β-Diketone Derivative That Forms a Fluorescent Eu^{3+} Complex in an Aqueous Solution

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A new β-diketone-type ligand for Eu^{3+}, *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-bis(3-phenylpropane-1,3-dione) was synthesized and the fluorescence property of its complex with Eu^{3+} was studied. The ligand was found to form a stable fluorescent complex with Eu^{3+} in an aqueous solution.

Keywords europium; β-diketone derivative; fluorescence; ligand; time-resolved fluoroimmunoassay

Time-resolved fluoroimmunoassay (TR-FIA) using Eu^{3+} as the probe has been gaining much attention as an alternative method for non-radioisotopic (non RI) immunoassay with a sensitivity comparable to RI techniques.[1)] The TR-FIA is based on the unique spectroscopic properties of Eu^{3+}, *i.e.*, a long emission wavelength of approximately 613 nm due to the $^5D_0 \rightarrow {}^7F_2$ transition, and a long fluorescence lifetime (0.6—1.0 ms). A number of ligands for Eu^{3+} that efficiently transfer its absorbed light energy intramolecularly to the metal ion have been reported not only as labels in TR-FIA but also as luminescent probes for biological macromolecules.[2)] For example, a macrobicyclic cryptate was synthesized,[3)] and more recently, a calixarene compound with ester functionalities at the lower rim was documented to exhibit a strong luminescence and a long luminescence lifetime upon complexing with a lanthanide ion.[4)] A β-diketone compound has been successfully employed in TR-FIA, in which 2-naphthoyltrifluoroacetone formed a fluorescent complex with Eu^{3+} in a micellar medium composed of Triton X-100 and the synergistic agent tri-*n*-octylphosphine oxide.[1a,5)] Since the fluorescence of the complex is quenched in an aqueous solution, the assay is composed of two steps involving the displacement of Eu^{3+} from the aqueous medium in which the immunological reaction is carried out. A single-step assay procedure was first realized using a novel chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA).[6)] BCPDA forms a stable fluorescent complex with Eu^{3+}, with the two heteroaromatic nitrogens and two carboxy groups as the coordination site, and its fluorescence is insensitive to aqueous quenching.

Stimulated by these works, we have reported in a previous paper[7)] the synthesis and fluorescence property of the Eu^{3+} complex of a bis-phenanthroline compound as an inclusion-type ligand. This ligand possesses a cavity suitable for binding Eu^{3+}, but the complexation is rather sluggish due to the rigid and preorganized ligand structure. We describe in this report our preliminary results on a new ligand for Eu^{3+}, *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-bis(3-phenylpropane-1,3-dione) (**1**, Chart 1) which, having a β-diketone structure as the primary coordination site, exhibits a strong fluorescence in aqueous solution.

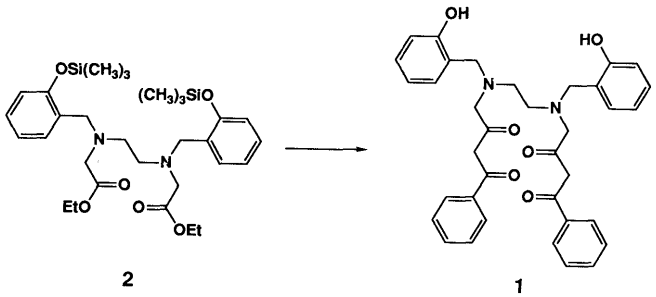

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Chart 1. Synthesis of Ligand **1**

Experimental

Europium chloride hexahydrate ($EuCl_3 \cdot 6H_2O$, 99.99%), samarium chloride hexahydrate ($SmCl_3 \cdot 6H_2O$, >99.99%) and terbium chloride hexahydrate ($TbCl_3 \cdot 6H_2O$, 99.999%) were obtained from Aldrich (Milwaukee, WI, U.S.A.) and were used without purification. ^{1}H-Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AC-200P operating at 200 MHz with tetramethylsilane as an internal standard. The splitting patterns were designated as follows: s, singlet; t, triplet; q, quartet; m, multiplet; br, broad. Fluorescence spectra were recorded on a Hitachi 650-60 spectrometer. IR spectra were taken in KBr disks on a Hitachi 270-30 spectrometer. Fast atom bombardment mass spectra (FAB-MS) were measured on a JEOL JMS-AX 505W. Uncorrected melting points were obtained on a Yamato MP-21 melting point apparatus.

***N,N'*-Bis(2-trimethylsilyloxybenzyl)ethylenediamine-*N,N'*-diacetic Acid, Diethyl Ester (2)** This ester was synthesized in a manner similar to that described by Mathias *et al.*[8a)] Briefly, *N,N'*-bis(2-hydroxybenzyl)ethylenediamine was prepared by a Schiff base formation reaction with ethylenediamine and salicylaldehyde (2 eq) followed by sodium borohydride reduction. Protection of the hydroxy groups with bis(trimethylsilyl)acetamide and the subsequent introduction of ethylcarboxymethyl groups ($-CH_2CO_2C_2H_5$) with ethyl bromoacetate afforded ester **2** in a 70% overal yield. mp 73—75 °C. IR ν_{max} cm^{-1}: 2980 (CH_2), 2950 (CH_2), 2840 (CH_2), 1750 (C=O), 1620 (C=C), 1590 (Ar), 1190 (C–O–C). ^{1}H-NMR ($CDCl_3$) δ: 1.27 (6H, t, Et), 2.73 (4H, s, $-NCH_2-$), 3.27 (4H, s, $-NCH_2Ar-$), 3.75 (4H, s, $-NCH_2CO-$), 4.20 (4H, q, Et), 6.73—7.20 (8H, m, Ar).

***N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-bis(3-phenylpropane-1,3-dione) (1)** β-Diketone formation was achieved by Claisen condensation[9)] of ester **2** with acetophenone in the presence of a base. Sodium hydride (60% dispersed in oil, 2.5 g, 62.5 mmol) was added in one portion to a stirred tetrahydrofuran solution (20 ml) containing ester **2** and acetophenone (2.4 g, 20 mmol), and the mixture was warmed at 60 °C for 1 h. After being cooled, the reaction was quenched by adding acetic acid (4 ml). The reaction mixture was concentrated *in vacuo* to leave a residue which was taken up with chloroform (100 ml) and washed with water. Concentrating the chloroform layer after drying ($MgSO_4$) afforded a crude product, which was recrystallized from methanol to give 2.2 g (37%) of compound **1**. mp 134—135 °C. IR ν_{max} cm^{-1}: 3050 (Ar), 2980 (CH_2), 2920 (CH_2), 2850 (CH_2), 1750 (C=O), 1620 (C=C), 1590 (Ar). ^{1}H-NMR ($CDCl_3$) δ: 2.77 (4H, s, $-NCH_2-$), 3.46 (4H, s, $-NCH_2Ar-$), 3.75 (4H, s, $-NCH_2CO-$), 6.10 (2H, s, $-COCH_2CO-$), 6.70—7.80 (18H, m, Ar), 12.5 (2H, br s, OH). FAB-MS *m/z*: 593 $(M+H)^+$. *Anal.* Calcd for $C_{36}H_{36}N_2O_6$: C, 72.95; H, 6.12; N, 4.73. Found: C, 72.55; H, 6.16; N, 4.74.

Results and Discussion

From a structural point of view, ligand **1** can be

considered to be a hybrid molecule composed of a hydrophilic complexone-like derivative bearing catecholate functions and a hydrophobic β-diketone structure. L'Eplattenier *et al.*[8b] reported that the complexone, *N*,*N'*-bis-(2-hydroxybenzyl)ethylenediamine-*N*,*N'*-diacetic acid (HBED, non-trimethylsilylated free acid of ester **2** in Chart 1), which is a partial structure of ligand **1**, has an extremely high affinity for Fe^{3+}. β-Diketones, on the other hand, are well known to produce fluorescent complexes with Eu^{3+}, although their fluorescence is measurable only in a hydrophobic media.[5,10] For these reasons, we envisioned that unification of these two structural elements would give rise to an enhancement in the stability of the complex of β-diketone derivatives with Eu^{3+}, as well as in the fluorescence intensity of the complex. The catecholate functionalities would provide additional chelating sites and, at the same time, make the ligand more hydrophilic. Furthermore, the presence of a *N*,*N'*-bis(2-hydroxybenzyl)ethylenediamine unit is expected to mitigate the aqueous quenching of the fluorescence of the Eu^{3+} complex by displacing the coordinated water molecules, thereby enhancing its fluorescence in an aqueous medium. Ligand **1** was readily prepared in a moderate yield from diester **2** which was synthesized in a manner analogous to HBED,[8] under the conditions of Claisen condensation with sodium hydride which simultaneously removed the phenol-protecting trimethyl groups. Ligand **1** is sparsely soluble in water, with a solubility of less than 1 mM.

The absorpion spectra of ligand **1** (1×10^{-5} M) and its Eu^{3+} complex (1×10^{-5} M) in 50 mM Tris buffer at pH 8.2 shows that the two spectra are very similar. Although a slight bathochromic shift (*ca.* 5 nm) of the absorption maximum (λ_{max}) of the free ligand upon complexation with Eu^{3+} occurred, the similarity between the spectra appeared to indicate that the absorption of the free ligand (λ_{max}= 325 nm) corresponded to the π–π^* transition of the aromatic portions of the ligand, not a ligand-to-metal charge transfer absorption that quenches the fluoresence of Eu^{3+}.[2]

The addition of Eu^{3+} to the nonfluorescent ligand **1** in an aqueous solution gives rise to an intense and narrow emission at 614 nm, which is characteristic of the $^5D_0\rightarrow{}^7F_2$ transition of Eu^{3+} (Fig. 1b). Since the excitation maximum (λ_{ex}) of the excitation spectrum (Fig. 1a) is almost equal to the λ_{max} of the complex, it is obvious that a nonradiative energy transfer takes place from the lowest π–π^* transition state of the ligand to the excited state of the metal ion. There is also a minor peak in each spectra (at 310 nm in the excitation and 670 nm in the emission spectrum) which is due to Rayleigh scattering.

Figure 2 indicates the fluorescence emission spectra of ligand **1** in the presence of Eu^{3+}, Sm^{3+}, or Tb^{3+} in a buffered solution at pH 8.2. These spectra were measured with λ_{ex} at 334 nm since the excitation spectra of these metal complexes of ligand **1** were very similar. The prominent emission observed at 614 nm is the $^5D_0\rightarrow{}^7F_2$ transition of Eu^{3+}, along with a weak emission at 590 nm (nondegenerate $^5D_0\rightarrow{}^7F_0$ transition); significant fluorescence is not observed with either Sm^{3+} or Tb^{3+}. The sharp line of the $^5D_0\rightarrow{}^7F_2$ emission of Eu^{3+}, without splitting patterns, does

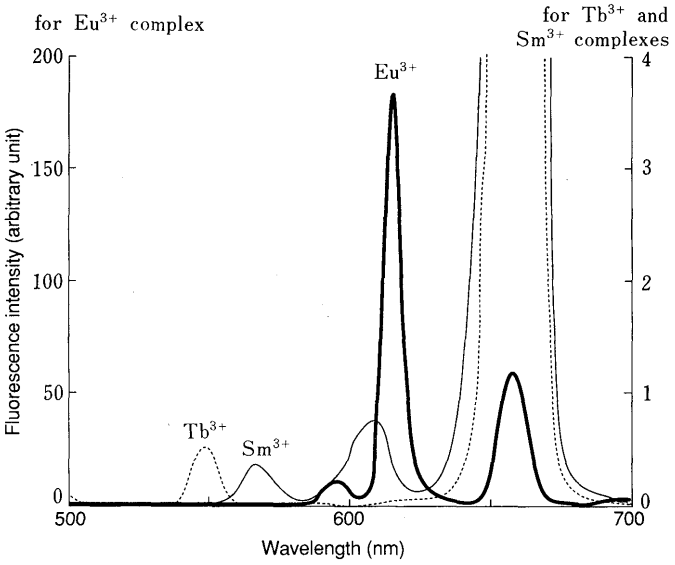

Fig. 2. Fluorescence Emission Spectra of the Complexes of Ligand **1** with Lanthanide Ions in 50 mM Tris Buffer at pH 8.2

Concentrations of ligand **2** and lanthanide ions are all 1×10^{-5} M. The spectra were measured with λ_{ex} at 334 nm.

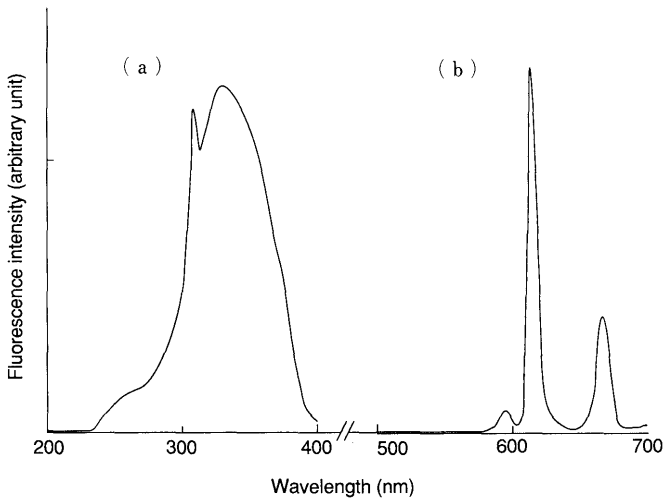

Fig. 1. Fluorescence Spectra of Eu^{3+}–Ligand **1** Complex in 50 mM Tris Buffer at pH 8.2

Concentrations of ligand **1** and Eu^{3+} are both 1×10^{-5} M. (a) excitation spectrum (λ_{em}=615 nm); (b) emission spectrum (λ_{ex}=334 nm).

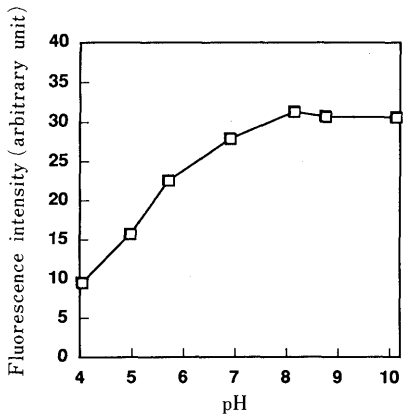

Fig. 3. pH Profile of the Fluorescence Intensity of Eu^{3+}–Ligand **1** Complex

The intensities were measured at 614 nm (λ_{ex}=334 nm) with Eu^{3+} and ligand **1** (1×10^{-6} M both) in 50 mM acetate (for pH 4.0—5.7) or 50 mM Tris buffer (for pH 6.0—10.2).

not provide any information as to the detailed nature of a ligand environment. The terbium complex, which often shows luminescence properties superior to Eu^{3+}, exhibits a weak emission at 550 nm ($^5D_4 \rightarrow {}^7F_5$ transition) because of the inefficient energy transfer from the triplet excited state of the ligand to the $4f$ level of the metal ion. This might be ascribed to the presence of a ligand-to-metal charge-transfer state since Tb^{3+} is similar in size to Eu^{3+} (ionic radius of 1.06 Å compared to 1.09 Å of Eu^{3+}) and also has similar coordination properties.[2b,11] Although Sm^{3+} was reported to be potentially usable probe in combination with 2-naphthoyltrifluoroacetone, a ligand currently in use for TR-FIA as described before,[10a] the Sm^{3+} complex of β-diketone ligand **1** does not indicate emissions with substantial intensities.

The fluorescence of the Eu^{3+} complex of ligand **1** was next examined as a function of pH (Fig. 3). As the ligand contains a pH-sensitive *N,N'*-di(2-hydroxybenzyl)ethylenediamine unit that possibly takes part in the complex formation, the fluorescence of the complex is largely affected by pH. The maximum fluorescence intensity is obtained at a pH above 8, where ligand **1** is thought to be neutral (H_2L); the fluorescence intensity at this pH range is found to be 1.4-fold larger than that of a BCPDA compelx of Eu^{3+} under the same conditions. The intensity decreases as the pH is lowered, probably because of the high proton affinities of the two basic nitrogens of the ligand, which makes the ligand less effective (H_3L^+/H_4L^{2+}) in binding the metal ion. The strong fluorescence at an alkaline pH is due to the coordination of the negatively charged phenolate group(s) to the metal ion to prevent hydroxide precipitation. The relatively constant fluorescence intensity over a physiological pH range is advantageous as a probe in TR-FIA.

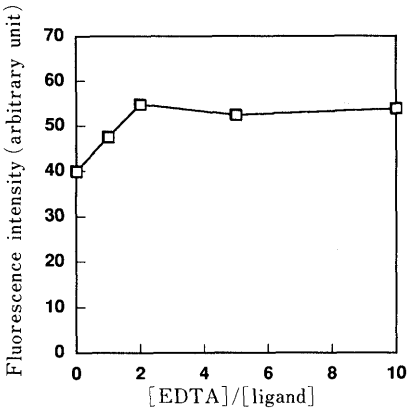

Fig. 4. Effect of EDTA on the Fluorescence Intensity of Eu^{3+}–Ligand **1** Complex

The intensities were measured at 614 nm (λ_{ex}=334 nm) with Eu^{3+} and ligand **1** (1×10^{-6} M both) in 50 mM Tris buffer at pH 8.2.

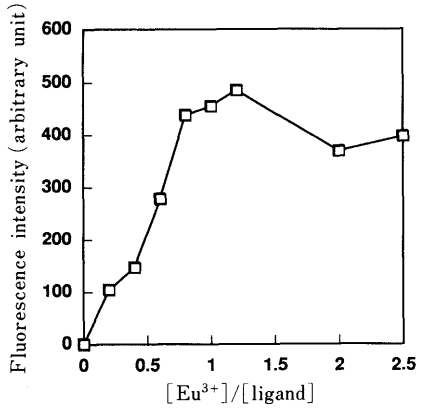

Fig. 5. Fluorescence Intensity of Eu^{3+}–Ligand **1** Complex as a Function of Eu^{3+}/Ligand Molar Ratio

The intensities were measured at 614 nm (λ_{ex}=334 nm) with ligand **1** (1×10^{-5} M) in 50 mM Tris buffer at pH 8.2 in the presence of EDTA (1×10^{-4} M).

The enhancement in the fluorescence intensity of the complex by the addition of EDTA was observed (Fig. 4). The intensity increased when EDTA of up to 2 eq was added to a buffered solution (pH 8.2) of the complex. When EDTA was added prior to the complex formation, the same enhancement effect as above was observed. Since EDTA is among the most common chelating oxygen ligands used in the formation of a stable water-soluble complex with Eu^{3+}, which is of great importance in ion-exchange separation,[11] we concluded that EDTA is effective in suppressing the hydrolysis of the aqua ion of Eu^{3+} (*e.g.*, $[Eu(H_2O)_3]^{3+}$) to form a hydrated species such as $[Eu(H_2O)_2(OH)]^{2+}$, although the exact mechanism of this EDTA-dependent enhancement of the fluorescence is not clear. The fact that the fluorescence intensity of Eu^{3+}–ligand **1** complex is not decreased by the addition of EDTA, with which the complex formation is considered to be much faster than with ligand **1**, and that the EDTA complex is nonfluorescent, indicate that the complex is kinetically inert.

In order to examine the composition of the complex, the fluorescence intensities were measured at various Eu^{3+}/ligand molar ratios at pH 8.2 in the presence of EDTA (Fig. 5). Although a slight decrease in the fluorescence intensity was seen at a higher molar ratio, the intensity reached a maximum with 1 eq of Eu^{3+} to the ligand, and no further increase was observed by further addition of the metal ion. Thus, the complex most likely exists in an aqueous solution as a 1 : 1 Eu^{3+}/ligand complex. Further examination of an exact coordination mode of this ligand is underway.

In conclusion, β-diketone ligand **1** forms a complex with Eu^{3+} that fluoresces at 614 nm in an aqueous solution and has a sensitivity comparable to BCPDA. Since most of the Eu^{3+} ligands, except BCPDA, that have been previously developed are not applicable to homogeneous immunoassay in an aqueous solution, ligand **1** seems to have potential value as a probe for TR-FIA, although functionalizing the molecule to incorporate a reactive group to be labeled to macromolecules is a step that is still required.

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