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Published on Web 05/23/2007

Cocktails of Tb^{3+} and Eu^{3+} Complexes: A General Platform for the Design of Ratiometric Optical Probes

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Abstract: Fluorescent and luminescent reporters that signal molecular events of interest by modulating the ratio of peaks in their emission profile have advantages over reporters that simply modulate their emission intensity, since ratiometric measurement is concentration-independent and allows them to be effective in complex contexts, such as living cells or sensor microarrays. We herein describe a general platform for the design of ratiometric probes based on a heterometallic Tb^{3+}/Eu^{3+} bis-lanthanide ensemble, consisting of a mixture, or "cocktail", of otherwise identical heterometalated chelates. The chelate contains an organic photon antenna that sensitizes the Tb^{3+}/Eu^{3+} luminescence. The contributions of the two metals to the composite luminescence spectrum can be tuned to the same relative scale by adjusting the stoichiometry of the cocktail, allowing subtle changes in their ratio to be accurately measured. Importantly, the ratio responds to chemical and environmental changes experienced by the photon antenna, making the system an ideal platform for the design of chemical and enzymatic probes. As proofs of concept, we describe a ratiometric probe for esterase activity and a polarity-responsive ratiometric sensor.

Introduction

The rational design of molecules that exhibit fluorescent switching is a pursuit of great interest, since these switches can function as reporters of chemical and enzymatic events.[1−3] A fluorescent reporter can signal an event (e.g., the binding of an analyte or a chemical transformation) in two conceptually different ways: (1) by increasing (*fluorogenic*) or decreasing (*fluorolytic*) the intensity of its emission, or (2) by modulating the ratio of peaks in its emission profile (*fluoromorphic*). Since calibration of the output signal is required for quantification, the former method yields quantitative information only in cases where the precise concentration of the reporter is known; otherwise, such fluorogenic/fluorolytic reporting strategies can be relied upon only for observing qualitative trends in signal change. On the other hand, the latter method allows ratiometric measurement of the event—this measurement is independent of probe concentration, thereby extending its quantitative utility to more complex applications, such as imaging living cells and tissues.[4] Modulation of a fluorescent reporter's radiative lifetime rather than the strength or position of its emission maximum is also a viable optical reporting strategy, although it generally requires a more complex experimental setup.[5]

Previous Approaches to Ratiometric Probes. In principle, ratiometric reporting strategies can be evolved by coupling fluorogenic or fluorolytic reporters to a second dye in one of two scenarios: (1) the dynamic emission response of a reporter dye is calibrated by a second dye whose emission remains constant with respect to the parameter of interest,[6] or (2) the excited state of the responsive dye is coupled to that of a second dye, such that their interaction is gated by the event of interest (intramolecular quenching or resonance energy transfer).[3,7] However, dual-dye systems do not offer the perfect solution, since the first scenario requires two sets of excitation and emission wavelengths to be monitored simultaneously, which necessitates a more complex instrumental setup and potentially limits the temporal resolution of consecutive data collection. If

(1) For a general review, see: (a) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566. For a recent account of supramolecular sensors, see: (b) Anslyn, E. V. *J. Org. Chem.* **2007**, *72*, 687−699. For reviews of fluorescent enzyme activity reporters, see: (c) Lawrence, D. S. *Acc. Chem. Res.* **2003**, *36*, 401−409. (d) Baruch, A.; Jeffery, D. A.; Bogyo, M. *Trends Cell. Biol.* **2004**, *14*, 29−35. (e) Goddard, J.-P.; Reymond, J.-L. *Trends Biotechnol.* **2004**, *22*, 363−370.

(2) (a) Yee, D. J.; Balsanek, V.; Sames, D. *J. Am. Chem. Soc.* **2004**, *126*, 2282−2283. (b) Chen, G.; Yee, D. J.; Gubernator, N. G.; Sames, D. *J. Am. Chem. Soc.* **2005**, *127*, 4544−4545. (c) Tremblay, M. S.; Sames, D. *Org. Lett.* **2005**, *7*, 2417−2420. (d) Froemming, M. K.; Sames, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 637−642. (e) Yee, D. J.; Balsanek, V.; Bauman, D. R.; Penning, T. M.; Sames, D. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13304−13309. (f) Tremblay, M. S.; Zhu, Q.; Martí, A. A.; Dyer, J.; Halim, M.; Jockusch, S.; Turro, N. J.; Sames, D. *Org. Lett.* **2006**, *8*, 2723−2726. (g) Tremblay, M. S.; Sames, D. *Chem. Commun.* **2006**, 4116−4118.

(3) For recent reviews of fluorescent protein-based reporters, see: (a) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906−918. (b) Tsien, R. Y. *FEBS Lett.* **2005**, *579*, 927−932. (c) Giepmans, B. N. G.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y. *Science* **2006**, *312*, 217−224.

(4) Demchenko, A. P. *FEBS Lett.* **2006**, *580*, 2951−2957.

(5) Excimer formation induces useful changes in fluorescence lifetimes; for recent examples, see: (a) Martí, A. A.; Li, X.; Jockusch, S.; Li, Z.; Raveendra, B.; Kalachikov, S.; Russo, J. J.; Morozova, I.; Puthanveettil, S. V.; Ju, J.; Turro, N. J. *Nucleic Acids Res.* **2006**, *34*, 3161−3168. (b) Yang, C. J.; Jockusch, S.; Vicens, M.; Turro, N. J.; Tan, W. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17278−17283. For reviews on time-resolved measurement of resonance energy transfer, see: (c) Wallrabe, H.; Periasamy, A. *Curr. Opin. Biotechnol.* **2005**, *16*, 19−27. (d) Selvin, P. R. *IEEE J. Sel. Top. Quant. Electron.* **1996**, *2*, 1077−1087.

(6) Woodroofe, C. C.; Lippard, S. J. *J. Am Chem. Soc.* **2003**, *125*, 11458−11459.

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the two dyes are not covalently linked,[6,7d] nonhomogeneous distribution of the two dyes inside a cell may limit the spatial dimensions over which the signal can be accurately calibrated. Although dual-dye resonance energy transfer systems offer the convenience of a single excitation wavelength, they still suffer from a limitation inherent to all dual-dye systems, namely that each dye has a different set of susceptibilities to photobleaching and reversible environmental quenching.[4] In contrast, a single dye with responsive dual emission offers the advantage of monitoring conversion of the reporter ratiometrically using a single excitation wavelength.[4,8] Although rare, examples of rationally designed fluoromorphic reporters with single excitation wavelengths have been reported, usually based on perturbation of the equilibrium between distinctly emissive ground-state[8a] or excited-state[4,8b] tautomers.

Design of a General Ratiometric Sensing Strategy Based on Hetero-bis-lanthanide Ensembles. As part of a program directed at the design and application of novel fluorescent[2a−e] and luminescent lanthanide[2f,g] switches, we wanted to develop responsive, ratiometric reporters based on the luminescent lanthanide ions,[9] since their sharp, long-wavelength emission bands are ideal for biological applications, and because their long radiative lifetimes allow background-free measurement using time-gating.[10] Because many organic chromophores are capable of efficiently sensitizing lanthanide luminescence via energy transfer, sensitized lanthanide complexes are a flexible class of optical reporters and are thus an attractive platform for the design of responsive optical switches.

The sharp lines of the lanthanide emission spectra are essentially atomic and are consequently inert to shifts in wavelength. As a result, a ratiometric signaling strategy based on lanthanide luminescence is difficult to envision. Some ratiometric sensors have been designed on the basis of modulating the relative intensities of these lines;[11] however, these changes generally result from a change in the symmetry of the ligand sphere of a coordinatively unsaturated complex—such changes are difficult to predict and are known to be unreliable in anion-rich biological contexts.[2f,12] We sought a more robust approach based on a heterometallic bis-lanthanide ensemble, wherein luminescence from two lanthanide ions with distinct

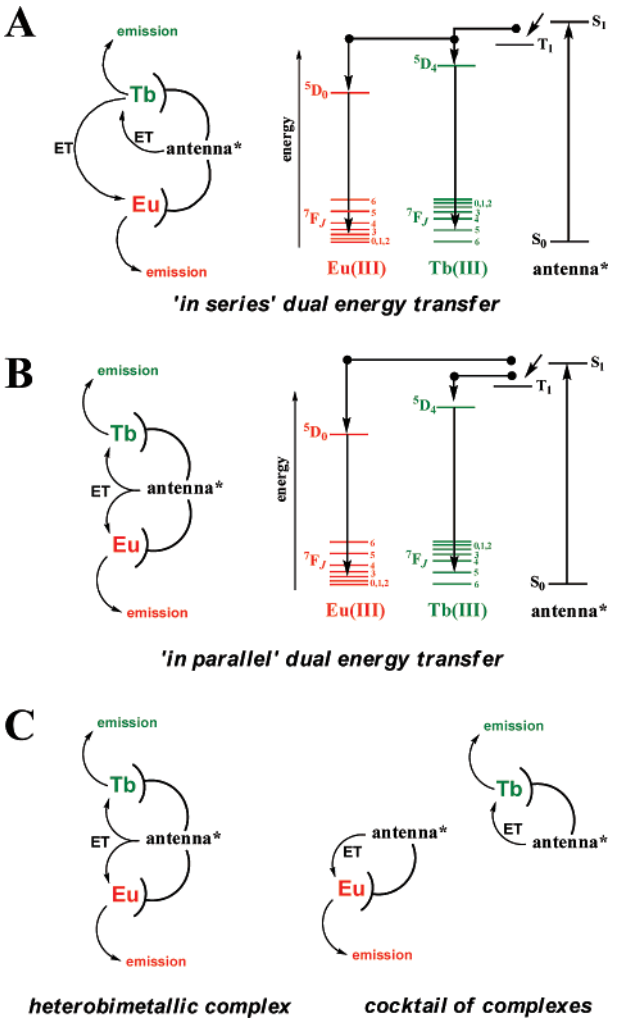


Figure 1. Schematic representations and simplified Jabłoński diagrams depicting dual-emissive bis-lanthanide ensembles sensitized by *in series* (A) or *in parallel* (B) energy transfer originating from the excited state of an organic photon antenna. (C) Since the *in parallel* mode does not involve interaction between the metals, a heterobimetallic complex and a cocktail of monometallic complexes will have similar optical output.

emission profiles would be combined and the relative contribution from each lanthanide varied as a function of an event of interest. Since emission from both lanthanides will originate from the same excited state (the organic photon antenna), their emission ratio is independent of concentration, photobleaching effects, and power fluctuation of the excitation source.

Our first design was based on a heterometallic bis-lanthanide complex—a single molecule containing discretely incorporated Tb^{3+} and Eu^{3+} ions—that exhibited a ratiometric Tb^{3+}/Eu^{3+} emission profile as a function of solvent polarity.[2g] In this system, we sensitized Tb^{3+} luminescence and hoped to observe significant energy transfer[13] from Tb^{3+} to Eu^{3+} and to vary the ratio of the emission by perturbing the metal−metal energy transfer; the overall dual energy transfer is analogous to an electronic circuit wired in series (Figure 1A). Unfortunately, Eu^{3+} emission could not be readily observed on the same relative scale as Tb^{3+} luminescence, such that perturbation of their ratio would be difficult to measure with the desired sensitivity. This

(7) For a recent review, see: (a) Kikuchi, K.; Takakusa, H.; Nagano, T. *Trends Anal. Chem.* **2004**, *23*, 407−415. For recent examples, see: (b) Albers, A. E.; Okreglak, V. S.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 9640−9641. (c) Coskun, A.; Akkaya, E. U. *J. Am. Chem. Soc.* **2006**, *128*, 14474−14475. (d) Bozym, R. A.; Thompson, R. B.; Stoddard, A. K.; Fierke, C. A. *ACS Chem. Biol.* **2006**, *1*, 103−111. (e) Wichmann, O.; Wittbrodt, J.; Schultz, C. *Angew. Chem., Int. Ed.* **2006**, *45*, 508−512. (f) Haidekker, M. A.; Brady, T. P.; Lichlyter, D.; Theodorakis, E. A. *J. Am. Chem. Soc.* **2006**, *128*, 398−399. (g) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Lynch, P. L. M. *New J. Chem.* **1996**, *20*, 871−880.

(8) (a) Chang, C. J.; Jaworski, J.; Nolan, E. M.; Sheng, M.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1129−1134. (b) Henary, M. M.; Wu, Y.; Fahrni, C. J. *Chem. Eur. J.* **2004**, *10*, 3015−3025.

(9) For general reviews, see: (a) Parker, D.; Dickins, R. S.; Puschmann, H.; Crossland, C.; Howard, J. A. K. *Chem. Rev.* **2002**, *102*, 1977−2010. (b) Bünzli, J.-C. G.; Piguet, C. *Chem. Soc. Rev.* **2005**, *34*, 1048−1077. For a review on lanthanide-based switches, see: (c) Gunnlaugsson, T.; Leonard, J. P. *Chem. Commun.* **2005**, 3114−3131. A key paper missing from review 9c: (d) Lee, K.; Dzubeck, V.; Latshaw, L.; Schneider, J. P. *J. Am. Chem. Soc.* **2004**, *126*, 13616−13617. For a recent example of a lanthanide-based protease probe, see: (e) Terai, T.; Kikuchi, K.; Iwasawa, S.-y.; Kawabe, T.; Hirata, Y.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 6938−6946.

(10) Dickson, E. F. G.; Pollak, A.; Diamandis, E. P. *J. Photochem. Photobiol. B* **1995**, *27*, 3−19.

(11) (a) Bretonniere, Y.; Cann, M. J.; Parker, D.; Slater, R. *Org. Biomol. Chem.* **2004**, *2*, 1624−1632. (b) Parker, D.; Yu, J. *Chem. Commun.* **2005**, 3141−3143. (c) Pal, R.; Parker, D. *Chem. Commun.* **2007**, 474−476.

(12) Duimstra, J. A.; Femia, F. J.; Meade, T. J. *J. Am. Chem. Soc.* **2005**, *127*, 12847−12855.

(13) Heterobimetallic Tb^{3+}/Eu^{3+} complexes have been prepared as statistical mixtures, and energy transfer has been estimated to be efficient: Piguet, C.; Bünzli, J. -C. G. *Chem. Soc. Rev.* **1999**, *28*, 347−358.

led us to explore a dual energy transfer analogous to a parallel circuit, where the excited-state energy of the photon antenna would be proportioned between the metals through direct sensitization of both Tb^{3+} and Eu^{3+} (Figure 1B). While the series circuit design requires the two lanthanides to exist within the same molecule, the parallel circuit design does not; in fact, a mixture of Eu^{3+} and Tb^{3+} complexes outfitted with the same antenna chromophore would produce an emission response similar to that of a bis-complex functioning in the parallel circuit mode (Figure 1C). Since Eu^{3+} and Tb^{3+} complexes have nearly identical behavior in solution, *a "cocktail" of complexes is expected to be homogeneous with respect to all important properties*—solubility, localization in a biological matrix, and the ability to serve as an enzyme substrate—and to be distinguishable only by their photophysical properties. The latter rationale has been substantiated by Bornhop in the clever design of bimodal (luminescent/magnetic) imaging agents based on mixtures of Eu^{3+} and Gd^{3+} complexes, which are both taken up by cells.[14] Because the stoichiometry of the bis-lanthanide ensemble is not restricted, the composition of the cocktail can be "tuned" depending on the inherent bias of the antenna chromophore to sensitize Tb^{3+} and Eu^{3+} emission, thus allowing the two lanthanide profiles to be detected with comparable sensitivity. Finally, the cocktail of luminescent complexes could function as a ratiometric probe of physical, chemical, or enzymatic events occurring at the antenna chromophore, since changes in its electronic structure will be manifested in the relative efficiencies of energy transfer to Tb^{3+} and Eu^{3+}.

To the best of our knowledge, there is only a single report demonstrating the ratiometric sensing ability of a mixture of Tb^{3+} and Eu^{3+} complexes, wherein binding of urate to the lanthanide center induces selective quenching of the Tb^{3+} excited state ($^5D_4$).[15] This example differs mechanistically and in scope from our proposed strategy, since it relies on a specific electronic interaction between the analyte and the metal center, and because it involves the formation of a stable ternary complex with the analyte.

Results and Discussion

The well-known carbostyril 124 (cs124) (Figure 2A) has the ability to sensitize both Tb^{3+} and Eu^{3+} emission[16] and serves as a suitable scaffold for the design of antenna chromophores that could readily sensitize a Tb^{3+}/Eu^{3+} cocktail. Also, chemical derivatization of cs124 has been shown to alter the degree to which it sensitizes Tb^{3+} and Eu^{3+}, so it should be amenable to the design of probes exhibiting ratiometric luminescence responses to chemical/enzymatic transformations.[17] Cs124 itself shows a strong preference to sensitize Tb^{3+} luminescence (Figure 2B). Not unexpectedly, the emission spectrum of a 1:1 mixture of Tb:DTPA-cs124 and Eu:DTPA-cs124 is dominated by Tb^{3+} luminescence (Figure 2C, green spectrum). However, by simply adjusting the composition of the cocktail, the relative luminescence intensities of the two lanthanides can be detected with comparable sensitivity, allowing subtle changes in their emission ratio due to environmental or chemical perturbation to be detected (Figure 2C, black spectrum). Since the cocktail is adjusted by reducing the proportion of the brighter component (Tb^{3+} in this case), a consequence of adjusting the stoichiometry is that the overall luminescence intensity is decreased.

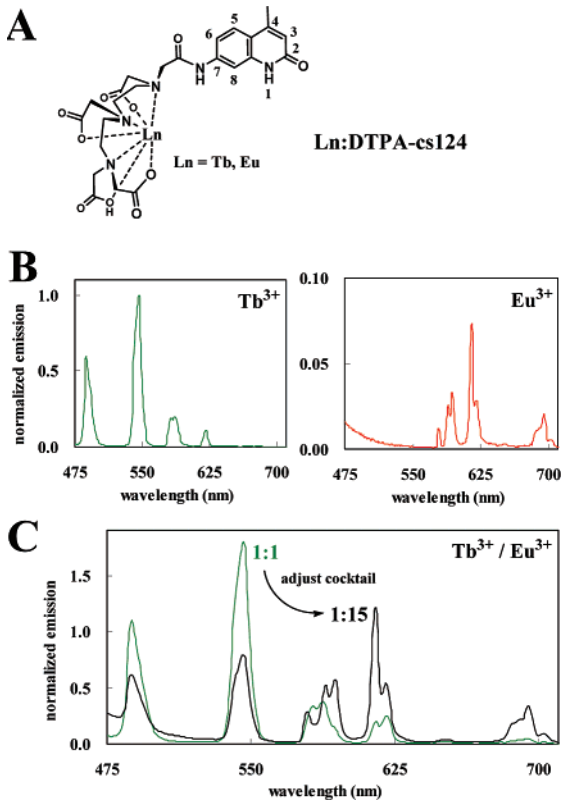
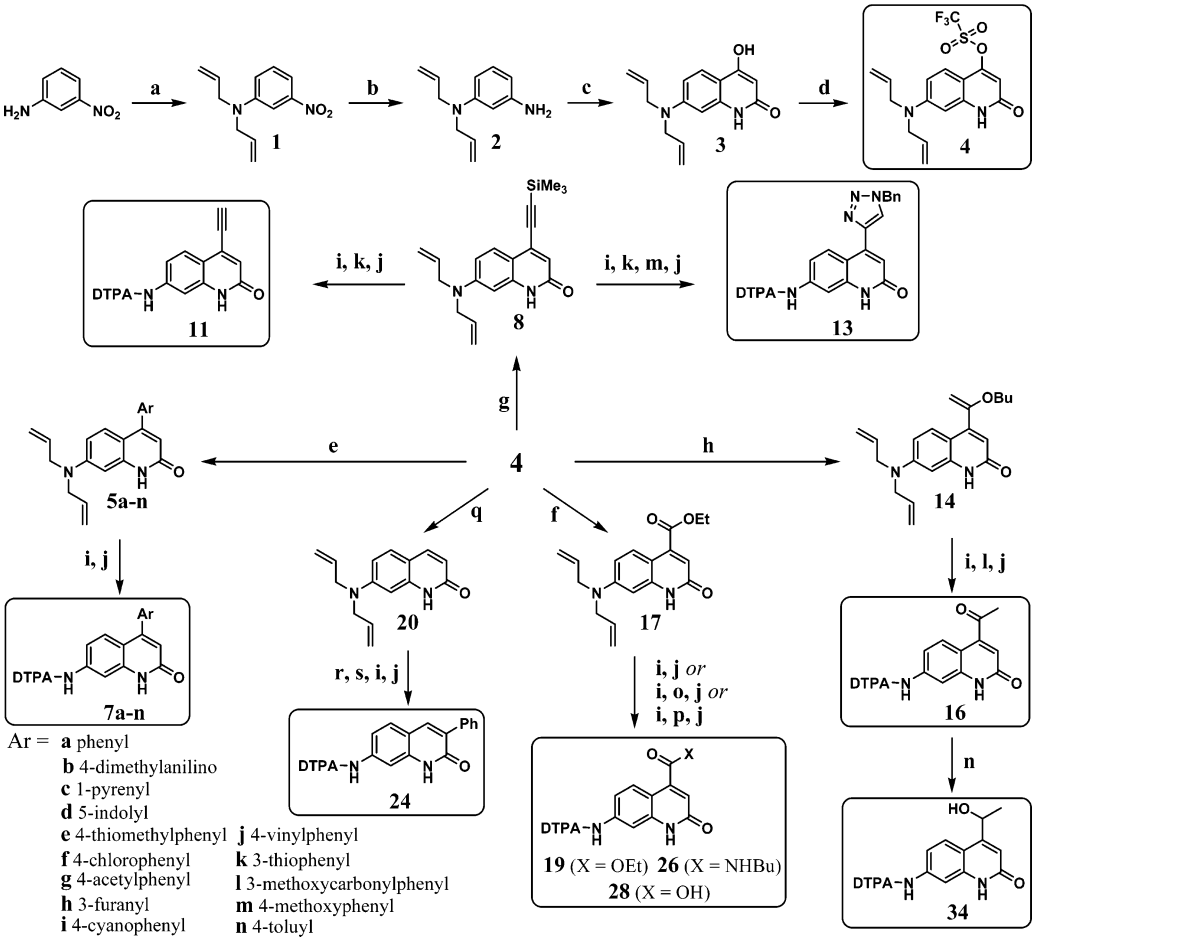


Figure 2. (A) Structure and numbering of the diethylenetriamine pentaacetic acid (DTPA) conjugate of carbostyril 124 (cs124). (B) Emission spectra of Tb:DTPA-cs124 and Eu:DTPA-cs124 normalized to the Tb^{3+} emission maximum, showing the strong preferential sensitization of Tb^{3+} (note the difference in scale) [25 μM DTPA-cs124, 50 μM Tb^{3+} (green spectrum) or 50 μM Eu^{3+} (red spectrum)]. (C) A 1:1 Tb^{3+}/Eu^{3+} cocktail (green spectrum) is adjusted to bring the Eu^{3+} luminescence on scale with Tb^{3+} luminescence (black spectrum) [25 μM DTPA-cs124, 50 μM Ln^{3+}, where $Ln^{3+} = Tb^{3+}/Eu^{3+}$ 1:1 (green spectrum) or 1:15 (black spectrum)]. All spectra were taken in 10 mM HEPES, 100 mM NaCl (pH 7.4) at 25 °C; excitation at 340 nm.

Divergent Synthesis of Cs124 Derivatives. The rational development of responsive "switches" based on a cocktail of cs124 complexes requires understanding how functional group changes on the cs124 scaffold affect the relative sensitization of Tb^{3+} and Eu^{3+}. In particular, we wanted to explore chemical functionality at the 3- and 4-positions of the cs124 core, since chemical changes at these positions produce robust fluorescent switches in the analogous coumarin structures,[2a] and since derivatization of cs124 at positions 1, 5, 6, and 8 resulted in only modest luminescence changes.[17b] However, no general method for the preparation of cs124 derivatives exists; reported analogues have been prepared by relatively harsh methods, which are typically low-yielding and intolerant of many functional groups that we wish to explore.[17,18] In an effort to devise a general, divergent entry into functionalized carbostyrils, we developed the versatile synthetic intermediate **4**, which serves as a competent coupling partner in many Pd-catalyzed cross-coupling reactions and can be used to prepare derivatives with

(14) Manning, H. C.; Goebel, T.; Thompson, R. C.; Price, R. R.; Lee, H.; Bornhop, D. J. *Bioconjugate Chem.* **2004**, *15*, 1488−1495.
(15) Poole, R. A.; Kielar, F.; Richardson, S. L.; Stenson, P. A.; Parker, D. *Chem. Commun.* **2006**, 4084−4086.
(16) Li, M.; Selvin, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 8132−8138.
(17) (a) Chen, J.; Selvin, P. R. *J. Photochem. Photobiol. A* **2000**, *135*, 27−32. (b) Ge, P.; Selvin, P. R. *Bioconjugate Chem.* **2004**, *15*, 1088−1094.
(18) Strohmeier, G. A.; Fabian, W. M. F.; Uray, G. *Helv. Chim. Acta* **2004**, *87*, 215−226.

Scheme 1. Divergent Synthesis of 3- and 4-Substituted Carbostyrils via Triflate **4**[a]



[a] Reagents and conditions: (a) allyl bromide, Na_2CO_3, MeCN, 80 °C, 24 h; (b) Fe^0, EtOH/AcOH, 110 °C, 4 h; (c) bis(2,4,6-trichlorophenyl)malonate, PhMe, 110 °C, 2 h; (d) $PhN(SO_2CF_3)_2$, NEt_3, DMF, 3 h; (e) $ArB(OH)_2$, $PdCl_2(dppf)$, Na_2CO_3, DMF/H_2O, 80−100 °C, 1 h; (f) ROH, $Pd(OAc)_2$, DPPF, DMF, CO (1 atm), 70 °C, 3 h; (g) Me_3SiCCH, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF, room temperature, 1 h; (h) butyl vinyl ether, $Pd(OAc)_2$, dppp, NEt_3, DMF, 80 °C, 12 h; (i) $Pd(PPh_3)_4$, *N*,*N*′-dimethylbarbituric acid, THF, 50 °C, 12 h; (j) DTPA bis-anhydride, NEt_3, DMF, room temperature, 3 h; (k) K_2CO_3, MeOH/CH_2Cl_2 (2:1), 0 °C to room temperature, 2 h; (l) 3 N HCl/AcOH (2:3), room temperature, 1 h; (m) BnN_3, $CuSO_4$, tris-benzyltriazolyl amine (TBTA), NaAsc, *t*-BuOH/H_2O/DMSO (2:2:1), 70 °C, 20 h; (n) $NaBH_4$, MeOH, room temperature, 10 min; (o) *n*-butylamine, AcOH, 100 °C, 12 h; (p) 2 M NaOH, EtOH, 100 °C, 4 h; (q) $(i\text{-Pr})_3SiH$, $PdCl_2(PPh_3)_2$, NEt_3, DMF, 85 °C, 24 h; (r) NBS, CH_2Cl_2, room temperature, 1 h; (s) $PhB(OH)_2$, $PdCl_2(dppf)$, Na_2CO_3, DMF/H_2O, 80−100 °C, 1 h.

diverse substituents at the 4-position of the cs124 scaffold (Scheme 1). Suzuki, Sonogashira, Heck, and carbonylation reactions were used to introduce aryl/heteroaryl, alkyne, enol, and ester functional groups, respectively.[19] Elaboration of the diallylamino quinolinones via two- or three-step procedures involving acylation with DTPA bis-anhydride as the final step furnished the final ligands.[20] Alternatively, reductive detriflation of **4** gave compound **20**, which could be brominated and functionalized at the 3-position. Tb^{3+} and Eu^{3+} complexes of the DTPA conjugates were prepared by metalation with $TbCl_3$ or $EuCl_3$ at neutral pH.[19]

Photophysical Characterization of the Tb^{3+}/Eu^{3+}:DTPA-Carbostyril Series. Photophysical characterization of the series revealed that preferential Tb^{3+} to Eu^{3+} sensitization could be altered by chemical changes at the 4-position; spectra of representative compounds show that a wide range of luminescence properties can be obtained (Figure 3). While the Tb^{3+}/

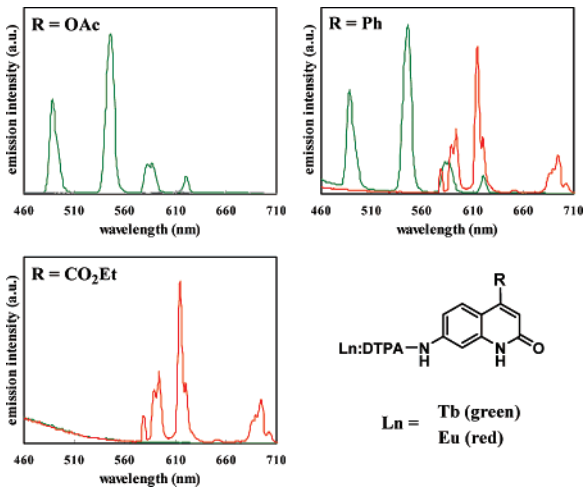


Figure 3. Luminescence spectra of representative 4-substituted Ln:DTPA-cs124 derivatives, demonstrating that the inherent bias for Tb^{3+} or Eu^{3+} sensitization can be varied by chemical derivatization [25 μM substrate, 50 μM Tb^{3+} (green spectra) or Eu^{3+} (red spectra), pH 7.4 (10 mM HEPES, 100 mM NaCl), 25 °C; excitation at 340 nm].

(19) See Supporting Information for detailed synthetic procedures and characterization.

(20) While the acyclic DTPA chelate is not ideal for many applications due to its kinetic lability, it is more than sufficient for exploratory photophysical studies and was chosen in the interest of synthetic brevity.

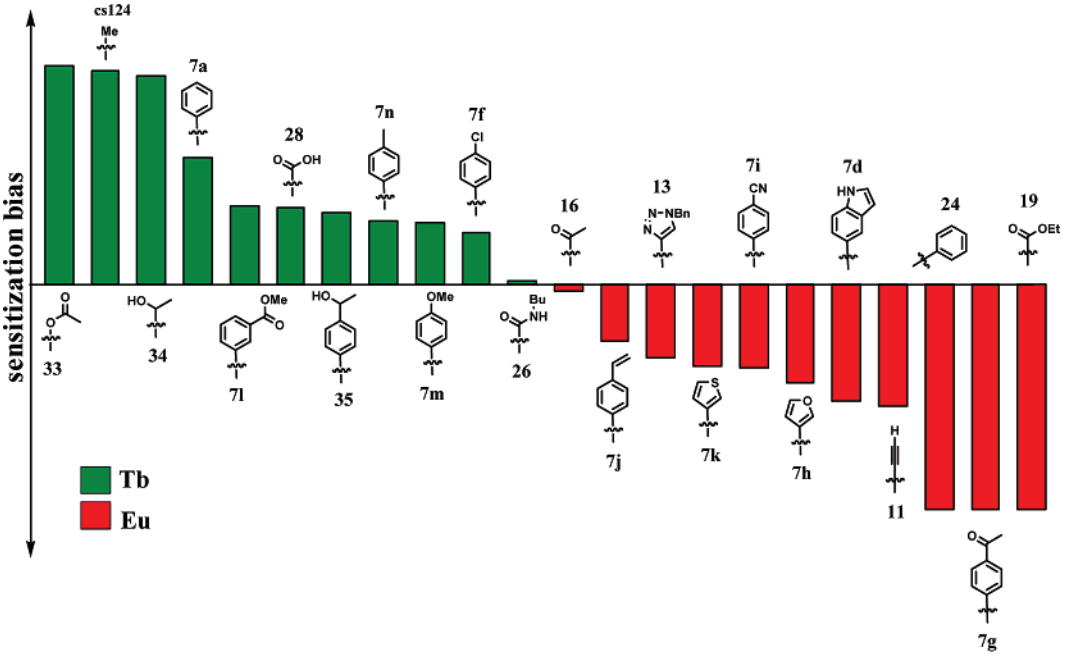


Figure 4. Tb−Eu sensitization biases for the DTPA-carbostyril series. The luminescence properties of cs124 are effectively modulated by chemical derivatization.

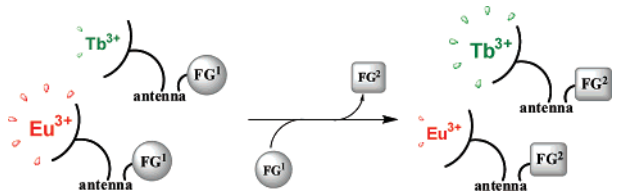


Figure 5. Schematic representation of a responsive luminescent cocktail undergoing a chemical/enzymatic functional group transformation ($FG^1 \rightarrow FG^2$) — the accompanying change in sensitization bias of the cocktail allows quantitative, ratiometric measurement of conversion.

Eu^{3+} (or Eu^{3+}/Tb^{3+}) ratio is the simplest parameter for quantifying preferential sensitization, the "sensitization bias" (eq 1) is more descriptive and convenient for comparing the entire series; it takes into account the difference in absolute sensitization between Tb^{3+} and Eu^{3+}, and normalizes it by the average intensity of Tb^{3+} and Eu^{3+} luminescence.

$$\text{sensitization bias} = \frac{\text{Tb} - \text{Eu}}{{}^1/_2(\text{Tb} + \text{Eu})} \qquad (1)$$

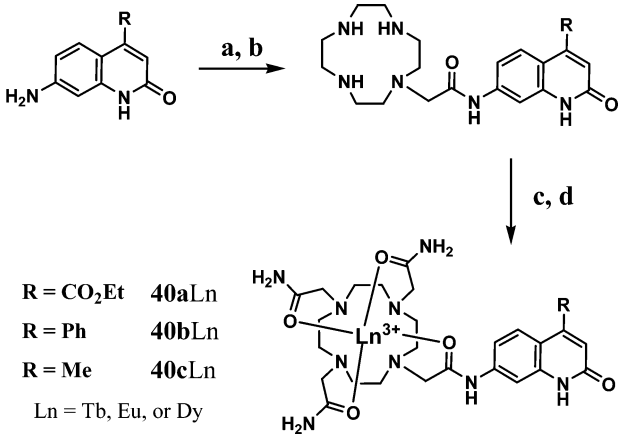
where Tb and Eu represent the intergrated emission intensity from 530 to 560 nm and from 605 to 635 nm, respectively. Derivatization of cs124 had only subtle effects on its absorption maximum (λ_{max}) and molar absorptivity (ϵ), but the sensitization bias and average luminescence intensity were significantly affected (Table S1, Supporting Information). Importantly, this series of compounds demonstrates that chemical derivatization of the carbostyril scaffold provides a wide range of dual-emissive, supramolecular ensembles, many of which are related through chemical transformations and can be developed into ratiometric reporters (Figure 4).

Several luminescent switches based on functional group transformations can be envisioned from the DTPA:cs124 derivatives and related chemical space (Figure 5). The 4-acetyl derivative **16** and its reduction product **34** constitute an excellent switch—the change in the Tb^{3+}/Eu^{3+} emission ratio is greater than 25-fold.[21] Ketone **16** is also related to the 4-acetoxy derivative **33**[22] by Baeyer−Villiger-type oxygen insertion; theoretically, a 60-fold enhancement in Tb^{3+}/Eu^{3+} emission ratio would accompany such a transformation. We observed only a modest difference in sensitization bias between **11** and **13**, a chemical switching pair related by the popular "click chemistry" bioconjugation reaction.[23] Substitution at the 3-position was not investigated in any detail, since the 3-phenyl derivative **24** sensitized only Eu^{3+} luminescence, suggesting that even weakly electron-withdrawing substituents in this position lower the relevant energy donor state below the Tb^{3+} threshold and thus outside the useful range for a Tb^{3+}/Eu^{3+} cocktail.

Encouragingly, the relatively subtle differences in electronic structure between ester **19**, amide **26**, and carboxylic acid **28** manifested themselves as large differences in preferential sensitization of Tb^{3+} and Eu^{3+}. Many important enzymatic processes involve the interconversion of these three species (e.g., esterase- and protease-catalyzed hydrolysis, esterification, and peptide bond formation). Worthy of mention is the fact that the chromophores themselves (i.e., apo chelates **19**, **26**, and **28**) *do not show significant changes in fluorescent emission*—their emission maxima occur at 419, 426, and 443 nm, respectively, and the emission intensity at these maxima spans a modest range of less than 2-fold (Figure S11, Supporting Information). Since the fluorescence intensity change is accompanied by a shift in wavelength, ratiometric measurement should be possible; in practice, however, broad molecular fluorescence makes it difficult to accurately determine emission ratios over such a short wavelength span, particularly in microscopy applications where the bandwidths of emission filters are typically several tens of nanometers. This highlights an important feature of the Tb^{3+}/Eu^{3+} cocktail method: since each of the two luminescent

(21) The Tb/Eu emission ratio is defined as the integrated Tb luminescence divided by the integrated Eu luminescence; photophysical parameters for all compounds can be found in the Supporting Information.
(22) See Supporting Information for the preparation of this compound.
(23) See a recent review and references therein: Lutz, J.-F. *Angew. Chem., Int. Ed.* **2007**, *46*, 1018−1025.

Scheme 2. Synthesis of DOTAm Complexes[a]



[a] Reagents and conditions: (a) $ClCH_2C(O)Cl$, NEt_3, DMF, 0 °C, 30 min. (b) cyclen, $CHCl_3$/DMF (3:1), RT, 12 h. (c) $BrCH_2CONH_2$, DiPEA, DMF, 0 °C, 12 h. (d) $LnCl_3$, EtOH/0.1M triethylammonium acetate (pH 5), RT, 12 h.

metals has several sharp maxima, most possessing baseline resolution with respect to one another, it is possible to integrate the emission intensity for each metal in a window as large as 30−40 nm without overlap between the two signals. For example, the Tb/Eu ratio can be conveniently measured by integrating the $Tb^{3+}[^7F_5]$ and the $Eu^{3+}[^7F_4]$ transitions (530−560 nm and 680−710 nm, respectively). It is important to note that the kinds of optical switches that can be developed within this framework involve modulating the relevant electronic excited states of the photon antenna (probably the triplet state, T_1) and cannot rely simply on the relief (or exaggeration) of a quenching process, such as photoinduced electron transfer (PET). Such switching mechanisms, which constitute the majority of the fluorescent sensor literature,[1a] are expected to quench both Tb^{3+} and Eu^{3+} emission without any significant change in their ratio.[9e]

Development of a Ratiometric Esterase Activity Reporter. We chose the ester **19**/acid **28** pair to demonstrate the effectiveness of this strategy in monitoring a dynamic, enzyme-catalyzed chemical transformation in a continuous manner. The Tb^{3+}/Eu^{3+} emission ratios are 0.35 and 1.62 for ester **19** and acid **28**, respectively,[24] which should provide a comfortable 4−5-fold contrast window in which to observe the transformation. Compound **40a**Ln, an analogue of **19** in which the DTPA chelate has been replaced by the macrocyclic DOTAm chelate, was prepared as an enzyme substrate, since the latter possesses superior complex stability.[2f,g,20] Although DTPA and DOTAm complexes exhibit different preferential sensitization of Tb^{3+} and Eu^{3+} due to the sensitivity of the Eu^{3+} emission lines to chelate symmetry, the overall trends are the same; by adjusting the composition of the cocktail, these differences can be accounted for, and thus the information in Figure 4 is directly applicable to the corresponding DOTAm complexes.[25] DOTAm analogues were prepared in a four-step sequence from the intermediate amines described above (Scheme 2). The homogeneity of the DOTAm cocktails is supported by reverse-phase

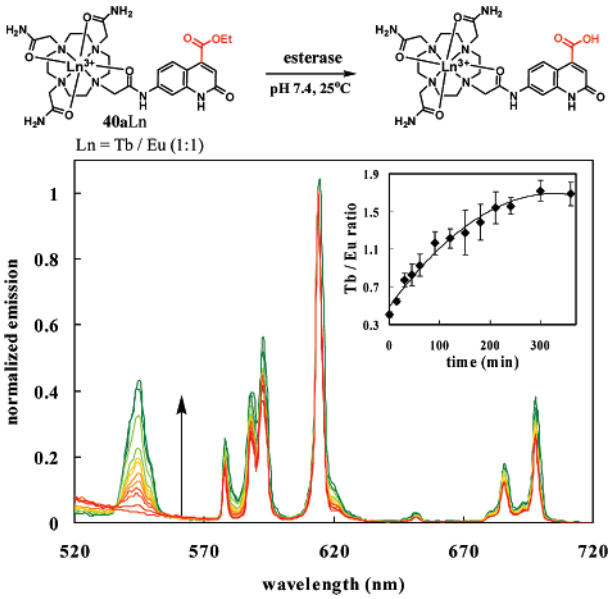


Figure 6. Enzymatic hydrolysis of DOTAm substrate **40a**Ln (Ln = 1:1 Tb/Eu) catalyzed by hog liver esterase [69 μM substrate, 0.5 mg/mL enzyme, pH 7.4, 10 mM HEPES, 25 °C; excitation at 340 nm; error bars represent standard deviations derived from five experiments].

HPLC; the complexes coelute at a retention time that is readily distinguishable from that of the apo chelate.[26]

Indeed, a cocktail of **40a**Tb and **40a**Eu allowed continuous monitoring of enzyme-catalyzed hydrolysis (Figure 6). Specifically, incubation of **40a**Tb/Eu (1:1) with hog liver esterase (EC 3.1.1.1) (pH 7.4, 25 °C) gave rise to a time-dependent, 4-fold increase in the Tb^{3+}/Eu^{3+} emission ratio. Uncatalyzed hydrolysis of the substrate did not occur to any significant extent during the course of the assay but did occur at longer time scales (<25% after 24 h). These encouraging results demonstrated the feasibility of using a Tb^{3+}/Eu^{3+} complex cocktail to provide a ratiometric readout of a dynamic, enzyme-catalyzed transformation in real time.

Development of a Ratiometric Polarity Sensor. In addition to responding to chemical transformations, luminescence from the bis-lanthanide ensemble could also be used to probe physical changes in the environment, such as polarity. As a proof of principle, a cocktail of **40b**Tb and **40b**Eu (1:4) was subjected to a range of solvent polarity. Titration with the nonionic surfactant Triton X-100 induced a dramatic change in Tb^{3+}/Eu^{3+} emission ratio, spanning a range from 10.2 to almost zero at high surfactant concentrations (Figure 7).

Although Tb^{3+} and Eu^{3+} are by far the most studied of the luminescent lanthanides,[9] several others also exhibit sensitized emission in the visible[27] and near-infrared regions.[28] A DOTAm conjugate of the parent sensitizer cs124 (**40c**Ln) efficiently sensitizes Dy^{3+} luminescence (481 and 574 nm), which occurs in a useful wavelength region and exhibits narrow bandwidths characteristic of lanthanide emission. A cocktail of **40c**Dy and

(24) Although ester **19** does not sensitize Tb luminescence at all, residual ligand fluorescence occuring between 530 and 560 nm causes this number to be nonzero.
(25) Richardson, F. *Chem. Rev.* **1982**, *82*, 541−552. See Supporting Information for further discussion of this point.
(26) See Supporting Information for details of the HPLC experiment.
(27) (a) Petoud, S.; Cohen, S. M.; Bünzli, J.-C. G.; Raymond, K. N. *J. Am. Chem. Soc.* **2003**, *125*, 13324−13325. (b) Petoud, S.; Muller, G.; Moore, E. G.; Xu, J.; Sokolnicki, J.; Riehl, J. P.; Le, U. N.; Cohen, S. M.; Raymond, K. N. *J. Am. Chem. Soc.* **2007**, *129*, 77−83.
(28) (a) Faulkner, S.; Beeby, A.; Dickins, R. S.; Parker, D.; Williams, J. A. G. *J. Fluoresc.* **1999**, *9*, 45−49. (b) Werts, M. H. V.; Woudenberg, R. H.; Emmerink, P. G.; van Gassel, R.; Hofstraat, J. W.; Verhoeven, J. W. *Angew. Chem., Int. Ed.* **2000**, *39*, 4542−4544. (c) Comby, S.; Imbert, D.; Chauvin, A.-S.; Bünzli, J.-C. G. *Inorg. Chem.* **2006**, *45*, 732−743.

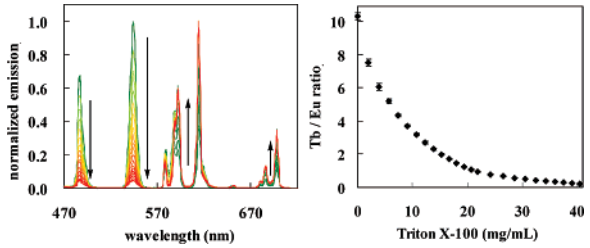


Figure 7. Solvent-polarity-dependent, ratiometric luminescence response of **40b**Ln (Ln = 1:4 Tb/Eu) [50 μM, deionized H_2O, titrated with Triton X-100 (100 mg/mL), 25 °C; excitation at 340 nm; error bars represent standard deviations derived from three experiments].

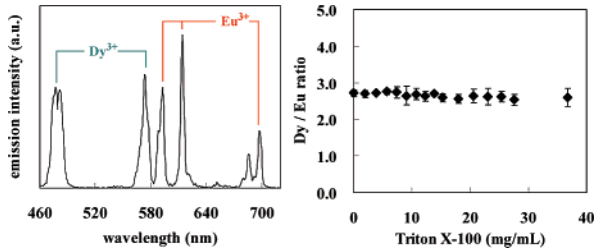


Figure 8. No change in the luminescence of a cocktail of **40c**Dy and **40c**Eu (1:9) is observed upon titration with Triton X-100 [50 μM, deionized H_2O, titrated with Triton X-100 (100 mg/mL), 25 °C; excitation at 340 nm; error bars represent standard deviations derived from three experiments].

40c Eu (1:9) demonstrates the complementarity and minimal overlap of the two lanthanide spectra (Figure 8). Interestingly, the Dy^{3+}/Eu^{3+} emission ratio was inert to polarity changes induced by the addition of nonionic surfactant.[29] The reduced sensitivity of Dy^{3+} to environmental changes suggests a potential role for it as a replacement for Tb^{3+} in applications of the cocktail strategy where fluctuating Tb^{3+} emission due to environmental changes may convolute the ability to read out a chemical or enzymatic event. Also, since the Dy^{3+} band centered at 574 nm occurs conveniently between the dominant Tb^{3+} and Eu^{3+} bands (centered at 545 and 613 nm, respectively), the possibility exists for simultaneous multi-variable readout using a tris-lanthanide cocktail consisting of all three ions.

Conclusion

We have described a general strategy for the rational design of ratiometric, luminescent probes based on a heterometallic bis-lanthanide platform. The cocktail of Eu^{3+} and Tb^{3+} (or Dy^{3+}) complexes exhibits a highly structured, information-rich composite luminescence spectrum with several sharp, baseline-resolved emission lines that blanket the visible region from 470 to 705 nm. The relative intensities of the emission lines can be tuned by adjusting the stoichiometry of the cocktail without disrupting its physical homogeneity, since the lanthanide chelates have nearly identical properties in solution. Since the luminescence of the mixture is sensitized by a single antenna, changes in its electronic structure can manifest themselves as changes in the relative ratio of luminescence from each metal. When these changes are coupled to chemical/enzymatic processes or environmental changes, a ratiometric reporting strategy emerges that is independent of probe concentration and — as a result of having a single excitation wavelength — is independent of excitation source fluctuation. In addition, the narrow bandwidths and baseline resolution of the signals render their independent measurement particularly amenable to optical microscopy. To demonstrate the feasibility of these concepts, we have designed a ratiometric reporter of esterase activity and a polarity-responsive probe. The photophysical mechanisms underlying the observed ratiometric transformations require more detailed study, but they most likely result from a combination of two factors: alteration of the energy of the antenna's donor state, which in turn affects the partitioning of energy transfer between the two metals, and the differential sensitivity of the lanthanide excited states to environmental changes. One limitation of the system is the relatively narrow energy range required for sensitization of both Tb^{3+} and Eu^{3+}—the allowable electronic changes that a chromophore can experience and stay within this range may restrict the types of chemical and enzymatic transformations for which reporting cocktails can be developed. However, the success of **40a**Tb/Eu in monitoring enzyme-catalyzed ester hydrolysis suggests that, within this range, the lanthanide cocktail strategy may provide greater sensitivity to subtle electronic changes. Another limitation is the ultraviolet excitation required by these chromophores; light of this wavelength can be damaging to biomatter and is not ideal for many biological applications. Nevertheless, two-photon excitation of chromophores capable of sensitizing Tb^{3+} and Eu^{3+} may be a viable way to address this challenge.[30]

Experimental Section

Chemical Synthesis. Detailed synthetic procedures and characterization data for all DTPA and DOTAm conjugates can be found in the Supporting Information. Purification of final chelates was done by preparative reverse-phase HPLC using increasing linear gradients of 90% MeCN/H_2O in H_2O (0.1% trifluoroacetic acid).

Metalation of Chelates. Metalation of DTPA chelates (**7a**−**n**, **11**, **13**, **14**, **16**, **19**, **24**, **26**, **28**, **34**) was carried out by incubating the chelate (25 μM in 10 mM HEPES, 100 mM NaCl, pH 7.4) with the appropriate $LnCl_3 \cdot 6H_2O$ salt (50 μM) for 2 h. These solutions were used directly for exploratory photophysical measurements. Complexes used for enzyme and polarity assays (**40a**−**c**Ln) were prepared by metallating the DOTAm chelates (1 equiv) with the appropriate $LnCl_3 \cdot 6H_2O$ salt (4 equiv) in 1:1 EtOH/0.1 M triethylammonium acetate buffer (pH ~5). After 12 h at room temperature, the complexes were purified by preparative HPLC as described above, giving white solids after lyophilization. All complexes were pure by HPLC and gave satisfactory mass spectral data (see Supporting Information).

Photophysical Characterization of the DTPA-Carbostyril Series. Molar extinction coefficients were determined in 10 mM HEPES, 100 mM NaCl, pH 7.4 buffer using five different concentrations in the range 20−100 μM from two independently weighed stock solutions. Emission spectra were measured following excitation at 340 nm for all compounds. Sensitization bias was determined by eq 1 using the integrated Tb^{3+} (530−560 nm) and Eu^{3+} emission (605−635 nm); data were processed using Microsoft Excel. Complete photophysical data for the series can be found in the Supporting Information, Table S1.

Esterase Assay. A cocktail of **40a**Tb and **40a**Eu (1:1) (69 μM) was treated with hog liver esterase (EC 3.1.1.1, Fluka catalog no. 46058, 0.5 mg/mL) in pH 7.4 HEPES (10 mM) at 25 °C, and emission spectra were taken at various time points (Figure 6). Samples were excited at 340 nm, and emission was measured from 400 to 750 nm. The Tb/Eu

(29) This resistance to polarity-induced luminescence change is not a property of the cs124 chromophore; Tb:cs124 complexes exhibit polarity-dependent emission similar to that of **40b**Tb.

(30) (a) Werts, M. H. V.; Nerambourg, N.; Pélégry, D.; Grand, Y. L.; Blanchard-Desce, M. *Photochem. Photobiol. Sci.* **2005**, *4*, 531−538. (b) Piszczek, G.; Maliwal, B. P.; Gryczynksi, I.; Dattelbaum, J.; Lakowicz, J. R. *J. Fluoresc.* **2001**, *2*, 101−107.

emission ratio was calculated using the integrated emission intensity from 535 to 555 nm for Tb and the integrated emission intensity from 680 to 705 nm for Eu. Uncatalyzed hydrolysis of the substrate does not occur to any significant extent during the course of the assay but does occur at longer time scales (~25% after 24 h). Compound **40a**Ln is not an optimized substrate for this enzyme; due to the relatively high concentration of enzyme required to effect this transformation over the course of 4−5 h, detailed kinetic parameters were not measured.

Polarity Assay. The Tb/Eu emission ratio (defined as above for the esterase assay) from a cocktail of **40b**Tb and **40b**Eu (1:4) (50 μM) was measured at a variety of concentrations of Triton X-100 (polyethylene glycol *tert*-octylphenyl ether), which was added incrementally as a stock solution (100 mg/mL in H_2O) and mixed thoroughly before measurement (Figure 7). The same procedure was used for a cocktail of **40c**Dy and **40c**Eu (1:9).

Acknowledgment. The authors thank the G. Harold & Leila Y. Mathers Charitable Foundation for generous financial support. M.H. is the recipient of the Novartis Graduate Fellowship in Organic Chemistry.

Supporting Information Available: Synthetic procedures and characterization for all compounds and additional photophysical data. This material is available free of charge via the Internet at http://pubs.acs.org.

JA070867Y