Synthesis and characterization of coumarin-based europium complexes and luminescence measurements in aqueous media†

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A series of new ligands suitable for the formation of luminescent lanthanide complexes in water is described. The chelates are designed for analyte labeling and play the role of fluorescent donor in homogeneous time-resolved fluorescence assays using LEDs as a light source for excitation at 370 nm. Ligands **3–7** are constructed from a coumarin nucleus, for lanthanide sensitization, and different aminomethylenecarboxy moieties are introduced in positions 7 and 5, 6, or 8 of the sensitizer. A reactive spacer arm under biocompatible conditions (maleimide, azide) is introduced at position 3 for ultimate bioconjugation purposes. The synthesis and characterization of the ligands are described, together with the preparation of their corresponding europium complexes. Photophysical properties of the complexes are investigated in water by means of UV-vis and luminescence spectroscopy.

Introduction

The rational design of molecules that exhibit fluorescence properties has gained much interest over the past decade. Among them, luminescent lanthanide complexes with remarkable physical and chemical properties have been developed and found many applications, especially in the field of bioanalytical chemistry.[1,2] For instance, the time-resolved fluorescence resonance energy transfer (TR-FRET) technique takes advantage of the attracting properties of lanthanide chelates. Indeed, their long-lived excited states along with characteristic narrow emission bands make them ideal for fluorescent background discrimination and delayed emission signal detection. Long decay times result from forbidden transitions involving 4f orbitals and, as a consequence, the molar absorption coefficient of these complexes are very low and typically less than 1 M^{-1} cm^{-1}.[3,4] Most of the time, effective excitation cannot be performed directly, but *via* a light-harvesting antenna that acts as a sensitizer. During that process (termed the "antenna effect"), UV/visible light energy is collected by an allowed antenna-centered absorption. Antenna excitation is followed by a non-radiative intramolecular energy transfer from the excited states of the antenna to Ln(III), resulting in a radiative metal-centered luminescence.[5,6] The luminescent properties of lanthanide ions, such as characteristic narrow-line-like emission bands, depend on how efficiently their excited state(s) can be populated and non-radiative deactivation paths minimized. Excitation mainly depends on antenna efficiency (high extinction coefficient, efficient $S_1 \rightarrow T_1$ intersystem crossing rates, proximity to the metal centre) whereas deactivation is a complex phenomenon. A main route of non-radiative decay occurs by energy transfer from the excited state of Ln(III) to the O–H vibrational oscillator.[7] Thus a good shielding of the metal from the surrounding O–H oscillators of the solvent is key for building an efficient luminescent probe that can be finely tuned by ligand design. The lanthanide ions usually possess high coordination requirements and, for Eu(III) and Tb(III), a coordination number of nine is very common. Although such high coordination is difficult to fulfil using a single coordinating ligand, the use of functionalized macrocyclic ligands has come close.[8-13] In such complexes, the antenna is tethered to the chelate so as it can efficiently sensitize Ln(III).

In the course of our efforts to develop portable point-of-care testing devices (POCT),[14,15] we have been interested in designing lanthanide-based probes to be excited by light-emitting diodes (LEDs) for steady-state and time-resolved fluorescence spectroscopy. The luminescent lanthanide chelate is ultimately expected to play the role of FRET donor in homogenous time-resolved fluorescence (HTRF) assays.[2,16-18] LEDs as an excitation source offer excellent stability, signal-to-noise ratio, power efficiency and economy, and thus are especially suitable for portable instrumentation manufacturing. Although commercially available LEDs cover the whole visible range, only a few can be found with emission wavelengths below 360–370 nm and specific fluorophores are required. At the end of the 1990s, Selvin developed quinolinone-based lanthanide complexes that proved to be suitable for bioanalytical applications but required excitation at 337 nm.[19-22] More recently, azaxanthone chromophores have been described as europium sensitizers to be excited at 360–405 nm.[23-26] Coumarins may also be interesting chromophores with regards to their photochemical and photophysical properties. With emission maximum in the range 350–450 nm, they are ideally suited for the sensitization of lanthanides.[27–30] Though coumarins so far have scarcely been used in the design of lanthanide chelates, some reports describe their conjugation with crown ethers and cryptands or aminopolycarboxylic chelates and their use as fluorescent probes for applications in metal ion detection,[31,32] time-resolved

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† Electronic supplementary information (ESI) available: ^{13}C-NMR spectra of **3b**, **3c**, **4a**, **5a**, **6a**, **7a**, **7d**, **8–16**, **18–21**, **25–28**, **30**, and **31**; [Eu(**7a**)] emission spectrum.

fluoroimmunoassays,[33–35] site-specific protein labeling,[36] distance measurements in biomolecules,[37] DNA sensing,[38] or enzyme activity reporting.[39] Most of these coumarin-based probes suffer from poor stability and relatively low emission quantum yield in aqueous media. This stresses the necessity to develop additional work based on ligand design for improving their properties, and for allowing their use in bioanalytical applications.

Recent work by Lakowicz *et al.* validated the use of LEDs as light sources in bioanalytical studies.[30] The authors used compound [Eu(**1**)], a coumarin-sensitized europium probe resulting from the consecutive functionalization of diethylenetriamine pentaacetic acid dianhydride with 7-amino-4-(trifluoromethyl)coumarin, the metal sensitizer, and dodecylamine, the membrane anchor required for their study (Fig. 1). Thus, the efficiency of a microscope-based fluorimeter with UV/visible LED excitation could be demonstrated. These results prompted us to design compound [Eu(**2**)] which incorporates a metal chelator, a coumarin core as a sensitizer, and a functionalized spacer.[34] Functionalization of the spacer with a chemically reactive group (azide) was necessary to allow further conjugation under biocompatible conditions with the adequate biomolecule (AZT derivative) for final assay setup. Although compound [Eu(**2**)] can be satisfactorily excited at 360–370 nm and proved to be fairly stable in water and human plasma, the fluorescence quantum yield was modest. This particular result is attributed to the likely longer distance between the coumarin moiety and the metal centre in [Eu(**2**)], compared to [Eu(**1**)].

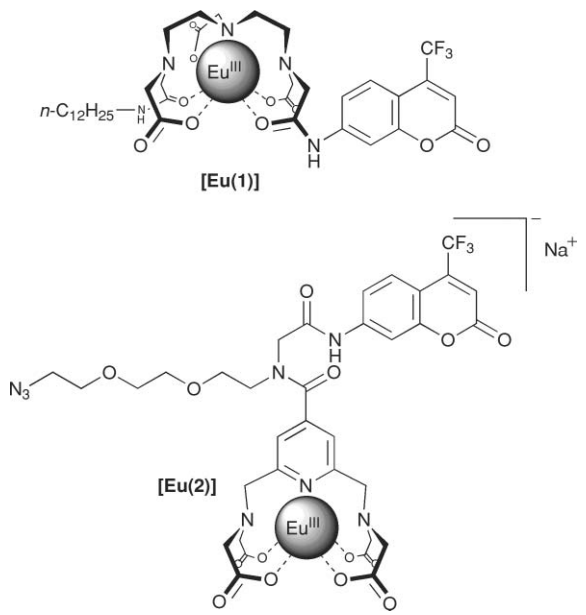


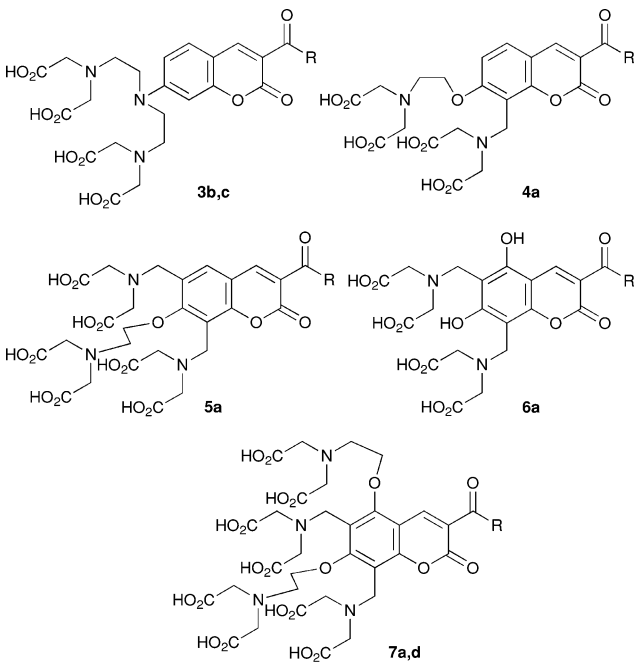
Fig. 1 Structure of 7-amino-4-(trifluoromethyl)coumarin-based europium sensitizers.

In order to develop HTRF donor probes with improved properties, we planned the relocation of an adequately functionalized coumarin core in the close proximity of the metal centre. Herein we describe our results concerning the design, synthesis, and evaluation of photophysical properties of a series of new functionalized chelating coumarins and of their corresponding europium complexes.

Results and discussion

Design and synthesis of the ligands

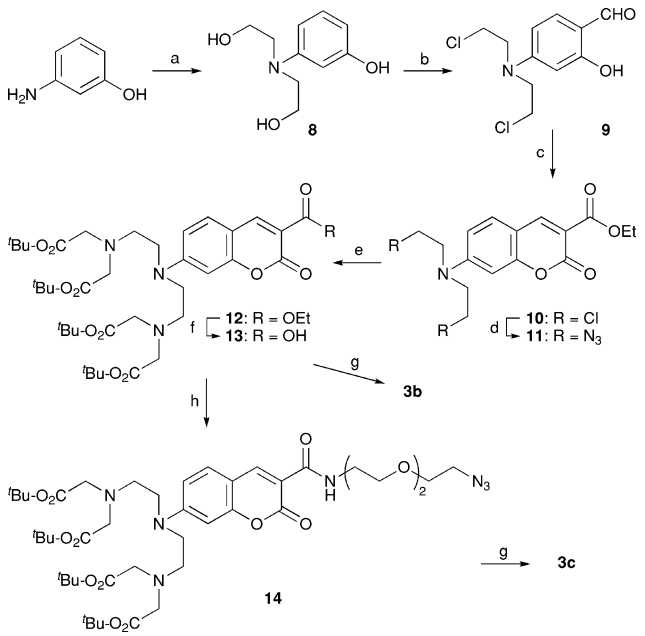
Previously developed ligand **2** has a T-shape or branched structure where 3 functional entities (*i.e.* metal-chelating moiety, antenna, and reactive spacer) are distributed radially (Fig. 1). Eu(III) complexation by the pyridine-2,6-diyl-bis(methyleneniminodiacetic acid) unit leads to a heptadentate pattern that has been previously described and used in DNA labeling applications.[40–44] In order to improve the quantum yield of metal sensitization, we designed compounds **3–7** differing from **2** by their "linear" geometry (Fig. 2). The coumarin antenna is inserted between the chelating part and the reactive spacer, playing to some extent the role of a scaffold. Iminodiacetic acid groups are directly connected to the coumarin aromatic ring through different linkers that can also participate in metal chelating. Compounds **3**, **4**, and **6** can supposedly lead to heptacoordinate Eu(III) chelates whereas **5** and **7** can potentially accommodate 10 and 14 different coordination bonds with lanthanide ions. On the opposite side of these molecules, the spacer R is connected at position 3 on the coumarin moiety. The reactive spacer displays either an azide or a maleimide group. Both functional groups have been extensively used for bioconjugation purposes and are fully compatible with ligand reactivity.[45–48] Not all the synthesized compounds have a reactive spacer. In some cases and for practical purposes, ethyl esters have been prepared in place of amide-linked reactive spacers, the photophysical evaluation of the compounds and their corresponding Eu(III) chelates being better with a "silent" spacer (*vide infra*).



a: R = OEt; **b**: R = OH; **c**:R = $NH(CH_2CH_2O)_2$-CH_2CH_2-N_3;
d: R = $NH(CH_2CH_2O)_2$-CH_2CH_2-*N*-maleimidyl.

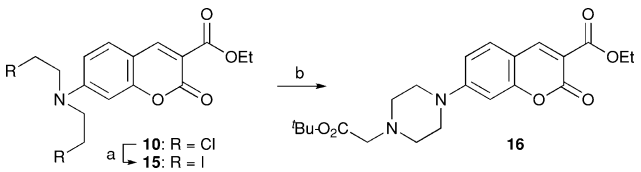
Fig. 2 Structure of ligands **3–7**.

The synthesis of ligands **3b** and **3c** was achieved according to Scheme 1. The coumarin scaffold was elaborated starting from 3-aminophenol. Bis-*N*-alkylation of the starting material



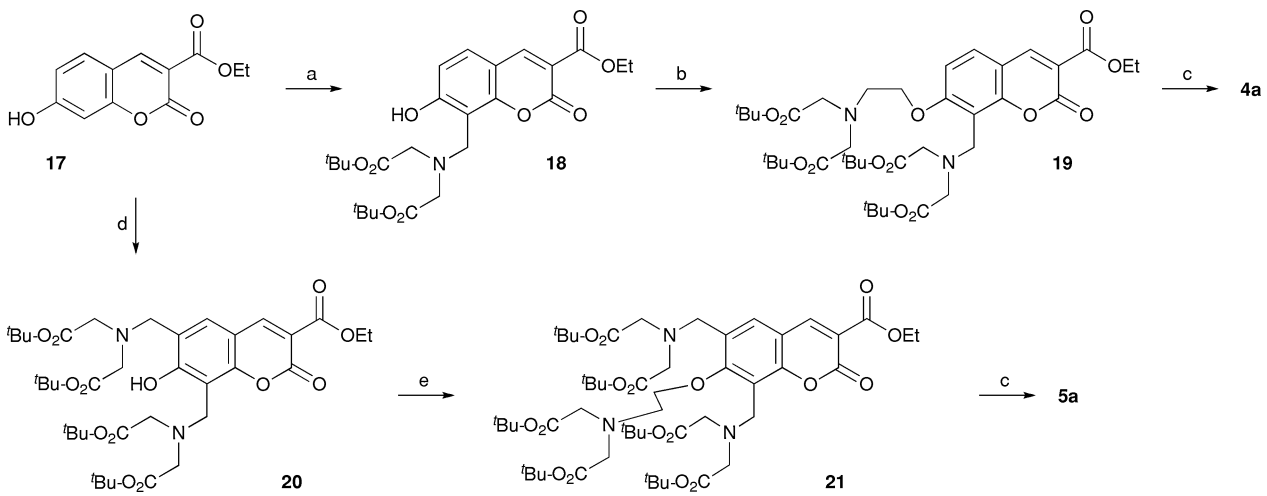
Scheme 1 Reagents and conditions: (a) ethylene oxide, AcOH, 88%; (b) $POCl_3$, DMF, 84%; (c) diethyl malonate, piperidine, EtOH, 94%; (d) NaN_3, NaI, DMF, 98%; (e) 1. PPh_3, THF; 2. aq. HCl; 3. $BrCH_2CO_2tBu$, Na_2CO_3, DMF, 51% (3 steps); (f) LiOH, quant.; (g) TFA, quant.; (h) DCC, NHS, $H_2N(CH_2CH_2O)_2CH_2CH_2N_3$, 68%.

with ethylene oxide in aqueous acetic acid[49] led to 3-[bis(2-hydroxyethyl)amino]phenol **8** in 88% yield and proved superior to the reaction with 2-chloroethanol reported by Li and Xie.[50] Simultaneous bis-chlorination and formylation of **8** using the Vilsmeier–Haack reagent formed *in situ* from DMF and phosphorus oxychloride was achieved in 84% yield. The resulting compound **9** was subjected to a classical Knoevenagel condensation reaction[51] to give coumarin **10** in a nearly quantitative yield. Nucleophilic displacement of the two chloride substituents with sodium azide was achieved quantitatively in DMF to afford compound **11**. Reduction of the latter under Staudinger conditions followed by reaction of the intermediate triamine compound with excess *t*-butyl bromoacetate led to pentaester **12** in 51% yield. Compound **12** was quantitatively converted into acid **13** upon treatment with lithium hydroxide. Introduction of a reactive spacer was achieved through condensation of **13** with 2-[2-(2-azidoethoxy)ethoxy]ethylamine,[52] and amide **14** was obtained in 68% yield. Direct treatment of tetra-*t*-butyl esters **13** and **14** with trifluoroacetic acid (TFA) afforded the two ligands **3b** and **3c**. We intended to transform bis-chlorocoumarin **10** into compound **12** using a one-step procedure (Scheme 2). All our attempts to displace the two chloride substituents by iminodiacetic acid di-*t*-butyl ester (directly or *via* intermediate iodide substitution) failed, and yielded dialkyl piperazine **16**.

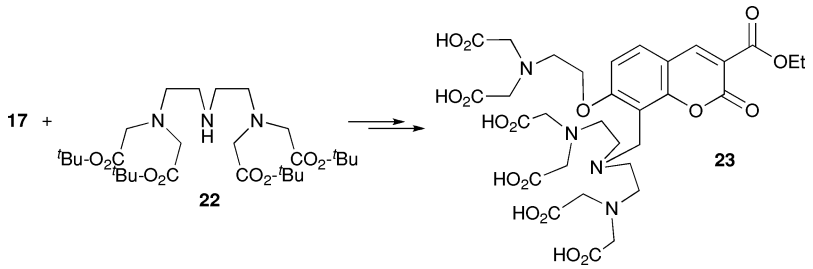


Scheme 2 Reagents and conditions: (a) NaI, acetone, quant.; (b) $HN(CH_2CO_2tBu)_2$, DMF, 62%.

Ligands **4a** and **5a** were prepared starting from 3-ethoxycarbonyl-7-hydroxycoumarin **17**[53] as a common precursor (Scheme 3). The iminodiacetate pendant arm was introduced *via* a Mannich reaction with formaldehyde and iminodiacetic acid di-*t*-butyl ester (Table 1). The latter compound was prepared according to a procedure described by Achilefu.[54] Compound **18** was obtained in 76% yield under the experimental conditions described by Regnier *et al.*[55] Alkylation of the phenol oxygen with *N*-(2-hydroxyethyl)iminodiacetic acid di-*t*-butyl ester[56] under the Mitsunobu reaction conditions yielded compound **19**, which was quantitatively converted into ligand **4a** upon treatment with TFA. Compound **5a** was prepared in a similar manner except that the initial Mannich reaction was conducted with 2 molar equivalents of iminodiacetic acid di-*t*-butyl ester and excess formaldehyde, in *N*-methylmorpholine (NMM). The introduction of the second aminomethylene pendant arm at position 6 on the



Scheme 3 Reagents and conditions: (a) 1.1 eq HCHO, 1.0 eq $HN(CH_2CO_2tBu)_2$, 76%; (b) PPh_3, DIAD, $HOCH_2CH_2N(CH_2CO_2tBu)_2$, 47%; (c) TFA, quant.; (d) 2.5 eq HCHO, 2.0 eq $HN(CH_2CO_2tBu)_2$, 13%; (e) PPh_3, DIAD, $HOCH_2CH_2N(CH_2CO_2tBu)_2$, 43%.



Scheme 4 Tentative route to **23**.

Table 1 Reaction conditions for the preparation of coumarins **18** and **20** from **17**

Entry	Amine (eq)	HCHO (eq)	Solvent	T (°C)	**18** (%)[a]	**20** (%)[a]
1	2	2	EtOH	78	86	0
2	2	12	EtOH	78	79	0
3	4	12	*t*BuOH	83	82	0
4	2	2	NMM	115	54	6
5	2	2.5	NMM	115	62	13
6	2	6[b]	NMM	115	56	11

[a] Isolated yield. [b] A larger excess in aqueous formaldehyde did not improve the conversion into **20**.

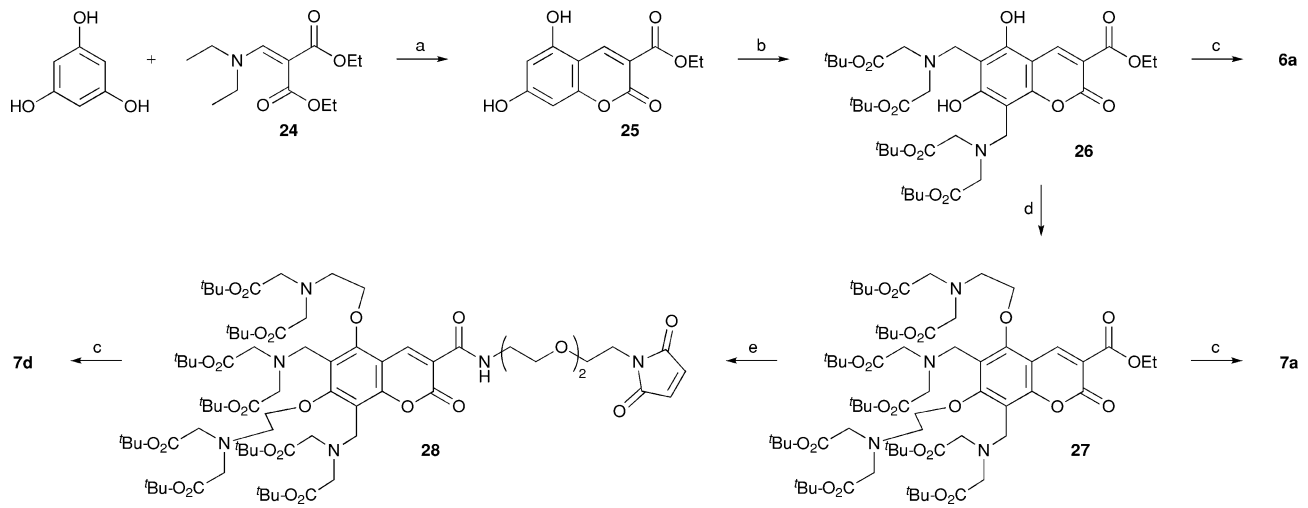
coumarin nucleus proved to be difficult and was achieved in a modest yield following a procedure described by Hinshaw *et al.*[57] (Table 1, entry 5). Alkylation of phenol oxygen in compound **20** and subsequent removal of all *t*-butyl protecting groups in **21** led to ligand **5a**.

Due to the relatively easy introduction of the iminodiacetic acid di-*t*-butyl ester moiety at position 8 on 3-ethoxycarbonyl-7-hydroxycoumarin **17** *via* the Mannich condensation reaction, we were interested in using a similar strategy to prepare coumarin **23** (Scheme 4). However, despite our efforts, we failed to find any evidence for the condensation reaction between coumarin **17** and *iso*-diethylenetriamine pentaacetic acid tetra-*t*-butyl ester **22**.[54] This result may be attributed to some steric hindrance introduced by the two bulky substituents at the central nitrogen atom in triamine **22**.

Ligands **6a**, **7a**, and **7d** were elaborated from phloroglucinol (Scheme 5). Reaction of the starting triol with diethyl (*N*,*N*-diethylaminomethylene)malonate **24** (prepared according to Momose *et al.*[58]) provided 3-ethoxycarbonyl-5,7-dihydroxycoumarin **25**[59] in 81% yield. The presence of a second hydroxyl group at position 5 on the coumarin nucleus makes compound **25** much more reactive than the corresponding monohydroxycoumarin **17**, and the double Mannich reaction with formaldehyde and iminodiacetic acid di-*t*-butyl ester proceeded quantitatively to yield compound **26**. The latter was further bis-alkylated with *N*-(2-hydroxyethyl)iminodiacetic acid di-*t*-butyl ester under Mitsunobu reaction conditions. Ethyl ester hydrolysis in **27**, followed by carboxylic acid activation and reaction with 8-(*N*-maleimidyl)-3,6-dioxaoctan-1-ol **31** afforded compound **28**. Trifluoroacetic acid treatment of **26**, **27**, and **28** gave quantitatively ligands **6a**, **7a**, and **7d**, respectively.

The reactive spacer **31** was prepared as described in Scheme 6, starting from 1-amino-3,6-dioxaoctan-1-ol.[60] Protected amino-alcohol **29**[61] was transformed into *N*-alkyl maleimide **30** by reacting with maleimide under Mitsunobu reaction conditions.[62] Removal of the carbamate protecting group was achieved under standard conditions.

Europium complexes with ligands **3**–**7** were prepared from stoichiometric amounts of ligand and europium chloride hexahydrate, in water (pH 7.0).[63,64]

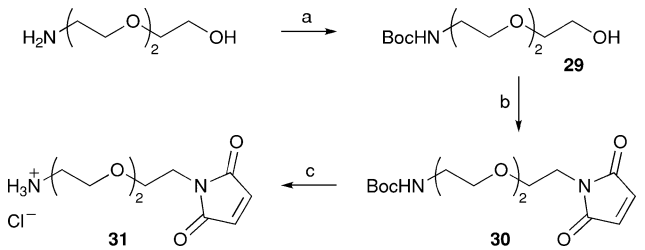


Scheme 5 Reagents and conditions: (a) AcOH, 81%; (b) 2.0 eq HCHO, 2.0 eq $HN(CH_2CO_2tBu)_2$, 97%; (c) TFA, quant.; (d) PPh_3, DIAD, $HOCH_2CH_2N(CH_2CO_2tBu)_2$, 51%; (e) 1. LiOH; 2. DCC, NHS, DIPEA, *N*-maleimidyl-$(CH_2CH_2O)_2CH_2CH_2NH_3^+Cl^-$ (**31**), 53% (2 steps).

Table 2 Spectroscopic properties of compounds **3–7** measured in H_2O, pH 7.0. Φ_F, τ_F, and k_F are fluorescence quantum yield, fluorescence lifetime, and fluorescence rate constant, respectively

Compound	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	λ_{phos} (nm)	Φ_F (%)	τ_F (ns)	k_F (10^6 s^{-1})
3c	396	394	453	481/549	36 (45)[a]	2.4	150
4a	342	344	401	479	16[b]	0.9	170
5a	402/332/302	402	443	482	2.5 (3.1)[c]	3.5	9
6a	405/366	411	457	491	1.8	3.1	10
7a (**7d**)[c]	407/318	407	451	481	6.0	3.8	16

[a] Measured at pH 5.0. [b] Measured at λ_{ex} 350 nm. [c] Spectroscopic data for **7a** and **7d** are identical.



Scheme 6 Reagents and conditions: (a) $(Boc)_2O$, quant.; (b) PPh_3, DIAD, maleimide, 74%; (c) TFA, 81%.

Photophysical properties

The photophysical data for compounds **3c**, **4a**, **5a**, **6a**, and **7a** are summarized in Table 2. Since all compounds have the same chromophore system, the fluorescence spectra are all very similar to each other, displaying one broad band with a maximum around 450 nm. The only exception is **4a**, which also differs from the others in other spectroscopic features. Considering the absorption spectra, no clear trend can be found. Coumarins **3c**, **5a**, **6a**, and **7a** all display a longest wavelength absorption band at 400 nm that corresponds to the fluorescence emission, as evident from the excitation spectra. However, in the absorption spectra of **5a**, **6a**, and **7a**, additional absorption bands can be observed that have no counterpart in the excitation spectra (Fig. 3). Therefore, different species have to be present in aqueous solution that likely result from different protonated forms. According to the excitation spectra, the fluorescence is due to the deprotonated species that predominates at pH 7. Compared to those of the other coumarins, absorption and fluorescence of compound **4a** are shifted about 50 nm towards shorter wavelengths. As expected from the two hydroxyl groups attached to the chromophore system, the absorption spectrum of **6a** is pH-sensitive in the range investigated (Fig. 3). For all the coumarin compounds, phosphorescence emission around 480 nm could be observed at 80 K.

Fluorescence lifetime τ_F and quantum yield Φ_F are drastically influenced by type and position of the complexation arms on the coumarin core. The quantum yield of the 7-aminocoumarin derivative **3c** exceeds the values of the 7-alkoxycoumarins **4a**, **5a**,

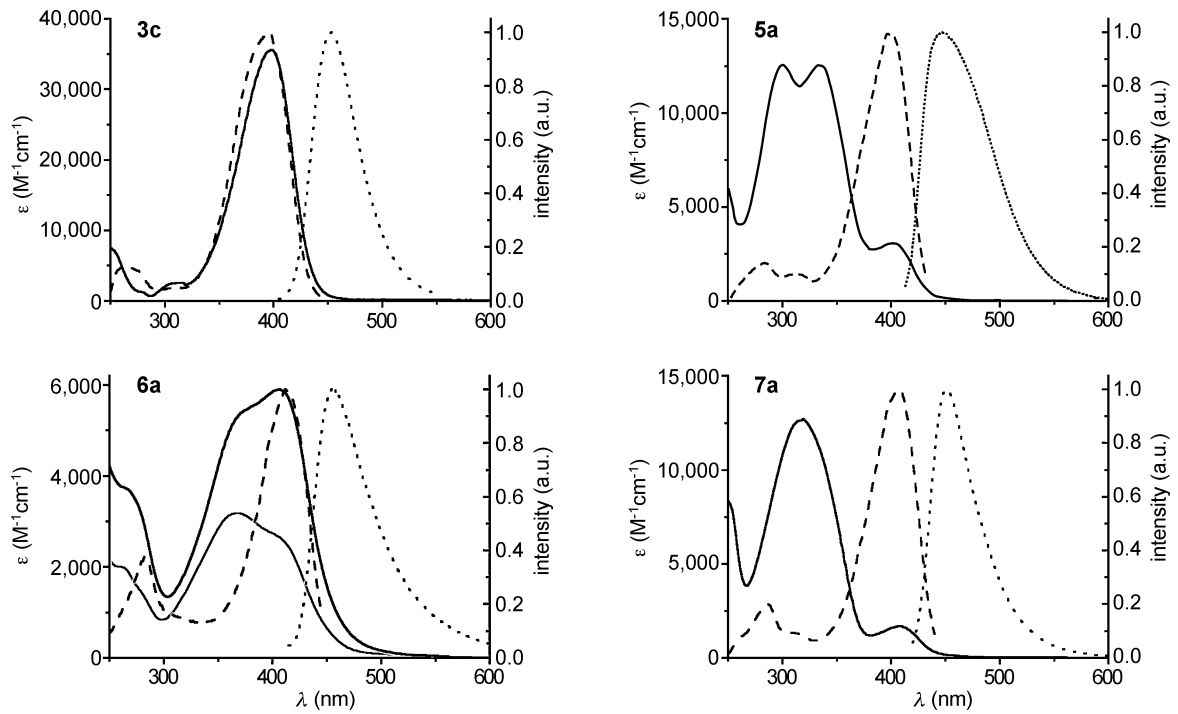


Fig. 3 Absorption (solid), fluorescence (dotted, λ_{ex} 400 nm) and excitation spectra (dashed, λ_{em} 450 nm) of compounds **3c**, **5a**, **6a**, and **7a** in water, pH 7.0 (grey line: pH 5.0).

Table 3 Spectroscopic properties of europium complexes obtained from ligands **3–7** measured in H_2O, pH 7.0

Ligand	λ_{exc} (nm)	Stoichiometry[a]		τ_{em} (ms)[b]		q[c]	Φ_F (%)[d]	
		pH 5	pH 7	H_2O	D_2O		Ligand	Eu^{3+}
3c	327	0.8 : 1	0.6 : 1	0.43	1.91	1.9	4.7	0.4
4a	365	1 : 1	1 : 1	0.40	2.08	2.1	4.3	2.8
5a	334	—	0.6–0.9 : 1[e]	0.58	2.47	1.4	1.0	0.9
7a	326	1 : 1	1 : 1	0.42	2.09	2.0	0.5	1.3

[a] [Eu]/[ligand], determined at ligand concentration of 10^{-4} M. [b] τ values are the mean of at least 3 independent measurements (±0.02 ms). [c] The number of coordinated water molecules at 300 K is obtained from Horrocks' empirical equation,[66] reviewed by Parker *et al.*:[67] $q = A[(1/\tau_{H_2O} - 1/\tau_{D_2O}) - 0.25]$ with $A = 1.2$ for Eu^{3+}. The estimated uncertainty in q is approximately ±0.5 water molecules. [d] Quantum yields of the complexes, separated in ligand centered and europium centered emission. The error in quantum yield values is estimated to be ±20%. [e] Depending on ligand concentration (see the main text).

6a, and **7a** by one order of magnitude. This observation is in agreement with data in the literature, since it is well established that an electron-donor substituent in the 7-position enhances the fluorescence intensity of coumarins.[65-67] Modification of the pH value induces a noticeable variation of Φ_F, as can be seen for coumarins **3c** and **5a**. This finding is consistent with the coumarin pH-sensitivity reported earlier by Goodwin and Kavanagh.[68] The results for τ_F are quite different. The fluorescence lifetime of **3c** (2.4 ns) is similar to the τ_F of **5a**, **6a**, and **7a** (3.1–3.8 ns), whereas the τ_F of **4a** is much shorter (0.9 ns). However, if we consider the fluorescence rate constant k_F calculated from these data, the results are more homogenous and confirm the trend that is already obvious from the Φ_F values. Interestingly, no pH effect can be observed for coumarin **6a** (for τ_F or Φ_F), although protolysis reactions are expected.

In the europium complexes, energy transfer from the ligand triplet state to the metal occurs and results in emission of the characteristic europium bands when the complexes are excited in the ligand absorption band (Table 3). However, for all the complexes, partial decomposition in water can be observed (data not shown). This is evident from the excitation spectra which show an absorption band at 410 nm (exactly at the same position as for the free ligands) when the wavelength of the emission channel is set at 450 nm, and show a blue-shifted absorption around 340 nm if the emission channel is set at 620 nm.

The stoichiometry of the Eu(III) complexes formed with ligands **3–7** was determined by a mole ratio method (Table 3).[69] Complex luminescence was monitored as a function of Eu^{3+} added to an aqueous solution of the ligand of interest. A sharp break in the titration curve reveals that a stoichiometric quantity of Eu(III) has been added. Fig. 4 shows a typical plot of the relative Eu(III) luminescence intensity *versus* equivalents of Eu^{3+} added to the ligand **7a**. Experiments were conducted at three different ligand concentrations (10^{-4}, 10^{-5}, and 10^{-6} M). The stoichiometry of complex [Eu(**3c**)] appears to be 1 : 2, probably revealing that poorly nucleophilic aniline nitrogen does not participate in the chelation of Eu^{3+}. Complex [Eu(**5a**)] shows a 1 : 1 stoichiometry in the micromolar concentration range. Surprisingly measurements at higher ligand concentration (10^{-5} and 10^{-4} M) reveal a 1 : 2 stoichiometry that is in agreement with the law of mass action. A mixture of both complexes is obtained at intermediate ligand concentration. Complexes [Eu(**4a**)] and [Eu(**7a**)] have a 1 : 1 stoichiometry. Stoichiometry of [Eu(**7a**)] does not vary with ligand concentration, as is the case for [Eu(**5a**)], although the two ligands only differ in an additional chelating arm at position 5 for **7a**.

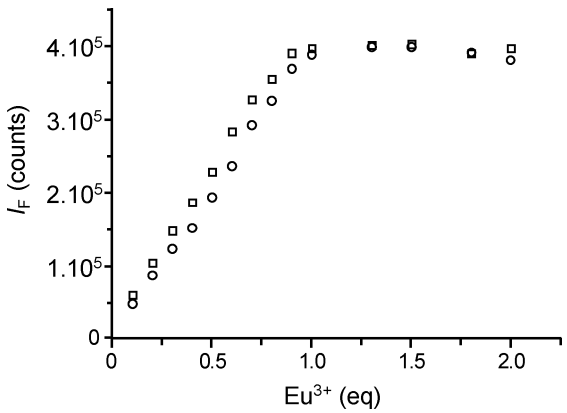


Fig. 4 Intensity of the $^5D_0$–$^7F_2$ transition peak as a function of Eu^{3+} added to a solution of ligand **7a** (10^{-6} M) in water, pH 5.0 (square) and pH 7.0 (circle).

Given the predominance of metal fluorescence quenching by O–H oscillators in aqueous media, the number of water molecules q in the inner coordination sphere may be derived from the difference in the rate of quenching in H_2O and D_2O.[70] We thus measured luminescence lifetime τ_{em} of the europium complexes in water and deuterium oxide at pH 7. According to Horrocks' empirical equation[7] (reviewed by Parker *et al.*[71]) about 2 water molecules remain bound to the metal in complexes [Eu(**3c**)], [Eu(**4a**)], and [Eu(**7a**)] (Table 3). The water content of [Eu(**5a**)] appears significantly lower (1.4 molecules). From these results we can conclude that whatever the number of donor atoms in the ligand (6 to 12), not all water molecules can be removed from the inner coordination sphere of the lanthanide cation, either in the 1 : 1 or in the 2 : 1 complexes. However for a 2 : 1 europium complex with **5a** (high ligand concentration, *vide supra*), one (0.7) additional water molecule could be displaced. From that, it can be assumed that the chelating arm at position 5 in **7a** is not available for intramolecular cation complexation and that formation of a 2 : 1 complex is hindered due to the steric needs of that substituent.

For demonstrating the potentiality of complex [Eu(**7a**)] as a donor in TR-FRET experiments, we used the diffusion-enhanced fluorescence energy transfer method.[72] The donor fluorescence lifetime was decreased dose-dependently by addition of the acceptor dye Cy5 (Fig. 5). The Stern–Volmer constant K_D was calculated as 7×10^7 M^{-1} s^{-1} according to the equation $F_0/F = \tau_0/\tau = 1 + K_D[Q]$ (where F_0 and τ_0, and F and τ, are respectively the fluorescence intensity and lifetime of the donor in the absence

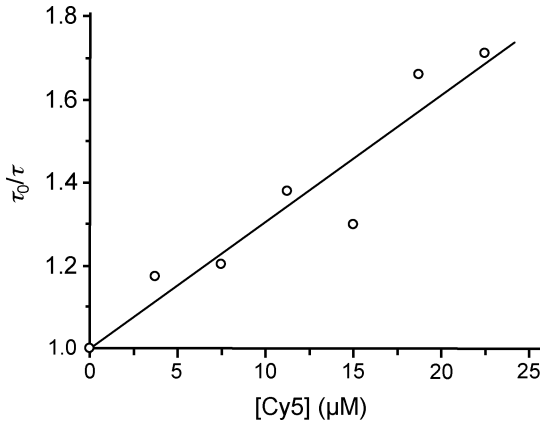


Fig. 5 Stern–Volmer plot for Cy5 quenching of [Eu(**7a**)].

and in the presence of the acceptor; [Q] is the concentration of the acceptor). Such a value does reflect a high efficiency of fluorescence energy transfer from [Eu(**7a**)] to Cy5.[3] In addition, the linearity of the Stern–Volmer plot indicates that only one type of quenching occurs in the aqueous environment.

Conclusions

A series of 7 coumarin-based ligands have been prepared. These ligands have been designed so they can potentially accommodate 6 to 14 coordination bonds to lanthanide cations and were synthesized in 4 to 8 steps involving either Knoevenagel or Mannich condensation. The europium(III) complexes have been prepared from each characterized ligand and the resulting photophysical properties have been investigated in water. Throughout the ligand series, dodecadentate ligand **7a** emerges. The related ligand **5a** lacking a chelating arm at the 5-position was revealed to be more efficient at isolating the metal centre from the surrounding water molecules. However, in that case, the stoichiometry of the complex is 2 : 1, which might indicate that the additional chelating arm in **7a** does not directly participate in metal chelation but prevents formation of a 2 : 1 complex, probably due to high steric hindrance introduced by the chelating moiety at the 5-position. The potential of [Eu(**7a**)] as a fluorescent donor in time-resolved FRET experiments has been assessed by a diffusion-enhanced fluorescence energy transfer method. Additional work is required for evaluating possible displacement of ligand donor atoms or inner coordination sphere water molecules by coordinating groups of proteins or endogenous anions present in biological samples. This is currently underway, and maleimide-functionalized europium complex [Eu(**7d**)], which presents similarly attractive features as [Eu(**7a**)], is currently being evaluated in a homogenous time-resolved fluorescence assay setup. Bioanalytical results will be reported in due course.

Experimental

Materials

Starting materials were purchased from Sigma-Aldrich and Alfa Aesar and used without purification. All reactions were carried out with reagent-grade solvents. When required, solvents were dried according to standard procedures as described elsewhere.[61] Silica gel for flash chromatography (silica gel 60, 0.040–0.063 mm, 230–400 mesh ASTM) and TLC plates (0.25 mm silica gel 60 precoated plates, F_{254}) were from E. Merck. Melting points were determined using a Stuart SMP3 apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker WP-200-Sy and Avance-DPX-300 instruments, and chemical shifts δ are reported in ppm relative to their standard reference (^{1}H: $CHCl_3$ at 7.27 ppm, HDO at 4.63 ppm, CD_2HOD at 3.31 ppm; ^{13}C: $CDCl_3$ at 77.0 ppm, CD_3OD at 49.0 ppm). Mass spectra (MS) were recorded on a ZQ Waters/Micromass spectrometer using electrospray ionization (ESI) mode. Mass data are reported in mass units (m/z). High-resolution mass spectra (HRMS) were recorded on a Bruker Daltonics MicrOTOF-Q spectrometer in ESI mode. IR spectra were recorded on a Perkin-Elmer 1600-FTIR spectrometer in the ATR mode and absorption values ν are in wavenumbers (cm^{-1}). Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad.

Luminescence measurements

Absorption spectra were recorded on a Cary 500 UV–vis–NIR spectrophotometer (Varian Inc., Palo Alto, CA, USA). Absorption maxima λ_{max} are given in nm and molar extinction coefficients ε are in M^{-1} cm^{-1}. For spectroscopic measurements, fluorophore stock solutions were prepared in water (10^{-3} M) and diluted to 10^{-4}–10^{-6} M. Solutions were adjusted to pH 5 and pH 7 with HCl or NaOH, respectively. Spectroscopic data for the europium complexes were obtained from ligand solutions in 10^{-3} M europium chloride. All spectra were recorded in 1 cm quartz cells. Stationary fluorescence and excitation spectra were obtained with a Fluoromax 3 fluorimeter (Jobin Yvon, Edison, NJ, USA). All spectra were corrected using the correction functions incorporated in the spectrometer software. Absolute fluorescence quantum yields were measured with an integration sphere system (Hamamatsu) at an excitation wavelength of 400 nm. Fluorescence lifetime measurements were performed with a FLS920 fluorimeter (Edinburgh Instruments Ltd, Livingston, UK). A frequency-doubled titanium sapphire laser system (Tsunami 3960, Spectra Physics, Mountain View, USA) set at 400 nm was used as the excitation light source. The original repetition rate of 80.2 MHz was reduced to 500 kHz with a pulse picker (Pulse Select, APE, Berlin, Germany). Fluorescence emission was detected with a multichannel plate (ELDY EM1-132/300, Europhoton, Berlin, Germany), providing a time response of ~100 ps. Phosphorescence lifetime data were obtained with a Fluoromax P fluorimeter (Jobin Yvon, Edison, NJ, USA). For phosphorescence measurements at 80 K, the samples were prepared in a 5 × 5 mm home-built monolithic quartz cell, deoxygenated by flushing the samples for 15 min with argon, and then cooled to 80 K with an Optistate DN1704 cryostat (Oxford Instruments, Wiesbaden, Germany) fitted with an external controller (ITC4, Oxford Instruments). The stoichiometry of the Eu(III) complexes was determined by a mole ratio method as previously reported.[69] TR-FRET experiments with ligand **7a** (10^{-4} M) were realized in the presence of $EuCl_3$ (5×10^{-4} M), with Cy5 as an acceptor (concentration ranging up to 22.5×10^{-6} M). Excitation was achieved at 350 nm with an OPO system (Nd YAG Laser, Spectra-Physics, Mountain View, CA, USA; OPO GWU-Lasertechnik, Erftstadt, Germany) working at a 20 Hz repetition rate, with an average pulse energy

of *ca.* 15 μJ. Detection was achieved at 600–700 nm using an iCCD camera system (iStar DH720, Andor, Belfast, UK) attached to an imaging monochromator (MS257, LOT-Oriel, Darmstadt, Germany).

Synthesis and characterization

7-(*N*,*N*-Bis{2-[bis(carboxymethyl)amino]ethyl}amino)-3-carboxycoumarin (3b). Compound **13** (96 mg, 0.13 mmol) was stirred in CH_2Cl_2/TFA 1 : 1 (3 mL) at room temperature for 7 h. Water (5 mL) was added and the solution was brought to pH 7 with 1 N NaOH, washed with CH_2Cl_2 (3 × 5 mL) and evaporated to dryness. The residue was triturated with MeOH, filtered and dried to yield **3b** (68 mg, 100%) as a yellow hygroscopic powder. ^{1}H-NMR (D_2O, 200 MHz) δ 8.22 (s, 1H); 7.55 (d, J = 8.3 Hz, 1H); 6.90 (d, J = 8.3 Hz, 1H); 6.78 (bs, 1H); 3.93 (bs, 4H); 3.80 (s, 8H); 3.48 (bs, 4H). ^{13}C-NMR (D_2O, 50 MHz) δ 170.9; 163.2; 162.7; 156.5; 151.1; 147.3; 131.6; 118.0; 115.1; 111.6; 110.7; 99.3; 58.1; 52.2; 45.9. IR (film) ν 3391 (b); 2987; 2900; 1682; 1606. UV-vis λ_{max} 375 (H_2O, pH 6.5). MS (ESI) m/z 524 $[M + H]^+$; 546 $[M + Na]^+$. ESI-HRMS for $C_{22}H_{25}N_3O_{12}$: calcd. 546.1330 $[M + Na]^+$, found 546.1313.

3-{*N*-2-[2-(2-Azidoethoxy)ethoxy]ethylaminocarbonyl}-7-(*N*,*N*-bis{2-[bis(carboxymethyl)amino]ethyl}amino)coumarin (3c). Compound **3c** (13 mg, 100%) was obtained as a yellow hygroscopic powder from **14**, following the same procedure as for **3b**. ^{1}H-NMR (CD_3OD, 300 MHz) δ 8.61 (s, 1H); 7.56 (d, J = 8.1 Hz, 1H); 6.89 (d, J = 8.1 Hz, 1H); 6.74 (s, 1H); 4.01 (m, 10H); 3.89 (bs, 4H); 3.69 (bs, 8H); 3.44 (bs, 4H); 3.37 (bs, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 169.3; 164.7; 163.1; 157.0; 152.5; 149.0; 132.5; 111.8; 111.6; 110.5; 98.8; 70.2; 70.1; 69.4; 65.8; 56.7; 52.5; 49.4; 46.0; 39.7. IR (film) ν 3387 (b); 2972; 2101. UV-vis λ_{max} 403 (H_2O, pH 6.5). MS (ESI) m/z 681 $[M + H]^+$; 703 $[M + Na]^+$. ESI-HRMS for $C_{28}H_{37}N_7O_{13}$: calcd. 680.2522 $[M + H]^+$, found 680.2518.

7-{2-[Bis(carboxymethyl)amino]ethoxy}-8-[bis(carboxymethyl)-aminomethyl]-3-ethoxycarbonylcoumarin (4a). Compound **19** (30 mg, 39 μmol) was stirred in TFA (0.3 mL) for 3 h at rt. The resulting mixture was coevaporated 3 times with $CHCl_3$ (5 mL) and the residue was triturated in CH_2Cl_2. The precipitate was filtered and dried under vacuum to yield **4a** (21 mg, 70%) as a yellow hygroscopic solid (TFA salt). Mp 189 °C. ^{1}H-NMR (CD_3OD, 300 MHz) δ 8.70 (s, 1H); 7.92 (d, J = 7.2 Hz, 1H); 7.19 (d, J = 7.2 Hz, 1H); 4.83 (bs, 2H); 4.50 (m, 2H); 4.36 (q, J = 7.2 Hz, 2H); 4.17 (bs, 4H); 3.99 (bs, 4H); 3,66 (m, 2H); 1.39 (t, J = 7.2 Hz, 3H).^{13}C-NMR (CD_3OD, 75 MHz) δ 172.8; 170.5; 165.1; 164.9; 163.2 (q, J = 36.3 Hz); 158.5; 157.8; 151.4; 136.2; 118.7 (q, J = 289.7 Hz); 116.5; 114.8; 116.6; 108.0; 67.4; 63.7; 57.4; 57.1; 56.1; 56.0; 15.4. IR (film) ν 3473; 2987; 1738; 1605. UV-vis λ_{max} 365 (H_2O, pH 6.5). MS (ESI) m/z 539 $[M + H]^+$; 561 $[M + Na]^+$. ESI-HRMS for $C_{23}H_{26}N_2O_{13}$: calcd. 539.1508 $[M + H]^+$, found 539.1499.

6,8-Bis[bis(carboxymethyl)aminomethyl]-7-{2-[bis(carboxymethyl)amino]ethoxy}-3-ethoxycarbonylcoumarin (5a). Compound **5a** (61 mg, 86%) was obtained as a yellow hygroscopic solid (TFA salt) from **21**, following the same procedure as for **4a** (reaction time 15 h). ^{1}H-NMR (D_2O, 300 MHz) δ 8.73 (s, 1H); 8.07 (s, 1H); 4.78 (s, 2H); 4.62 (s, 2H); 4.37 (m, 2H); 4.27 (q, J = 6.9 Hz, 2H); 4.22 (s, 4H); 4.09 (s, 4H); 4.03 (s, 4H); 3.91 (m, 2H); 1.25 (t, J = 6.9 Hz, 3H). ^{13}C-NMR (D_2O, 75 MHz) δ 169.3; 169.1; 168.8; 164.0; 163.4; 157.6; 156.5; 149.9; 138.5; 121.5; 117.4; 116.7; 112.7; 71.1; 63.5; 57.2; 55.4; 53.8; 48.0; 47.6; 47.5; 13.7. IR (film) ν 3391; 2987; 2904; 1682; 1614; 1384. UV-vis λ_{max} 402 (H_2O, pH 6.5). MS (ESI) m/z 684 $[M + H]^+$; 706 $[M + Na]^+$. ESI-HRMS for $C_{28}H_{33}N_3O_{17}$: calcd. 684.1883 $[M + H]^+$, found 684.1877.

6,8-Bis[bis(carboxymethyl)aminomethyl]-5,7-dihydroxy-3-ethoxycarbonylcoumarin (6a). Compound **6a** (38 mg, 72%) was obtained as an orange hygroscopic solid (TFA salt) from **26**, following the same procedure as for **4a** (reaction time 3 h). ^{1}H-NMR (D_2O, 300 MHz) δ 8.93 (s, 1H); 4.73 (s, 4H); 4.35 (q, J = 6.9 Hz, 1H); 3.90 (s, 8H); 1.39 (t, J = 6.9 Hz, 3H). ^{13}C-NMR (CD_3OD, 75 MHz) δ 169.8; 165.8; 165.1; 163.5 (q, J = 35.2 Hz); 160.2; 158.1; 157.1; 147.7; 118.8 (q, J = 291.0 Hz); 111.6; 109.8; 105.3; 104.6; 69.7; 67.0; 65.1; 63.4; 15.4. IR (film) ν 3084; 2973; 1731; 1614; 1574. UV-vis λ_{max} 408 (H_2O, pH 6.5). MS (ESI) m/z 541 $[M + H]^+$; 563 $[M + Na]^+$. ESI-HRMS for $C_{22}H_{24}N_2O_{14}$: calcd. 541.1300 $[M + H]^+$, found 541.1291.

5,7-Bis{2-[bis(carboxymethyl)amino]ethoxy}-6,8-bis[bis(carboxymethyl)aminomethyl]-3-ethoxycarbonylcoumarin (7a). Compound **7a** (60 mg, 89%) was obtained as a yellow hygroscopic solid from **27**, following the same procedure as for **4a** (reaction time 15 h). ^{1}H-NMR (CD_3OD, 300 MHz) δ 9.00 (s, 1H); 4.91 (s, 2H); 4.83 (s, 2H); 4.61 (s, 2H); 4.41–4.38 (m, 4H); 4.07 (s, 4H); 3.94 (s, 4H); 3.83 (s, 4H); 3.78 (s, 4H); 3.38 (m, 4H); 1.39 (t, J = 7.2 Hz, 3H). ^{13}C-NMR (CD_3OD, 75 MHz) δ 174.6; 174.1; 172.6; 171.5; 166.2; 165.2; 164.7 (q, J = 35,5 Hz); 162.0; 159.2; 157.8; 146.1; 118.7; 118.2; 118.1 (q, J = 289.6 Hz); 114.0; 112.4; 78.0; 76.0; 63.9; 57.4; 57.1; 56.8; 56.6; 15.4. IR (film) ν 3441; 2980; 1731; 1596; 1393. UV-vis λ_{max} 397 (H_2O, pH 6.5). MS (ESI) m/z 860 $[M + H]^+$; 882 $[M + Na]^+$. ESI-HRMS for $C_{34}H_{42}N_4O_{22}$: calcd. 859.2363 $[M + H]^+$, found 859.2353.

5,7-Bis{2-[bis(carboxymethyl)amino]ethoxy}-6,8-bis[bis(carboxymethyl)aminomethyl]-3-(2-[2-{2-(*N*-maleimidyl)ethoxy}-ethoxy]ethylaminocarbonyl)coumarin (7d). Compound 7d (32 mg, 91%) was obtained as a yellow hygroscopic solid from 28, following the same procedure as for **4a** (reaction time 60 h). ^{1}H-NMR (CD_3OD, 300 MHz) δ 9.04 (s, 1H); 6.79 (s, 2H); 4,83 (bs, 2H); 4.70 (bs, 2H); 4.50 (bs, 2H); 4.44 (bs, 2H); 4.10 (s, 4H); 4.01 (s, 4H); 3.97 (s, 4H); 3.94 (s, 4H); 3.68–3.61 (m, 16H). ^{13}C-NMR (CD_3OD, 75 MHz) δ 172.9; 172.5; 170.7; 165.1; 163.3; 161.9; 160.7; 157.9; 143.9; 135.4; 119.3; 118.1; 115.5; 112.0; 76.0; 74.8; 71.4; 71.3; 70.3; 68.9; 56.4; 56.3; 56.2; 55.8; 55.7; 55.6; 40.8; 38.2. IR (film) ν 3359; 2973; 1728; 1392. UV-vis λ_{max} 397 (H_2O, pH 6.5). MS (ESI) m/z 1042 $[M + H]^+$; 1064 $[M + Na]^+$. ESI-HRMS for $C_{42}H_{51}N_6O_{25}$: calcd. 1041.3055 $[M + H]^+$, found 1041.3052.

3-Bis(2-hydroxyethyl)aminophenol (8). Ethylene oxide (13.5 g, 0.3 mol) was condensed using a cold trap in a mixture of 3-aminophenol (3.3 g, 30.2 mmol) and aqueous AcOH 50% (50 mL) at 0 °C. The solution was allowed to warm to rt and was stirred for 24 h. The crude mixture was coevaporated twice with toluene, dissolved in AcOEt and washed with water and brine. The organic layer was dried over $MgSO_4$, reduced under vacuum, and the residue was purified by silica gel chromatography (AcOEt) to yield **8** (5.24 g, 88%) as a yellow oil. TLC R_f 0.35 (AcOEt). ^{1}H-NMR

(CD_3OD, 300 MHz) δ 6.96 (t, $J = 8.1$ Hz, 1H); 6.23 (dd, $J = 8.1$, 2.1 Hz, 1H); 6.19 (d, $J = 1.8$ Hz, 1H); 6.11 (dd, $J = 8.1$, 1.8 Hz, 1H); 3.70 (t, $J = 6.0$ Hz, 4H); 3.48 (t, $J = 6.0$ Hz, 4H). ^{13}C-NMR (CD_3OD, 75 MHz) δ 159.2; 150.7; 130.9; 105.2; 104.5; 100.3; 60.5; 55.1. MS (ESI) m/z 198 $[M + H]^+$.

4-Bis(2-chloroethyl)amino-2-hydroxybenzaldehyde (9). Freshly distilled phosphorus oxychloride (1,80 mL, 19,0 mmol) was added dropwise to anhydrous DMF (3 mL) at 0 °C. The mixture was stirred for 20 min at rt before compound **8** (1.08 g, 5.5 mmol) in DMF (3 mL) was added. The resulting mixture was heated at 70–80 °C for 2 h and the orange solution was cooled down to rt, dropped on iced water (50 mL), and stirred for another 2 h period, maintaining pH at 6–7 by addition of aqueous NaOH. The mixture was then extracted with ether and the organic layer was dried over $MgSO_4$, reduced under vacuum, and the residue was purified by silica gel chromatography (Et_2O/*n*-hexane 40 : 60) to yield **9** (1.21 g, 84%) as a grey-purple powder. Mp 69 °C. TLC R_f 0.35 (Et_2O/*n*-hexane 50 : 50). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 11.53 (s, 1H); 9.59 (s, 1H); 7.37 (d, $J = 8.8$ Hz, 1H); 6.31 (dd, $J = 8.8$, 2.4 Hz, 1H); 6.13 (d, $J = 2.4$ Hz, 1H); 3.82 (t, $J = 6.0$ Hz, 4H); 3.68 (t, $J = 6.0$ Hz, 4H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 192.9; 164.2; 153.1; 135.7; 112.7; 104.4; 97.9; 53.3; 39.9. IR (film) ν 2930; 2848; 1627. MS (ESI) m/z 359 $[M + H]^+$.

7-Bis(2-chloroethyl)amino-3-ethoxycarbonylcoumarin (10). Piperidine (50 µL, 0.50 mmol) was added to a mixture of compound **9** (125 mg, 0.48 mmol) and diethyl malonate (72 µL, 0.48 mmol) in EtOH (1 mL). A yellow suspension immediately formed that was stirred for 2 h at rt. The solvent was removed under vacuum and the residue was purified by silica gel chromatography (CH_2Cl_2/Et_2O 100 : 0 to 50 : 50) to yield **10** (160 mg, 94%) as a yellow powder. Mp 157 °C. TLC R_f 0.4 (CH_2Cl_2/Et_2O/*n*-hexane 1 : 1 : 1). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 8.45 (s, 1H); 7.45 (d, $J = 8.8$ Hz, 1H); 6.67 (dd, $J = 8.8$, 2.4 Hz, 1H); 6.54 (d, $J = 2.4$ Hz, 1H); 4.39 (q, $J = 7.1$ Hz, 2H); 3.86 (t, $J = 6.2$ Hz, 4H); 3.70 (t, $J = 6.2$ Hz, 4H); 1.40 (t, $J = 7.1$ Hz, 3H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 163.7; 157.9; 157.4; 151.6; 148.9; 131.3; 111.5; 109.5; 109.0; 97.9; 61.4; 53.4; 39.9; 14.3. IR (film) ν 1729; 1693; 1614; 1584. UV-vis λ_{max} 402 (H_2O, pH 7.0). MS (ESI) m/z 359 $[M + H]^+$; 381 $[M + Na]^+$. ESI-HRMS for $C_{16}H_{17}Cl_2NO_4$: calcd. 358.0607 $[M + H]^+$, found 358.0606.

7-Bis(2-azidoethyl)amino-3-ethoxycarbonylcoumarin (11). Compound **10** (4.20 g, 11.7 mmol) was stirred in anhydrous DMF (40 mL) with NaN_3 (2.30 g, 35.4 mmol) and NaI (1.12 g, 7.5 mmol) at 90 °C for 6 h. The mixture was cooled to rt, added with Et_2O (50 mL), and washed with water (3 × 50 mL). The organic layer was dried over $MgSO_4$ and reduced under vacuum to yield analytically pure **11** (4.26 g, 98%) as an orange-yellow powder. Mp 91 °C. TLC R_f 0.4 (AcOEt/*n*-hexane 1 : 1). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 8.45 (s, 1H); 7.44 (d, $J = 8.8$ Hz, 1H); 6.69 (dd, $J = 8.8$, 2.6 Hz, 1H); 6.54 (d, $J = 2.6$ Hz, 1H); 4.38 (q, $J = 7.1$ Hz, 2H); 3.72–3.55 (m, 8H); 1.39 (t, $J = 7.1$ Hz, 3H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 163.7; 157.9; 157.6; 151.9; 148.9; 131.2; 111.4; 109.7; 108.9; 97.9; 61.4; 50.7; 48.6; 14.3. IR (film) ν 2979; 2099; 1764; 1698; 1618; 1514; 1412; 1205. MS (ESI) m/z 372 $[M + H]^+$; 394 $[M + Na]^+$. ESI-HRMS for $C_{16}H_{17}N_7O_4$: calcd. 372.1415 $[M + H]^+$, found 372.1410.

7-(*N*,*N*-Bis{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethyl}-amino)-3-ethoxycarbonylcoumarin (12). Compound **11** (0.46 g, 1.25 mmol) and triphenyl phosphine (0.66 g, 2.5 mmol) were stirred at rt in anhydrous THF (5 mL) for 3 h. The resulting mixture was added with 1 N HCl (7 mL) and refluxed for 15 h. Solvent was removed under vacuum. The residue was coevaporated twice with MeOH, dissolved in anhydrous DMF (8 mL), and Na_2CO_3 (1.09 g, 10.3 mmol) and *tert*-butyl bromoacetate (1.1 mL, 7.4 mmol) were added. The suspension was stirred at 70–80 °C for 12 h. Ethyl acetate (25 mL) was added and the organic layer was washed with water, brine, and was dried over $MgSO_4$. Solvent was removed under vacuum and the residue was purified by silica gel chromatography (AcOEt/*n*-hexane 30:70) to yield **11** (0.49 g, 51%) as a yellow oil. TLC R_f 0.6 (AcOEt/*n*-hexane 1 : 1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.41 (s, 1H); 7.32 (d, $J = 8.9$ Hz, 1H); 6.73 (dd, $J = 8.9$, 1.9 Hz, 1H); 6.50 (d, $J = 1.9$ Hz, 1H); 4.37 (q, $J = 7.1$ Hz, 2H); 3.60 (t, $J = 7.2$ Hz, 4H); 3.47 (s, 8H); 2.91 (t, $J = 7.2$ Hz, 4H); 1.47 (bs, 36H); 1.39 (t, $J = 7.1$ Hz, 3H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 170.3; 164.1; 158.2; 158.0; 153.2; 149.0; 130.9; 109.7; 109.2; 107.8; 96.7; 81.2; 61.0; 56.6; 51.1; 50.2; 28.0; 14.2. IR (film) ν 2977; 1732; 1616; 1587. MS (ESI) m/z 777 $[M + H]^+$. ESI-HRMS for $C_{40}H_{61}N_3O_{12}$: calcd. 776.4328 $[M + H]^+$, found 776.4326.

7-(*N*,*N*-Bis{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethyl}-amino)-3-carboxycoumarin (13). Compound **12** (0.15 g, 0.19 mmol) was stirred in EtOH (10 mL) with NaOH 1 N (0.19 mL, 0.19 mmol) for 20 h at rt. Solvent was removed under vacuum and the residue was filtered over a short silica gel cartridge (AcOEt/MeOH 100 : 0 to 50 : 50) to yield carboxylic acid **13** (0.14 g, 97%) as a yellow glassy solid. TLC R_f 0.1 (AcOEt). ^{1}H-NMR ($CDCl_3$/CD_3OD 1 : 1, 200 MHz) δ 8.55 (s, 1H); 7.42 (d, $J = 8.8$ Hz, 1H); 6.79 (dd, $J = 8.8$, 1.0 Hz, 1H); 6.57 (d, $J = 1.0$ Hz, 1H); 3.61 (t, $J = 8.4$ Hz, 4H); 3.44 (s, 8H); 2.89 (t, $J = 8.4$ Hz, 4H); 1.45 (bs, 36H). ^{13}C-NMR ($CDCl_3$/CD_3OD 1 : 1, 75 MHz) δ 171.5; 169.6; 164.4; 158.2; 153.9; 150.1; 131.8; 112.3; 111.2; 109.6; 97.4; 82.1; 82.1; 57.2; 51.8; 50.8; 28.4. IR (film) ν 3330; 2979; 2935; 1727; 1614; 1582. MS (ESI) m/z 749 $[M + H]^+$. ESI-HRMS for $C_{38}H_{57}N_3O_{12}$: calcd. 770.3834 $[M + Na]^+$, found 770.3818.

3-(2-[2-{2-(Azido)ethoxy}ethoxy]ethylaminocarbonyl)-7-(*N*,*N*-bis{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethyl}amino)coumarin (14). NHS (17 mg, 0.15 mmol), 2-[2-(2-azidoethoxy)-ethoxy]ethylamine[52] (28 mg, 0.16 mmol), and DCC (55 mg, 0.26 mmol) were successively added to carboxycoumarin **13** (81 mg, 0.11 mmol) in anhydrous Et_2O (4 mL). The reaction mixture was stirred at rt for 20 h, reduced under vacuum, and the residue was purified by silica gel chromatography (Et_2O) to yield compound **14** (67 mg, 68%) as a yellow oil. TLC R_f 0.3 (Et_2O). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 8.98 (bs, 1H); 8.64 (s, 1H); 7.35 (d, $J = 8.9$ Hz, 1H); 6.73 (dd, $J = 8.9$, 1.6 Hz, 1H); 6.51 (d, $J = 1.6$ Hz, 1H); 3.65–3.50 (m, 14H); 3.42 (s, 8H); 3.36 (t, $J = 5.4$ Hz, 2H); 2.88 (t, $J = 8.4$ Hz, 4H); 1.43 (s, 36H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.3; 163.0; 162.3; 157.4; 152.8; 147.7; 130.9; 110.5; 110.0; 108.5; 96.6; 81.1; 70.5; 69.9; 69.7; 56.5; 51.0; 50.6; 50.2; 39.3; 28.0. IR (film) ν 2976; 2106; 1735; 1616. MS (ESI) m/z 905 $[M + H]^+$. ESI-HRMS for $C_{44}H_{69}N_7O_{13}$: calcd. 904.5026 $[M + H]^+$, found 904.5003.

7-Bis(2-iodoethyl)amino-3-ethoxycarbonylcoumarin (15). Coumarin **10** (0.11 g, 0.31 mmol) was stirred with NaI (97 mg, 0.64 mmol) in refluxing acetone for 16 h. The reaction mixture was reduced under vacuum and the residue was purified by chromatography (AcOEt/*n*-hexane 30 : 70 to 70 : 30) to yield **15** (0.16 g, 98%) as a yellow powder. Mp 160 °C. TLC R_f 0.6 (Et_2O). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.43 (s, 1H); 7.44 (d, J = 9.0 Hz, 1H); 6.60 (dd, J = 9.0, 2.1 Hz, 1H); 6.84 (d, J = 2.1 Hz, 1H); 4.37 (q, J = 7.2 Hz, 2H); 3.84 (t, J = 8.4 Hz, 2H); 3.25 (t, J = 8.4 Hz, 2H); 1.39 (t, J = 7.2 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 163.6; 157.9; 157.4; 150.9; 148.8; 131.3; 111.4; 109.3; 109.1; 97.6; 61.4; 53.8; 14.3; –0.7. IR (film) ν 3071; 2981; 2921; 1732; 1587 MS (ESI) m/z 542 $[M + H]^+$; 564 $[M + Na]^+$.

7-[(4-*tert*-Butoxycarbonylmethyl)piperazin-1-yl]-3-ethoxycarbonylcoumarin (16). Yellow powder. Mp 127 °C. TLC R_f 0.6 (AcOEt). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.43 (s, 1H); 7.40 (d, J = 7.9 Hz, 1H); 6.80 (dd, J = 7.9, 2.1 Hz, 1H); 6.64 (d, J = 2.1 Hz, 1H); 4.37 (q, J = 6.9 Hz, 2H); 3.49 (m, 4H); 3.20 (s, 2H); 2.76 (m, 4H); 1.47 (s, 9H); 1.39 (t, J = 6.9 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 169.2; 164.1; 158.1; 158.0; 155.2; 149.1; 130.8; 111.6; 111.5; 109.4; 99.7; 81.7; 61.5; 59.7; 52.3; 47.0; 28.3; 14.5. IR (film) ν 2966; 2923; 2850; 1732; 1590. MS (ESI) m/z 417 $[M + H]^+$; 439 $[M + Na]^+$. ESI-HRMS for $C_{22}H_{28}N_2O_6$: calcd. 417.2020 $[M + H]^+$, found 417.2018.

8-[*N*,*N*-Di(*tert*-butoxycarbonylmethyl)aminomethyl]-3-ethoxycarbonyl-7-hydroxycoumarin (18). Aqueous formaldehyde (37%, 0.19 mL, 2.50 mmol) was added to 3-ethoxycarbonyl-7-hydroxycoumarin **17** (0.50 g, 2.10 mmol) and iminodiacetic acid di-*tert*-butyl ester (0.53 g, 2.10 mmol) in *tert*-butanol (10 mL). The mixture was refluxed for 15 h, solvent was removed under vacuum, and the residue was purified by silica gel chromatography (CH_2Cl_2) to yield compound **18** (0.79 g, 76%) as a yellow solid. Mp 113 °C. TLC R_f 0.6 (Et_2O). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.47 (s, 1H); 7.42 (d, J = 8.5 Hz, 1H); 6.87 (d, J = 8.5 Hz, 1H); 4.38 (q, J = 7.2 Hz, 2H); 4.20 (s, 2H); 3.41 (s, 4H); 1.49 (s, 18H); 1.39 (t, J = 7.2 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.0; 165.0; 163.6; 156.9; 155.5; 149.9; 130.8; 115.3; 112.7; 110.8; 108.5; 82.6; 61.6; 55.6; 48.1; 28.3; 14.5. IR (film) ν 3307 (b); 3227; 2979; 2930; 1753; 1599. MS (ESI) m/z 492 $[M + H]^+$. ESI-HRMS for $C_{25}H_{33}NO_9$: calcd. 492.5382 $[M + Na]^+$, found 492.5369.

7-{2-[Bis(*tert*-butoxycarbonylmethyl)amino]ethoxy}-8-[bis(*tert*-butoxycarbonylmethyl)aminomethyl] - 3 - ethoxycarbonylcoumarin (19). A mixture of DIAD (0.15 mL, 0.74 mmol) and PPh_3 (0.17 g, 0.61 mmol) in anhydrous THF (4 mL) was added by portion to coumarin 18 (0.20 g, 0.40 mmol) and *N*-(2-hydroxyethyl)iminodiacetic acid di-*tert*-butyl ester (0.12 g, 0.40 mmol) in THF (4 mL) until starting material completely disappeared. The reaction mixture was stirred for 2 h at rt before the solvent was removed under reduced pressure. The residue was triturated with Et_2O and the precipitate was filtered off. The filtrate was reduced under vacuum and the residue was purified by chromatography (AcOEt/n-hexane 50 : 50) to yield 19 (0.14 g, 47%) as a yellow oil. TLC R_f 0.3 (AcOEt/n-hexane 50 : 50). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.41 (s, 1H); 7.44 (d, J = 8.7 Hz, 1H); 6.90 (d, J = 8.7 Hz, 1H); 4.35 (q, J = 7.1 Hz, 2H); 4.23 (t, J = 6.1 Hz, 2H); 4.15 (s, 2H); 3.56 (s, 4H); 3.51 (s, 4H); 3.19 (t, J = 6.1 Hz, 2H); 1.41 (s, 18H); 1.36 (s, 18H); 1.35 (t, J = 7.1 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171.5; 171.2; 164.1; 163.3; 157.1; 155.9; 149.6; 130.6; 116.1; 114.7; 112.4; 109.7; 81.7; 81.1; 68.9; 62.1; 57.5; 56.6; 53.9; 46.7; 28.7; 14.8. IR (film) ν 2978; 2929; 1731; 1605. MS (ESI) m/z 764 $[M + H]^+$; 786 $[M + Na]^+$. ESI-HRMS for $C_{39}H_{58}N_2O_{13}$: calcd. 763.4012 $[M + Na]^+$, found 763.3992.

6,8-Bis[*N*,*N*-di(*tert*-butoxycarbonylmethyl)aminomethyl]-3-ethoxycarbonyl-7-hydroxycoumarin (20). Aqueous formaldehyde (0.48 mL, 5.8 mmol) was added to iminodiacetic acid di-*tert*-butyl ester (1.15 g, 4.6 mmol) in MeOH/*t*-BuOH (10 mL). The resulting solution was stirred at rt for 90 min. Then solvent was removed under vacuum and the residue was coevaporated twice with anhydrous MeOH (4 mL). Coumarin **17** (0.55 g, 2.3 mmol) was then added, followed by *N*-methylmorpholine (1.5 mL, 13.3 mmol) and the reaction mixture was stirred in a sealed vessel at 115 °C for 5 h and for 16 h at rt. Volatile was removed under reduced pressure and the residue was dissolved in AcOEt, washed with water and brine, dried over $MgSO_4$, and purified by flash chromatography (CH_2Cl_2/AcOEt 80 : 20 to 50 : 50) to yield compound **20** (0.23 mg, 13%) as a yellow solid. Mp 115 °C. TLC R_f 0.35 (CH_2Cl_2/AcOEt 80 : 20). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.49 (s, 1H); 7.69 (s, 1H); 4.37 (q, J = 7.2 Hz, 2H); 4.20 (s, 2H); 3.97 (s, 2H); 3.47 (s, 4H); 3.34 (s, 4H); 1.47 (s, 36H); 1.38 (t, J = 7.2 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.2; 169.7; 162.6; 163.1; 156.5; 154.3; 149.4; 130.0; 123.6; 112.1; 110.1; 108.5; 81.7; 80.8; 61.0; 55.3; 55.0; 51.5; 47.0; 27.9; 27.8; 13.9. IR (film) ν 2977; 2929; 1730; 1613; 1135. MS (ESI) m/z 750 $[M + H]^+$; 772 $[M + Na]^+$. ESI-HRMS for $C_{38}H_{56}N_2O_{13}$: calcd. 749.3855 $[M + H]^+$, found 749.3841.

6,8-Bis[*N*,*N*-di(*tert*-butoxycarbonylmethyl)aminomethyl]-7-{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethoxy}-3-ethoxycarbonylcoumarin (21). DIAD (47 µL, 0.24 mmol) was added dropwise at rt to a stirred solution of *N*-(2-hydroxyethyl)iminodiacetic acid di-*tert*-butyl ester (0.55 mg, 0.19 mmol), coumarin 20 (118 mg, 0.16 mmol), and PPh_3 (62 mg, 0.24 mmol) in anhydrous THF (2 mL). The reaction mixture was stirred for 3 h, the solvent was removed under vacuum and the residue was purified by chromatography (toluene/CH_2Cl_2/AcOEt 3 : 5 : 0.5 to 3 : 5 : 2) to yield compound 21 (69 mg, 43%) as a yellow oil. TLC R_f 0.6 (toluene/CH_2Cl_2/AcOEt 3 : 5 : 2). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.49 (s, 1H); 7.92 (s, 1H); 4.38 (q, J = 7.2 Hz, 2H); 4.17 (t, J = 6.1 Hz, 2H); 4.15 (s, 2H); 3.99 (s, 2H); 3.55 (s, 4H); 3.53 (s, 4H); 3.42 (s, 4H); 3.19 (t, J = 6.1 Hz, 2H); 1.45 (s, 36H); 1.40 (s, 18H); 1.38 (t, J = 7.2 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.6; 170.5; 170.4; 163.2; 162.7; 156.4; 154.4; 148.9; 130.6; 130.1; 120.6; 115.9; 114.3; 81.0; 80.8; 80.7; 74.8; 61.6; 56.4; 55.5; 55.3; 54.2; 51.7; 46.7; 28.2; 28.1; 14.2. IR (film) ν 2977; 2931; 1728; 1139. MS (ESI) m/z 1021 $[M + H]^+$; 1043 $[M + Na]^+$. ESI-HRMS for $C_{52}H_{81}N_3O_{17}$: calcd. 1020.5639 $[M + H]^+$, found 1020.5620.

5,7-Dihydroxy-3-ethoxycarbonylcoumarin (25). Diethyl (*N*,*N*-diethylaminomethylene)malonate (1.92 g, 7.90 mmol) was added to anhydrous phloroglucinol (1.00 g, 7.90 mmol) in AcOH (25 mL). The reaction mixture was refluxed for 8 h, then cooled down to rt. The precipitate was filtered and washed with $CHCl_3$ to yield coumarin **25** (1.60 g, 81%) as an orange powder. Mp > 250 °C (dec.). TLC R_f 0.4 (AcOEt). ^{1}H-NMR (CD_3OD, 300 MHz) δ 8.85 (s, 1H); 6.24 (d, J = 1.8 Hz, 1H); 6.22 (d, J = 1.8 Hz, 1H); 4.33 (q, J = 7.1 Hz, 2H); 1.37 (t, J = 7.1 Hz, 3H). ^{13}C-NMR (DMSO-d_6,

50 MHz) δ 166.7; 163.9; 159.4; 158.8; 157.6; 145.5; 109.2; 102.4; 99.4; 94.9; 61.5; 15.1. IR (film) ν 3296 (b); 1726; 1592; 1356. UV-vis λ_{max} 362 (MeOH, pH 7.0). MS (ESI) m/z 251 $[M + H]^+$; 273 $[M + Na]^+$. ESI-HRMS for $C_{12}H_{10}O_6$: calcd. 273.0370 $[M + Na]^+$, found 273.0351.

6,8-Bis[*N*,*N*-bis(*tert*-butoxycarbonylmethyl)aminomethyl]-5,7-dihydroxy-3-ethoxycarbonylcoumarin (26). Aqueous formaldehyde (37%, 15 µL, 0,40 mmol) was added to a solution of coumarin **25** (50 mg, 0.20 mmol) and iminodiacetic acid di-*tert*-butyl ester (0.97 mg, 0.40 mmol) in *tert*-BuOH (1 mL). The reaction mixture was refluxed for 5 h and reduced under vacuum to yield compound **26** (0.19 g, 97%) as a yellow powder. That compound was used without further purification. Analytical sample was obtained by preparative TLC (CH_2Cl_2/AcOEt 6 : 4). Mp 130 °C. TLC R_f 0.8 (CH_2Cl_2/Et_2O 8 : 2). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.98 (s, 1H); 4.35 (q, $J = 7.2$ Hz, 2H); 4.07 (s, 2H); 4.01 (s, 2H); 3.41 (s, 4H); 3.38 (s, 4H); 1.49 (s, 36H); 1.37 (t, $J = 7.2$ Hz, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.6; 170.4; 164.1; 158.3; 157.9; 155.2; 146.1; 110.1; 105.1; 102.6; 99.9; 82.9; 82.8; 61.6; 55.9; 55.6; 48.8; 48.1; 28.6; 14.9. IR (film) ν 2977; 2929; 1731; 1614; 1133. MS (ESI) m/z 766 $[M + H]^+$. ESI-HRMS for $C_{38}H_{56}N_2O_{14}$: calcd. 765.3804 $[M + H]^+$, found 765.3788.

5,7-Bis{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethoxy}-6,8-bis[*N*,*N*-bis(*tert*-butoxycarbonylmethyl)aminomethyl]-3-ethoxycarbonylcoumarin (27). Compound **27** (42 mg, 51%) was obtained as a yellow glassy solid, starting from **26** and following the same procedure as for **19**. TLC R_f 0.7 (*n*-hexane/AcOEt 1 : 1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.06 (s, 1H); 4.38 (q, $J = 7.2$ Hz, 1H); 4,29 (m, 2H); 4.19 (m, 2H); 4,12 (bs, 2H); 4.00 (bs, 2H); 3.59 (s, 4H); 3.56 (s, 4H); 3.54 (s, 4H); 3.44 (s, 4H); 3.22 (m, 4H); 1.44-1.40 (m, 75H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171.3; 171.2; 170.9; 165.3; 164.1; 158.2; 157.0; 155.8; 145.8; 123.7; 117.7; 116.0; 110.6; 81.6; 81.4; 81.3; 81.1; 76.5; 62.2; 57.6; 56.9; 56.1; 55.1; 55.0; 54.8; 48.2; 47.5; 28.8; 14.9. IR (film) ν 2977; 2930; 1729; 1595; 1142. MS (ESI) m/z 1308 $[M + H]^+$; 1330 $[M + Na]^+$. ESI-HRMS for $C_{66}H_{106}N_4O_{22}$: calcd. 1307.7371 $[M + H]^+$, found 1307.7349.

5,7-Bis{2-[bis(*tert*-butoxycarbonylmethyl)amino]ethoxy}-6,8-bis[*N*,*N*-bis(*tert*-butoxycarbonylmethyl)aminomethyl]-3-(2-[2-{2-(*N*-maleimidyl)ethoxy}ethoxy]ethylaminocarbonyl)coumarin (28). Coumarin **27** (0.37 g, 0.28 mmol) in *tert*-BuOH (5 mL) was sonicated for 5 min with NaOH 1 N (0.56 mL, 0.56 mmol) and stirred for 30 h at rt. Then HCl 1 N (0.56 mL, 0.56 mmol) was added and the reaction mixture was evaporated to dryness. The residue was suspended in anhydrous CH_2Cl_2 (2 mL), DCC (0.07 g, 0.34 mmol) in CH_2Cl_2 (2 mL) was added at 0 °C, followed by NHS (0.04 g, 0.34 mmol), and the solution was stirred for 20 h at rt. Compound **31** (0.12 g, 0.34 mmol) in anhydrous CH_2Cl_2 (2 mL) and *N*,*N*-di-*iso*-propyl-ethylamine (58 µL, 0.34 mmol) were added to the previously activated ester and the reaction mixture was stirred at rt for 5 h, reduced under vacuum, and purified by silica gel chromatography (*cyclo*-hexane/AcOEt 6 : 4) to yield coumarin **28** (0.22 g, 53%) as a yellow oil. TLC R_f 0.3 (*cyclo*-hexane/AcOEt 1 : 1). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 9.29 (s, 1H); 8.95 (bs, 1H); 6.71 (s, 2H); 4.30–4.15 (m, 4H); 4.13 (s, 2H); 4.02 (s, 2H); 3.78–3.66 (m, 10H); 3.60 (m, 10H); 3.56 (s, 4H); 3.54 (s, 4H); 3.45 (s, 4H); 3.26–3.21 (m, 4H); 1.45 (s, 18H); 1.43 (s, 18H); 1.39 (s, 36H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171.4; 171.3; 171.2; 171.0; 165.0; 162.5; 161.6; 158.4; 155.1; 145.4; 134.8; 124.1; 117.6; 116.3; 111.6; 81.4; 81.2; 81.1; 76.3; 71.2; 70.7; 70.4; 68.6; 57.6; 56.9; 56.1; 55.1; 54.8; 49.8; 47.5; 40.2; 37.8; 28.8. IR (film) ν 3323; 2923; 2851; 1714; 1625; 1145. MS (ESI) m/z 1491 $[M + H]^+$; 1513 $[M + Na]^+$. ESI-HRMS for $C_{74}H_{116}N_6O_{25}$: calcd. 1489.8063 $[M + H]^+$, found 1489.8039.

***tert*-Butyl 2-[2-(2-maleimidoethoxy)ethoxy]ethylcarbamate (30).** A freshly prepared mixture of DIAD (1.20 mL, 6.0 mmol) and PPh_3 (1.20 g, 4.5 mmol) in anhydrous THF (10 mL) was added dropwise to a solution of protected aminoalcohol **29** (0.75 g, 3.0 mmol) and maleimide (0.73 g, 7.5 mmol) in THF (5 mL) at rt. The reaction mixture was stirred for 2 h, reduced under vacuum, and triturated with Et_2O. Precipitated Ph_3PO was removed by filtration, the filtrate was reduced under vacuum, and the residue was purified by chromatography (*cyclo*-hexane/AcOEt 9 : 1) to yield **30** (0.73 g, 74%) as a colorless oil. TLC R_f 0.3 (*cyclo*-hexane/AcOEt 7 : 3). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.64 (s, 2H); 5.05 (m, 1H); 3.64 (t, $J = 5.4$ Hz, 2H); 3.55 (t, $J = 5.4$ Hz, 2H); 3.48 (m, 4H); 3.40 (t, $J = 5.1$ Hz, 2H); 3.18 (td, $J = 5.1$, 5.1 Hz, 2H); 1.35 (s, 9H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171.1; 156.4; 134.6; 79.5; 70.6; 70.3; 68.2; 40.8; 37.5; 28.8. IR (film) ν 3368; 2975; 2929; 2871; 1701; 1101. MS (ESI) m/z 329 $[M + H]^+$; 351 $[M + Na]^+$.

[2-(2-Maleimidoethoxy)ethoxy]ethylamine hydrochloride (31). Compound **30** (0.14 g, 0.42 mmol) was stirred in TFA/$CHCl_3$ (3 : 4, 4 mL) for 2 h at rt. Volatile was removed under reduced pressure and the residue was coevaporated twice with $CHCl_3$, dissolved in Et_2O, and washed with HCl 10%. The organic layer was dried over $MgSO_4$ and evaporated to dryness to yield **31** (0.11 g, 81%) as a colorless glassy solid. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.74 (s, 2H); 3.72–3.68 (m, 4H); 3.62–3.57 (m, 6H); 3.20–3.16 (m, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171.6; 134.9; 70.7; 70.3; 68.7; 67.2; 40.3; 37.8. MS (ESI) m/z 229 $[M + H]^+$.

General procedure for the preparation of europium complexes

$EuCl_3 \cdot 6H_2O$ 0.1 M (1 eq) was added to a solution of the appropriate ligand (20 mg, 1 eq) in water (1 mL) and the resulting mixture was stirred for 20 h at rt. The solution was brought to pH 7 with NaOH 0.2 M and was lyophilized. The residue was triturated with MeOH (2 × 2 mL) and the precipitate was collected after filtration or centrifugation.

[Eu(3b)]. Yellow powder (91%). UV-vis λ_{max} 329 (H_2O, pH 6.5). ESI-HRMS for $C_{22}H_{22}EuN_3O_{12}$: calcd. 674.0489 $[M + H]^+$, found 674.0460.

[Eu(3c)]. Yellow powder (79%). UV-vis λ_{max} 327 (H_2O, pH 6.5). ESI-HRMS for $C_{28}H_{34}EuN_7O_{13}$: calcd. 830.1500 $[M + H]^+$, found 830.1468.

[Eu(4a)]. Yellow powder (93%). UV-vis λ_{max} 341 (H_2O, pH 6.5). ESI-HRMS for $C_{23}H_{24}EuN_2O_{13}$: calcd. 689.0485 $[M + H]^+$, found 689.0469.

[Eu(5a)]. Yellow powder (81%). IR (film) ν 3391; 1592; 1393. UV-vis λ_{max} 334 (H_2O, pH 6.5). ESI-HRMS for $C_{28}H_{30}EuN_3O_{17}$: calcd. 834.0863 $[M + H]^+$, found 834.0832.

[Eu(6a)]. Yellow powder (66%). ESI-HRMS for $C_{22}H_{21}EuN_2O_{14}$: calcd. 691.0278 $[M + H]^+$, found 691.0259.

[Eu(7a)]. Yellow powder (86%). IR (film) ν 3379; 1614; 1393. UV-vis λ_{max} 326 (H_2O, pH 6.5). ESI-HRMS for $C_{34}H_{39}EuN_4O_{22}$: calcd. 1009.1341 $[M + H]^+$, found 1009.1319.

[Eu(7d)]. Yellow powder (75%). UV-vis λ_{max} 326 (H_2O, pH 6.5). ESI-HRMS for $C_{42}H_{49}EuN_6O_{25}$: calcd. 1191.2032 $[M + H]^+$, found 1191.2011.

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