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Synthesis of a coumarin-based europium complex for bioanalyte labeling

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Abstract—A coumarin-based europium chelate ready-to-use for analyte labeling and homogeneous time-resolved fluorescence measurements has been designed. Compound **1** displays three functional elements: an azide reactive spacer arm, a coumarin sensitizer, and a seven-coordinate europium complex. That complex can be excited at 370 nm by inexpensive UV-LEDs as a light excitation source.

Accurate, fast, affordable analysis of biological samples is of prime interest for medicine and research. Currently, most often sample preparation procedures involve handling steps prone to introducing artifacts, and analysis methods commonly require skilled technicians and expensive equipment. The recent developments in micro fluidics and miniaturized lab-on-a-chip-like devices are extremely attractive for biomedical analysis and point-of-care testing (POCT) has revolutionized the continuum of patient care process.[1,2] However, monitoring of non-treated biological samples is still challenging in most of the cases as well as assay sensitivity. An improvement of the latter thus results in reduced sampling (i.e., blood collection, bone marrow aspiration...) and consequently in enhanced comfort of the patient. Homogeneous proximity-based immunoassay techniques are good candidates for monitoring non-treated biological samples. Early setup involving radioactive labels (scintillation proximity assay[3,4]) has increasingly been replaced with fluorescent labels, mostly because of safety issues.[5] In these techniques, the signal of the labeled component is modulated by binding and no laborious separation steps are needed. Consequently such assays are faster and easier to perform than heterogeneous assays, for example, dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA)[6] or enzyme-linked immunosorbent assay (ELISA).[7] An additional improvement is met with techniques based on time-resolved fluorescence that allow to get free from short-lived intrinsic fluorescence of biological samples (in the ns time range). Assays based on homogeneous time-resolved fluorescence (HTRF) all involve luminescent lanthanide chelates.[8–10]

Lanthanide ions, especially Eu^{III} and Tb^{III}, display luminescence lifetimes greater than 0.1 ms in aqueous solution under ambient conditions.[11] Such long decay times result from forbidden transitions involving 4f orbitals and, as a result, the molar absorption coefficients are very low (less than $10\ M^{-1}\ cm^{-1}$ in aqueous media).[12] Because of their weak absorption, lanthanides are usually not directly excited and sensitizers (antennas) are required to significantly populate the lanthanide excited vibrational states. UV/vis light energy is collected by allowed antenna-centered absorptions, followed by non-radiative intramolecular energy transfer from antenna excited states to Ln^{III}, resulting in a radiative metal-centered luminescence.[13,14] Under these conditions, luminescent Ln^{III} complexes have found many useful applications especially in the field of bioanalytical chemistry.[9,10] Recently there has been a growing interest in the use of inexpensive light-emitting diodes (LEDs) as a light source in place of expensive and cumbersome lasers, for both steady-state and time-resolved fluorescence spectroscopy.[15] However while high performance LEDs are now available to wavelengths down to 360 nm, a rapid fall-off in their quantum efficiency occurs toward shorter wavelengths.[16]

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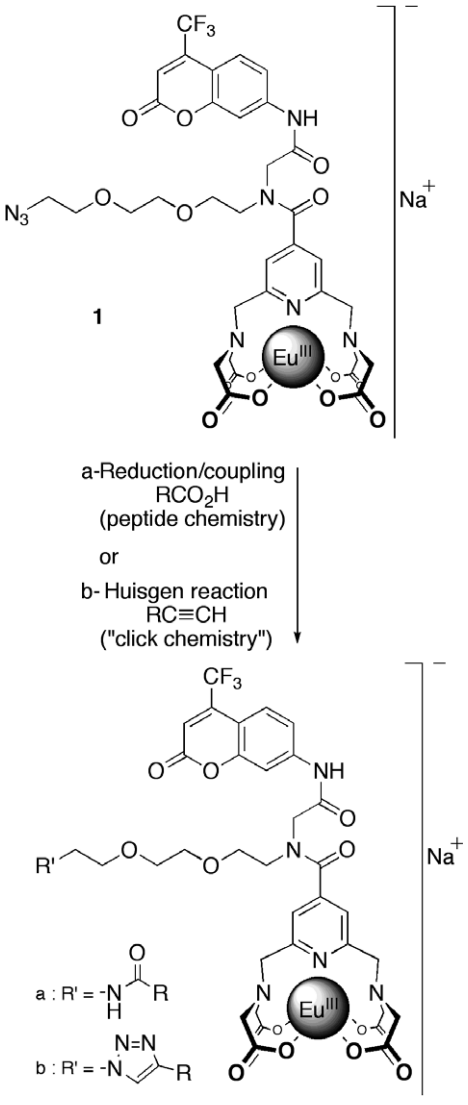
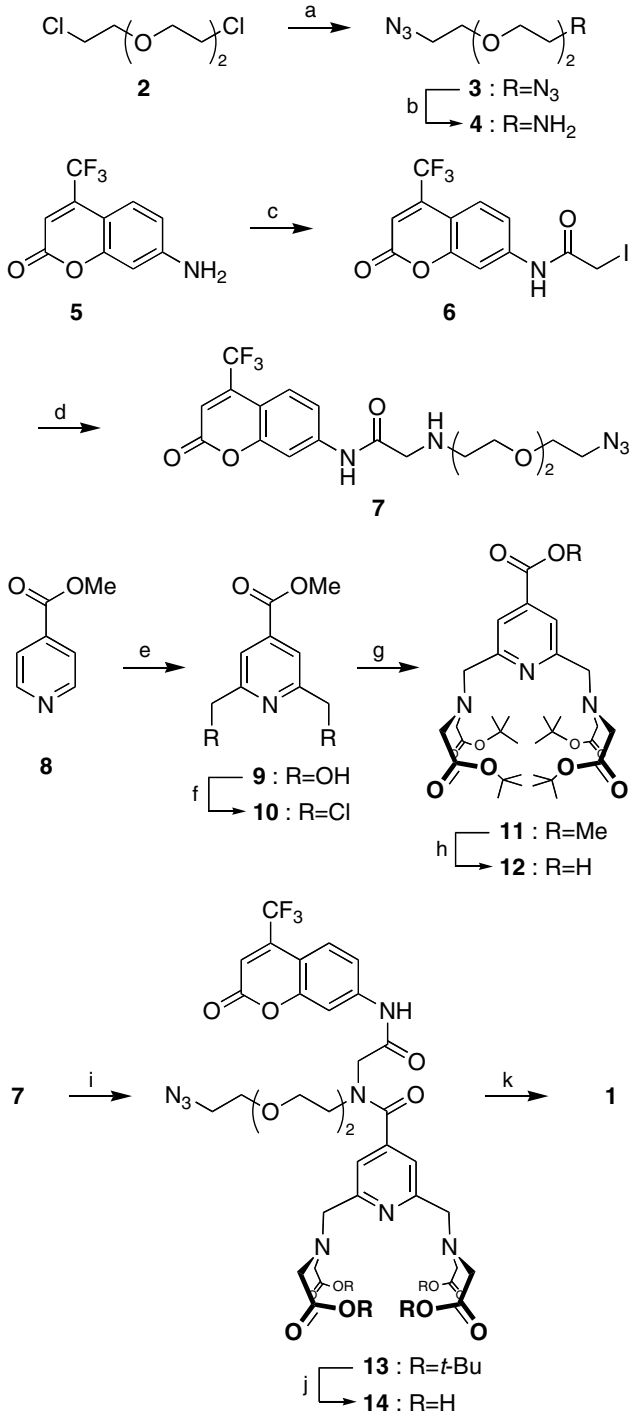
In the course of our research program focused on the design of POCT devices for molecular diagnostics based on HTRF measurements, we aimed to develop ready-to-use specific probes to be excited at 360–370 nm, for bioconjugation and labeling (Scheme 1). The target compounds display 3 functional elements: (i) a chemically reactive spacer for conjugation to the bioanalyte (or competitor), (ii) a coumarin antenna for proper excitation of the lanthanide cation through non-radiative intramolecular energy transfer, and (iii) a lanthanide chelate moiety for HTRF detection. Methods for bioconjugation have been intensively investigated over the past 2 decades.[17] Coupling reactions between carboxylic acids and amines (peptide chemistry) most often satisfactorily fulfill requirements for successful labeling experiment. Recently, new bioconjugation methods have appeared involving the 1,3-dipolar cycloaddition reaction of azides and alkynes (Huisgen reaction), and are getting more and more popular under the 'click chemistry' designation.[18–21] Applying that coupling strategy involves prior introduction of an azide or alkyne functionality onto the bioanalyte (or competitor) when missing in the initial structure. Consequently, we designed the spacer arm in target structure **1** to be hydrophilic, eliminating the solubility issue, and terminated with an azide functionality. That group can be further reduced in situ (i.e., by Staudinger reaction in the presence of a water-soluble phosphine) prior to coupling with acid compound, or directly involved in click chemistry. The antenna has been selected so that excitation can be achieved at 360–370 nm. 7-Amino-4-(trifluoromethyl)coumarin **5** does absorb in the blue and UV spectral region and thus perfectly suits to the selected LED excitation source.[22] Furthermore, coumarin emission at ca. 490 nm in aqueous media is compatible with Eu^{III} sensitization through a non-radiative energy transfer process.[15] Connection of the coumarin antenna to the metal chelate moiety and the spacer arm can be achieved through acylation of the amino group without affecting its spectroscopic properties. Finally, the choice of the chelating motif is crucial as the complex has to be stable in biological media which are glutted with potential metal chelating competitors (i.e., phosphoesters, proteins, urea…). Indeed, partial europium ligand displacement would invariably result in metal ion leaching out of the complex with subsequent decrease of lanthanide fluorescence as Eu^{III} is not in the close vicinity of the coumarin sensitizer any more. Examining the abundant literature dedicated to lanthanide complexes (for recent reviews, see[9,10,23,24]), we selected the *N*,*N*′-[2,6-pyridinediyl bis(methylene)]bis[*N*-(carboxymethyl) glycine] core that should provide a stable chelate in competitive biological media.[25,26]



Scheme 1. Design of the coumarin-based europium probes.

The synthesis of compound **1** was realized using a convergent approach as described in Scheme 2. The spacer arm was prepared in 2 steps starting from commercially available bis-1,2-(2-chloroethoxy)ethane **2**.

Quantitative conversion into bis-1,2-(2-azidoethoxy) ethane **3** and monoreduction using a Staudinger reaction under biphasic conditions afforded the heterobifunctional azido amine **4**. The antenna was elaborated starting from 7-amino-4-(trifluoromethyl) coumarin **5**[27] that was acylated with bromoacetyl bromide. The resulting intermediate bromoacetamide was converted into corresponding iodide **6** under standard conditions. Alkylation of primary amine **4** with iodoacetamide **6** led to compound **7**. The metal chelating group was prepared starting from methyl isonicotinate **8**. Double addition of hydroxymethyl radical[28,29] onto protonated nicotinate yielded bis-2,6-hydroxymethyl compound **9** in 51% yield. Methanesulfonylation in standard conditions produced a complex mixture of methanesulfonylated and chlorinated products. Conducting the reaction in refluxing dichloromethane allowed quantitative transformation of **9** into bis-chloride **10**. The latter compound treated with nitrilodiacetic acid di-*tert*-butyl ester[30] quantitatively afforded pentaester **11** that was regioselectively hydrolyzed using lithium hydroxide to yield carboxylic acid **12**. Amide coupling between amino compound **7** and acid **12** produced fully protected probe precursor **13**. Subsequent treatment with trifluoroacetic acid yielded tetra-acid **14**.[31] which was finally converted into lanthanide chelate **1**. upon addition of europium chloride hexahydrate.[32]



Scheme 2. Synthesis of the europium chelate **1**. Reagents and conditions: (a) NaN_3 (3 equiv), CH_3CN, NaI cat., △, 48 h, 99%; (b) PPh_3 (1 equiv), aq 1 N HCl, Et_2O/THF (9:1 v/v), rt, 2 h, 77%; (c) 1-$BrCH_2COBr$ (1.1 equiv), Et_3N (1.1 equiv), CH_2Cl_2, 0 °C, 3 h. 2-NaI (1.5 equiv), acetone, △, 2 h, 98% (2 steps); (d) **4** (3.0 equiv), CH_3CN, △, 2 h, 65%; (e) $(NH_4)_2S_2O_8$ (10 equiv), H_2SO_4 cat., MeOH, △, 6 h, 51%; (f) MsCl (2.1 equiv), Et_3N (2.0 equiv), CH_2Cl_2, △, 15 h, 99%; (g) $HN(CH_2CO_2$-*t*-$Bu)_2$ (2.5 equiv), Na_2CO_3 (2.5 equiv), NaI cat., CH_3CN, Δ, 15 h, 99%; (h) LiOH (1.1 equiv), H_2O/THF (1:6 v/v), rt, 8 h, 89%; (i) **12** (1.0 equiv), *i*-Pr_2NEt (3.0 equiv), BOP (1.6 equiv), CH_2Cl_2, rt, 16 h, 50%; (j) $CHCl_3$/TFA (8:2 v/v), rt, 20 h, 99%; (k) $EuCl_3 6\,H_2O$ (1.0 equiv), MeOH, pH 7 (NaOH 1 N), rt, 1 h, 99%.

Europium chelate **1** is soluble in water at physiological pH (pH 7.4–7.8) and exhibits strong absorbance at 360–370 nm (Fig. 1). Excitation in the range of coumarin absorption band of **1** causes the well-known, structured emission of the Eu^{3+} ion (ca. 596, 616, and 699 nm), showing that energy transfer from ligand-centered to metal-centered levels does take place. Fluorescence properties of compound **1** appeared stable in pure water, in phosphate-buffered saline (PBS), and in human plasma (Fig. 2). No decrease in the characteristic europium fluorescence intensity was observed over 30 min incubation periods in these media. Incubation of complex **1** in the presence of fluoride ions did not increase fluorescence intensity suggesting that the seven coordinate complex with the metal ion provides thermodynamic stability and minimal fluorescence quenching by water.[13,14]

As a first example of application for compound **1**, coupling with an AZT derivative has been achieved to offer an alternative tracer for enzyme immunoassay of the antiviral nucleoside.[33] Thus, azido compound **1** was reduced into primary amine **15** using tris(2-carboxy-eth-

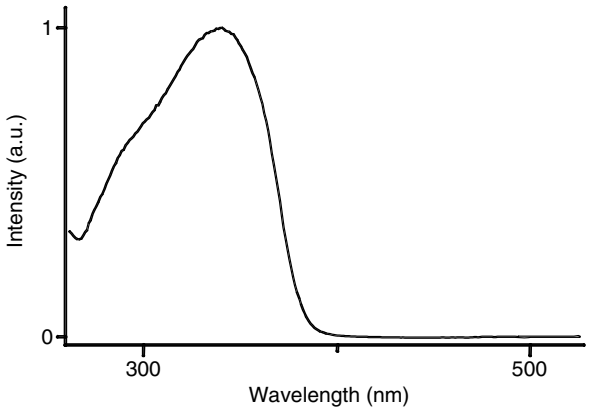


Figure 1. Absorption spectra of coumarin europium complex **1** in water (pH 7.8).

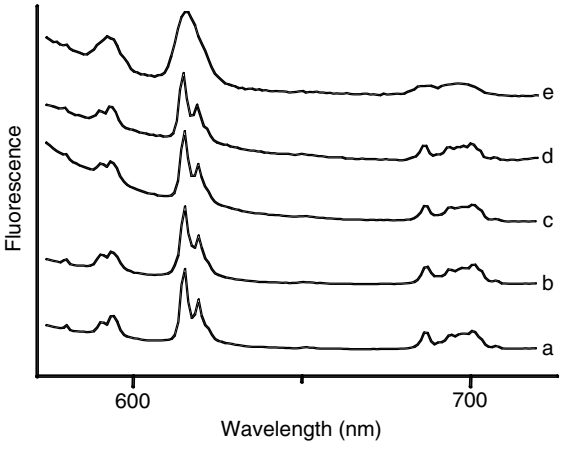
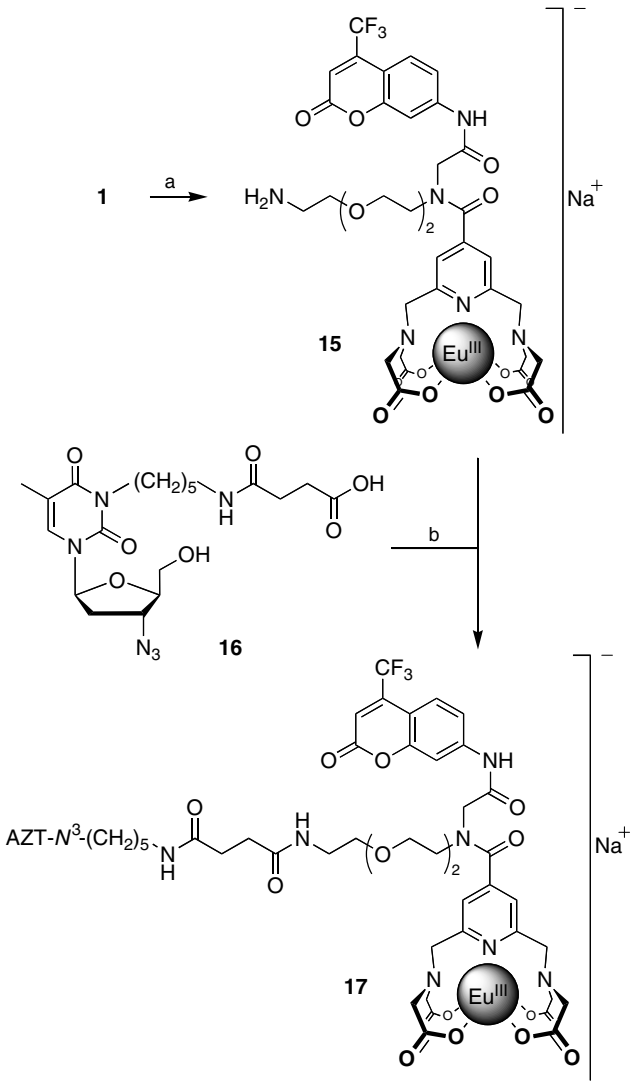


Figure 2. Characteristic europium emission bands for compound **1** in different media (λ_{exc} = 370 nm): (a) H_2O (pH 7.8); (b) H_2O, 50 mM NaF (pH 7.8); (c) PBS (pH 6.6); (d) Standard human plasma (Dade Behring, diluted 1/10); (e) Eu emission bands for conjugate **17** (H_2O, pH 7.8).



Scheme 3. Synthesis of labeled AZT.[35] (a) TCEP, H_2O; (b) DCC, NHS.

yl)phosphine hydrochloride in water and conjugation to modified nucleoside **16**[34] was realized via classical NHS activation of the terminal carboxylic acid function (Scheme 3). AZT fluorescent conjugate **17** exhibits characteristic lanthanide emission bands indicating that the spectroscopic features of the label are preserved (Fig. 2e).

In summary, we have described the design, synthesis, and preliminary evaluation of a coumarin-based europium chelate for homogeneous time-resolved fluorescence measurements with excitation by UV-LEDs at 370 nm. Complete determination of its physical properties and evaluation in applications for quantitative point-of-care HTRF immunoassays are currently underway and will be published in due course.

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31. Selected spectral data for **14**. ^{1}H NMR (D_2O, 200 MHz) 3.76 (br s, 2H, C$\underline{H_2}$N$_3$); 4.00 (br s, 8H, C$\underline{H_2}$ CO$_2$H); 4.23–5.09 (m, 14H, C$\underline{H_2}$C$\underline{H_2}$O, C$\underline{H_2}$CH$_2$ N$_3$, NC$\underline{H_2}$py); 5.18 (s, 2H, NC$\underline{H_2}$CONH); 7.23 (s, 1H, CF$_3$C=C$\underline{H}$); 7.71–7.88 (m, 1H, H$_{ar}$); 8.09–8.16 (m, 4H, H$_{ar}$). ^{13}C NMR (CD_3OD, 50 MHz) δ (ppm): 50.3; 51.6; 51.8; 56.5; 57.6; 69.5; 70.8; 71.0; 71.4; 108.3; 110.4; 115.3; 117.3; 123.2; 124.1 (q, J = 161.0 Hz); 126.9; 142.8 (q, J = 53.0 Hz); 144.4; 151.7; 156.4; 156.7; 160.7; 168.9; 170.2; 177.7. MS (ESI) *m/z* 840 $[M+H]^+$; 862 $[M+Na]^+$.
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35. Coupling procedure: complex **1** (10.7 mg, 10.6 μmol) and TCEP-HCl (3.5 mg, 12 μmol) are stirred for 15 h at rt in H_2O/MeOH 1:1 (1 mL). The reaction mixture is filtered and solvent is removed under vacuum. Analysis for intermediate **15**: MS (ESI) *m/z* 960.1, 962.1 $[M-Na]^-$.

The residue is solubilized in H_2O/MeCN 1:5 (6 mL) and preformed NHS-ester of AZT derivative [**16** (27.9 mg, 62 μmol), NHS (8.2 mg, 71 μmol), and DCC (14.4 mg, 71 μmol) stirred for 3 h at rt in MeCN/CH_2Cl_2 2:1 (3 mL)] is added. The mixture is stirred overnight at rt, evaporated, and the residue is triturated with CH_2Cl_2. The filtrate is reduced under vacuum and purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O 100:0:0–0:50:50) to yield **17** (5.5 mg, 37%). MS (ESI) *m*/*z* 1252.3, 1254.3 [M-azidodeoxyribose-Na]$^-$; 1393.3, 1395.3 [M−Na]$^-$.