}C substitution was determined with a JMOD pulse sequence, differentiating signals of methyl and methine carbons pointing "down" (−) from methylene and quaternary carbons pointing "up" (+). Infrared (IR) spectra were recorded as thin-film on sodium chloride plates or KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer with frequencies given in reciprocal centimeters (cm^{-1}). UV–visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence spectroscopic studies were performed in a semi-micro fluorescence cell (Hellma®, 104F-QS, 10 × 4 mm, 1400 μL) with a Varian Cary Eclipse

spectrophotometer. Mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source. The purified peptides were characterised by MALDI-TOF mass spectrometry on a Voyager DE PRO in the reflector mode with CHCA as a matrix. The syntheses of Eu(III) chelate dimer **18** and FRET substrate **21** are described in the ESI.†

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps. *System A*: RP-HPLC (Zorbax Eclipse XDB-C_8 column, 5 µm, 4.6 × 150 mm) with CH_3CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.0) as the eluents [100% TFA (5 min), linear gradient from 0 to 45% (30 min) and 45 to 90% (15 min) of CH_3CN] at a flow rate of 1 mL min^{-1}. UV detection was achieved at 285 nm. *System B*: System A with triethylammonium acetate (TEAA, 100 mM, pH 7.0) as aqueous buffer. *System C*: RP-HPLC (Waters Xterra MS C_{18} column, 5 µm, 7.8 × 100 mm) with CH_3CN and 0.1% aq. acetic acid (aq. AcOH, 0.1%, v/v, pH 3.8) as the eluents [95% AcOH (5 min), linear gradient from 5 to 65% (30 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. *System D*: System C with the following gradient [95% AcOH (5 min), linear gradient from 5 to 50% (30 min) of CH_3CN]. *System E*: RP-HPLC (Zorbax Eclipse XDB-C_8 column, 5 µm, 4.6 × 150 mm) with CH_3CN and aq. AcOH as the eluents [100% AcOH (5 min), linear gradient from 0 to 60% (30 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV–visible detection was achieved at 650 nm. *System F*: System C with the following gradient [100% AcOH (5 min), linear gradient from 0 to 70% (70 min) of CH_3CN]. *System G*: RP-HPLC (Waters Xterra MS C_{18} column, 5 µm, 7.8 × 100 mm) with CH_3CN and triethylammonium bicarbonate buffer (TEAB 50 mM, pH 7.5) as the eluents [100% TEAB (5 min), linear gradient from 0 to 10% (10 min) and 10 to 70% (120 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. *System H*: System G with the following gradient [100% TEAB (5 min), then linear gradient from 0 to 10% (10 min) and 10 to 60% (120 min)] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. *System I*: RP-HPLC (Zorbax Eclipse XDB-C_8 column, 5 µm, 4.6 × 150 mm) with CH_3CN and TEAB buffer (50 mM, pH 7.5) as the eluents [100% TEAB (5 min), linear gradient from 0 to 60% (30 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV detection was achieved at 260 nm.

Tetramethyl ester[15] 5

Compound **4** (0.7 g, 1.11 mmol) was suspended in dry DMF (5 mL). KI (0.205 g, 1.22 mmol) and DIEA (3.11 mL, 17.81 mmol) were sequentially added and the resulting mixture was de-aerated with argon. Methyl bromoacetate (0.85 mL, 8.90 mmol) was added and the resulting reaction mixture was stirred overnight under an argon atmosphere. The reaction was checked for completion by TLC (cyclohexane–ethyl acetate–Et_3N 45 : 45 : 10) and the mixture was evaporated to dryness. The residue was dissolved in dichloromethane (75 mL) and washed with sat. $NaHCO_3$ (1 × 50 mL) and brine (2 × 50 mL), dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (30 g, dry loading) with a step gradient of ethyl acetate (0–40%) in cyclohexane–triethylamine (9 : 1) as the mobile phase, giving 0.48 g (0.65 mmol) of compound **5** as an orange oil (yield 58%). R_f 0.78 (cyclohexane–ethyl acetate–Et_3N 45 : 45 : 10); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.61 (s, 2H), 8.47 (d, J = 7.9 Hz, 2H), 7.82–7.52 (m, 8H), 4.11 (s, 4H, –CH_2–N(CH_2–CO_2CH_3)$_2$), 3.65 (s, 8H, N–CH_2–CO_2CH_3), 3.63 (s, 12H, N–CH_2–CO_2CH_3).

Full-protected AP_3-terpyridine ligand 6

Compound **5** (0.46 g, 0.63 mmol) and *N*-propargyltrifluoroacetamide (0.14 g, 0.95 mmol) were dissolved in a mixture of dry DMF–triethylamine (8 : 2, 4.4 mL) and de-aerated with argon for 10 min. CuI (19 mg, 0.1 mmol) and $PdCl_2(PPh_3)_2$ (35 mg, 0.05 mmol) were added and the mixture was stirred overnight at 65–70 °C (oil bath temperature). After 5.5 h, further amounts of *N*-propargyltrifluoroacetamide (71 mg, 0.47 mmol), CuI (9.5 mg, 0.05 mmol), $PdCl_2(PPh_3)_2$ (17.5 mg, 0.025 mmol), DMF (1.75 mL) and Et_3N (0.46 mL) were added. The reaction was checked for completion by TLC (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10) and the mixture was evaporated to dryness. Purification by chromatography on a silica gel column (30 g, loading with dichloromethane) with a step gradient of ethyl acetate (0–100%) in cyclohexane–triethylamine (9 : 1) gave 0.25 g (0.31 mmol) of compound **6** as an orange oil (yield 49%). R_f 0.36 (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.63 (s, 2H), 8.48 (d, J = 7.9 Hz, 2H), 7.83–7.53 (m, 8H), 4.38 (d, J = 5.2 Hz, 2H, –C≡C–CH_2–$NHCOCF_3$), 4.12 (s, 4H, –CH_2–N(CH_2–CO_2CH_3)$_2$), 3.65 (s, 8H, N–CH_2–CO_2CH_3), 3.63 (s, 12H, N–CH_2–CO_2CH_3); ^{19}F-NMR (282 MHz, $CDCl_3$): δ −76.15 (s, 3F, $COCF_3$); MS (ESI+): m/z 806.20 $[M + H]^+$, 828.07 $[M + Na]^+$, calcd for $C_{40}H_{39}F_3N_6O_9$ 804.79.

Tetra(*tert*-butyl) ester[36] 7

Compound **4** (0.57 g, 0.91 mmol) was suspended in dry DMF (4 mL). KI (0.165 g, 1.0 mmol) and DIEA (2.53 ml, 14.5 mmol) were sequentially added and the resulting mixture was de-aerated with argon. *tert*-Butyl bromoacetate (1.1 mL, 7.25 mmol) was added and the resulting reaction mixture was stirred overnight under an argon atmosphere. The reaction was checked for completion by TLC (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10) and the mixture was evaporated to dryness. The residue was dissolved in dichloromethane (50 mL) and washed with aq. 5% $NaHCO_3$ (1 × 50 mL) and brine (1 × 50 mL), dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (20 g, dry loading) with a step gradient of ethyl acetate (0–20%) in cyclohexane–triethylamine (9 : 1) as the mobile phase, giving 0.58 g (0.64 mmol) of compound **7** as an orange oil (yield 70%). R_f 0.96 (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.62 (s, 2H), 8.47 (d, J = 7.1 Hz, 2H), 7.82–7.56 (m, 8H), 4.09 (s, 4H, –CH_2–N(CH_2–CO_2t-Bu)$_2$), 3.48 (s, 8H, N–CH_2–CO_2t-Bu), 1.39 (s, 36H, N–CH_2–CO_2tBu); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ 27.9 (12C), 55.5 (4C), 59.6 (2C), 80.8 (4C), 118.3 (2C), 119.4 (2C), 123.1 (2C), 128.7 (2C), 131.8 (2C), 137.2 (2C), 137.6 (2C), 148.5, 154.9, 155.9, 158.5 (2C), 170.4 (4C).

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Full-protected AP_3-terpyridine ligand 8

Compound **7** (0.165 g, 0.18 mmol) and *N*-tritylpropargylamine (65 mg, 0.22 mmol) were dissolved in dry triethylamine (1.6 mL) and de-aerated with argon for 10 min. CuI (1 mg, 5.2 μmol) and $Pd(Ph_3)_4$ (5 mg, 4.3 μmol) were sequentially added and the resulting mixture was stirred at 80 °C overnight under an argon atmosphere. The reaction was checked for completion by TLC (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10) and the mixture was evaporated to dryness. Purification by chromatography was made on a silica gel column (40 g, dry loading) with a step gradient of ethyl acetate (0–10%) in cyclohexane–triethylamine (9 : 1) as the mobile phase. 67 mg (0.60 mmol) of compound **8** were obtained as a yellow foam (yield 33%). R_f 0.71 (cyclohexane–ethyl acetate–Et_3N 55 : 35 : 10); IR (CH_2Cl_2): ν_{max} 755, 1143, 1218, 1260, 1368, 1581, 1736, 2978 cm^{-1}; ^1H-NMR (300 MHz, $CDCl_3$): δ 8.72 (s, 2H), 8.54 (d, $J = 7.1$ Hz, 2H), 7.89–7.81 (m, 4H), 7.71 (d, $J = 7.0$, 2H), 7.56–7.52 (m, 8H), 7.34–7.20 (m, 9H), 4.17 (s, 4H, –CH_2–N(CH_2–CO_2*t*-Bu)$_2$), 3.55 (s, 8H, N–CH_2–CO_2*t*-Bu), 3.24 (bd, $J = 4.2$ Hz, 2H, –C≡C–CH_2–NHTrt), 1.46 (s, 36H, N–CH_2–CO_2*tBu*); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ 27.9 (12C), 34.2 (1C), 55.5 (4C), 59.6 (2C), 70.8, 80.7 (4C), 118.3, 119.4, 123.0, 126.3, 126.8, 127.0, 127.6, 127.7, 128.4, 131.9, 137.2, 145.0, 148.8, 155.0, 155.8, 158.5, 170.4 (4C); MS (ESI+): *m/z* 1120.40 $[M + H]^+$, 1141.60 $[M + Na]^+$, calcd for $C_{69}H_{78}N_6O_8$ 1119.43.

AP_3-Terpyridine ligand 9

A mixture of TFA–$PhSCH_3$–H_2O (90 : 5 : 5, 3 mL) was cooled to 4 °C and added to compound **8** (0.158 g, 0.14 mmol). The reaction mixture was stirred for 5 h at room temperature. Thereafter, the mixture was evaporated to dryness and co-evaporated once with cyclohexane. The resulting oily residue was dissolved in TFA (1 mL) and precipitated with cold Et_2O (10 mL). The resulting brown solid was collected by centrifugation and purified by chromatography on a RP-C_{18} silica gel column (16 g) with a step gradient of MeOH (0–30%) in aq. TFA as the mobile phase. The product-containing fractions were lyophilised to give the compound **9** as a beige amorphous powder (57 mg, yield 41%). IR (KBr): ν_{max} 1395, 1586, 1632 (broad), 3078, 3401 (broad) cm^{-1}; ^1H-NMR (300 MHz, D_2O + 1 N NaOD): δ 8.16 (s, 2H), 8.05–7.97 (m, 4H), 7.86 (d, $J = 7.5$ Hz, 2H), 7.73 (d, $J = 7.1$ Hz, 2H), 7.53 (d, $J = 7.9$ Hz, 2H), 4.05 (s, 4H, –CH_2–N(CH_2–CO_2H)$_2$), 3.74 (s, 2H, –C≡C–CH_2–NH_2), 3.38 (s, 8H, N–CH_2–CO_2H); HPLC (system A): $t_R = 18.1$ min, purity >95%; UV–visible (recorded during the HPLC analysis): $\lambda_{max} = 255$, 288 nm; MS (ESI+): *m/z* 653.6 $[M + H]^+$, calcd for $C_{34}H_{32}N_6O_8$ 652.67; Anal.: found C 48.15; H 2.91; N 8.43. $C_{34}H_{32}N_6O_8 \cdot 3C_2HF_3O_2$ (TFA) requires C 48.30; H 3.55; N 8.45%.

Eu (TMT)-AP_3 chelate 3

Compound **9** (46.7 mg, 0.047 mmol) was introduced into a Reacti-Vial™ (Pierce, #13222) and dissolved in deionised water (1.16 mL). 290 μL of an aq. solution of $NaHCO_3$ (55.3 mg in 0.98 mL of deionised water) were added in order to adjust the pH at 6.5. 150 μL of an aq. solution of $EuCl_3 \cdot 6H_2O$ (48.3 mg in 0.39 mL of deionised water) were added and the reaction mixture was protected from light and stirred at room temperature for 2 h. After several min, further 50 μL of aq. $NaHCO_3$ were added to again reach pH 6.5. The reaction was checked for completion by UV–visible spectrophotometry. The mixture was dissolved with deionised water (~1 mL) and purified by chromatography on a RP-C_{18} silica gel column (8 g) with a step gradient of MeOH (0–16%) in water as the mobile phase. The product-containing fractions were lyophilised to give the compound **3** as a white amorphous powder (38.7 mg, quantitative yield). IR (KBr): ν_{max} 1404, 1602 (broad), 2926, 3435 (broad) cm^{-1}; HPLC (system B): $t_R = 17.5$ min, purity >95%; UV–visible (water): λ_{max} (ε) = 238 (25 000 L mol^{-1} cm^{-1}), 298 (42 000 L mol^{-1} cm^{-1}); MS (ESI+): *m/z* 801.0 and 803.0 $[M + H]^+$, 823.1 and 825.1 $[M + Na]^+$; MS (ESI−): *m/z* 799.2 and 801.2 $[M - H]^-$, calcd for $C_{34}H_{28}N_6O_8Eu$ 800.60.

Iodoacetamido derivative 11

Compound **3** (22.0 mg, 0.027 mmol) was introduced into a Reacti-Vial™ and dissolved in borate buffer (0.8 mL, 50 mM, pH 8.1). 200 μL of a solution of Sulfo-SIAB reagent in borate buffer (19.2 mg, 0.038 mmol) were added. The reaction mixture was protected from light and stirred at room temperature for 2 h. After 30 min, further amounts of borate buffer (1 mL) and 3 N NaOH (12 μl) were added to again reach pH ~8. The reaction was checked for completion by RP-HPLC (system B). Finally, the reaction mixture was quenched with a solution of pentylamine in borate buffer (19.1 μl in 2.0 mL), stirred for 30 min and purified by RP-C_{18} silica gel column (5 g) with a step gradient of acetonitrile (0–20%) in water. The product-containing fractions were lyophilised to give the compound **11** as a white amorphous powder (19.9 mg, yield 68%). HPLC (system B): $t_R = 25.5$ min, purity >95%; MS (ESI+): *m/z* 1132.0 and 1134.1 $[M + 2Na]^+$; MS (ESI−): *m/z* 1086.0 and 1087.9 $[M - H]^-$, calcd for $C_{43}H_{34}IN_7O_{10}Eu$ 1087.66.

Optical properties of Eu (TMT-AP_3) chelate 3

The absorption spectrum of **3** was recorded (220–800 nm) in deionised water (concentration: 3.0 μM) at 25 °C. Emission spectra were recorded (350–800 nm) under the same conditions (concentration: 11.0 μm) after excitation at 298 nm (excitation and emission slit = 5 nm). The luminescence quantum yield of **3** was measured in deionised water at 25 °C by a relative method using $Ru(bpy)_3Cl_2$ as a standard ($\Phi = 0.028$, according to ref. 37). Lifetime (average luminescence decay time) 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.

Synthesis and characterisation of fluorogenic caspase-3 substrate Ac-Cys(Eu (TMT)-AP_3)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 (16)

Preparation of Cy 5.0 carboxylic acid, succinimidyl ester. Cy 5.0 carboxylic acid (3.2 mg, 4.88 μmol) was introduced into a Reacti-Vial™ and dissolved in 85 μL of dry DMF. 40 μL of a solution of TSTU reagent in dry DMF (1.47 mg, 4.88 μmol) and 1.35 μL of Et_3N (9.88 μmol) were added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h.

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Synthesis of Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 (14). 5.79 mg of crude peptide Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys-NH_2 **13** (6.09 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in 220 μL of dry DMF and 0.83 μL of Et_3N (6.09 μmol) was added. After complete solubilisation by vortexing, the resulting solution was added to the crude reaction mixture containing the succinimidyl ester of Cy 5.0 dye. Thereafter, a further amount of Et_3N (2.7 μL, 19.52 μmol) was added and the reaction mixture was protected from light and stirred at room temperature overnight. The reaction was checked for completion by RP-HPLC (system C). Finally, the reaction mixture was quenched by dilution with 1 mL of water and 1mL of aq. AcOH 0.1% and purified by RP-HPLC (system D, 2 injections). The product-containing fractions were lyophilised to give the peptide–Cy 5.0 conjugate **14** as a blue amorphous powder. Quantification was achieved by UV–visible measurements at $\lambda_{max} = 650$ nm of the Cy 5.0 dye by using the ε value 250 000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification: 62%). HPLC (system D): $t_R = 24.2$ min; MS (MALDI-TOF, positive mode, CHCA matrix): *m/z* 1476.5 $[M + H]^+$; MS (MALDI-TOF, negative mode, CHCA matrix): *m/z* 1473.4 $[M - H]^-$, calcd exact mass for $C_{66}H_{94}N_{10}O_{20}S_4$ 1475.80.

Removal of *tert*-butylthio group. Peptide Ac-Cys(S*t*-Bu)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 **14** (4.5 mg, 3.05 μmol) was introduced into a Reacti-Vial™ and dissolved in 110 μL of DMF. 340 μL of a solution of DTT (10.5 mg in 380 μL, 60.9 μmol) in sodium bicarbonate buffer (0.1 M, pH 8.5) were added. The reaction mixture was protected from light and stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system E). Finally, the reaction mixture was quenched by dilution with aq. AcOH 0.1% (2 mL) and purified by RP-HPLC (system F, 2 injections). The product-containing fractions were lyophilised to give the free sulfhydryl containing peptide–Cy 5.0 conjugate **15** as a blue amorphous powder. Quantification was achieved by UV–visible measurements at $\lambda_{max} = 650$ nm of the Cy 5.0 dye by using the ε value 250 000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification: 52%). HPLC (system E): $t_R = 22.5$ min; HPLC (system F): $t_R = 29.3$ min.

Synthesis of Ac-Cys(Eu (TMT)-AP_3)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 (16). Peptide Ac-Cys-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 **15** (2.22 mg, 1.6 μmol) was introduced into a Reacti-Vial™ and dissolved in 100 μL of sodium bicarbonate buffer (0.1 M, pH 8.5). 260 μL of a solution of iodoacetyl derivative **11** (3.54 mg, 3.25 μmol) in sodium bicarbonate buffer (0.1 M and 4% (v/v) DMF, pH 8.5) were added. The reaction mixture was protected from light and stirred at room temperature for 4 h. The reaction was checked for completion by RP-HPLC (system G). Finally, the reaction mixture was quenched by dilution with TEAB buffer (2.65 mL) and purified by RP-HPLC (system H, 3 injections). The product-containing fractions were twice lyophilised to give the peptide Ac-Cys(Eu (TMT)-AP_3)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH_2 **16** as a blue amorphous powder. A stock solution of fluorogenic caspase-3 substrate **16** was prepared in HPLC grade water and UV–visible quantification was achieved at λ_{max} of the Cy 5.0 dye by using the ε value 250 000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification: 40%). HPLC (system G): $t_R = 40.8$ min; HPLC (system H): $t_R = 43.1$ min; HPLC (system I): $t_R = 19.0$ min, purity >95%; UV–visible (water): λ_{max} (ε) = 297, 652 (250 000 L mol^{-1} cm^{-1}); MS (MALDI-TOF, positive mode, CHCA matrix): *m/z* 2350.7 $[M + H]^+$; MS (MALDI-TOF, negative mode, CHCA matrix): *m/z* 2346.9 $[M - H]^-$, 2771.2 $[M - 6H + Na + 4HTEA]^-$, calcd exact mass for $C_{105}H_{115}N_{17}O_{30}S_3Eu$ 2348.38.

in vitro peptide cleavage by recombinant human caspase-3

A 1.0 μM solution of peptide **16** (or **21**) was prepared in 50 μL of caspase-3 buffer (100 mM NaCl, 40 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% (w/v) sucrose and 0.1% (w/v) CHAPS, pH 7.2) and transferred into an ultra-micro fluorescence cell (Hellma®, 105.51-QS, 3 × 3 mm, 45 μL). 2 μL of human recombinant caspase-3 (3.2 10^{-3} U) were added and the resulting mixture was incubated at 37 °C. After excitation at 298, 308, 336 and 653 nm (excitation slit = 5 nm), fluorescence at 616 and 672 nm (excitation slit = 5 nm) was monitored over time with measurements recorded every 5 s.

Antibody labelling using the Eu (TMT)-AP_3 chelate

7 μL of an aq. solution (0.81 mg in 250 μL of deionised water) of chelate **3** (23.3 nmol) was added to 500 μL of an aq. solution of anti c-*myc* 9E10 mAb[38] (0.7 mg mL^{-1}) in phosphate buffer pH 6.5. The mixture was protected from light and stored for 2 h at 4 °C. Thereafter, 0.9 μL of glutaraldehyde (50% in water) were added. The resulting mixture was stored at 4 °C and periodically vortexed every 30 min over 4 h. Thereafter, $NaBH_4$ (2.0 mg) was added. The mixture was again stored at 4 °C and periodically vortexed every 15 min over 1 h. Finally, the mixture was purified on a Sephadex® G-25 column (7 mL of gel, elution with phosphate buffered saline (0.01 M phosphate buffer, 0.015 M NaCl, pH 7.4)). The labelling level was determined by UV–visible spectroscopy. The UV absorbance at $\lambda = 325$ nm ($\varepsilon = 22\,750$ L mol^{-1} cm^{-1} for **3** and no significant absorbance for mAb) enabled to determine the concentration of Eu (TMT)-AP_3 chelate within the protein conjugate. At $\lambda = 280$ nm, it was possible to determine the mAb concentration after subtraction of the residual UV absorbance of **3** (ε 17 100 L mol^{-1} cm^{-1}) by using the following relation: [mAb] = Abs ($\lambda = 280$ nm)/1.48 and 150 000 Da as the average molecular weight of mAb. The luminescence quantum yield of this conjugate was measured in PBS buffer at 25 °C by a relative method using Eu (TMT-AP_3) chelate **3** as a standard.

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