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COMMUNICATIONS

Published on Web 07/27/2006

# "*Cymothoe sangaris*": An Extremely Stable and Highly Luminescent 1,2-Hydroxypyridinonate Chelate of Eu(III)

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The use of fluorescent assays is widespread and essential for the detection of a range of analytes, such as DNA,[1] proteins,[2] or other biologically active molecules[3] typically present at very low concentrations (e.g., nano- or picomolar) and often in the presence of a complicated matrix of other chemical species (e.g., whole blood, serum, saliva). Due to their specific luminescent properties (sharp emission bands, large Stokes shift, and long-lived luminescence), lanthanide cations, such as Eu(III), are attractive as reporters for these assays.[4,5] Fluorescence detection is an exquisitely sensitive analytical tool, rapidly approaching the single molecule level.[6] Using both spectral and temporal discrimination of the luminescent signal from background autofluorescence enables highly sensitive assays.[7,8] The Dissociation Enhanced Lanthanide Fluoro Immuno Assay (DELFIA) techniques commercialized by Wallac and based on polyaminocarboxylate chelates of Eu(III)[9] have held a prominent place in biological assay systems, often yielding more sensitive results when compared to traditional ELISA techniques.[10,11] However, DELFIA is inherently limited since these assays require conversion of the nonfluorescent Ln(III) chelate into a highly fluorescent form, by addition of a sensitizing $\beta$-diketonate solution. Hence the fluorescent signal cannot be traced until the end of an assay. To counteract this limitation, efforts have turned to the development of luminescent lanthanide chelates that can be utilized directly and/or covalently bound to a given biomolecule of interest for homogeneous assays.[12−18]

Of these compounds, perhaps the most well-known is the Lehn cryptand, a trisbipyridyl macrobicycle which effectively sensitizes Eu(III) ($\Phi_{Eu} = 0.02$) and protects against nonradiative deactivation by solvent interactions. This complex is also kinetically inert toward metal ion exchange, a desirable feature for biological applications, such as luminescence immunoassays. However, lifetime measurements have shown the presence of chelated water, which necessitates the addition of a competitor, such as fluoride, to saturate the lanthanide and displace bound solvent to achieve optimum results.[19,20] Differing chromophores in a variety of other topologies (e.g., podand, macro(bi)cyclic, cryptand, or polyamino-carboxylate derivatives) have also been investigated.[21,22] Nonetheless, the preparation of a ligand scaffold, wherein the lanthanide cation is effectively sensitized while also forming a stable complex in dilute aqueous solution remains a significant challenge. Herein we report the stability and photophysics of the 1,2-hydroxypyridinonate chromophore (noted[23−25] to sensitize Ln(III) ions) as a tetradentate derivative, 5LIO-1,2-HOPO, which forms an $ML_2$ complex with Eu(III). This ligand simultaneously fulfills both criteria of high thermodynamic stability and excellent photophysical properties.

The 5LIO-1,2-HOPO ligand is readily prepared by reaction of the thiazolide intermediate prepared[26] from commercially available 6-bromopicolinic acid with the terminal primary amines of 2-(2-aminoethoxy)ethylamine (5LIO), yielding the protected ligand (Scheme 1). Deprotection and complexation with Eu(III) gave the desired complex in good yield, and X-ray quality crystals were grown by vapor diffusion of ether into a methanolic solution of the tetramethylammonium salt.

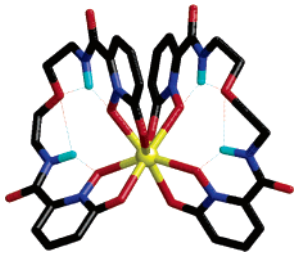
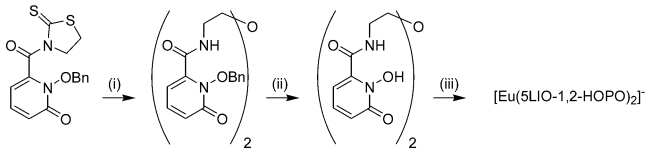


***Figure 1.*** X-ray crystal structure of $Me_4N[Eu(5LiO\text{-}1,2\text{-}HOPO)_2]$. $Me_4N$ cation and selected H atoms are omitted for clarity.

***Scheme 1*** [a]




[a] Conditions: (i) 1 equiv of 2-(2-aminoethoxy)ethylamine/$CH_2Cl_2$, 85% yield; (ii) HCl + AcOH, 90% yield; (iii) 4 equiv of pyridine, 0.5 equiv of $EuCl_3 \cdot 6H_2O$, MeOH, 89% yield.

The $ML_2$ complex (Figure 1, Table S1) with Eu(III) is eight coordinate. Trivalent lanthanide cations display variable coordination numbers of eight, nine, or higher, and the energy difference between these various forms is quite small. Shape analysis as discussed elsewhere[26,27] showed the bicapped trigonal prismatic ($C_{2v}$) geometry as the best fit to the differing idealized eight-coordinate polyhedra. The presence of four intramolecular H-bond interactions (avg. N−H···O = 1.80 Å) between phenolic oxygen and adjacent amide protons imparts additional stability to the metal complex, as noted previously for related compounds.[26,28] Also evident is a weaker interaction (avg. N−H···O = 2.63 Å) between amide protons and the ether oxygens of the linker. One 1,2-HOPO chelating group from each ligand also forms an intermolecular $\pi$-stacked structure with a distance of ca. 3.4 Å between planes. The two corresponding Eu−phenolate bond lengths for these chelators are slightly elongated (2.46 and 2.43 Å, respectively) compared to that of the corresponding Eu−carbonyl (avg. = 2.38 Å).

Solution thermodynamic experiments assessed the stability of the $[Eu(5LIO\text{-}1,2\text{-}HOPO)_2]^-$ complex. The free ligand is quite acidic with p$K_a$ values of 4.19(3) and 5.79(1) attributed to the N-hydroxyl groups. Hence, under physiological conditions, the ligand is twice deprotonated and readily available for Eu(III) chelation. Competition titrations with DTPA determined the stability of the $[Eu(5LIO\text{-}1,2\text{-}HOPO)_2]^-$ chelate, yielding a pEu of 18.64(10). A model with three stability constants ($\beta_{110}$, $\beta_{120}$, and $\beta_{121}$) was applied to fit the data from spectrophotometric titration experiments performed in the absence of competitor. Analysis of these data shows that the $[Eu(5LIO\text{-}1,2\text{-}HOPO)_2]^-$ complex predominates in



**10.1021/ja062597+ CCC: $33.50** 

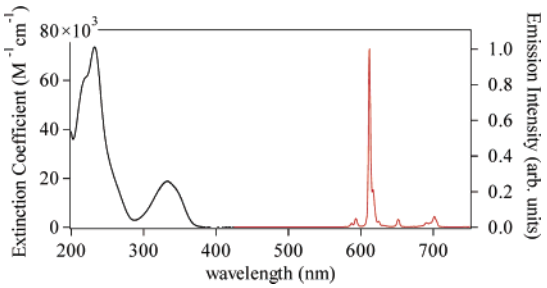



***Figure 2.*** Electronic absorption spectrum (left) and steady-state emission spectrum (right) ($\lambda_{ex}$ = 330 nm, 5 nm band-pass) for the [Eu(5LIO-1,2-HOPO)$_2$]$^-$ complex in 0.1 M Tris buffer at pH 7.4.

***Table 1.*** Protonation Constants of 5LIO-1,2-HOPO and Formation Constants of ML, ML$_2$, and ML$_2$H Complexes with Eu(III)

| | | | |
|---|---|---|---|
| p$K_1$ | 4.19(3) | $\beta_{110}$ | 12.46(2) |
| p$K_2$ | 5.79(1) | $\beta_{120}$ | 22.85(10) |
| *p*Eu | 18.64(10) | $\beta_{121}$ | 25.21(3) |

***Table 2.*** Summary of Experimental and Calculated[29] Photophysical Parameters for the [Eu(5LIO-1,2-HOPO)$_2$]$^-$ Complex

| | | | | |
|---|---|---|---|---|
| $\epsilon_{max}$ | 233 nm | 73860 M$^{-1}$ cm$^{-1}$ | $k_{rad}$ (calcd) | 0.609 ms$^{-1}$ |
| | 333 nm | 19250 M$^{-1}$ cm$^{-1}$ | | |
| $\tau_{obs}$ | ($H_2O$) | 727 ± 1.2 $\mu$s | $k_{nonrad}$ (calcd) | 0.807 ms$^{-1}$ |
| | ($D_2O$) | 1012 ± 2.5 $\mu$s | | |
| $\Phi_{tot}$ | ($H_2O$) | 0.215 ± 0.03 | $\Phi_{Eu}$ (calcd) | 0.43 |
| $\tau_{rad}$ | (calcd) | 1640 $\mu$s | $\eta_{sens}$ (calcd) | 0.49 |

solution to a limiting concentration of ca. $10^{-10}$ M. With decreasing concentration, the effective pH range (where the $ML_2$ complex predominates) becomes smaller until at ca. $10^{-11}$ M the ML complex becomes the dominant species (Figure S3, Supporting Information). The excellent stability of the [Eu(5LIO-1,2-HOPO)$_2$]$^-$ complex, as determined by its pEu, we ascribe to the 5LIO backbone, which forms an extensive intramolecular hydrogen bonding network.

Electronic absorption and emission spectra for the complex are shown in Figure 2, and relevant photophysical parameters determined experimentally (and calculated from the emission spectrum as discussed elsewhere[29]) are summarized in Table 2. The absorption spectrum is typical of the 1,2-HOPO chromophore as previously reported[30] and is red shifted upon deprotonation or complexation to the metal. The luminescence spectra are also typical of those for the Eu(III) cation in a low symmetry environment. Notably, the $^5D_0 \rightarrow {}^7F_2$ hypersensitive transition is very intense, resulting in an exceptional quantum yield and almost pure red luminescence ($\lambda_{em}$ = 612 nm). The corresponding luminescence lifetime was evaluated as ca. 727 $\mu$s (Figure S6, Supporting Information), indicating the metal center is well protected from deactivation by solvent. This is confirmed by measurements in $D_2O$, which yield a lifetime of ca. 1012 $\mu$s. Applying the recently improved Horrocks equation[31] to account for outer sphere solvent molecules

$$q = A'(k_H - k_D - B)$$

where $A'$ and $B$ are empirical constants of 1.11 and 0.31 ms$^{-1}$ for Eu(III), and $k_D$ and $k_H$ represent the observed luminescence decay rate constants, $k_{obs}$, in deuterated and nondeuterated solvent, respectively, yielded an apparent $q$ of 0.1 ± 0.1. We take this noninteger value to be effectively zero, in accordance with the structure determined crystallographically, with the small residual likely due to the presence of the four slowly exchanging amide protons.

As a result of their highly luminescent properties with Eu(III) and excellent thermodynamic stability, 1,2-HOPO-based chelators seem promising for applications in highly sensitive biological assays. Our efforts are now turning toward the synthesis of polydentate ligands with other topologies by using higher denticity linkers (e.g., H22 = *N*,*N*,*N*′,*N*′-tetrakis(2-aminoethyl)ethane-1,2-diamine). Removing amide NH oscillators via methylation has also proven[13] to be a method of improving the radiative decay, although in this case this modification may negatively impact the complex structure and stability. These efforts are ongoing, together with the preparation of functionalized derivatives suitable for bioconjugation.

**Acknowledgment.** This work was partially supported by the NIH (Grant HL69832) and the Director, Office of Science, Office of Advanced Scientific Computing Research, Office of Basic Energy Sciences (U.S. Department of Energy) under contract DE-AC02-05CH11231. This technology is licensed to Lumiphore, Inc. in which some of the authors have a financial interest. Financial support was provided to C.J. by the German Research Foundation (DFG). The authors thank Amanda Samuel for experimental assistance.

**Supporting Information Available:** Detailed synthesis of 5LIO-1,2-HOPO, experimental data for potentiometric, spectrophotometric, and spectrofluorometric titrations, additional photophysical data, and X-ray crystallographic files (in CIF format). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA062597+