Luminescence Properties of Terbium(III) Complexes with 4-Substituted Dipicolinic Acid Analogues

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The spectral properties of Tb(III) complexes with a series of 4-substituted analogues of dipicolinic acid (2,6 pyridinedicarboxylic acid) were compared. Analogues in which the 4-substituent was −H, −OH, −Cl, −Br, $-NH_2$, or −NHAc all sensitized emission from bound terbium ions when excited with ultraviolet light. Molar extinction coefficients of the complexes at the wavelengths of maximum absorbance were in the range (2.2×10^3 to 8.3×10^3) M^{-1} cm^{-1} with magnitudes in the following order: NHAc > NH_2 > OH > H > Br > Cl. When excitation spectra of Tb(III) complexes were measured under identical conditions for each ligand, the maximum Tb(III) emission intensities were in the following order: NH_2 > NHAc > OH > H > Cl ~ Br. Measurements of Tb(III) emission decay kinetics revealed lifetimes in the range 1.0−2.0 ms. Relative quantum yields for energy transfer were ordered as follows: NH_2 > OH > NHAc > Cl > H ~ Br. Energy transfer efficiency was not enhanced when Br was substituted for Cl at the 4-position, consistent with energy transfer from excited singlet, rather than triplet states. The efficient sensitization of Tb(III) emission by the analogues with nitrogen at the 4-position suggests that this class of compounds will be most useful for developing millisecond luminescence probes for bioanalytical studies.

Introduction

Energy transfer from aromatic ligands to bound Tb(III) or Eu(III) results in dramatically enhanced luminescence from the bound lanthanide ions.[1] Because of their large Stokes shifts and millisecond-range excited state lifetimes, organic donor−lanthanide acceptor complexes are useful in energy transfer studies of biological systems[2] and in delayed luminescence immunoassays (reviewed in ref 3).

Chelating agents commonly used for enhancement of Tb(III) and Eu(III) luminescence include bidentate ligands, such as β-diketonates,[4] tridentate pyridines, such as dipicolinic acid and chelidamic acid,[5] and multidentate polyaminocarboxylate chelators.[6] The bi- and tridentate ligands form lanthanide complexes with low stability constants[7] but are very efficient sensitizers of Tb(III) and Eu(III) luminescence. Tb(III) luminescence is enhanced more than 10^4-fold by dipicolinic acid,[5] and Eu(III) luminescence is similarly enhanced by diketonates.[8] In both cases, atoms involved in the conjugated π-electron systems are directly coordinated to the metal ions. In contrast, the poly(amino carboxylate) ligands such as EDTA or DTPA form complexes with very high stability constants, but are much less efficient sensitizers of emission, even when substituted with aromatic groups, apparently due to the distance of the aromatic group from the bound metal ion.[9] However, within this last group of complexing agents, a number of bifunctional chelating agents have been developed that can be used for covalent coupling of lanthanide-complexing groups to macromolecules. Similarly reactive derivatives of the efficient sensitizers would be highly desirable, and some progress has been made in this area using derivatives of 1,10-phenanthroline-2,9-dicarboxylic acid[10] and 2,2′-bipyridine.[11] Unfortunately, the sensitization efficiency of these compounds is apparently not as great as that of dipicolinic acid. Perhaps the most promising analogues described so far are those based on 2,2′,2″,2‴-[4-(phenylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)tetrakis(acetic acid)[12] which have been employed for direct time-resolved luminescence imaging in immunohistochemistry and *in situ* nucleic acid hybridization. The main drawbacks of this class of chelators are the bulky nonpolar phenylethynyl group, and their relatively low luminescence intensities.[13]

Because of the ease with which functional groups can be placed at the 4 position of dipicolinic acid, and the efficiency with which dipicolinic acid sensitizes lanthanide ion emission in aqueous solution, compounds of the type 4-**X**−2,6-pyridinedicarboxylic acid represent attractive intermediates for the formation of lanthanide energy transfer complexes. Despite extensive studies of lanthanide complexes with dipicolinic acid (DPA) and a few preliminary descriptions of syntheses of 4-substituted DPA analogues,[14] almost no information has been available on the chemistry or spectroscopy of other members of this class and their complexes with Tb(III) and Eu(III). The mechanisms of emission sensitization are still not fully understood, nor is the relationship between donor structure and efficiency of emission sensitization. Therefore it is not possible

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Scheme 1

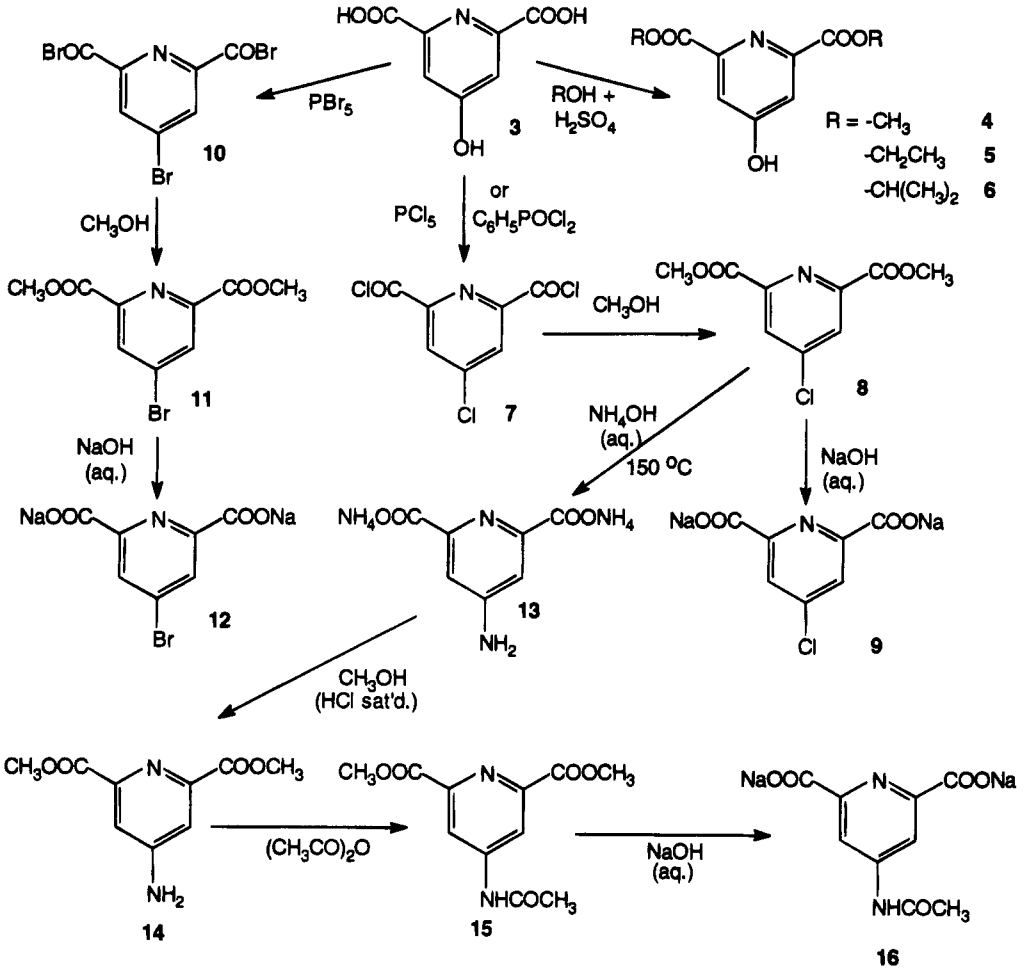
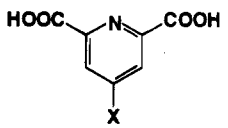


Chart 1



X =		
	H	1 (DPA)
	OH	3 (Chelidamic Acid)
	Cl	9
	Br	12
	NH_2	13
	$NHCOCH_3$	16

to predict with reliability which substituents will lead to more or less efficient energy transfer to bound lanthanides.

Here we describe the preparation and characterization of a series of highly purified alkyl esters of 4-substituted DPA analogues (Chart 1), as well as the UV absorbance and luminescence properties of Tb(III) complexes with their carboxylate forms. The spectral properties, including luminescence lifetime data, allow comparisons of the efficiencies of energy transfer from the different ligands, as well as the overall efficiencies of sensitized emission. Examples of the reactions used may be found in Scheme 1.

Experimental Section

Preparation of Ligands. All the solvents and reagents were of reagent grade and were used without any further purification, except for chelidamic acid (Sigma Chemical Co.) which was supplied and used as a dark brown powder of about 90% purity. In some experiments, chelidamic acid was purified as its methyl ester prior to derivatization, but this procedure did not improve product yields. Analtech silica plates (both HL and HLF) were used for monitoring the reactions and column fractions, with development by benzene: ethylacetate = 3:2 (A) or 3:7 (B). Melting points were recorded on a Thiele melting point apparatus and are uncorrected.

Dipicolinic Acid Dimethyl Ester (2). Dipicolinic acid, **1**, (2 g, 12 mmol) was refluxed with methanol in the presence of concentrated H_2SO_4 as a catalyst for 8 h. The solvent was removed under vacuum and the residue was diluted with water (25 mL) and extracted into EtOAc (3 × 25 mL). The combined EtOAc extracts were washed with water (3 × 25 mL), dried (Na_2SO_4) and evaporated under reduced pressure to obtain **2** (1.4 g, 60%): rf (A) 0.48, (B) 0.54; ^{1}H NMR ($CDCl_3$) δ 8.33 (d, J = 9 Hz, 2H, H3/H5), 8.06 (t, J = 9 Hz, 1H, H4), 4.04 (s, 6H, 2 × $COOCH_3$).

Alkyl Esters (4, 5, and 6) of Chelidamic Acid. Chelidamic acid, **3**, (1g, 5.4 mmol of a tautomeric mixture of 1,4 dihydro-4-oxo-2,6-pyridinedicarboxylic acid and 4 hydroxy-2,6-pyridinedicarboxylicacid) was refluxed with an excess of alcohol (methyl, ethyl, or isopropyl; 25 mL) in the presence of concentrated H_2SO_4 as a catalyst for 4–5 h. The solvent was evaporated under vacuum and the residue was diluted with water (25 mL) and extracted into EtOAc (3 × 25 mL). The combined EtOAc extracts were washed with water (3 × 25 mL), dried (Na_2SO_4), and evaporated under reduced pressure to obtain the corresponding esters. Dimethyl ester **4** (70%): rf (A), 0.12, (B), 0.25, mp 170–172 °C; ^{1}H NMR ($CDCl_3$) δ 7.25 (s, 2H, Ar–H), 3.98 (s, 6H, 2 × $COOCH_3$). Diethyl ester **5** (77%): rf (A), 0.2, mp 110–112 °C; ^{1}H NMR ($CDCl_3$) δ 7.26 (s, 2H, Ar–H), 4.43 (q, J = 7.0 Hz, 4H, 2 × CH_2) 1.4 (t, J = 7.0 Hz, 6H, 2 × CH_3). Diisopropyl ester **6** (54%): rf (A), 0.35, mp 145–146 °C; ^{1}H NMR (acetone-d_6) δ 7.56 (s, 2H, Ar–H), 5.18 (septet, J = 6.3 Hz, 2H, 2 × CH), 1.31 (d, J = 6.3 Hz, 12H, 4 × CH_3).

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4-Chloro-2,6-dimethylpyridinedicarboxylate (8). This compound was prepared by two methods.

(1) By the Action of Phenylphosphonic Dichloride: A mixture of phenylphosphonicdichloride (21.73 g; 112 mmol) and chelidamic acid, **3** (5.1 g; 28 mmol) was heated to 130−140 °C for 2 h while protected with a moisture guard tube. The reaction mixture was cooled to room temperature and anhydrous MeOH (100 mL) was added slowly over 25−30 min. The mixture was stirred at room temperature for 10 more minutes and then diluted with water (200 mL) and extracted with EtOAc (3 × 100 mL). The combined EtOAc extracts were washed with water (3 × 150 mL) and dried (Na_2SO_4) and the solvent was evaporated under vacuum to obtain a crude solid which was recrystallized from MeOH to yield **8** (4.3 g; 68.5%): mp 141−142 °C; ^{1}H NMR ($CDCl_3$) δ 8.31 (s, 2H, Ar−H), 4.05 (s, 6H, 2 × $COOCH_3$); ^{13}C NMR ($CDCl_3$) δ 163.7 (CO), 149.1 (CO), 146.4 (C_4), 127.9 (Ar−C_2, C_3, C_5, and C_6) and 53.1 (OCH_3).

(2) By the Action of PCl_5: A mixture of chelidamic acid, **3** (2.17 g; 12 mmol), PCl_5 (10.50 g; 48 mmol), and $CHCl_3$ (20 mL) was refluxed for 72 h. Then the solvent was distilled off and the residue was treated with anhydrous MeOH (25 mL). The reaction mixture was worked up as described in the previous section. The crude product upon crystallization from MeOH gave a pure compound **8** (1.92 g; 70%); mp 141−142 °C; identical to the product of the phenylphosphonic dichloride reaction by mixed melting point, TLC, and ^{1}H and ^{13}C NMR.

4-Bromo-2,6-dimethylpyridinedicarboxylate (11). This compound was synthesized following the same procedure as described in method 2 above for preparation of **8**, except that PBr_5 was used as the halogenating agent instead of PCl_5. The brown crude solid, upon crystallization from MeOH, yielded a pure white bromo derivative **11** (1.92 g; 70%); mp 175−177 °C. ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 2H, Ar−H), 4.04 (s, 6H, 2 × $COOCH_3$); ^{13}C NMR ($CDCl_3$) δ 163.9 (CO), 149.1 (CO), 135.0 (C_4), 131.2 (Ar−C_2, C_3, C_5, and C_6), and 53.4 (2 × OCH_3). Anal. Calcd for $C_9H_8NO_4Br$ (274): C, 39.41; H, 2.92; N, 5.01; Br, 29.19. Found: C, 39.21; H, 2.95; N, 5.16; Br, 29.38.

4-Amino-2,6-dimethylpyridinedicarboxylate (14). A mixture of 4-chloro-2,6-dimethylpyridine dicarboxylate, **8** (2 g; 8.7 mmol) and 28% aqueous NH_3 (10 mL) was heated in a bomb at 150 °C for 24 h. After being cooled to room temperature, the mixture was acidified with concentrated HCl to pH 2.5. It was then evaporated to dryness on a hot plate, and the solid obtained was refluxed with MeOH (100 mL) saturated with HCl(g) for 10 h. The solvent was evaporated under vacuum, and the concentrated solution was neutralized with Et_3N to pH 7. The white solid which precipitated upon neutralization was filtered, washed with MeOH, dried, and weighed (585 mg). The filtrate was concentrated on a rotary evaporator and chromatographed over SiO_2 (1 × 35 cm) with C_6H_6:EtOAc (1:1) as an eluant. A total of 15 fractions (50 mL each) were collected. Fractions 5−8 contained the desired pure compound (50 mg), thus leading to the total yield of 635 mg (35%). An analytical sample was obtained by recrystallizing the white solid from MeOH: mp 258−260 °C (decomposed); ^{1}H NMR (DMSO-d_6) δ 7.34 (s, 2H, Ar−H), 6.69 (br s, 2H, NH_2; exchanges with D_2O), 3.82 (s, 6H, 2 × $COOCH_3$); ^{13}C NMR (DMSO-d_6) δ 165.6 (CO), 157.5 (CO), 124.7 (C4), 111.4 (Ar−C2, C3, C5, and C6), and 51.66 (2 × OCH_3).

4-Acetamido-2,6-dimethylpyridinedicarboxylate (15). 4-Amino-2,6-dimethylpyridinedicarboxylate (**14**) (50 mg, 0.24 mmol), was suspended in Ac_2O (5 mL), and two drops of concentrated H_2SO_4 were added to it. The reaction mixture was stirred at room temperature for 2 h and then diluted with water (10 mL), heated on a water bath for 10 min, and extracted with EtOAc (3 × 10 mL). The combined EtOAc extracts were washed with water (4 × 20 mL) and dried (Na_2SO_4), and the solvent was removed under vacuum. The residue obtained was chromatographed over SiO_2 (1 × 21 cm) with C_6H_6:EtOAc (1:1) as eluant. The faster moving impurities were removed in the first four 25 mL fractions. The desired pure compound **15** was then eluted with EtOAc alone (4 × 25 mL). It gave a single spot on TLC and on recrystallization from MeOH gave a white crystalline solid (50 mg; 50%, mp 250−252 °C). ^{1}H NMR (DMSO-d_6) δ 10.75 (s, 1H, ArNH), 8.41 (s, 2H, Ar−H), 3.87 (s, 6H, 2 × $COOCH_3$), 2.10 (s, 3H, $NHCOCH_3$); ^{13}C NMR (DMSO-d_6) δ 170.9 ($COOCH_3$), 165.6 (CONH), 149.5 ($COOCH_3$), 148.9 (C4), 117.3 (Ar−C2, C3, C5, and C6); 53.5 (2 OCH_3); and 25.2 CH_3). GC/MS *m/e* (relative intensity): 253 (MH^+, 10), 237 (10), 222 (5), 195 (11), 194 (100), 178 (9), 162 (22), 152 (30), 134 (29), 120 (28), and 43 (60). Anal. Calcd for $C_{11}H_{12}N_2O_5$ (252): C, 52.38; H, 4.78; N, 11.11. Found: C, 52.52; H, 4.79; and N, 11.05.

Spectroscopic Measurements. ^{1}H and ^{13}C NMR spectra were recorded on a GE, QE-300 spectrometer. GC/MS was carried out on a Hewlett-Packard 5890/VG, Trio-1 instrument. Ultraviolet absorbance spectra were measured using a Hewlett Packard 8452 diode array spectrophotometer. Steady-state luminescence excitation and emission spectra were recorded using an Aminco-Bowman spectrofluorimeter modified as described.[15] Lifetime measurements were made using a UV-optimized continuous wave argon ion laser (Innova 200−15/3, Coherent Radiation, Palo Alto, CA). The optics for 275/305 nm output were used, with the two lines used together or with either selected using a diffraction grating. In some cases, the near UV optics were used with the 337 nm line selected. Lifetimes were found not to be dependent on the excitation wavelength. The emission was detected at right angles, after passing through a high pass filter and a 546 nm interference filter. Detection was by a red-sensitive photomultiplier (Hamamatsu R928) cooled using a thermoelectric housing (Products for Research R928/0115/0381). The photomultiplier output was amplified using one of three amplifiers: A Keithley 427 current amplifier (15 μs time constant), a Keithley 417 picoammeter, or a LeCroy 612AM amplifier. When the Keithley amplfiers were used, the output was fed directly into an analogue-to-digital convertor (DAS-16G, Metrabyte) installed in an 80386-based AT-type personal computer. When the 612AM amplifier was used, the output was fed into a Tennelec 454 constant fraction discriminator, and the fast current pulse output from the discriminator was converted to a voltage pulse using a Tennelec 862 time-to-amplitude convertor (TAC), with the Stop and Start inputs both supplied by the discriminator output, and with the Stop separated in time from the Start by means of a delay cable. The TAC voltage pulse was recorded by the DAS-16G. Recording of each decay was triggered by the amplified output of a photodiode positioned next to the excitation slit.

Lifetimes were determined from linear least squares analysis of the emission decays after background subtraction and conversion to logarithmic form as described[16] or by a nonlinear least squares fitting routine based on the parameter searching method[17] using a modified version of the program CURFIT[18] for fitting the decay to a sum of two exponentials.

Purified dimethyl esters were hydrolyzed to the corresponding dicarboxylic acids by gentle warming with 2 equiv of sodium hydroxide just before use for ultraviolet absorbance or luminescence spectroscopy (TLC was used to verify complete hydrolysis). For recording the luminescence spectra and lifetimes of Tb(III) complexes, $TbCl_3$ was added at a 3:1 molar ratio of ligand to metal ion, with total metal ion concentration equal to 23.3 μM in the presence of a pH 8.0 buffer containing 0.1 M Tris/Tris HCl [tris(hydroxymethyl)aminomethane]. The pH of each solution was adjusted with sodium hydroxide or hydrochloric acid as necessary to achieve a final value of 8.0.

The third stepwise formation constants (K_3) for the reactions $TbL_2^{-1} + L \rightleftharpoons TbL_3^{-3}$ were determined by monitoring the emission at 545 nm (the apparent Tb(III) emission maximum on the instrument used) from solutions containing 0.1 μM $Tb(NO_3)_3$, the pH 8.0 Tris buffer, and 4-X-DPA at total concentrations ranging from 0.1 to 275 μM. For these determinations it was assumed that the first two stepwise formation constants were significantly greater than 10^7 M^{-1} as found for DPA[7] and that formation of the bis complexes could be treated as nearly stoichiometric at total ligand concentrations of 0.4 μM and above. Free ligand concentrations then were calculated by subtracting 0.2 μM from total added ligand, and the intensity observed at 0.2 μM total ligand was treated as the intensity of the bis complexes by subtracting it from the total intensity at each ligand concentration to obtain the added signal due to the conversion of the bis to the tris complex. At high ligand concentrations inner filter effects were significant, and some Stern−Volmer quenching by excess ligand was observed, so the signal was

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Table 1. UV Absorbance and Luminescence Properties

	1	3	9	12	13	16
X	H	OH	Cl	Br	NH_2	NHAc
	Absorbance (Metal-Free)					
λ_{max} (nm)	270	282	274	276	284	248
ϵ (mM^{-1} cm^{-1})	4.84	8.69	2.44	2.88	8.87	8.66
	Absorbance (Tb(III) Complex)					
λ_{max} (nm)	270	274	272	274	274	266
ϵ (mM^{-1} cm^{-1})	5.29	5.99	2.27	3.0	7.36	8.22
	Tb(III) Luminescence					
λ_{max} (nm)	274	279	272	275	275	272
I^a	26.9	41.1	11.1	12.1	100	52.4
τ (ms)^b	2.00	1.00	1.77	1.70	1.58	1.47
Q_E	5.08	6.86	4.90	4.03	13.6	6.37
Q_T	2.5	6.9	2.76	2.37	8.61	4.33

[a] Relative values at ligand concentrations sufficient to ensure stoichiometric formation of TbL_3 complexes, after correction for inner filter effects. [b] Maximum values observed in titrations with ligands.

corrected for these effects according to $I_{corr} = I_{obs}F$ where I_{corr} is the corrected intensity used to calculate K_3, I_{obs} is the raw intensity value, and F is a combined correction factor:

$$F = (1 + K_q[L])\epsilon \ln(10)\,[L]l/(1\text{-}\exp(-\epsilon \ln(10)\,[L]l)) \quad (1)$$

K_q is the Stern–Volmer quenching constant (determined from titrations at free ligand concentrations high enough to ensure nearly stoichiometric formation of tris complexes), ϵ is the molar extinction coefficient at the wavelength of excitation, [L] is the ligand concentration and l is the pathlength (note that inner filter effects are observed only for excitation, as the ligands display neglible absorbance in the visible). These values were then used to determine $K_3 \equiv [TbL_3]/[TbL_2][L]$ by fitting to the data the function:

$$I([L]) = I_{TbL_2} + (I_{max} - I_{TbL_2})[L]/([L] + 1/K_3) \quad (2)$$

I_{TbL_2} is the corrected intensity observed at 0.2 μM total ligand, and I_{max} and K_3 were determined by a two-parameter nonlinear least squares fit to the data, as described.[19] K_3 values are reported ± standard deviations estimated according to $\sigma = (2/(\partial^2\chi^2/\partial K_3^2))^{1/2}$, using numerically calculated second partial derivatives at the best fit I_{max} values.[18,19] Values for I_{max} reported in Table 1 are corrected for inner filter effects, but not for ligand quenching, because the latter is a relatively small effect (at ligand concentrations sufficient to ensure that 99% of Tb(III) was in TbL_3 complexes, the average correction was 8.8%, with a range of 0% to 19% correction), and is largely compensated for in the estimation of energy transfer efficiency Q_T (see Discussion) through normalization by the luminescence lifetimes.

Results

Syntheses and Properties of 4-Substituted DPA Analogues. Although several of the compounds studied had been reported previously, only DPA itself and chelidamic acid had been prepared in high enough purity for characterization of their physical properties. Our initial attempts to purify the free acids by recrystallization and ion exchange chromatography met with mixed, and generally poor, results. However, purification of the esters by silica gel chromatography and recrystallization was found to be a generally useful procedure for obtaining highly purified analogues that could then be quantitatively converted to free acids just before use by mild alkaline hydrolysis. This procedure also avoids the formation of complexes with contaminating metal ions during purification, so that the lanthanide complexes can be formed quantitatively.

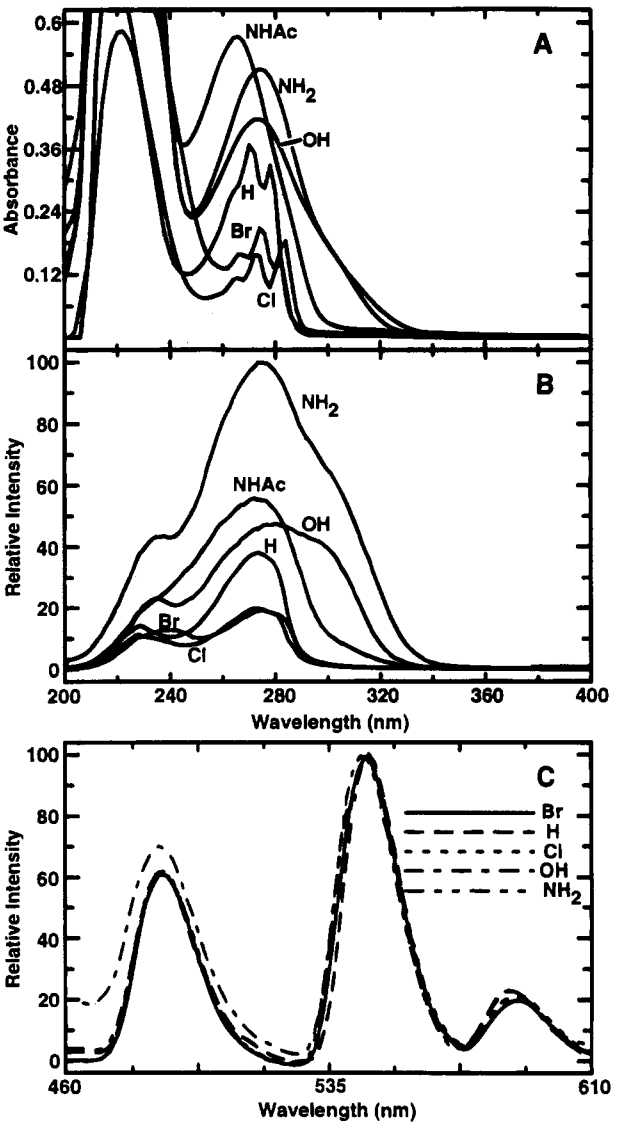

Figure 1. Absorbance and luminescence spectra of Tb(III) complexes with 4-substituted dipicolinic acid analogues. (A) Absorbance spectra: ligands with the indicated 4-substituents were present at 70 μM, $TbCl_3$ was added to 77 μM, and the pH was adjusted to 8.0. (B) Excitation spectra: ligands with the indicated 4-substituents were present at 70 μM, $TbCl_3$ was added to 23.3 μM, and the pH was adjusted to 8.0. Emission was detected at the Tb(III) λ_{max} of 545 nm. (C) Emission spectra: normalized by the peak intensity for each complex, under the same conditions as for the excitation spectra, with excitation at 268 nm.

UV Absorbance. All of the analogues absorbed strongly in the ultraviolet. Their molar extinction coefficients at wavelengths of maximal absorbance in the 240–340 nm range are given in Table 1. The red-shifting and strengthening of the principal absorbance band of the 4-amino anlogue as compared to DPA are consistent with the effects of nitrogen substitution at the same position on pyridine, which has been attributed to a shifting of the energy of the π–π^* transition.[20] Like 4-aminopyridine, 4-aminodipicolinic acid, **14**, displays a weak purple fluorescence. Quantitative complexation by Tb(III) resulted in significant perturbation of the absorbance spectra (Figure 1A, Table 1), indicating that the energies and probabilities of the electronic transitions involved are both affected by complexation of the metal ions.

Excitation and Emission Spectra of Tb(III) Complexes. Figure 1B shows the excitation spectra of the different analogues

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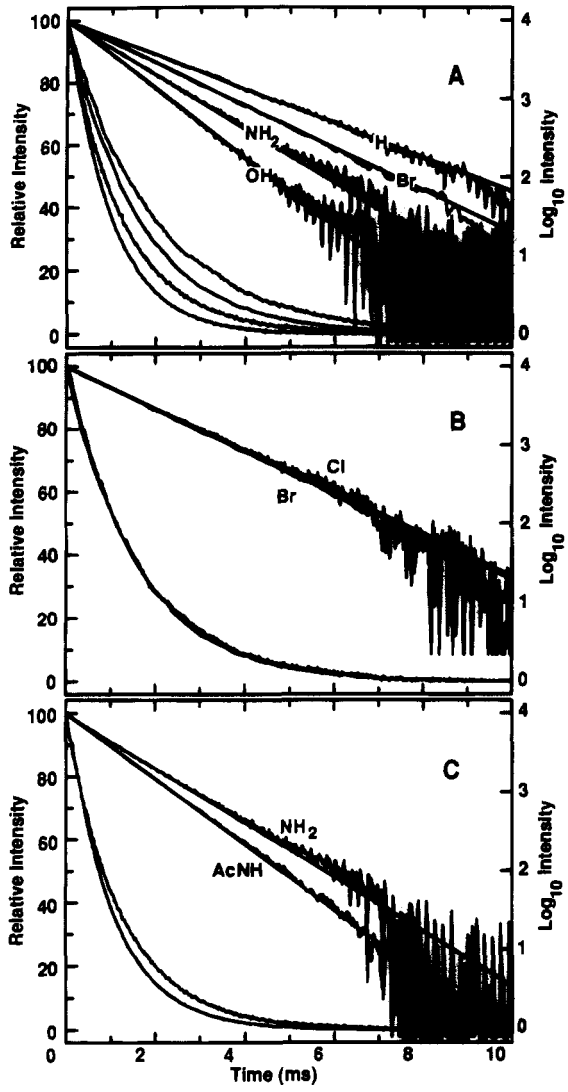

Figure 2. (A) Luminescence decay kinetics of Tb(III) complexes. Measurements were made on samples prepared identically to those in Figure 1B. The straight lines superimposed on the raw data represent the best fit single exponential decays under these conditions (τ, in ms): H, 1.90; OH, 0.98; Cl, 1.70; Br, 1.65; NH_2, 1.27; NHAc 1.26. The 4-substituent is indicated on each decay curve. (B) Comparison of 4-Br and 4-Cl ligands. (C) Comparison of 4-NH_2 and 4-NHAc ligands.

complexed with Tb(III) at a ligand:metal ion stoichiometry of 3:1, with the ligands present at 70 μM. While the peak wavelengths above 240 nm are close to those observed in the absorbance spectra for the complexes, there are significant differences in the spectral shapes. These are probably due primarily to the uncorrected nature of the excitation spectra which were recorded on an instrument lacking ratio mode capabilities. Because of the instrument's very low excitation intensity below 240 nm, the excitation properties in this wavelength range remain to be determined; the longer wavelengths are those of most interest for practical applications. As was observed in the absorbance spectra, the strongest signals are obtained from the analogues bearing nitrogen at the 4-position. The order of maximum emission intensities is not identical to the order of maximum extinction coefficients, suggesting differences in energy transfer efficiencies. The emission spectra (Figure 1C) are typical of Tb(III) complexes, with the strongest emission line corresponding to the 546 nm $^5D_0-{}^7F_5$ transition. The 488 nm band of the 4-aminodipicolinate complex overlaps slightly with the violet emission from

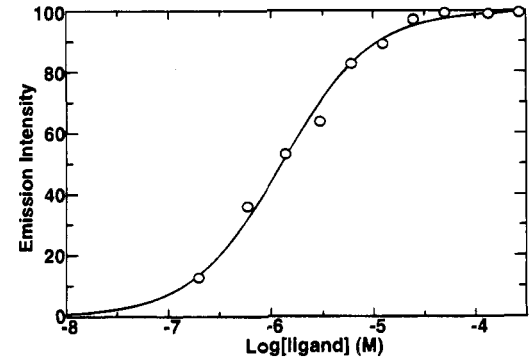

Figure 3. Equilibrium titration of $[Tb(4\text{-Cl-DPA})_2]^{-1}$ (0.1 μM) with 4-Cl-DPA. The curve drawn is the prediction of a nonlinear least squares fit to the data calculated as described in the text, with a stepwise formation constant, $K_3 = 1/(1.27\ \mu M)$. The emission intensity is plotted as a percentage of the maximum intensity from the theoretical fit, and has been corrected for inner filter effects and Stern–Volmer quenching by free ligand using a quenching constant of $4 \times 10^{-4}\ \mu M^{-1}$. Emission was detected at 545 nm.

the metal-free 4-aminodipicolinate, but otherwise the emission spectra are essentially indistinguishable.

Emission Decay Kinetics and Stabilities of Tb(III) Complexes. The emission decay kinetics of the Tb(III) complexes were in the ms range (Figure 2), with the lifetimes in the order H > Cl > Br > NH_2 > NHAc > OH (Table 1). The lifetime for $Tb(DPA)_3^{3-}$ of 2.00 ms agrees well with that previously reported.[21] Titrations of $Tb(NO_3)_3$ with ligands (*e.g.* Figure 3) allowed estimation of the formation constants K_3 for the tris complexes. The reciprocal values of these constants (in units of μM) were as follows: H, 1.22 (±0.11), OH; 2.59 (±0.12); Cl, 1.27 (±0.06); Br, 1.87 (±0.08); NH_2, 4.05 (±0.18); NHAc 2.36 (±0.11). The value for DPA, 1.22, agrees well with that determined from potentiometric titrations[7] of 1.20 (±0.08).

Discussion

Relative Quantum Yields for Energy Transfer and Sensitized Emission. The relative intensities in Figure 1B give an indication of the overall luminescence sensitivities of the different complexes, each of which is a product of the molar extinction coefficient, the quantum yield for energy transfer to the emissive excited state of the bound metal ion, and the quantum yield for emission from the metal ion's excited state. These are in the order NH_2 > NHAc > OH > H > Br ~ Cl. From these relative intensities, I, relative quantum yields for sensitized emission, Q_E, can be obtained by dividing by the molar extinction coefficients: $Q_E = I/\epsilon$. From Q_E, the relative quantum yields for energy transfer, Q_T, can be obtained by dividing each by the luminescence lifetime, τ, which is proportional to the emission quantum yield for the metal ion excited state: $Q_T = Q_E/\tau$. The resulting values are given in Table 1 and indicate that energy transfer efficiencies are in the order NH_2 > OH > NHAc > Cl > H ~ Br. The overall quantum yields for sensitized emission are in the order NH_2 > OH > NHAc > H > Cl > Br. Thus, while $Tb(DPA)_3^{3-}$ displays the longest lifetime, and therefore the highest metal ion emission quantum yield, it is 4-amino DPA that displays the most efficient energy transfer and overall efficiency of sensitized emission, as well as the greatest overall sensitivity for sensitized emission.

Implications for Analytical Applications. The finding that the energy transfer efficiency from the 4-Cl analogue is greater

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than that from the 4-Br analogue (Table 1) argues against an intramolecular heavy atom effect, and therefore against a triplet intermediate in the energy transfer process. This result is consistent with a report indicating a triplet-mediated pathway for energy transfer from an indole ring to Tb(III) but a nontriplet pathway for energy transfer from DPA,[22] based on oxygen sensitivity and heavy atom effects of Br^-, as well as with an earlier suggestion that ligand-to-lanthanide energy transfer could occur *via* an S1→ metal mechanism.[23] A lack of sensitivity to quenching by molecular oxygen would be a distinct advantage of DPA analogue-based chelates in bioanalytical applications, and in preliminary experiments we have not detected significant effects on luminescence intensities of either purging solutions of the Tb(III) complexes with nitrogen, or of bubbling air through the solutions.[24]

In addition to their strong sensitization of Tb(III) emission, the nitrogen-substituted analogues can be excited at less energetic wavelengths, which are less likely to induce undesirable photochemistry and autofluorescence in biological samples. These spectroscopic features, in addtion to the ease with which they can be synthesized and derivatized, make this nitrogen-bearing class of compounds the most attractive of those studied for incorporation of DPA moieties into macromolecules for ultrasensitive time resolved lanthanide luminescence-based immunoassays.

Preliminary experiments with Eu(III)[24] indicate that 4-aminodipicolinic acid and 4-acetamidodipicolinic acid are also very efficient sensitizing ligands for Eu(III). However, the situation with Eu(III) is considerably more complicated, due to the presence of two closely lying emissive states in Eu(III) complexes, with different decay kinetics, and possibly different and mixed energy transfer mechanisms. In addtion, Eu(III) displays greater sensitivity to quenching by N−H and O−H oscillators and to photoreduction. Thus, more work will be necessary before it can be determined to what extent the conclusions drawn for the Tb(III) complexes can be extended to applications using Eu(III).

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