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A Versatile Long-Wavelength-Absorbing Scaffold for Eu-Based Responsive Probes

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Dedicated to Professor József Rábai

Abstract: Coumarin-sensitized, long-wavelength-absorbing luminescent Eu^{III}-complexes have been synthesized and characterized. The lanthanide binding site consists of a cyclen-based chelating framework that is attached through a short linker to a 7-hydroxycoumarin, a 7-$B(OH)_2$-coumarin, a 7-*O*-(4-pinacolatoboronbenzyl)-coumarin or a 7-*O*-(4-methoxybenzyl)-coumarin. The syntheses are straightforward, use readily available building blocks, and proceed through a small number of high-yielding steps. The sensitivity of coumarin photophysics to the 7-substituent enables modulation of the antenna-absorption properties, and thus the lanthanide excitation spectrum. Reactions of the boronate-based functionalities (cages) with H_2O_2 yielded the corresponding 7-hydroxycoumarin species. The same species was produced with peroxynitrite in a $\times 10^6$–10^7-fold faster reaction. Both reactions resulted in the emergence of a strong $\approx$407 nm excitation band, with concomitant decrease of the 366 nm band of the caged probe. In aqueous solution the methoxybenzyl caged Eu-complex was quenched by $ONOO^-$. We have shown that preliminary screening of simple coumarin-based antennae through UV/Vis absorption spectroscopy is possible as the changes in absorption profile translate with good fidelity to changes in Eu^{III}-excitation profile in the fully elaborated complex. Taken together, our results show that the 7-hydroxycoumarin antenna is a viable scaffold for the construction of turn-on and ratiometric luminescent probes.

Keywords: coumarins · cyclen · europium · fluorescent probes · lanthanides · reactive oxygen species

Introduction

Lanthanide-based luminescent probes have come a long way since their first applications as spectroscopically active Ca substitutes in proteins.[1] Complexes of increasing sophistication are applied to monitor biologically relevant targets in vitro and in cellulo.[2] The popularity of lanthanide-based probes can be ascribed to their exceptionally long excited-state lifetimes, which enables the filtering-out of sample autofluorescence and scattered excitation light.[2] Whereas lanthanide absorption coefficients are typically $\approx$1–10, multidentate chelating frameworks that can shield the metal from solvent O–H oscillators in combination with light-harvesting substituents ("antennae") can increase the brightness of the complex by several orders of magnitude.[2]

Responsive lanthanide probes can be obtained by analyte-dependent regulation of antenna photophysics,[2b–d,f,3] the antenna-lanthanide energy-transfer,[2b–d,f,3] or the Ln-emission lifetime.[4] Turn-on probes are preferred to those for which detection is accompanied by a change in signal intensity due to their inherently larger sensitivity. However, such probes require calibration before they can report on analyte concentrations. Additional factors, for example, antenna bleaching, and fluctuations in excitation light intensity and probe concentration can complicate the situation. These unpredictable variations can be eliminated by the use of ratiometric probes.

Of all the known antennae a few stand out as particularly attractive: Fluorescein and rhodamine are good sensitizers for the near infrared (NIR) emitting Yb and Nd,[5] and strategies exist to transpose the rich chemistry of fluorescein-based responsive probes into the lanthanide probes domain.[6] A major drawback is the awkward synthesis of fluorescein derivatives.[7] Additionally, most laboratories are not equipped to perform NIR emission spectroscopy, and fluorescein does not sensitize the visible-emitting lanthanides (Eu, Tb). Parker and co-workers have developed Eu-sensitizing azaxanthones and azathioxanthones.[8] Precursors to these antennae are readily available on multigram scale in a two-step process from substituted (thio)phenols and

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various 2-chloronicotinic acids.[8b] Complexes incorporating such antennae have been used for ratiometric oxygen detection in vitro, as well as the monitoring of changes in intracellular bicarbonate concentration and lysosomal pH.[8j,n,o] However, the drawback of these probes is that the sensing mechanism seems to limit them to the handful of reported analytes.

Esterified, amidated, or carbamoylated phenol or aniline antennae can undergo chemoselective ester, amide, or carbamate cleavage with concomitant changes in the absorption/excitation spectrum, and/or lanthanide sensitization efficiency.[9] Reactivity-based turn-on or ratiometric probes have been constructed to detect H_2O_2 and to measure the activity of enzymes such as phosphatases or peptidases.[9] Analyte-dependent installation of the OH group has been reported for the detection of OH$^{\bullet}$.[10] A serious limitation of these probes is their excitation maxima, which rarely extend beyond 300 nm. Such high-energy light can be damaging to biomolecules, and is poorly suited for in vivo work.

This work was carried out to investigate coumarin-sensitized Eu-complexes for reactivity-based analyte detection. In addition to absorbing at >320 nm and emitting light at >400 nm with high fluorescence quantum yield, coumarins are also easily synthesized from readily available precursors,[11] and possess appreciable two photon absorption.[12] Coumarin absorption and emission spectra are sensitive to the electronic properties of the 7-substituent, with electron-donating groups affecting a bathochromic shift (Figure 1).[11]

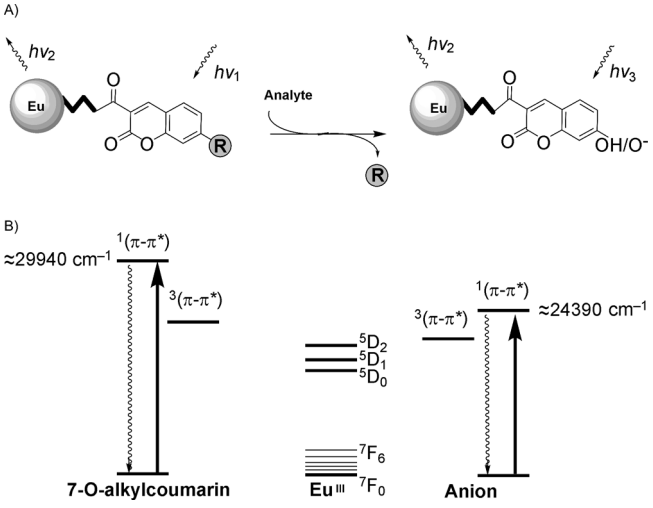


Figure 1. a) Responsive luminescent probes based on analyte-modulated changes in the photophysical properties of the coumarin antenna. b) Energy diagram of 7-O-alkylated and 7-phenolate coumarins and the most likely accepting levels of Eu^{III}. The location of the coumarin triplet-states in relation to the Eu^{III} $^5D_2$ level is not known.

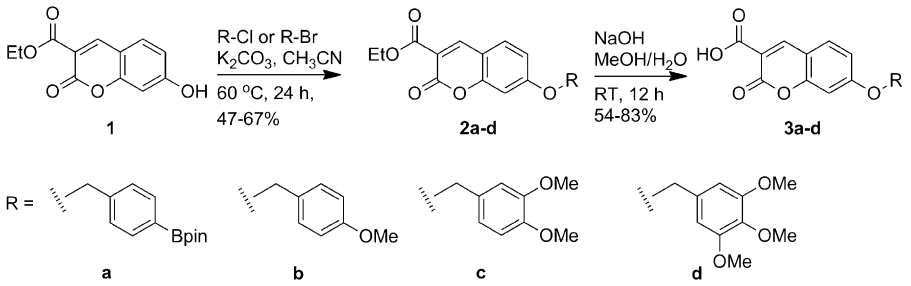
If coupled to lanthanide emission, such changes offer the possibility of ratiometric luminescent probe development. Such a design was supported by literature data that placed the absorption maxima of 7-*O*-alkylcoumarins at 330–360 nm, and that of the corresponding phenolate at ≈370–410 nm.[13] Additionally, the redshift in absorption maximum upon OH deprotonation would enable the construction of Eu^{III}-based pH indicators. Encouragingly, the triplet energy levels of 7-*O*-alkyl, 7-OH and 7-phenolate coumarins were all reported to be above ≈ 20000 cm^{-1}.[14–16] Efficient sensitization requires an antenna triplet-lanthanide excited state energy gap of ≥ 2000 cm^{-1}. This puts the red end of the excitation of Eu^{III} complexes at around 430 nm, given that the most likely Eu^{III} acceptor level is at 17200 cm^{-1}.[2f] Coumarins,[17,18] and their N-analogous carbostyrils[19] have been employed for the sensitization of both Eu^{III} and Tb^{III}. Responsive probes incorporating coumarin antennae have been reported that rely on selective coumarin excited state quenching by nucleotides, dsDNA, or a proximal ketone.[18] Our group has reported a strategy relying on the in situ analyte-induced formation of the sensitizing coumarin antenna.[20] However, the potential of controlling the coumarin excited state through the 7-substituent to regulate lanthanide emission has not been explored so far.

We decided to demonstrate the feasibility of our approach by the creation of responsive luminescent probes for reactive oxygen species (ROS). ROS and reactive nitrogen species (RNS) are attractive targets due to their roles in the emergence of a variety of pathologies (e.g., neurodegenerative diseases, asthma, stroke) on the one hand, and essential physiological functions on the other.[21] Key to such discoveries was the development of tools to monitor ROS with high sensitivity and selectivity.[22] In addition to the ones detecting HO$^{\bullet}$[10] and H_2O_2[9,20,23] mentioned above, lanthanide-based probes specific for NO,[24] $ONOO^-$,[25] $^1O_2$[26] are known.

Results and Discussion

Preliminary screening of alkylated coumarins: 7-Hydroxycoumarin derivatives O-alkylated with H_2O_2-cleavable 4-*p*-pinacolatoboron benzyl bromide, or one of three other benzyl bromides with differing electron-rich moieties were prepared. It was expected that by fine-tuning the substitution pattern in the benzyl ether we could discover caging groups that can be selectively cleaved by one of the ROS in preference of the others. Alternatively, differential responses to ROS might be exploited to obtain "fingerprints" for the different species. Such a strategy is reminiscent of the pattern-based recognition of peptides,[27a,b] terpenes,[27c] flavonoids[27d], and plastic explosives[27e] based on UV/Vis absorption changes. Alkylated coumarins **3a–d** were prepared from 7-hydroxycoumarin **1** (Scheme 1). Alkylation with the appropriate benzyl bromides or chlorides in the presence of K_2CO_3 afforded **2a–d** in good yields. Exposure to NaOH in a mixture of H_2O and MeOH yielded the carboxylic acids after aqueous/organic work-up and precipitation from methanolic solutions with Et_2O.

Methanolic solutions of **2a–d** and **3a–d** were treated with various ROS or RNS (H_2O_2, $O_2^{\bullet-}$, HO$^{\bullet}$, NO_2^-, the Supporting Information, Figures S1–S3). Boronate-caged **2a** and **3a**



Scheme 1.

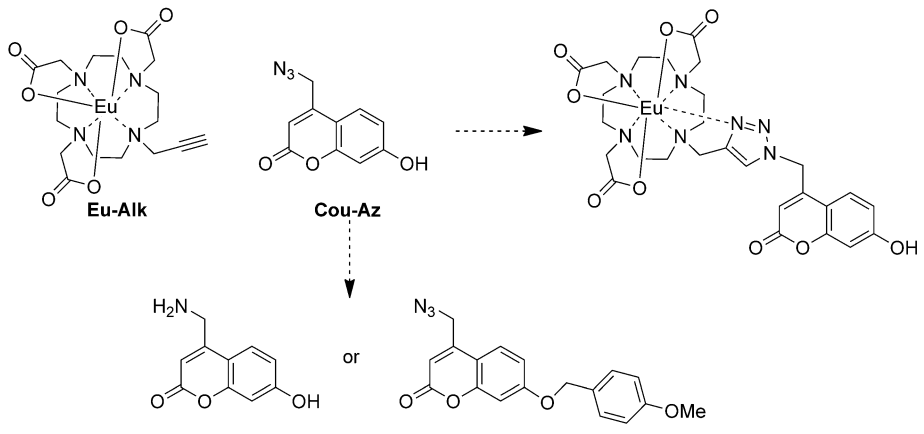
reacted with H_2O_2, as evidenced by a decrease in absorption at 332 nm and a concomitant increase at 387 nm. The spectrum of dimethoxybenzyl-caged **3c** did not change appreciably under these conditions, that of **3d** decreased by 25% in the presence of KO_2. Exposure of 4-methoxybenzyl-caged **3b** resulted in a decrease in absorption at 332 nm and the emergence of a shoulder that slowly developed into a new peak at 447 nm. Based on these results we decided to incorporate 7-(4-pinacolatoboronbenzyl)-coumarin and 7-(4-methoxybenzyl)-coumarin antennae into the Eu complexes.

Coumarin-appended lanthanide complexes: One of our aims was a readily accessible core structure that can serve as the starting point for luminescent probe development. Thus we developed a scalable, high yielding and short synthetic route. Initial attempts involved the synthesis of 4-azidomethyl-7-hydroxycoumarin (**Cou-Az**)[28] with the plan of introducing it into alkynyl-lanthanide complex **Eu-Alk**[29a] (Scheme 2) through a Cu-catalyzed azide–alkyne cycloaddition.[29] This route was particularly attractive due to its high convergence. However, upon exposure to a variety of cycloaddition reaction conditions, only complete decomposition of **Cou-Az** was observed. The lability of **Cou-Az** was confirmed by its rapid decomposition when we attempted to reduce the azide to the amine [PPh_3/H_2O, H_2/Pd(C)].

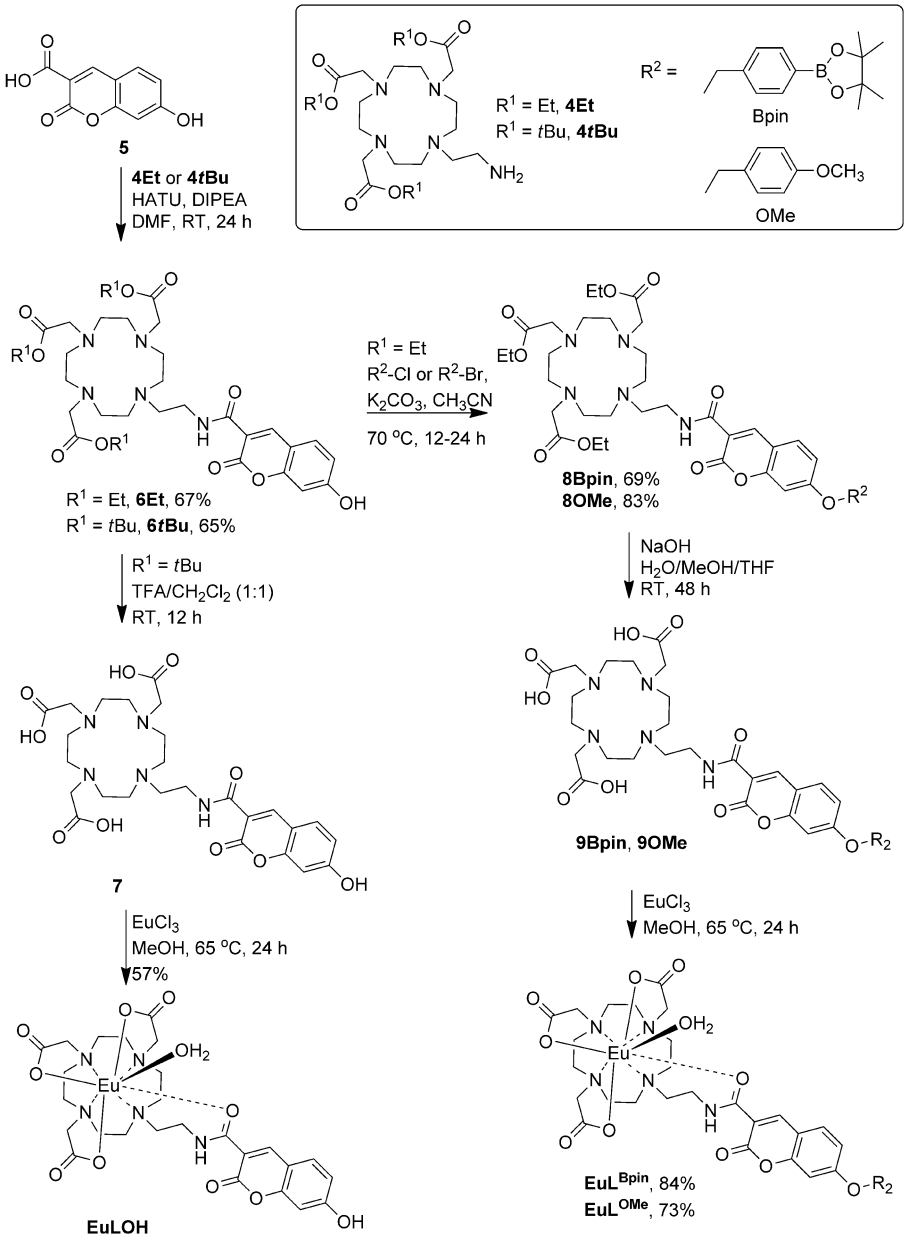
Cyclen derivatives **4Et** and **4*t*Bu**[30,31] were chosen as alternative scaffolds (Scheme 3). Both of these structures contain protected carboxylic acids that can later stabilize the lanthanide complex, as well as a primary amine that could serve as the point of attachment for the antennae. Additionally, they are readily prepared on a multigram scale (see the Supporting Information for the synthesis of **4Et**).[30] The primary amines in **4Et** or **4*t*Bu** and the carboxylic acid in **5** were reacted with HATU (1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) as the coupling reagent in the presence of DIPEA. Other activation methods (acid chloride, EDCI/HOBt) were inferior to HATU/DIPEA, resulting in either no product formation, or irreproducible yields. Key intermediates **6Et** and **6*t*Bu** were isolated in 67 and 65% yields, respectively. Exposure of **6*t*Bu** to a 1:1 mixture of TFA and CH_2Cl_2 for 18 h cleaved the *tert*-butyl esters quantitatively. Heating with $EuCl_3$ in MeOH, followed by precipitation with Et_2O afforded **EuL^{OH}** in 57% yield as a pale yellow solid (a discussion of the coordination environment of the Eu^{III} is given below).

The 7-hydroxycoumarin-bearing **6Et** was functionalized at the 7-OH position. Alkylation with 4-pinacolatoboron benzyl bromide or 4-methoxybenzyl chloride afforded **8Bpin** or **8OMe** cleanly in good yield (69–83%). Overalkylated quaternary ammonium products were not seen either by ESI-MS analysis or ^{1}H NMR analysis. The reaction could be monitored by TLC analysis despite the small differences in the polarities of starting materials and products by following the disappearance of the characteristic blue 7-OH coumarin-fluorescence. This diversification step on an advanced intermediate enables the efficient synthesis of a broad variety of complexes. Cleavage of the ethyl esters was possible with an excess of NaOH in a mixture of H_2O/MeOH/THF. Treatment of **9Bpin** or **9OMe** with $EuCl_3$ in hot MeOH, followed by precipitation with diethyl ether and freeze-drying from water yielded **EuL^{Bpin}** or **EuL^{OMe}** in 84 and 73% yield, respectively.

The synthesis of analogous complexes equipped with two photon absorbing 6,8-dibromocoumarin antennae[32] was attempted starting from 3,5-dibromo-2,4-dihydroxybenzaldehyde (the Supporting Information, Scheme S1).[33] However, this route was ultimately abandoned due to the instability of some of the intermediates. A discussion of the synthesis is given in the Supporting information.



Scheme 2.

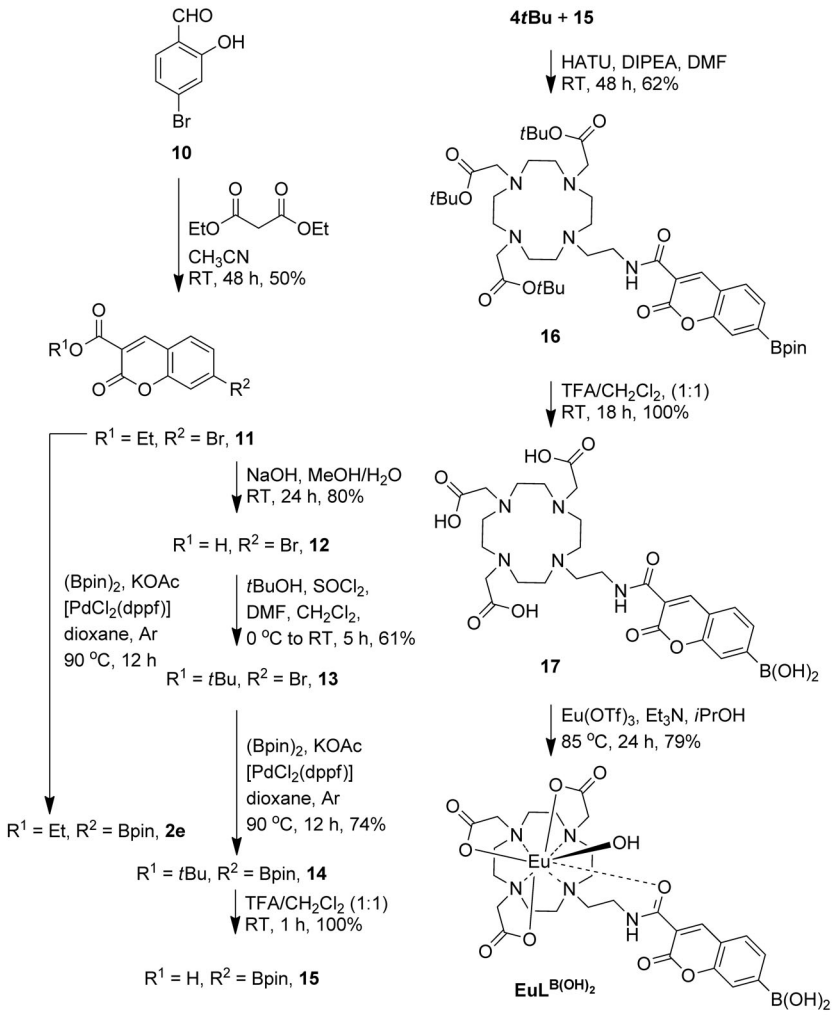


Scheme 3.

The effect of masking the 7-OH functionality as a boronic acid on the photophysical properties of the coumarin antenna and the corresponding Eu complex was also studied. Coumarin-7-boronic acid-functionalized **EuL$^{B(OH)2}$** was prepared as shown in Scheme 4. The base-labile ethyl ester in 7-bromocoumarin **11** was replaced with acid-cleavable *tert*-butyl ester in a two-step procedure. This was deemed necessary for synthetic tractability, as the boronate ester moiety might be destroyed when exposed to high concentrations of aqueous base. Whereas not detrimental per se, a mixture of poorly defined boronic acid and partially protected boronate ester species was expected to complicate further synthetic steps. Hydrolysis with aqueous NaOH provided carboxylic acid **12** in 80% yield after acidification of the reaction mixture and an aqueous/organic work-up. The crude product was >95% pure as shown by ^{1}H NMR analysis. In situ acid chloride formation with $SOCl_2$ and catalytic DMF,[34] followed by the addition of excess *tert*-butanol afforded **13** in 61% yield as a pale yellow solid. Alternative methods for the *tert*-butyl ester formation were less satisfactory. EDCI/DMAP mediated esterification[35] with *tert*-butanol gave the isolated **13** in 29–41% yield (48% based on recovered starting material). Reaction of **12** with *tert*-butyl trichloroacetimidate and catalytic $BF_3{\cdot}OEt_2$[36] afforded **13** in 54% yield, however, the purification of this product was tedious due to the presence of co-eluting impurities. Alternative carboxylic acid activation with carbonyl-diimidazole in DMF in the presence of DBU and *tert*-butanol[37] afforded 76% of a compound that was tentatively assigned as the decarboxylated derivative of **12**.

Miyaura borylation[38] (conditions: $(Bpin)_2$, $PdCl_2(dppf)$, KOAc, dioxane, and 90°C) provided **14** in good yield. Cleavage of the *tert*-butyl ester was quantitative in 1 h with TFA/CH_2Cl_2 (1:1). We found it necessary to dilute the reaction mixture with an equal volume of toluene prior to removal of the volatile components to avoid hydrolysis of the boronate ester. Carboxylic acid **15** was then coupled to **4*t*Bu** in the presence of HATU and DIPEA to afford **16** in 62% yield after column chromatography. Quantitative cleavage of the *tert*-butyl esters to obtain **17** necessitated overnight exposure of **16** to a 1:1 mixture of TFA and CH_2Cl_2, which resulted in complete hydrolysis of the boronate ester to the boronic acid. Treatment of ligand **17** with $Eu(OTf)_3$ gave **EuL$^{B(OH)2}$** in 79% yield after precipitation from MeOH with Et_2O.

Photophysical characterization of lanthanide complexes: The UV/Vis absorption spectra of coumarins, ligands and Eu-complexes were recorded in MeOH (the Supporting Information, Figures S4 and S5). All 7-O-benzylated species had strong ($\varepsilon \approx \times 10^4$) absorption bands in the 335–348 nm



Scheme 4.

region, attributed to a π–π* transition. The λ_{max} of this band depended on the 3-substituent (carboxylic acid, ethyl ester, or amide). The absorption spectra of coumarin-phenols **7** and **EuL^{OH}** and that of **EuL$^{B(OH)2}$** were pH-sensitive. Increasing the pH resulted in the emergence of a redshifted peak attributed to the phenolate[39] or boronate anion. For **EuL^{OH}** this new peak emerged at ≈405 nm, with an isosbestic point at 375 nm. The pK_a of **EuL^{OH}** was determined to be ≈6.8 by spectrophotometric titration (the Supporting Information, Figure S6), a typical value for a 3-carboxy-7-hydroxycoumarin.[39c] The spectra of the O-benzylated ligands and their Eu-complexes were similar to that of their respective antennae, but blueshifted by 10–12 nm (Table 1).

The excitation spectra of all Eu complexes were recorded in HEPES buffer (Figure 2). The spectra were similar to the absorption spectra, consisting of a single peak around 360 nm for **EuL^{Bpin}** and **EuL^{OMe}**. The excitation spectrum of **EuL^{OH}** consisted of a broad peak centered at 356 nm below pH 6.5. Above pH 7 this peak disappeared, and a new peak at 408 nm from the anion emerged. This redshifted peak extended up to ≈430 nm (Figure 3 and the Supporting Information, Figures S7 and S8). This shows that Eu can be sensitized by both the neutral and anionic forms of 7-hydroxycoumarin. It is worth noting that the pK_a of 7-hydroxycoumarins are easily variable between ≈3.7 and ≈7.8, which provides a straightforward way to increase the proportion of the long-wavelength-absorbing phenolate at physiological pH.[39c]

The lifetimes of the complexes in H_2O and D_2O were measured (Table 2). From this, the number of Eu-bound water molecules (q) were calculated using the equation $q = 1.2 \times [1/\tau_H - 1/\tau_D - (0.25 + 0.075x)]$, in which x = exchangeable NH-oscillators.[42,43] All complexes have similar hydration states ($q < 1$), which indicates that in addition to the three carboxylates, the amide carbonyl carbon in the linker moiety can also coordinate to the metal. The hydration state does not change upon cleavage of the H_2O_2-responsive boronate cages.

The quantum yields of the uncomplexed ligands and the Eu-complexes were determined (Table 2). The overall Eu quantum yields were 0.39, 0.45, and 2.70% for **EuL^{Bpin}**, **EuL^{OMe}**, and

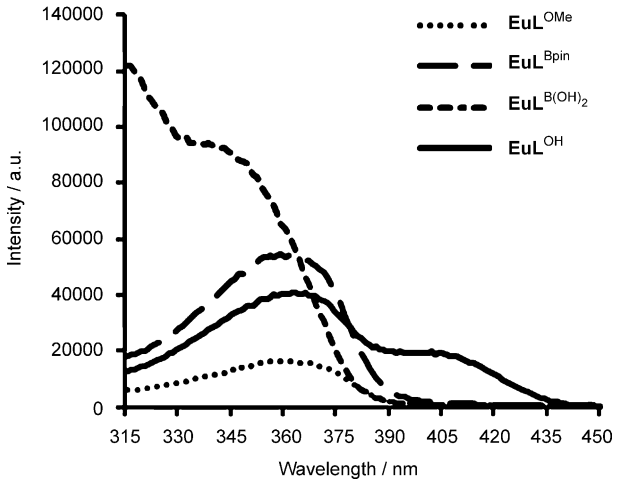


Figure 2. Excitation spectra of **EuL^{Bpin}**, **EuL^{OMe}**, **EuL$^{B(OH)2}$** and **EuL^{OH}** (λ_{em} = 700 nm, Eu complex (10 μM) in HEPES (10 mM), pH 7, RT).

Table 1. Absorption and emission data for Eu complexes and their precursors.[a]

Entry	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{em} [nm] (λ_{ex}/[nm])
1	351[b]	–	406[b]
5	339, 380[c]	4.21, 3.80[c]	450 (385)[c]
6Et	368, 409[d]	3.86, 4.09	407 (370)[d]
EuL^{OH}	355	4.04	410, 578, 587, 592, 615, 653, 700 (320)[a] 407, 578, 587, 592, 615, 653, 700 (373)[e]
2a	348	4.20	407 (370)
3a	347	4.20	407 (374)
8Bpin	338	4.00	407 (363)
EuL^{Bpin}	290, 334	3.72, 3.88	450, 578, 587, 615, 700 (367)[f]
2b	351	3.98	407 (373)
3b	350	4.20	413 (372)
8OMe	338	4.01	404 (363)
EuL^{OMe}	284, 338	3.76, 3.94	411, 578, 591, 615, 700 (359)[e]
14	297, 335	3.99, 3.68	422 (350)
15	297, 335[d]	4.28, 4.02	425 (365)
17	302, 336	3.91, 3.75	409 (367)
EuL$^{B(OH)2}$	312, 346, 408	3.85, 3.84, 3.25	443, 578, 587, 592, 595, 615, 688, 700 (346)[e]

[a] In MeOH. [b] From Ref. [40]. [c] From Ref. [41]. [d] In DMSO. [e] In HEPES (10 mM) at pH 7. [f] In H_2O.

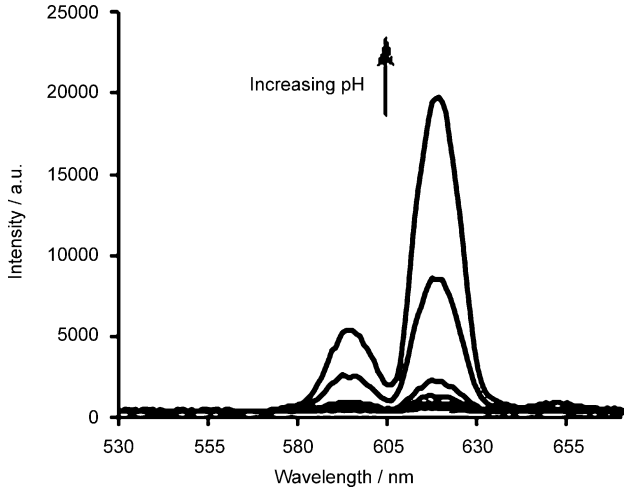


Figure 3. pH-dependence of the emission spectrum of **EuL^{OH}** (λ_{ex} = 410 nm, **EuL^{OH}** (10 μM) in HEPES (10 mM), RT, delay 50 μs, sample window 1050 μs, pH values: 4.0, 5.0, 6.4, 7.0, 7.5, 8.0, 8.3, 9.3, 10).

EuL^{OH}, respectively. These values are typical for coumarin-sensitized Eu-complexes,[17a] and are in the range reported for several well-performing responsive probes.[8c,9a,c,20,44] The coumarin fluorescence increased dramatically when going from O-alkylated- to free hydroxyl 7-substituents. This change is analogous to that observed for 7-substituted coumarins carrying modestly and strongly electron-donating groups. The same effect is seen upon phenol deprotonation. Both these observations are explained by the contribution of the electron-donating 7-substituent on the charge-transfer character of the $^1(\pi-\pi^*)$ level. From 2.60% (**EuL^{Bpin}**) and 0.90% (**EuL^{OMe}**), coumarin fluorescence quantum yield rose to 74.9% (**EuL^{OH}**). The increase in coumarin quantum yield upon Eu^{III} complexation compared with the free ligand may be down to a change in coumarin OH pK_a upon coordination of the amide carbonyl to the metal; the coumarin phenolate is significantly more emissive than the neutral form. Eu complexation decreased the coumarin fluorescence compared with that of the free ligand for **EuL^{Bpin}** and **EuL^{OMe}**, in the latter case quite dramatically, from 14.6 to 0.90%. This decrease could be due to the external heavy-atom effect of Eu.[45] Photoinduced electron-transfer from the antenna to Eu^{III} forming the relatively accessible Eu^{II} may also contribute to a diminished antenna quantum yield.[45]

Table 2. Photophysical properties of Eu complexes.

Entry	τ_{H_2O}[ms] (k[ms^{-1}])[a]	τ_{D_2O} [ms] (k[ms^{-1}])[a]	q	Φ [%][e]
EuL^{OH}	0.59 ± 0.02 (1.69) 0.41 ± 0.02[c]	0.66 ± 0.02 (1.52)	−0.2 (0.2)[c,d]	2.70[f] 74.9[g] (68.3)[h]
EuL$^{B(OH)2}$	1.15 ± 0.03 (0.870) 1.14 ± 0.02[b]	3.49 ± 0.30 (0.287) 3.12 ± 0.27 (0.321)	0.3 (0.6)[c]	n.d.[i]
EuL^{Bpin}	1.11 ± 0.04 (0.901) 1.02 ± 0.03[b]	2.61 ± 0.14 (0.383)	0.2 (0.5)[c]	0.39[f] 2.60[g] (3.95)[h]
EuL^{OMe}	1.16 ± 0.04 (0.862) 0.81 ± 0.04[b]	2.50 ± 0.17 (0.400)	0.2 (0.5)[c]	0.45[f] 0.90%[g] (14.6)[h]

[a] λ_{ex} = 338, λ_{em} = 614 nm in the solvent indicated. [b] Under an Ar atmosphere. [c] Calculated without correction for the proximal NH-oscillators by using $q = 1.05 \times (1/\tau_H - 1/\tau_D)$. [d] λ_{ex} = 363 nm. [e] In methanol, λ_{ex} = 360 nm. [f] Eu-quantum yield. [g] Coumarin quantum yield in complex. [h] Coumarin quantum yield in ligand. [i] n.d. = not determined.

The instrumentation available for us did not enable the determination of the triplet levels of the antennae. However, a lower estimate for the anionic triplet state of 19300 cm^{-1} (518 nm) can be obtained by monitoring the sensitivity of Eu-emission to quenching by oxygen. The presence of atmospheric oxygen did not appreciably affect the europium emission intensities and lifetimes of **EuL^{OH}**, **EuL^{Bpin}** and **EuL^{OMe}**. This invariance indicates that if energy transfer happens from the coumarin triplet state, it is fast and irreversible. Thus the triplet state is >2000 cm^{-1} above the 17300 cm^{-1} Eu^{III} excited state (the Supporting Information, Figure S9–S11, Table 2). The literature values of (22000 ± 300) cm^{-1} for the triplet state of 3-chloro-4-methyl-7-methoxycoumarin (analogous to the antenna in **EuL^{Bpin}** and **EuL^{OMe}**),[15] and 21320 cm^{-1} for 7-hydroxycoumarin[14] are consistent with this finding.

The Eu-emission intensity of **EuL$^{B(OH)2}$** decreased in the presence of oxygen compared with that of a deareated solution, indicating the sensitivity of the antenna excited state to quenching by triplet oxygen (the Supporting Information, Figure S12). A possible explanation is that the triplet state of **EuL$^{B(OH)2}$** lies below that of **EuL^{OH}**, and thus closer to the Eu excited state, making the back energy-transfer from the lanthanide to the antenna possible. The detailed photophysi-

cal investigation of all Eu complexes is in progress, and will be reported separately.

Reactions of Eu-complexes with ROS: Aryl boronates are well-established H_2O_2-selective switches. Boronate cages are often incorporated into ROS-sensitive prodrugs,[46] prochelators,[47] and fluorescent H_2O_2 probes[9a,22a–e] that rely on the formation of the corresponding phenols when exposed to H_2O_2. The addition of H_2O_2 to a 1 mM solution of **EuL^{Bpin}** yielded a gradual decrease in Eu emission when the excitation was performed at 361 nm, and an increase upon excitation at 397 nm (Figure 4), indicative of the formation of

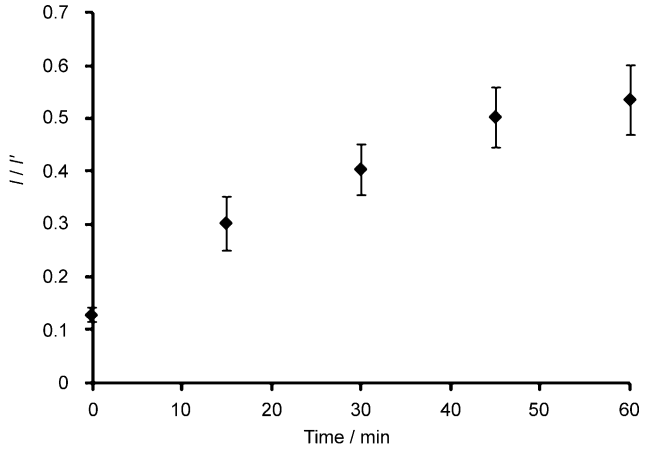
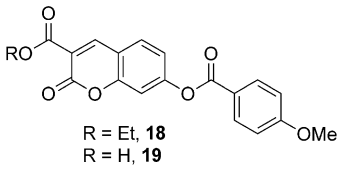


Figure 4. Changes in the ratio of the 698 nm Eu emission intensity with 361 nm and 397 nm excitation wavelengths in a sample of **EuL^{Bpin}** in the presence of 1 mM H_2O_2. I: intensity (a.u.) with $\lambda_{ex}=397$ nm, I': intensity (a.u.) with $\lambda_{ex}=361$ nm (**EuL^{Bpin}** (10 μM), pH 7 HEPES buffer (10 mM), RT).

EuL^{OH}. The complex **EuL$^{B(OH)2}$** behaved similarly to **EuL^{Bpin}**. The ratio of the 689 nm-peak intensities (excitation at 320 and 400 nm) increased from 0.54 to 1.50 in the presence of 100 μM H_2O_2 (the Supporting Information, Figures S13 and S14). These data reflect the differences in the excitation spectrum of **EuL$^{B(OH)2}$** on the one hand and **EuL^{OH}** on the other. The former is excitable at 320–340 nm, and not at 400 nm. **EuL^{OH}** is readily excitable at ≈400 nm and only modestly at 320–360 nm.

NMR/ESI studies: We followed the reactions of alkylated coumarins **2a** and **2b** with ROS by ESI-MS spectrometry and ^{1}H NMR spectroscopy. In accordance with the literature, the UV/Vis spectroscopic studies and the fluorescence measurements, compound **2a** reacted with H_2O_2 to afford the corresponding phenol **1**. The initial product arising from the reaction of **2a** and KO_2 in DMSO was tentatively identified as **18** (Scheme 5) by ESI-MS (m/z $[M+K+H_2O]^+$ 409.3, $[M+Na+H_2O]^+$ 393.3; the Supporting Information, Figures S15 and S16). In methanolic solution this species slowly hydrolyzed to the corresponding free acid **19** (m/z $[M+Na]^+$ 363.1), and to 7-OH coumarin **1** (m/z $[M+Na]^+$ 257.0). ^{1}H NMR spectroscopy in CD_3OD clearly showed the



Scheme 5.

disappearance of $CH_3CH_2O_2C$-signals (t, $\delta\approx1.30$ ppm and q, ≈3.77 ppm) and emergence of signals associated with free EtOH (t, $\delta\approx1.17$ ppm and q, ≈3.62 ppm).[48] ^{1}H NMR spectroscopy confirmed that **2b** did not react with H_2O_2 even under forcing conditions (1 mM H_2O_2, several hours).

We subjected **EuL^{Bpin}** and **EuL$^{B(OH)2}$** to a range of ROS and RNS (H_2O_2, OCl^-, *t*BuOOH, ·OH, NO_2^-, NO_3^-, $ONOO^-$, $^1O_2$, NO, O_2^-, Figure 5 and the Supporting Information, Figures S17–S19). Both probes displayed good selectivity for H_2O_2 over most ROS. Peroxynitrite was an exception, producing an instantaneous, robust decrease in the $\lambda_{abs}=360$ nm absorption, with a concomitant increase at $\lambda_{abs}=405$ nm (Figure 5 and the Supporting Information, Fig-

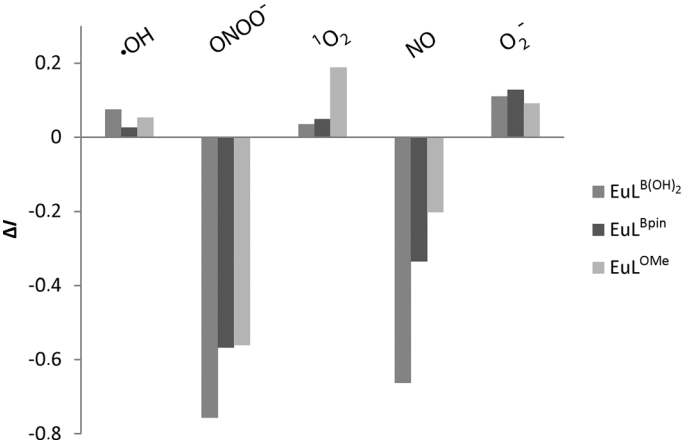


Figure 5. Reaction of Eu complexes with highly reactive ROS/RNS at pH 7.5 in the presence of catalase (Eu complex (10 μM), ROS/RNS (200 μM) in HEPES buffer (200 μL, 10 mM, pH 7.5, containing 0.1 mg mL^{-1} catalase), $\lambda_{ex}=356$, $\lambda_{em}=615$ nm, 50 μs delay, 1050 μs sample window). ·OH was generated in the Fenton system from $FeSO_4$ and H_2O_2. A stock solution of peroxynitrite was generated by mixing aqueous solutions of H_2O_2 (0.65 M) and KNO_2 (0.6 M), the resulting solution was treated with MnO_2 to eliminate excess H_2O_2. The concentration of peroxynitrite was determined by measuring its absorption at 302 nm ($\varepsilon=1670$). Singlet oxygen was prepared by mixing equimolar amounts of H_2O_2 and NaOCl in water. NO was generated from diethylenetriamine/nitric oxide adduct. The superoxide solution was generated from a mixture of xanthine (0.1 M) and xanthine oxidase (0.1 unit mL^{-1}).

ure S20). These data, in combination with ESI-MS analysis of the crude reaction mixture (m/z found: 728.7695; calcd for [**EuL^{OH}**+H]$^+$: 728.1434; m/z found: 750.7965; calcd for [**EuL^{OH}**+Na]$^+$: 750.1254) suggest that the product of the reaction of $ONOO^-$ is **EuL^{OH}**, the same as that with H_2O_2. In fact, Kalyanaraman and co-workers have recently found

that peroxynitrite reacted at least a million fold faster than H_2O_2 with alkyl and aryl boronates giving the corresponding alcohols.[49] Building on these findings, Yu et al. have developed the first $ONOO^-$-selective fluorescent probe consisting of pyreneboronic acid. This probe could be used to monitor $ONOO^-$ levels in PMA-stimulated macrophages.[50]

Peroxynitrite induced concentration-dependent changes in the Eu-emission of **EuL^{Bpin}** and **EuL$^{B(OH)2}$** at low micromolar concentrations (Figure 6 and the Supporting Information, Figure S20). The Eu-emission from methoxybenzyl ether-functionalized **EuL^{OMe}** was quenched by $ONOO^-$ (the Supporting Information, Figure S20). This result is interesting, as **EuL^{OMe}** does not react with H_2O_2, thus giving a potentially very selective response to a single ROS/RNS. Whereas a slow reaction was observed for the corresponding 7-methoxybenzyl coumarin antenna **2b** in DMSO with excess KO_2, the corresponding Eu complex was inert to this ROS in aqueous buffers, presumably due to the rapid dismutation of $O_2^{\bullet-}$. Quenching of Eu-emission was concentration dependent, and persisted long after the lifetime of $ONOO^-$ under the experimental conditions ($\tau_{1/2} \approx 1$ s at pH 7.4).[51] This suggests that **EuL^{OMe}** undergoes a reaction with $ONOO^-$, however, we have so far been unable to identify the reaction product. There is one known lanthanide-based $ONOO^-$-responsive ratiometric system, which consists of a mixture of methoxyphenyl-terpyridine Tb^{III}/Eu^{III} complexes. Sensing was achieved by selective Tb-emission quenching through a charge-transfer mechanism.[25]

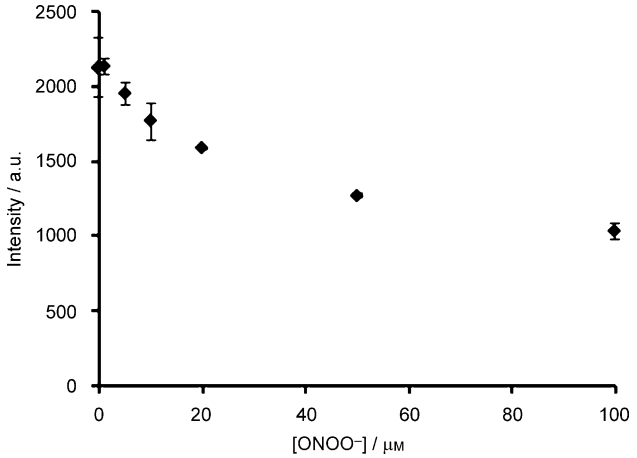


Figure 6. The Eu-intensity change of **EuL^{Bpin}** in the presence of various concentrations of $ONOO^-$ (30 min incubation time, **EuL^{Bpin}** (10 μM), HEPES buffer (10 mM), pH 7.5, $\lambda_{ex} = 356$, $\lambda_{em} = 615$ nm, 50 μs delay, 1050 μs sample window). A stock solution of peroxynitrite was generated by mixing aqueous solutions of H_2O_2 (0.65 M) and KNO_2 (0.6 M), the resulting solution was treated with MnO_2 to eliminate excess H_2O_2. The concentration of the peroxynitrite stock solution was determined by measuring its absorption at 302 nm ($\varepsilon = 1670$).

Pseudo first-order rate constants were determined for the reactions of **2a** and **2e** with excess H_2O_2 by following the changes in the coumarin absorption at 405 nm (the Supporting Information, Figures S21 and S22). Pinacolboronatobenzyl-caged **2a** reacted 5.2-fold faster than aryl pinacolatoboron-based **2e** ($0.900\ M^{-1}s^{-1}$ ($R^2 = 0.96$) vs. $0.173\ M^{-1}s^{-1}$, ($R^2 = 0.96$), respectively). These results are in line with those of Cohen and co-workers, who reported similar differences between boronates attached through benzyl ether self-immolative linkers and aryl boronates.[52] Pseudo-first-order rate constants for the reactions of **2a** and **2e** were measured using a stopped-flow instrument in fluorescence emission mode (the Supporting Information, Figures S23 and S24). This was required as nitrite absorbs at 354 nm, which interfered with the coumarin absorption measurements. Furthermore, it was necessary to use peroxynitrite as the limiting reagent, as at high peroxynitrite concentrations a decrease in emission output was recorded after an initial fluorescence emission intensity increase (the Supporting Information, Figure S25). The decrease in coumarin fluorescence is presumably due to uncontrolled oxidation of the relatively electron-rich hydroxycoumarin core, and is similar to that observed previously with other fluorescent redox indicators.[49b] Under these conditions, pseudo-first-order rate constants of $3.00 \times 10^3\ M^{-1}s^{-1}$ ($R^2 = 0.95$) and $1.68 \times 10^5\ M^{-1}s^{-1}$ ($R^2 = 0.97$) were obtained for **2a** and **2e**, respectively, corresponding to a 1 million-fold faster reaction for **2e** with $ONOO^-$ compared with that with H_2O_2. The change in order of reactivity with $ONOO^-$ compared to that with H_2O_2 presumably reflects a change in rate-determining step from C–B bond oxidation to quinone methide elimination for **2a**.

Peroxynitrite is a strong, but surprisingly selective oxidant with involvement in inflammation, stroke and neurodegeneration.[53] Low concentrations of peroxynitrite have also been shown to modulate cell signaling.[54] Based on the above results, **EuL^{Bpin}** and **EuL$^{B(OH)2}$** are selective probes for $ONOO^-$. However, it should be noted that the different intracellular lifetimes (H_2O_2: ≈10 ms[55] vs. $ONOO^-$: 10–20 ms),[56] concentrations (H_2O_2: low micromolar to nanomolar[57] vs. $ONOO^-$: nanomolar),[53,54] and localization profiles of these two species mean that H_2O_2 could potentially interfere with the detection of $ONOO^-$.

Conclusion

Europium complexes equipped with coumarin antennae have been prepared in short, concise, and scalable syntheses. The complexes can be rendered analyte-responsive through modulation of the electronic properties of the 7-position of the coumarin antenna. We have demonstrated this principle by the introduction of ROS/RNS-cleavable caging groups. Preliminary screening of potential antennae was possible as changes in the coumarin absorption/emission properties translated well to changes in the corresponding Eu-complex photophysics. This way both ratiometric and turn-on probes could be accessed. 7-Boronate or 7-*O*-(4-pinacolatoboronbenzyl) cages were rapidly cleaved by peroxynitrite to the corresponding phenol, furnishing the first ratiometric single-component luminescent probes for this RNS. We wish to point out that the design is not limited to the analytes used

in this work. A large number of biologically and environmentally relevant species install phenols selectively (for example, enzymes, metal ions ($Pd^{II/0}$, Hg^{II}), small molecules (H_2S)),[20,58] making the current ligands potentially very versatile.

Experimental Section

General Procedures: ^1H NMR (300 MHz, 400 MHz or 500 MHz) and ^{13}C NMR (75 MHz, 100 MHz or 125 MHz) spectra were recorded on a Varian 300, a Varian 400 or a Bruker 500 MHz instrument, respectively. Chemical shifts were referenced to residual solvent peaks and are given as follows: chemical shift (δ, ppm), multiplicity (s, singlet; br, broad singlet; d, doublet, t, triplet; q, quartet; m, multiplet), coupling constant (Hz), integration. IR experiments were performed on a PerkinElmer Spectrum-100 FT-IR spectrometer equipped with an ATR accessory. HR-ESI-MS analyses were performed on a Bruker MicroTOF ESI mass spectrometer. For accurate mass determination of Eu complexes the ^{153}Eu isotope was used. Compounds **1**,[13d] **4*t*Bu**,[30] **Eu-Alk**,[29] **Cou-Az**,[28] 4-bromosalicylaldehyde,[59] **S1**[33] and **S7**,[30] were synthesized following literature methods. All other chemicals were from commercial sources and used as received. Microwave heating was performed with a Biotage Initiator instrument. Titrations, selectivity measurements and kinetic experiments were carried out at least twice (independent), either representative or averaged data are shown.

Chromatography: Preparative chromatography was performed using silica (230–400 mesh). Thin layer chromatography was performed on silica-coated aluminum plates. Samples were visualized by UV-light (254 and 356 nm), or staining with $KMnO_4/K_2CO_3$ or cerium ammonium molybdate.

Photophysical measurements: Absorption spectra and fluorescence spectra were collected at room temperature in the solvent indicated at the experiment. UV/Vis absorption spectroscopy was performed on a Varian Cary 300 instrument; (sh) denotes shoulder to a peak. Steady state and time-resolved emission spectra were collected at room temperature using a Horiba Scientific FluoroMax 4 instrument equipped with a flash lamp (Tables 1 and 2, and S1 (the Supporting Information), Figures 2, 4, and the Supporting Information, S1–S5, S9–S13), or on a SpectraMax GEMINI EM Dual Scanning Microplate Spectrofluorometer in fluorescence scan or time-resolved fluorescence scan mode (Figures 3, 5, 6, and the Supporting Information, S6–S8, S14, and S17–S20). Hydration states (q) were determined according to the method developed by Horrocks,[42] and Parker and Beeby.[43] Emission intensity changes were fitted to single exponential decays using the instrument's software (FluorEssence Version 3.5.1.20, based on Origin 8.1090). Fitting to double exponential was less suitable based on R^2 and χ. Quantum yields were determined using the optically dilute method in methanol with coumarin 2 ($\Phi = 0.97$)[60] as the reference. Quantum yield measurements with this method carry an uncertainty of approximately $\pm 10\,\%$.[61]

Kinetics: Pseudo-first-order rate constants for the reactions of $\mathbf{EuL^{Bpin}}$, and $\mathbf{EuL^{B(OH)2}}$ with H_2O_2 were determined following the procedure of Major Jourden et al.[56] Pseudo-first-order rate constants for the reactions of **2b** and **2e** with peroxynitrite were carried out on an Applied Photophysics SX19 stopped flow machine as described in detail in the Figure legends.

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Fluorescent Probes

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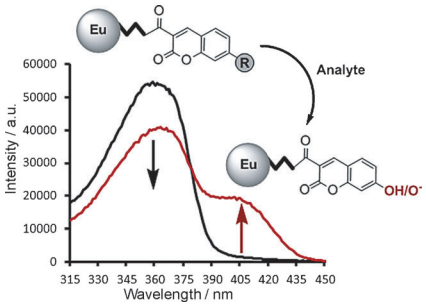
A Versatile Long-Wavelength-Absorbing Scaffold for Eu-Based Responsive Probes



A visible change: Eu complexes can be rendered analyte-responsive by exploiting the sensitivity of a coumarin antenna to the nature of the 7-substituent (see figure). A boronate switch installed in the 7-position afforded complexes that responded rapidly to μM concentrations of peroxynitrite; the product of this reaction being the corresponding phenol. The analogous reaction with H_2O_2 proceeds at a significantly lower rate. As the reaction shifts the absorption to longer wavelengths, the construction of ratiometric probes is possible.

