A New Ligand for Europium(III) That Forms a Stable Fluorescent Complex in Aqueous Solution

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A new macrocyclic ligand for Eu^{3+}, 24,30-diphenyl-8,19-(1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7*H*,18*H*-dibenzo[*b*,*k*][1,4,7,10,13,16]hexaazacyclooctadecine)diacetic acid (1), was synthesized and its fluorescence characteristics were examined, particularly to compare with 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) that has proved to be useful as a label in time-resolved fluoroimmunoassays. The complex of macrocycle 1 with Eu^{3+} was found to have a higher fluorescence intensity as well as a longer fluorescence lifetime than that of BCPDA.

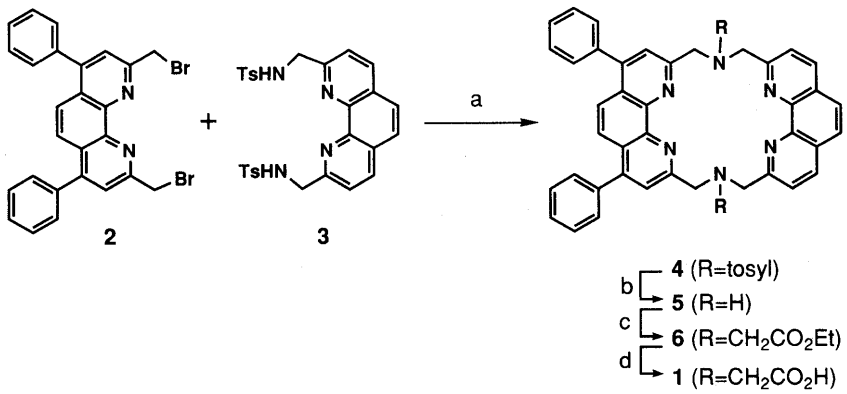
Keywords phenanthrolinophane derivative; macrocycle; europium chelator; stable fluorescence; aqueous medium

A number of analytical methods alternative to radioimmunoassay (RIA) have been extensively explored for immunological assays since RIA has several drawbacks as health hazards and the need for special disposal. Among various non-RIA techniques, time-resolved fluoroimmunoassay (TR-FIA) using Eu^{3+} complex as a label is gaining much attention because of its high sensitivity equivalent to RIA. TR-FIA is based on the unique spectroscopic properties of Eu^{3+} chelates of long fluorescence lifetimes (600—1000 μs) and large Stokes shifts (*ca.* 290 nm) with an emission wavelength at or near 615 nm.[1,2] Two types of approaches have been exploited: i) One is by Hemmilä and co-workers,[1] in which isothiocyanatophenylethylenediaminetetraacetic acid (isothiocyanatophenyl-EDTA) is utilized to bridge an antibody and Eu^{3+}. Although the method using this label has been used successfully, it suffers from the limitation that Eu^{3+} must be released by lowering pH from the nonfluorescent Eu^{3+}–EDTA complex in order to be re-complexed by a second chelator of β-diketone type, 2-naphthoyltrifluoroacetone (NTFA), in a micellar solution composed of Triton X-100 and the synergistic agent tri-*n*-octylphosphine oxide (TOPO) to produce a higher fluorescence. It therefore requires an extra step to the procedure and, as a result, is vulnerable to contamination. ii) The second approach, proposed by Diamandis and co-workers,[2,3] is more straightforward in the sense that it allows a direct quantification of Eu^{3+} in an aqueous solution after the immunoreaction using a chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). BCPDA forms a stable fluorescent complex with Eu^{3+} with two heteroaromatic nitrogens and two carboxy groups as the coordination sites, and its fluorescence is insensitive to aqueous quenching. The fluorescence intensity, however, is sensitive to the molar ratio of Eu^{3+}/BCPDA of the complex when the ratio is smaller than 1.0. A complex formation of Eu^{3+} with more than two molecules of BCPDA, which is unlikely in a labeled form, is suggested depending on the molar ratio.[3]

In an effort to circumvent these disadvantages, we synthesized a new macrocyclic chelator (**1**, Chart 1) that was designed to form a stable 1 : 1 complex with Eu^{3+} exhibiting a notable emission in aqueous solution. We wish to describe herein the synthesis and the fluorescence characteristics of macrocycle **1**, particularly in comparison with BCPDA.

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 as purchased. BCPDA was prepared according to the method described by Evangelista *et al.*[3] A stock



reagents and conditions: (a) K_2CO_3, DMF, 50 °C; (b) HOAc, H_2SO_4, 100 °C; (c) ethyl bromoacetate, K_2CO_3, DMF, 50 °C; (d) KOH, EtOH, reflux

Chart 1. Synthetic Route to Macrocycle **1**

solution of macrocycle **1** was prepared as 1×10^{-4} M aqueous solution. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured on a Bruker AC-200P operating at 200 and 50 MHz, respectively, with tetramethylsilane as an internal standard. The splitting patterns were designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Fluorescence spectra were recorded on a Hitachi 650-60 spectrometer. Time-resolved fluorescence measurements were performed on an Arcus 1230 fluorometer (LKB Wallac, Turku, Finland). IR spectra were taken in KBr disks on a Hitachi 270-30. 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.

4,7-Diphenyl-2,9-bis(bromomethyl)-1,10-phenathroline (2) Dibromide **2** was prepared by the method reported by Chandler *et al.*[4]

2,9-Bis(*p*-toluenesulfonamidomethyl)-1,10-phenanthroline (3) To a stirred solution of 2,9-bis(aminoethyl)-1,10-phenanthroline diperchlorate[4] (2.2 g, 5.0 mmol) in pyridine (20 ml) was added *p*-toluenesulfonyl chloride (2.3 g, 12.0 mmol) at 0 °C, and the stirring was continued for 3 h at 22 °C. Water (100 ml) was added and the reaction mixture was partitioned between chloroform and water. The chloroform layer was dried ($MgSO_4$) and concentrated to give a colored residue, which was chromatographed on silica gel (1% methanol in chloroform) to afford 1.1 g (40%) of ditosylate **3** as a white powder. mp 114 °C (dec.). IR (cm^{-1}): 3270 (NH), 3070 (Ar), 2940 (CH_3), 2870 (CH_3), 1630 (Ar), 1600 (Ar), 1540 (Ar), 1330 (SO_2), 1160 (SO_2). ^{1}H-NMR ($CDCl_3$) δ: 2.28 (6H, s, CH_3), 4.57 (4H, d, J=6.0 Hz, CH_2), 6.67 (2H, t, J=6.0 Hz, NH), 7.15 (2H, d, J=8.3 Hz, Ar), 7.62 (2H, d, J=8.3 Hz, Ar), 7.77 (6H, m, Ar), 8.19 (2H, d, J=8.3 Hz, Ar). FAB-MS *m/z*: 547 $(M+H)^+$.

24,30-Diphenyl-8,19-bis(*p*-toluenesulfonyl)-1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7*H*,18*H*-dibenzo[*b*,*k*][1,4,7,10,13,16]hexaazacyclooctadecine (4) A mixture of dibromide **2** (520 mg, 1.0 mmol), ditosylate **3** (550 mg, 1.0 mmol) and potassium carbonate (2.0 g, 14.5 mmol) in *N*,*N*-dimethylformamide (DMF, 20 ml) was stirred at 22 °C for 12 h, and the reaction mixture was filtered. The addition of water (100 ml) resulted in precipitation of the product, which was washed with methanol and dried *in vacuo* to yield 820 mg (91%) of tosylate **4** as an off-white powder. mp 154 °C (dec.). IR (cm^{-1}): 3040 (Ar), 2940 (CH_3), 2870 (CH_3), 1630 (Ar), 1600 (Ar), 1540 (Ar), 1350 (SO_2), 1160 (SO_2). ^{1}H-NMR ($CDCl_3$) δ: 2.52 (6H, s, CH_3), 4.73 (4H, br s, CH_2), 5.37 (4H, br s, CH_2), 7.25—7.90 (28H, m, Ar). ^{13}C-NMR ($CDCl_3$) δ: 21.61 (CH_3), 56.98 (CH_2), 122.90—156.80 (Ar). FAB-MS *m/z*: 903 $(M+H)^+$, 925 $(M+Na)^+$.

24,30-Diphenyl-1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7*H*,18*H*-dibenzo[*b*,*k*][1,4,7,10,13,16]hexaazacyclooctadecine (5) A solution of tosylate **4** (770 mg, 0.85 mmol) in acetic acid (4 ml) and sulfuric acid (6 ml) was heated at 120 °C for 10 h, and poured onto ice water (10 ml). Formed precipitates were collected and dried *in vacuo* to furnish 550 mg (82%) of amine **5** as a salt of equivalent sulfuric acid. mp 225 °C (dec.). IR (cm^{-1}): 3050 (Ar), 2950 (CH_2), 2850—2500 (NH_2), 1630 (Ar), 1600 (Ar), 1520 (Ar), 1120 (CN). ^{1}H-NMR (DMSO-d_6) δ: 4.62 (8H, s, CH_2), 7.61 (10H, s, Ar), 7.78 (6H, d, J=8.3 Hz, Ar), 8.00 (2H, s, Ar), 8.59 (2H, d, J=8.2 Hz, Ar). ^{13}C-NMR (DMSO-d_6) δ: 51.03 (CH_2), 122.82—152.58 (Ar). FAB-MS *m/z*: 594 $(M+H)^+$, 616 $(M+Na)^+$.

24,30-Diphenyl-8,19-[1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7*H*,18*H*-dibenzo[*b*,*k*][1,4,7,10,13,16]hexaazacyclooctadecine)-diacetic Acid Ethyl Ester (6) A mixture of amine **5** (500 mg, 0.84 mmol), ethyl bromoacetate (2 ml, 18.0 mmol) and potassium carbonate (2 g, 14.5 mmol) in DMF (20 ml) was stirred at 22 °C for 12 h. The reaction mixture was filtered and the filtrate was concentrated. Diethyl ether (10 ml) was added to an oily residue to form precipitates which were recrystallized from ethanol, yielding 170 mg (33%) of ester **6** as a white powder. mp 194—201 °C (dec.). IR (cm^{-1}): 3070 (Ar), 3000 (CH_2), 2940 (CH_2), 2860 (CH_2), 1740 (C=O), 1630 (Ar), 1600 (Ar), 1580 (Ar), 1210 (C–O–C). ^{1}H-NMR ($CDCl_3$) δ: 1.26 (6H, t, J=7.1 Hz, CH_3), 3.90 (4H, s, CH_2CO), 4.18 (4H, q, J=7.1 Hz, CH_2), 4.46 (8H, s, CH_2N), 7.50 (10H, s, Ar), 7.58 (2H, s, Ar), 7.71 (2H, d, J=8.3 Hz, Ar), 7.82 (4H, s, Ar), 8.33 (2H, d, J=8.2 Hz, Ar). ^{13}C-NMR ($CDCl_3$) δ: 14.09 (CH_3), 59.01 (CH_2N), 61.00 (CH_2), 64.00 ($\underline{CH_2}CO$), 123.67—158.34 (Ar), 172.67 (CO). FAB-MS *m/z*: 805 $(M+K)^+$.

24,30-Diphenyl-8,19-(1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7*H*,18*H*-dibenzo[*b*,*k*][1,4,7,10,13,16]hexaazacyclooctadecine)-diacetic Acid (1) A solution of ester **6** (150 mg, 0.20 mmol) and potassium hydroxide (200 mg, 3.5 mmol) in ethanol (20 ml) was refluxed for 2 h. The solvent was stripped off to leave an oily residue to which water (20 ml) was added to prompt crystallization. A formed solid was collected, washed with water and dried *in vacuo* to furnish 110 mg (70%) of acid **1** as an off-white powder. mp 170 °C (dec.). IR (cm^{-1}): 3400 (OH), 3060 (Ar), 2960 (CH_2), 2920 (CH_2), 2850 (CH_2), 1600 (CO_2), 1400 (CO_2). ^{1}H-NMR ($CDCl_3$) δ: 3.47 (4H, s, CH_2CO), 4.26 (4H, m, CH_2), 4.42 (4H, m, CH_2), 7.49 (12H, s, Ar), 7.67 (2H, d, J=7.9 Hz, Ar), 7.79 (4H, m, Ar), 8.32 (2H, s, Ar). ^{13}C-NMR ($CDCl_3$) δ: 29.61 (CH_2), 61.26 (CH_2N), 124.02—158.88 (Ar), 175.30 (CO). FAB-MS *m/z*: 749 $(M+K)^+$. *Anal.* Calcd for $C_{44}H_{32}K_{1.5}N_6O_4$: C, 68.82; H, 4.27; N, 10.94. Found: C, 69.39, H, 3.99, N, 10.71.

Results and Discussion

Although Eu^{3+} is luminescent with the emission wavelength (λ_{em}) at approximately 615 nm, the luminescence in an aqueous solution is quenched by a water molecule which, being a strong ligand for Eu^{3+}, nonradiatively deactivates the excited ion. For this quenching to be minimized, the suitable ligand should have strong coordination sites for Eu^{3+} that has variable coordination numbers (8 and 9 being most common[5]), as well as an organic structure which absorbs light and effectively transfers its energy to the metal ion. Macrocycle **1** consists of two phenanthroline rings that form an intramolecular cavity with the size of approximately 2.3 Å (by CPK model) which is suitable for the inclusion of Eu^{3+}. The two carboxymethyl groups are attached to strengthen the binding with Eu^{3+} held in the plane of the ring by coordinating axially; the two phenyl groups are to increase the efficiency of light absorption, and also, in a subsequent study, to provide a site for incorporating a functionality in order for the complex to be covalently linked to proteins.

As outlined in Chart 1, macrocycle **1** was synthesized over 4 steps from dibromide **2** and ditosylate **3** that were readily prepared according to the method described in literature.[4] Cyclization of dibromide **2** with ditosylate **3** proceeded smoothly under basic conditions to afford tosylate **4** in excellent yield, which was subjected to detosylation followed by carboxymethylation, ultimately giving ester **6**. Hydrolysis of ester **6** with potassium hydroxide gave macrocycle **1** as a potassium salt. Upon the addition of Eu^{3+} to macrocycle **1** in a neutral buffer, a strong fluorescence from Eu^{3+} with λ_{em} at 619 nm emerged, whereas the absorption intensity of macrocycle **1** (ε=37100 at λ_{max}=278 nm) slightly increased (ε=44200 at λ_{max}=279 nm). In contrast to BCPDA, the complex formation appears to be slow, taking account of the observation that it takes approximately 1 h for the fluorescence intensity to reach its maximum when equivalents of Eu^{3+} and macrocycle **1** are incubated at 22 °C. Inclusion of Eu^{3+} by this ligand was also evidenced by FAB-MS measurement, in which a peak corresponding to a monocation of the 1 : 1 complex of Eu^{3+}–macrocycle **1** (m/z=861 $[M-2H+Eu]^+$) was observed.

Fluorescence measurements of macrocycle **1** were then carried out at pH 7.5 with Eu^{3+}, Tb^{3+} and Sm^{3+}, respectively, as potentially usable probes for TR-FIA (Fig. 1). The Eu^{3+} complex presents sharp emissions at 619 and 594 nm which originate from the $^5D_0 \rightarrow {}^7F_1$ and $^5D_0 \rightarrow {}^7F_0$ transitions of Eu^{3+}, respectively; the Tb^{3+} complex luminesces at 548 nm due to the $^5D_4 \rightarrow {}^7F_5$ transition.[5] It is known that the de-activation process of a complex consists of a singlet–triplet intersystem crossing of the ligand molecule and a subsequent energy transfer from the triplet state to the excited 4*f* level of the metal ion.[5] Thus, the fact that neither Tb^{3+} nor Sm^{3+} complex exhibits a substantial fluorescence indicates that an effi-

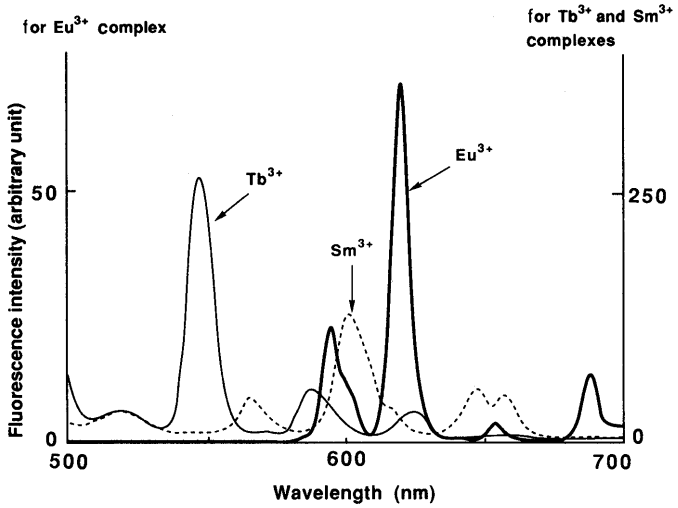


Fig. 1. Fluorescence Spectra of Macrocycle **1** with Lanthanide Ions in 50 mM Acetate Buffer at pH 7.5

Concentrations of macrocycle **1** and lanthanide ions were 1×10^{-6} and 1×10^{-5} M, respectively.

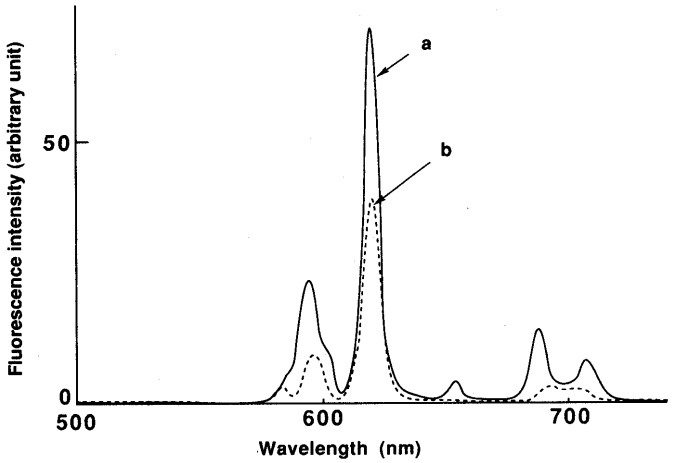


Fig. 2. Fluorescence Spectra of Macrocycle **1** and 4,7-Diphenyl-1,10-phenanthroline-2,9-dicarboxylic Acid in the Presence of Eu^{3+} in 50 mM Acetate Buffer at pH 7.5

Concentration of Eu^{3+} was 1×10^{-5} M. a: macrocycle **1** (1×10^{-6} M); b: 4,7-diphenyl-1,10-phenanthroline-2,9-dicarboxylic acid (1×10^{-6} M).

cient intramolecular energy transfer from the triplet excited state of the ligand to the 4*f* level of the metal ion is most probable with Eu^{3+}.

Figure 2 compares the fluorescence intensity of macrocycle **1** with that of 4,7-diphenyl-1,10-phenanthroline-2,9-dicarboxylic acid, a BCPDA derivative which bears two hydrogens instead of two chlorosulfonyl groups, under identical conditions of pH 7.5. The Eu^{3+}–macrocycle **1** complex fluorescence at 619 nm with the emission intensity being twice as large as that of the BCPDA derivative and, in addition, a larger Stokes shift (329 nm compared to 276 nm of BCPDA).

We next examined an effect of pH and of detergents in order to maximize the emission intensity of the complex. As shown in the pH profile in Fig. 3, the emission intensity depends on the type of buffer used, but the maximum intensity is obtained at pH 5—6. It may be reasoned that at acidic pHs where the carboxy groups are protonated, the chelate formation is sluggish, and at alkaline pHs an

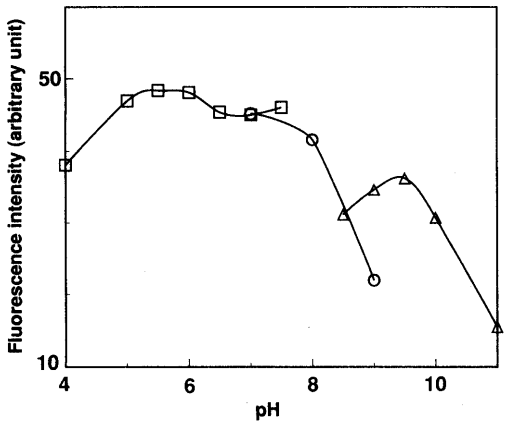


Fig. 3. pH Profile of the Fluorescence of Macrocycle **1** with Eu^{3+}

Emission wavelength was 619 nm. Concentrations of macrocycle **1** and Eu^{3+} were both 1×10^{-5} M. (□) 50 mM acetate buffer; (○) 50 mM Tris–acetic acid buffer; (△) 50 mM carbonate buffer.

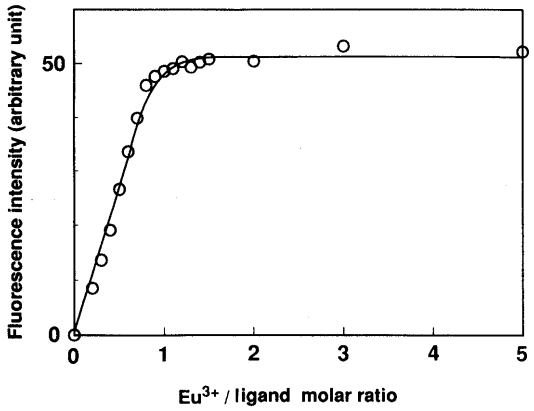


Fig. 4. Emission Intensity at Various Molar Ratios of Eu^{3+}–Macrocycle **1** Complex in 50 mM Acetate Buffer at pH 7.5

Concentration of **1** was 5×10^{-6} M.

insoluble hydroxide formation becomes predominant. The pH profile reflects the rate of the complex formation rather than the thermodynamic stability of the complex. Several types of detergents were examined in the hope of increasing the fluorescence by suppressing the aqueous quenching through a micelle formation. Both nonionic and cationic detergents were found to markedly enhance the emission intensity of the Eu^{3+}–macrocycle **1** complex. For example, 13-fold enhancement with Tween 80 and 10-fold with cetyltrimethylammonium chloride were observed in 50 mM acetate buffer at pH 6.0 with the concentration of the detergents being 3% (w/v), whereas no significant enhancement was observed with anionic detergents such as sodium dodecylsulfate under the same conditions.

The composition of the complex was investigated by varying the molar ratio of Eu^{3+}/macrocycle **1** (Fig. 4). As expected, the complex proved to be 1 : 1 Eu^{3+} to macrocycle **1** based on the observation that the emission intensity became constant when the ratio reached 1.0. Moreover, Fig. 4 demonstrates that, in the presence of excess macrocycle **1**, there are no complex formations of Eu^{3+} with more than 1 equiv. of the ligand molecule, which is the case in BCPDA. Once the complex is formed under these conditions, the emission intensity is not reduced even by the addition of a 100-fold molar excess of EDTA, whereas a drastic decrease

TABLE I. Comparison of Lifetimes and Relative Intensities of the Fluorescences from Eu(III) Complexes[a]

Ligand	Fluorescence lifetime (μs)	Relative intensity[b]
Macrocycle **1**	1144	0.64
BCPDA	400—760[c]	0.33
NTFA/dodecane	670[d]	—
NTFA/water–TOPO–Triton X-100	905[d]	—

a) [Ligand]/[Eu^{3+}] = $1 \times 10^{-5}/1 \times 10^{-4}$ M. *b*) The fluorescence intensity of quinine sulfate (1×10^{-5} M in water) was taken as 1.00. *c*) Ref. 3. *d*) Ref. 6.

can be observed in the case of BCPDA by adding more than a 2-fold excess of EDTA. This implies that the 1 : 1 Eu^{3+}–macrocycle **1** complex is kinetically more inert than BCPDA with a higher stability constant.

The fluorescence lifetime of the Eu^{3+}–macrocycle **1** complex was measured by the pulse technique and the results were compared with those of BCPDA and β-diketone ligand described in the literatures[3,6] (Table I). NTFA is the second chelator currently employed in the first approach (*vide supra*). After pulsed excitation, the decay of the fluorescence of the complex in 50 mM acetate buffer at pH 6.0 followed the first order kinetics, which gave a longer lifetime of 1144 μs than those reported for BCPDA. This longer lifetime may be attributed to a better shielding of Eu^{3+} from bulk water by the macrocyclic ligand. Since a lifetime of an Eu^{3+} complex is mainly determined by the rate of radiative emission from the excited level of Eu^{3+} and accordingly by the rate of radiationless de-activation *via* an energy transfer to the surrounding molecule, it is very sensitive to the chemical nature of the ligand environment.[5] It is therefore reasonable to assume that macrocycle **1** has a lesser number of water molecules in its inner coordination site than does BCPDA.

In summary, a new chelator, macrocycle **1**, was synthesized and demonstrated to form a stable 1 : 1 complex with Eu^{3+} which has a higher fluorescence intensity and a longer fluorescence lifetime than that of BCPDA, and these are favorable when used as a label in TR-FIA applications. Introducing a functionality to macrocycle **1** to be covalently linked to a macromolecule is currently being undertaken.

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