71. Synthesis and Luminescence of Novel Eu^{III} Complexing Agents and Labels with 4-(Phenylethynyl)pyridine Subunits

by **Harri Takalo*** and **Ilkka Hemmilä**

Wallac Oy, P.O. Box 10, FIN–20101 Turku

and **Timo Sutela** and **Martti Latva**

Department of Chemistry, University of Turku, FIN–20500 Turku

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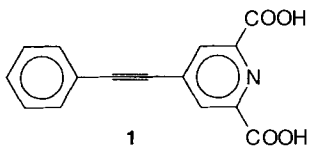
The synthesis of novel 4-(phenylethynyl)pyridine subunits containing H_2O-soluble complexing agents and their luminescence with Eu^{III} ions are reported. Ligands with high luminescence intensities as well as quantum yields were obtained. Also the prepared labeling reagents as antibody conjugates gave the highest quantum and luminescence yields reported for H_2O-soluble Eu^{III} labels.

Introduction. – Time-resolved fluorescence (TRF) combined with long-lifetime emitting lanthanide chelate labels is gradually gaining importance as an analytical tool, especially in connection with bioaffinity assays [1]. Applications based on TRF have already been commercialized in the field of clinical diagnostics in immunoassays and in DNA hybridization assays.

In commercial systems, the measurement comprises two steps, as the luminescence of the label is detected after an immunoreaction [2]. These technologies are not suitable for all applications, such as homogeneous assays, fluorescence imaging, immunohistochemistry, or *in situ* hybridization. If luminescent lanthanide chelates are to be used as labels in such applications, the chelate labels must fulfil strict requirements: *i*) high thermodynamic and kinetic stability, *ii*) hydrophilicity, *iii*) efficient cation emission, *i.e.*, high absorptivity at a suitable wavelength, efficient energy transfer from ligand to metal, and low nonradiative deactivation level of the metal excited state, *iv*) a structure allowing the formation of a covalent bond between the chelate and the target biomolecule, and *v*) the affinity and nonspecific binding properties of the labeled biomolecules have to be retained. In consequence of the requirements of optimal stability and luminescence, only a few viable chelate labels have been developed and tested [1].

Based on the synthesis and luminescence studies of many aromatic N-compounds, we have managed to develop applications employing the lanthanide chelates of 2,2′:6′,2″-terpyridine [3] and its thiazole [4] and triazole [5] analogues. Moreover, various bioapplications based on the Eu^{III} complexes of 4-(arylethynyl)pyridines have been elaborated [5–8] [10]. The Eu^{III} chelate of 2,2′,2″,2‴-{{4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic acid) [6] has been used as a luminescent label in TRF immunoassays [5], and microscopy [7], and its oligonucleotide modification in DNA hybridization assays [8]. Our study of the effects of ligand substituents through environmental changes on the luminescence properties of ligands **1** and **2** [9] has been



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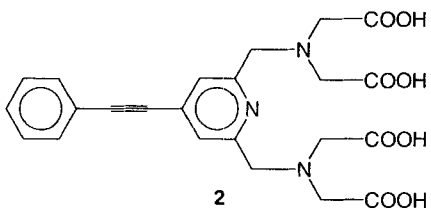

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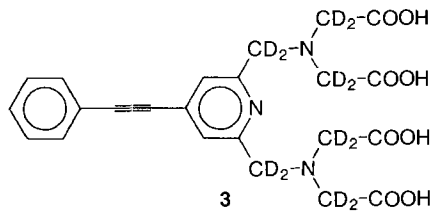

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applied to homogeneous immunoassays of haptenic antigens [10]. We have shown that variously substituted ligands give high luminescence with the Eu^{III} ion with readily changeable excitation wavelength, the luminescence is increased by increasing the absorptivity, and highly luminescent micelle-forming chelates can be prepared [11]. Unfortunately, only moderate luminescence can be obtained with the Tb^{III} ion. Although different derivatives of ligand **2** demonstrate high luminescence and have several bioapplications, better luminescence intensities will be needed.

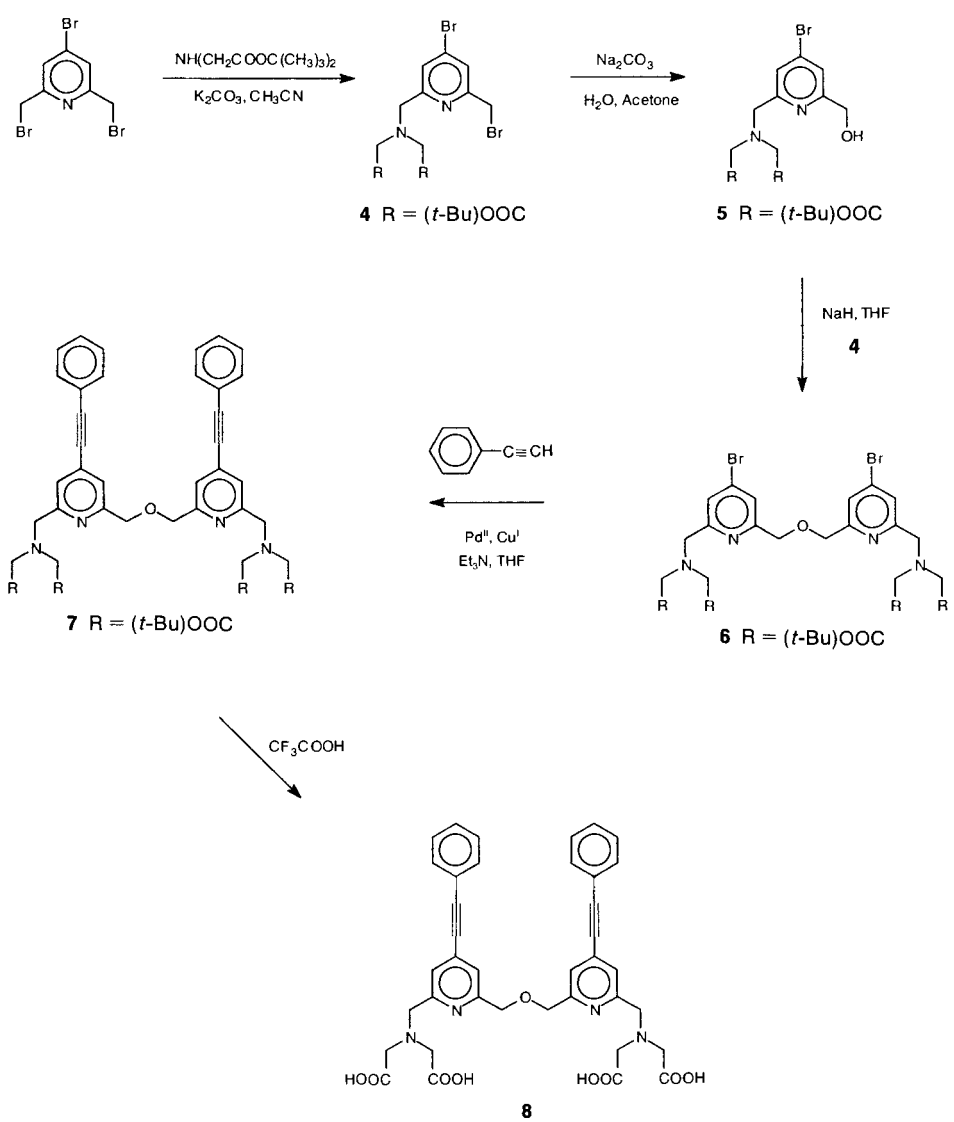
In principle, the europium luminescence of 4-(arylethynyl)pyridine chelates can be easily enhanced by a co-luminescence phenomenon [12], but practical applications are difficult to perform with stable luminescent lanthanide chelates. We have increased the luminescence of labeled biomolecules by using a dendrimeric labeling reagent with one activated binding arm or one hapten coupled to a dendrimeric structure having an exact known number of stable luminescent lanthanide chelates [13]. On the other hand, the synthesis of such dendrimeric labels contains many steps. Due to the large size of the labeling reagent molecules, some difficulties may be encountered in the purification of labeled biomolecules, *i.e.*, separation of protein from the labeling reagent.

The energy leakage through the vibrational manifold of O–H stretching is a well-documented phenomenon in luminescent chelates, and has also been observed with the Eu^{III} chelate of ligand **2** [14]. Quenching through C–H stretching quanta is seldom regarded as a problem. The study with the seven-dentate ligands **2** and **3** showed that the leakage through O–H stretching was so strong that no positive effect of deuterium could be found in H_2O. When the detrimental effect of H_2O was eliminated by replacing it with D_2O, the positive effect of deuterium replacement was clearly demonstrated. Besides being nine-dentate, Eu^{III} chelates with high quantum yields must have as few C–H groups as possible, or these groups must be replaced by C–D groups.

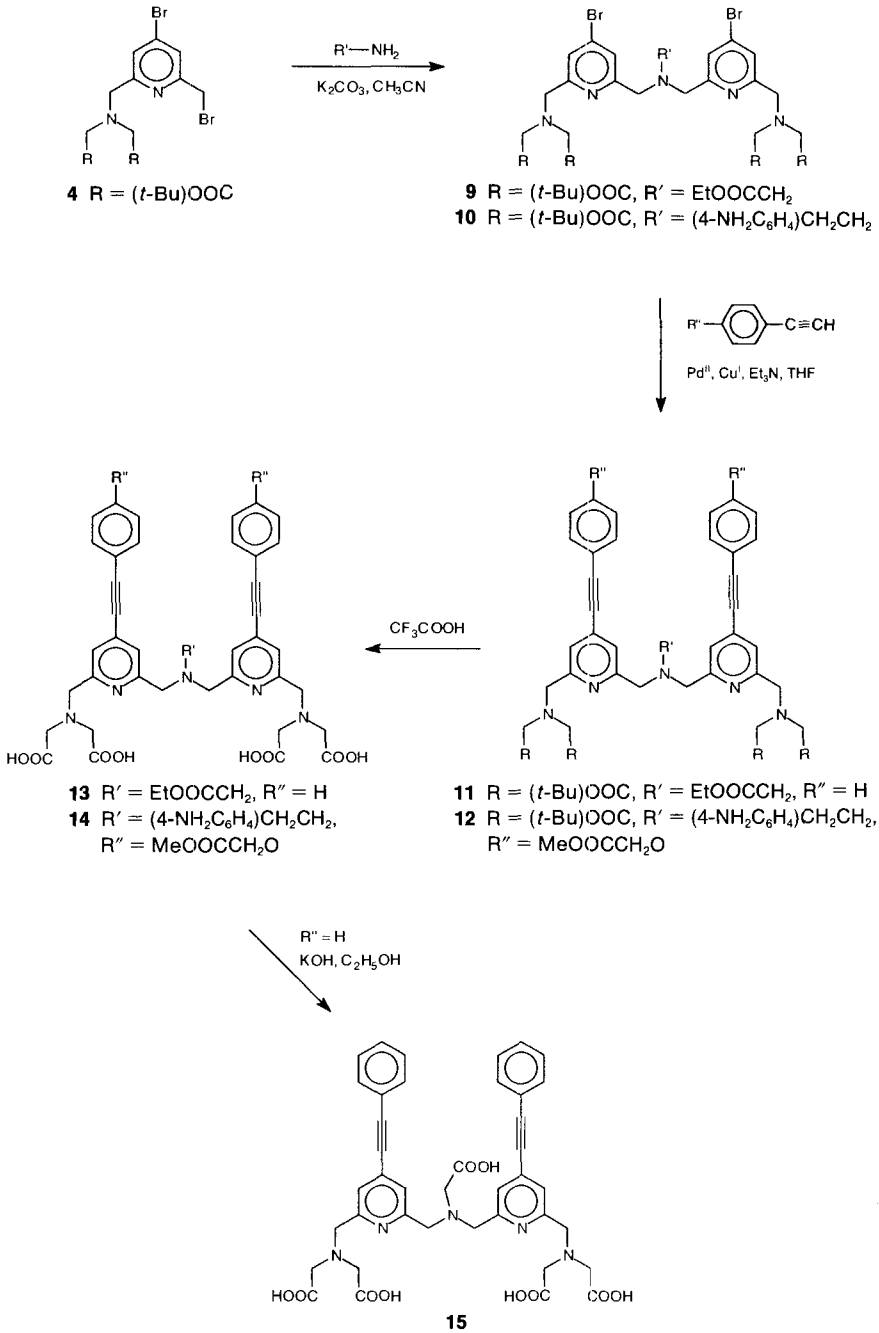
Sabbatini et al. [1b] have intensively studied ligands containing several separate 2,2′-bipyridines and obtained good luminescence properties with them. The aims of the present study were to prepare new H_2O-soluble ligands which contain two or three separate chromophors in the same ligand structure, to study the luminescence properties of their lanthanide chelates, and, finally, to develop novel highly luminescent Eu^{III} labels based on 4-(phenylethynyl)pyridine moieties.

Results and Discussion. – *Syntheses.* A moderate yield of compound **4** was obtained from the reaction of 4-bromo-2,6-bis(bromomethyl)pyridine [15] and di(*tert*-butyl) iminobis(acetate) (*Scheme 1*). The hydrolysis of the bromomethyl group with Na_2CO_3 in aqueous acetone gave the corresponding alcohol **5**. Compound **6** was prepared using the *Williamson* ether synthesis by coupling bromide **4** to alcohol **5**. Several by-products were formed in this coupling reaction, and only by using THF freshly dried with $LiAlH_4$, the desired ether **6** was obtained but in low yield. It seems that the Br-atom in the 4-position of the pyridine ring is responsible for this phenomenon. The Br-atoms of compound **6**

Scheme 1



Scheme 2



4 R = (*t*-Bu)OOC

9 R = (*t*-Bu)OOC, R′ = $EtOOCCH_2$

10 R = (*t*-Bu)OOC, R′ = $(4\text{-}NH_2C_6H_4)CH_2CH_2$

11 R = (*t*-Bu)OOC, R′ = $EtOOCCH_2$, R″ = H

12 R = (*t*-Bu)OOC, R′ = $(4\text{-}NH_2C_6H_4)CH_2CH_2$, R″ = $MeOOCCH_2O$

13 R′ = $EtOOCCH_2$, R″ = H

14 R′ = $(4\text{-}NH_2C_6H_4)CH_2CH_2$, R″ = $MeOOCCH_2O$

R″ = H, KOH, C_2H_5OH

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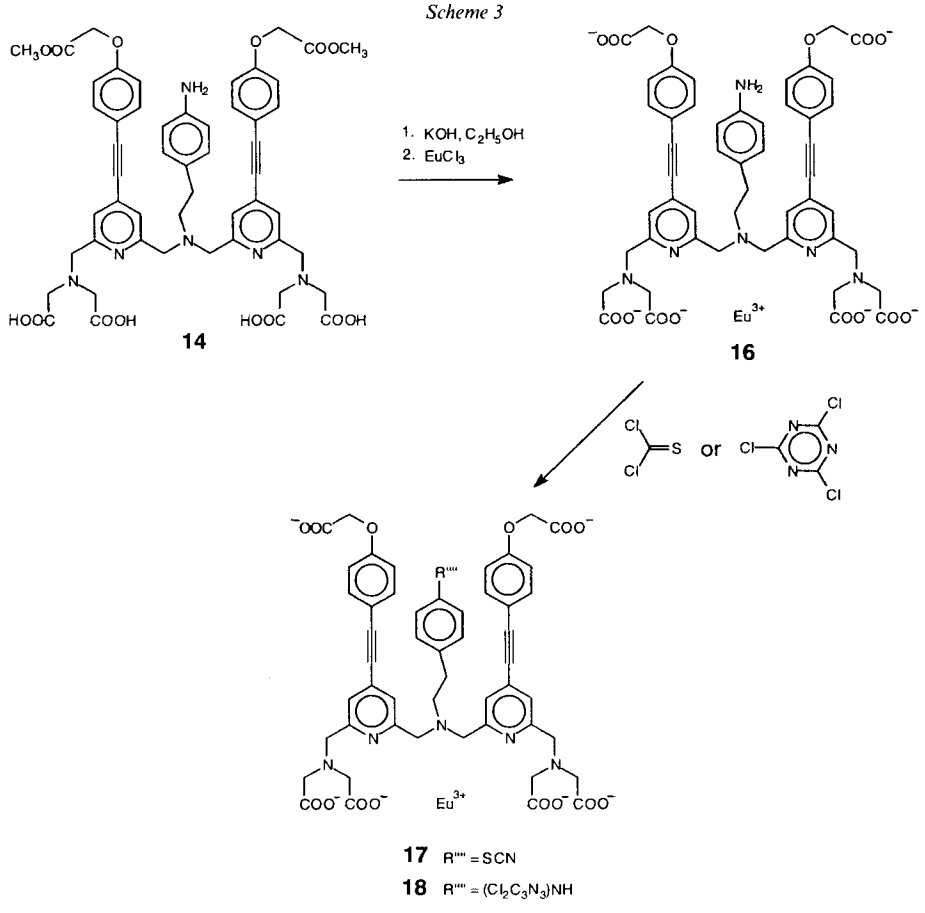
reacted with phenylacetylene in the presence of a catalytic amount of Pd^{II} catalyst and CuI, and finally, the tetra(*tert*-butyl) ester **7** was hydrolyzed to the target tetrakis(acetic acid) **8**.

The reaction of ethyl glycinate or 2-(4-aminophenyl)ethylamine with 2 equiv. of compound **4** yielded tetra(*tert*-butyl) esters **9** and **10**, respectively (*Scheme 2*). The organometallic coupling of aryl bromides **9** and **10** with the terminal acetylenes phenylacetylene and methyl (4-ethynylphenoxy)acetate, respectively, gave compounds **11** and **12**. Methyl (4-ethynylphenoxy)acetate was prepared from 4-iodophenol and ethyl bromoacetate *via* ethyl (4-iodophenoxy)acetate and {4-[(trimethylsilyl)ethynyl]phenoxy}-acetate (see *Exper. Part*). During the deprotection of the trimethylsilyl group, transesterification of ethyl to methyl ester also took place yielding the starting material for the synthesis of compound **12**. Ligand **15** was obtained after hydrolysis with CF_3COOH (→**13**) and KOH in EtOH.

After acid hydrolysis of compound **12**, the methyl-ester groups of compound **14** were saponified, and the Eu^{III} chelate was prepared by stirring the tetrakis(acetic acid) with $EuCl_3$ in a slightly acidic solution (*Scheme 3*). By its transformation to the isothiocyanato

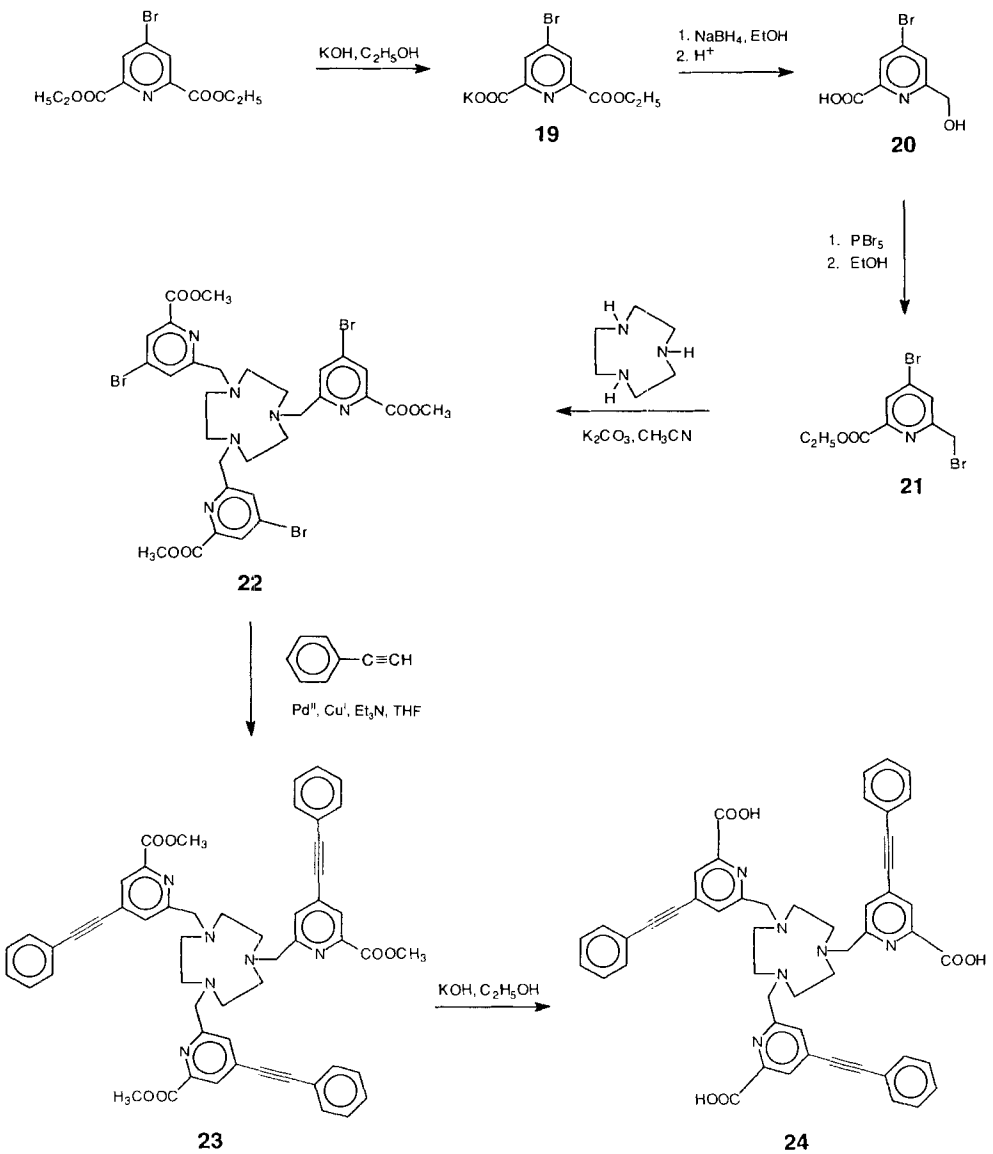
Scheme 3



group (chelate **17**), the NH_2 group of compound **16** was activated with thiophosgene in $H_2O/CHCl_3$, and the (4,6-dichloro-1,3,5-triazin-2-yl)amino derivative **18** was prepared using 2,4,6-trichloro-1,3,5-triazine.

A high yield of monopotassium salt **19** was obtained from diethyl 4-bromopyridine-2,6-dicarboxylate [16] using an equivalent amount of KOH in EtOH and without stirring the reaction mixture (*Scheme 4*). After $NaBH_4$ reduction in EtOH, the monoalcohol **20**

Scheme 4



was transformed into acid bromide with PBr_5, as bromination with PBr_3 was unsuccessful. Treatment with EtOH generated the corresponding ethyl ester **21**. Surprisingly, an ester exchange reaction took place during the flash chromatography (Et_3N/MeOH/$CHCl_3$) of the crude coupling product obtained from **21** and 1,4,7-triazacyclononane, yielding methyl ester **22**. The organometallic coupling reaction of **22** with phenylacetylene (→**23**) and saponification with KOH in EtOH yielded the target ligand **24**.

Luminescence. The excitation maxima (λ_{exc}), luminescence decay times (τ), quantum yields (Φ), and luminescence yields ($\varepsilon \cdot \Phi$) of the Eu^{III} chelates with ligands **8**, **13**, **15**, and **24**, and of chelates **16**–**18** in borate buffer are presented in *Table 1*. For comparison, the corresponding values of the Eu^{III} chelates with ligands **1**–**3** and the values of those with **2** and **3** in D_2O are also shown [14] [17].

Table 1. *Excitation Maxima* (λ_{exc}), *Luminescence Decay Times* (τ), *Quantum Yields* (Φ), *and Luminescence Yields* ($\varepsilon \cdot \Phi$) *of the Europium(III) Chelates of 4-(Phenylethynyl)pyridine Derivatives in a Borate Buffer* (pH 8.5). Concentrations: 10 and 1 μM for the ligand and Eu^{III} ion, respectively.

Ligand in $[Eu^{III}L]$ or $[Eu^{III}L]$	λ_{exc} [nm]	τ [μs]	Φ	$\varepsilon \cdot \Phi$
1[a])[b])	314	890	0.38	14210
2[b])	293	385	0.067	1410
2[c])	309	2220	0.13	2790
3[c])	306	2620	0.18	3790
8	315	1005	0.16	3895
13	313	1040	0.21	5000
15	310	1110	0.20	4870
16	333	65		320
17	322	1050	0.048	2050
17[d])	335	1060	0.14	5950
18[d])	336	1040	0.12	5190
24	321	850	0.22	12190

[a]) Ligand-to-metal ratio 3:1. [b]) See [17]. [c]) In D_2O; see [14]. [d]) In protein; ligand-to-metal ratio 1:1.

The excitation wavelengths of the Eu^{III} chelates are mainly somewhat over 300 nm. With chelates **16**–**18**, the maximum is shifted to a more convenient excitation wavelength (*i.e.*, over 330 nm) due to the additional ether functions in the *para*-positions of the two chromophores. The addition of chromophores decreases the solubility of ligands in H_2O, but these two ether groups together with two additional COOH groups increase the solubility of the label chelates **17** and **18**, and reduce the nonspecific binding of labeled biomolecules, whereas the affinity properties of the used biomolecules are almost unchanged.

To study the H_2O coordination number, the decay times (τ) for the new ligands with Eu^{III} should also be measured in D_2O. Anyhow, the variation in decay times in H_2O *vs.* D_2O seems to be quite small with an average of *ca.* 2000 μs (decay constant of 0.5 ms^{-1}) for Eu^{III} chelates [18], which is also demonstrated by the τ values of ligand **2**. Using the above average value, the decay times of the studied Eu^{III} chelates are mainly in accordance with anticipated values, *i.e.*, the chelates with ligands **8**, **13**, and **15** as well as chelates **17** and **18** contain *ca.* 0.4–0.5 coordinated H_2O molecules in the first coordination sphere. The value

for the chelate with ligand **24** is somewhat higher, *ca.* 0.7 coordinated H_2O molecules, being in accordance with the corresponding value for the chelate with ligand **1**. The additional COOH group between the chromophores in ligand **15** *vs.* **8** does not have any additional positive effect on the decay time. The decay time of the chelate with ligand **13** indicates that the additional COOH group does not participate in the chelation of the Eu^{III} ion or, alternatively, the COOH group replaces some other chelating part of the molecule. Chelate **16** has an exceptionally short decay time. The same phenomenon of amino groups has been observed with 2,2′:6′,2″-terpyridine derivatives [3a, b].

The differences in the luminescence properties of Eu^{III} complexes of ligands **1**–**3** are quite clear for obvious reasons. The energy leakage through the vibrational manifold of OH stretching is reflected in the values of the chelate with ligand **2** measured in borate buffer compared to those in D_2O. On the other hand, the quenching through CH stretching quanta causes the difference between ligands **2** and **3** in D_2O [14]. Moreover, the luminescence yield ($\varepsilon \cdot \Phi$) of the Eu^{III} complex with **1** (ligand-to-metal ratio 3:1) is over three-fold (*i.e.*, the number of separate chromophores in the complex) compared to the value of the complex with ligand **3**. These luminescence yields of ligands **1**–**3** with Eu^{III} in H_2O *vs.* D_2O indicate that better labels can be obtained by the addition of separate chromophores in one nine-dentate label or chelate. The novel nine-dentate ligands **8**, **13**, and **15** have two separate chromophores, and thus the luminescence yields of the chelates with ligands **13** and **15** especially are almost twice as high as the luminescence yield of the chelates with ligand **2** in D_2O, being in accordance with the number of chromophores. On the other hand, ligands **8**, **13**, and **15** contain several CH groups, which reduce luminescence yields. It seems that the Eu^{III} chelate of ligand **8** is the weakest one of these three ligands. As expected, the chelate of ligand **24** with three separate chromophores gives the highest quantum and luminescence yields of the new compounds, but the existence of many CH groups in close vicinity to the complexed Eu^{III} ion decreases to some extent its quantum and luminescence yields when compared to ligand **1**.

The ratios between the different emission lines are highly dependent on the ligand field [1a] and must also be taken into account in the development of various label reagents. This phenomenon is also observed with the studied ligands, *e.g.*, the emission line intensities at *ca.* 613 nm are *ca.* 65, 75, and 85% of the total emission produced for ligands **2**, **15**, and **24**, respectively. The luminescence properties of the prepared ligands with Tb^{III} ion were also measured, but unfortunately they were not equally promising. The Tb^{III} chelate with ligand **15** shows the highest luminescence yield ($\varepsilon \cdot \Phi = 1340$), but its decay time is really short ($\tau = 140$ μs). These results are in accordance with earlier experiments of 4-(phenylethynyl)pyridine derivatives [11]. Moreover, in our research on the correlations between the luminescence efficiencies of over 40 Eu^{III} and Tb^{III} complexes and their triplet-state energy levels, the most promising Eu^{III} complexes for labeling purposes are the novel ligands **15** and **24** [19].

In addition to the influence of the number of separate chromophores, coordinated H_2O molecules, ε, and CH groups on the luminescence properties of Eu^{III} chelates, we studied the effect of detergents in different buffers on the luminescence properties of ligand **1** with Eu^{III} ion (*Table 2*). Using a suitable buffer and cetyltrimethylammonium bromide (CTAB) as a detergent, the luminescence yield was enhanced up to the corresponding value of β-diketones [1a] [2]. As seen from *Table 2*, the choice of the buffer is also significant for the luminescence properties of the Eu^{III} chelate with ligand **1**.

Table 2. *The Effect of 0.1% Cetyltrimethylammonium Bromide* (CTAB) *in Different Buffers on the Decay Times* (τ) *and Luminescence Yields* ($\varepsilon \cdot \Phi$) *of Europium(III) Complexes with Ligand* **1**

Buffer (pH)	τ [μs]	$\varepsilon \cdot \Phi$	τ [μs]$_{CTAB}$	$\varepsilon \cdot \Phi_{CTAB}$
Acetate (6.0)	920	12950	1060	13950
Tris (7.75)	1030	17900	1310	26060
Borate (8.5)	890	14210	[a])	580

[a]) Not measured.

Table 3. *Luminescence Decay Times* (τ) *and Luminescence Yields* ($\varepsilon \cdot \Phi$) *of the Europium(III) Chelates of Prepared 4-(Phenylethynyl)pyridine Derivatives in Different Buffers.* Concentrations: 10 and 1 μM for the ligand and Eu^{III} ion, respectively.

Ligand in [Eu^{III}L] or [Eu^{III}L]	Acetate (pH 6.0)		*Tris* (pH 7.75)		Borate (pH 8.5)	
	τ [μs]	$\varepsilon \cdot \Phi$	τ [μs]	$\varepsilon \cdot \Phi$	τ [μs]	$\varepsilon \cdot \Phi$
8	1060	4080	995	3740	1005	3895
13	1090	4840	1070	4950	1040	5000
15	1365	5340	1130	5185	1110	4870
16	80	320	75	290	65	320
17	1020	2840	1000	2050	1050	2050
17[a])	1070	5820	1050	5660	1060	5950
18[a])	1060	4790	1050	4840	1040	5190
24	850	10000	830	10840	850	12190

[a]) In protein; ligand to metal ratio 1:1.

Unfortunately, a similar buffer effect is not observed with the other novel ligands (*Table 3*), with the exception of ligand **24**. These results indicate that under suitable conditions, 4-(phenylethynyl)pyridine-2,6-dicarboxylic acid is as powerful a ligand as 4,4,4-trifluoro-1-(naphth-2-yl)butane-1,3-dione, the main component in *Delfia*® enhancement solution (*Wallac Oy*, Turku, Finland). The latter ligand has the highest quantum and luminescence yields previously measured for Eu^{III} chelates ($\Phi = 0.69$ and $\varepsilon \cdot \Phi = 26320$) [20]. In the measurements, the β-diketone solution also contained detergents, *i.e.*, trioctylphosphine oxide and *Triton X-100*, to enhance the luminescence. Unfortunately, in our experiments, CTAB also decreased the luminescence in borate buffer. Thus, we have to conclude that the effect of detergent is not clear, and more experimental studies will be needed to clarify its role.

Our earlier studies with Eu^{III} labeling reagents have shown that the Eu^{III} chelates coupled to a protein have luminescence properties almost identical to their parent compounds [3a, b]. Thus, it is logical to prepare a labeling reagent containing ligand **13** or **15** instead of ligand **8**. The luminescence properties of both labeling reagents **17** and **18** coupled to protein were very good. The quantum yields were nearly the same and luminescence yields about twice as high as that of the chelate with ligand **2** in D_2O. On the other hand, the chelates containing **8**, **13**, and **15** had higher quantum yields but somewhat lower luminescence yields than the corresponding labeling reagents coupled to protein.

Only the amino and isothiocyanato groups in chelates **16** and **17** had a negative effect on the luminescence properties. The phenomenon is quite common and has also been

observed with other lanthanide chelates [3a, b] [4] [6]. Small modifications in the structure of labeling reagents may cause significant changes in their luminescence properties. In addition to the labeling reagents in *Table 1*, we also prepared (using 4-nitrophenylalanine) a derivative of chelate **17**, in which the connecting bridge between the two chromophores was $(4\text{-}SCNC_6H_4)CH_2CH(COOH)N(CH_2)_2$ instead of $(4\text{-}SCNC_6H_4)CH_2CH_2N(CH_2)_2$. It is unclear why this derivative did not give any luminescence with Eu^{III} ion before and after coupling to a biomolecule.

The preparation of a labeling reagent derived from a ligand with three separate chromophores (see **24**) needs a more sophisticated synthetic approach to find a realistic and practical preparation method as well as a suitable marker applicable to bioaffinity assays.

The highest reported quantum and luminescence yields of H_2O-soluble Eu^{III} chelates and labeling reagents have been observed for the above described chelates with 4-(phenylethynyl)pyridine ligands. Labeling reagents derived from ligand **24**, with three separate chromophors in one stable chelate structure, are under examination.

Experimental Part

General. See [4]. Moreover: IR Spectra: $\tilde{\nu}$ in cm^{-1}. Mass Spectra: *VG-7070E* mass spectrometer; *m/z* (rel. intensity).

Ethyl (4-Iodophenoxy)acetate. After the reaction of metallic Na (0.46 g, 20 mmol) with abs. EtOH (100 ml), 4-iodophenol (4.40 g, 20 mmol) was added, the mixture stirred for 0.5 h, and ethyl bromoacetate (4.34 g, 26 mmol) added. After refluxing for 5 h, the mixture was evaporated, the residue dissolved in $CHCl_3$ (130 ml), and the soln. washed with sat. $NaHCO_3$ soln. (2 × 40 ml), H_2O (2 × 40 ml), dried (Na_2SO_4), and evaporated. The product was purified by FC (silica gel, CH_2Cl_2): 5.00 g (78%). IR (KBr): 1754 (C=O), 1284 (C–O–C), 1212 (C–O). ^{1}H-NMR ($CDCl_3$): 1.30 (*t*, *J* = 7.1, 3 H); 4.27 (*q*, *J* = 7.1, 2 H); 4.59 (*s*, 2 H); 6.69 (*d*, *J* = 9.0, 2 H); 7.57 (*d*, *J* = 9.0, 2 H).

Ethyl {4-[(Trimethylsilyl)ethynyl]phenoxy}acetate. A mixture of ethyl (4-iodophenoxy)acetate (4.95 g, 15.5 mmol), bis(triphenylphosphine)palladium(II) chloride (220 mg, 0.31 mmol), CuI (120 mg, 0.62 mmol) in dry Et_3N (15 ml), and THF (20 ml) was deaerated with N_2. (Trimethylsilyl)acetylene (2.40 ml, 17.0 mmol) was added and the mixture stirred for 3.5 h at r.t. The mixture was filtered, the filtrate evaporated, the residue dissolved in $CHCl_3$ (50 ml), and the soln. washed with H_2O (2 × 20 ml), dried (Na_2SO_4), and evaporated. The product was purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3): 4.53 g (100%). IR (film): 2156 (C≡C), 1761 (C=O), 1250 (C–O–C), 1198 (C–O). ^{1}H-NMR ($CDCl_3$): 0.23 (*s*, 9 H); 1.29 (*t*, *J* = 7.1, 3 H); 4.27 (*q*, *J* = 7.1, 2 H); 4.61 (*s*, 2 H); 6.82 (*d*, *J* = 8.9, 2 H); 7.40 (*d*, *J* = 8.9, 2 H).

Methyl (4-Ethynylphenoxy)acetate. Ethyl {4-[(trimethylsilyl)ethynyl]phenoxy}acetate (4.67 g, 16 mmol) was dissolved in dry MeOH (30 ml) and deaerated with N_2. Dry K_2CO_3 (2.21 g, 16 mmol) was added to the mixture and stirred for 2.5 h at r.t. After filtration and evaporation, the residue was dissolved in CH_2Cl_2 (70 ml), washed with 5% $NaHCO_3$ soln. (40 ml) and H_2O (40 ml), dried (Na_2SO_4), and evaporated: 1.83 g (56%). IR (film): 3239 (C≡CH), 2105 (C≡C), 1753 (C=O), 1286 (C–O–C), 1217 (C–O). ^{1}H-NMR ($CDCl_3$): 3.00 (*s*, 1 H); 3.81 (*s*, 3 H); 4.64 (*s*, 2 H); 6.85 (*d*, *J* = 8.9, 2 H); 7.44 (*d*, *J* = 8.9, 2 H).

*Di(*tert*-butyl) 2,2'-{[4-Bromo-6-(bromomethyl)pyridin-2-yl]methylenenitrilo}bis(acetate)* (**4**). Dry K_2CO_3 (6.91 g, 50 mmol) and di(*tert*-butyl) iminobis(acetate) (2.45 g, 10 mmol) were added to a soln. of 2,6-bis(bromomethyl)-4-bromopyridine [15] (3.44 g, 10 mmol) and dry MeCN (80 ml) at 50°. After stirring for 4 h at 50°, the mixture was filtered, the filtrate evaporated, and the residue purified by FC (silica gel, toluene, then Et_2O/toluene 1:10): 2.44 g (48%). IR (film): 1738 (C=O), 1145 (C–O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 18 H); 3.48 (*s*, 4 H); 4.02 (*s*, 2 H); 4.47 (*s*, 2 H); 7.50 (*s*, 1 H); 7.82 (*s*, 1 H).

*Di(*tert*-butyl) 2,2'-{[4-Bromo-6-(hydroxymethyl)pyridin-2-yl]methylenenitrilo}bis(acetate)* (**5**). A soln. of **4** (1.05 g, 2.07 mmol), 20% Na_2CO_3 soln. (25 ml), and acetone (30 ml) was refluxed for 6.5 h and extracted with $CHCl_3$ (2 × 100 ml). The org. fraction was washed with H_2O (2 × 10 ml), dried (Na_2SO_4) and evaporated and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 1:1): 0.62 g (67%). IR (KBr): 1732 (C=O), 1145 (C–O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 18 H); 2.50 (br. *s*, 1 H); 3.48 (*s*, 4 H); 4.04 (*s*, 2 H); 4.70 (*s*, 2 H); 7.32 (*s*, 1 H); 7.76 (*s*, 1 H).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[Oxybis(methylene)bis(4-bromopyridine-6,2-diyl)]bis(methylenenitrilo)}tetrakis(acetate)* (**6**). A soln. of **5** (0.24 g, 0.54 mmol) in THF (freshly dried with $LiAlH_4$ and distilled; 2.5 ml) was deaerated with N_2. After addition of NaH (60–65% in oil; 22 mg, 0.54 mmol), the mixture was stirred for 30 min, and a soln. of **4** (0.27 g, 0.54 mmol) in dry THF was added. The mixture was stirred for 3 d at 40° and evaporated, the residue dissolved in $CHCl_3$ (20 ml), the soln. washed with H_2O (10 ml) and dried (Na_2SO_4), and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:2, then 5:3): 55 mg (11%). IR (film): 1738 (C=O), 1144 (C–O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.47 (*s*, 8 H); 4.02 (*s*, 4 H); 4.71 (*s*, 4 H); 7.57 (*s*, 2 H); 7.80 (*s*, 2 H).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{Oxybis(methylene)bis[4-(phenylethynyl)pyridine-6,2-diyl]bis(methylenenitrilo)}-tetrakis(acetate)* (**7**). A mixture of **6** (44 mg, 50 μmol), bis(triphenylphosphine)palladium(II) chloride (1.4 mg, 2 μmol), CuI (1.5 mg, 8 μmol) in dry Et_3N (0.75 ml), and THF (0.75 ml) was deaerated with N_2. After addition of phenylacetylene (33 μl, 0.30 mmol), the mixture was stirred for 24 h at 55° and evaporated. The product was purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3): 20 mg (43%). IR (film): 2216 (C≡C), 1737 (C=O), 1143 (C–O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.50 (*s*, 8 H); 4.05 (*s*, 4 H); 4.77 (*s*, 4 H); 7.32–7.41 (*m*, 6 H); 7.52 (*s*, 2 H); 7.54 (*dd*, J = 5, 1.5, 4 H); 7.67 (*s*, 2 H).

2,2′,2″,2‴-{Oxybis(methylene)bis[4-(phenylethynyl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis(acetic Acid) (**8**). A soln. of **7** (17 mg, 19 μmol) in CF_3COOH (0.5 ml) was stirred for 1.5 h at r.t. After evaporation without heating, the mixture was triturated with Et_2O and submitted to centrifugation: 9.0 mg (69%). UV (H_2O): 305 (sh), 289. UV ([Eu^{III} (**8**)], H_2O): 309, 295. IR (KBr): 2213 (C≡C), 1734, 1628 (C=O), 1388, 1198 (C–O). ^{1}H-NMR ((D_6)DMSO): 3.50 (*s*, 8 H); 3.98 (*s*, 4 H); 4.71 (*s*, 4 H); 7.47 (2*t*, J = 7.6, 6 H); 7.52 (*s*, 2 H); 7.63 (*s*, 2 H); 7.74 (*d*, J = 7.6, 4 H).

Tetrakis(acetates) **9** *and* **10**: *General Procedure.* Compound **4** (0.73 g, 1.43 mmol) was added to a mixture of ethyl glycinate hydrochloride or 2-(4-aminophenyl)ethylamine (0.72 mmol), $(i\text{-}Pr)_2EtN$ (1.0 ml, 5.72 mmol) or dry K_2CO_3 (1.0 g, 7.15 mmol for **10**), and dry MeCN (15 ml). After stirring for 4 h at 45–50°, the mixture was evaporated, the residue dissolved in $CHCl_3$ (40 ml), the soln. washed with H_2O (2 × 10 ml), dried (Na_2SO_4), and evaporated, and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[(Ethoxycarbonyl)methylimino]bis(methylene)bis(4-bromopyridine-6,2-diyl)-bis(methylenenitrilo)}tetrakis(acetate)* (**9**): Yield 54%. IR (KBr): 1738 (C=O), 1145 (C–O). ^{1}H-NMR ($CDCl_3$): 1.29 (*t*, J = 7.1, 3 H); 1.46 (*s*, 36 H); 3.47 (*s*, 10 H); 3.94 (*s*, 4 H); 4.01 (*s*, 4 H); 4.18 (*q*, J = 7.1, 2 H); 7.63 (*s*, 2 H); 7.75 (*s*, 2 H).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis(4-bromopyridine-6,2-diyl)-bis(methylenenitrilo)}tetrakis(acetate)* (**10**): Yield 52%. IR (film): 3465, 3372 (N–H), 1736 (C=O), 1143 (C–O). ^{1}H-NMR ($CDCl_3$): 1.46 (*s*, 36 H); 2.73 (*s*, 4 H); 3.47 (*s*, 8 H); 3.80 (*s*, 4 H); 3.99 (*s*, 4 H); 6.62 (*d*, J = 8.3, 4 H); 6.84 (*d*, J = 8.3, 4 H); 7.41 (*s*, 2 H); 7.71 (*s*, 2 H).

Tetrakis(acetates) **11** *and* **12**: *General Procedure.* A mixture of **9** or **10** (0.34 mmol), bis(triphenylphosphine)-palladium(II) chloride (10 mg, 14 μmol), CuI (5.3 mg, 28 μmol) in dry Et_3N (3.5 ml), and THF (3.5 ml) was deaerated with N_2. After addition of phenylacetylene or methyl (4-ethynylphenoxy)acetate (see above; 0.82 mmol), the mixture was stirred for 6–18 h at 55° and then filtered, the filtrate evaporated, the residue dissolved in $CHCl_3$ (30 ml), the soln. washed with H_2O (2 × 10 ml), dried (Na_2SO_4), and evaporated, and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3 or 2:5, resp.).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[(Ethoxycarbonyl)methylimino]bis(methylene)bis[4-(phenylethynyl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis(acetate)* (**11**): Yield 76%. IR (KBr): 2215 (C≡C), 1738 (C=O), 1144 (C–O). ^{1}H-NMR ($CDCl_3$): 1.29 (*t*, J = 7.1, 3 H); 1.46 (*s*, 36 H); 3.49 (*s*, 10 H); 4.00 (*s*, 4 H); 4.04 (*s*, 4 H); 4.19 (*q*, J = 7.1, 2 H); 7.30 (*t*, J = 7.6, 4 H); 7.33 (*t*, J = 7.6, 2 H); 7.52 (*d*, J = 7.6, 4 H); 7.62 (*s*, 2 H); 7.65 (*s*, 2 H).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{{4-[(methoxycarbonyl)-methoxy]phenyl}ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetate)* (**12**): Yield 68%. IR (film): 3340, 3370 (N–H), 2211 (C≡C), 1760, 1737 (C=O), 1280 (C–O–C), 1213, 1143 (C–O). ^{1}H-NMR ($CDCl_3$): 1.46 (*s*, 36 H); 2.80 (*s*, 4 H); 3.49 (*s*, 8 H); 3.82 (*s*, 6 H); 3.87 (*s*, 4 H); 4.02 (*s*, 4 H); 4.66 (*s*, 4 H); 6.68 (*d*, J = 8, 2 H); 6.82 (*d*, J = 8, 2 H); 6.91 (*d*, J = 8, 4 H); 7.47 (*d*, J = 8, 4 H); 7.48 (*s*, 2 H); 7.59 (*s*, 2 H).

Tetrakis(acetic Acids) **13** *and* **14**. A soln. of tetra(*tert*-butyl) esters **11** or **12** (0.13 mmol) in CF_3COOH (3.5 ml) was stirred for 1.5 h at r.t. After evaporation without heating, the mixture was triturated with Et_2O and filtered.

2,2′,2″,2‴-{[(Ethoxycarbonyl)methylimino]bis(methylene)bis[4-(phenylethynyl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis(acetic Acid) (**13**): Yield 98%. UV (H_2O): 304 (sh), 289. UV ([Eu^{III} (**13**)], H_2O): 312, 298. IR (KBr): 2212 (C≡C), 1734, 1628 (C=O), 1396, 1197 (C–O). ^{1}H-NMR ((D_6)DMSO): 1.21 (*t*, J = 7.3, 3 H); 3.58 (*s*, 8 H); 3.65 (*s*, 2 H); 4.06 (*s*, 4 H); 4.07 (*s*, 4 H); 4.10 (*q*, J = 7.3, 2 H); 7.41 (*t*, J = 7.3, 4 H); 7.48 (*t*, J = 7.3, 2 H); 7.59 (*d*, J = 7.3, 4 H); 7.62 (*s*, 4 H).

2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{{4-[(methoxycarbonyl)methoxy]phenyl}-ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**14**): Yield 80%. UV (H_2O): 314, 237. UV ([Eu^{III} (**14**)], H_2O): 318. IR (KBr): 2210 (C≡C), 1740, 1675 (C=O), 1207, 1179 (C–O). ^{1}H-NMR ((D_6)DMSO): 3.06 (*s*, 4 H); 3.55 (*s*, 8 H); 3.71 (*s*, 6 H); 4.04 (*s*, 4 H); 4.55 (*s*, 4 H); 4.89 (*s*, 4 H); 6.95 (*d*, *J* = 8, 2 H); 7.01 (*d*, *J* = 8.4, 4 H); 7.14 (*d*, *J* = 8, 2 H); 7.55 (*s*, 2 H); 7.55 (*d*, *J* = 8.4, 4 H); 7.72 (*s*, 2 H).

2,2′,2″,2‴-{[(Carboxymethyl)imino]bis(methylene)bis[4-(phenylethynyl)pyridine-6,2-diyl]bis(methylene-nitrilo)}tetrakis(acetic Acid) (**15**). A mixture of **13** (39 mg, 50 μmol) and 0.5M KOH in EtOH (5.5 ml) was stirred for 2 h at r.t. After evaporation, the residue was dissolved in cold H_2O (0.6 ml), acidified with 6M HCl (pH 1.5–2.0) and submitted to centrifugation: 26 mg (70%). UV (H_2O): 310, 293. UV ([Eu^{III} (**11**)], H_2O): 308, 295. IR (KBr): 2212 (C≡C), 1734, 1627 (C=O), 1395, 1214 (C–O). ^{1}H-NMR ((D_6)DMSO): 3.48 (*s*, 10 H); 3.93 (*s*, 4 H); 3.95 (*s*, 4 H); 7.39 (*t*, *J* = 7.3, 4 H); 7.46 (*t*, *J* = 7.3, 2 H); 7.53 (*s*, 2 H); 7.54 (*s*, 2 H); 7.58 (*d*, *J* = 7.3, 4 H).

{2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{[4-(carboxymethoxy)phenyl]ethynyl}-pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (**16**). A soln. of **14** (0.13 g, 0.10 mmol), 0.5M KOH in EtOH (6.5 ml), and H_2O (3.0 ml) was stirred for 3 h at 45°. After evaporation, the residue was dissolved in H_2O (2.3 ml) and the pH adjusted to 6.5 with 6M HCl. $EuCl_3$ (51 mg, 0.14 mmol) in H_2O (0.78 ml) was added within 15 min and the pH maintained at 5–7. After stirring for 4 h, the pH was raised to 8.5 with 1M NaOH, the precipitate removed by centrifugation, the filtrate treated with acetone, and the precipitate removed by centrifugation and washed with acetone. The solid material was dissolved in H_2O (3 ml), the soln. extracted with phenol (*ca.* 0.5–1.0 g), the phenol phase treated with H_2O (2 ml) and Et_2O (5 ml), the H_2O phase washed with Et_2O (2 × 5 ml) and treated with acetone, and the precipitate removed by centrifugation and washed with acetone: 0.12 g (100%). The product was used in the next step without further purification. UV (H_2O): 326, 329. IR (KBr): 2206 (C≡C), 1616 (C=O), 1400 (C–O).

{2,2′,2″,2‴-{[2-(4-Isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(carboxymethoxy)phenyl]-ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (**17**). An aq. soln. (0.95 ml) of **16** (40 mg, 34 μmol) was added within 20 min to a mixture of thiophosgene (21 μl, 0.27 mmol), $NaHCO_3$ (29 mg, 0.34 mmol), and $CHCl_3$ (0.95 ml). After stirring for 1 h, the H_2O phase was washed with $CHCl_3$ (3 × 3 ml), acetone was added to the aq. soln. and the product removed by centrifugation and washed with acetone. A small amount of the product was further purified by FC (*RP-2*, MeCN/H_2O 4:1). UV (H_2O): 325, 284, 268, 250, 224 (sh). IR (KBr): 2206 (C≡C), 2105 (S=C=N), 1616 (C=O), 1400 (C–O).

{2,2′,2″,2‴-{2-{4-{[(4,6-Dichloro-1,3,5-triazin-2-yl)amino]phenyl}ethylimino}bis(methylene)bis{4-{[4-(carboxymethoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (**18**). A mixture of 2,4,6-trichloro-1,3,5-triazine (14 mg, 77 μmol), acetone (0.1 ml), and H_2O (0.1 ml) was added to a soln. of **16** (9 mg, 7.7 μmol) and 0.1M NaOAc (0.2 ml). After stirring for 30 min, acetone was added to the mixture and the precipitate removed by centrifugation and washed with acetone. UV (H_2O): 324, 300, 243. IR (KBr): 2208 (C≡C), 1617 (C=O), 1406 (C–O).

Potassium Ethyl 4-Bromopyridine-2,6-dicarboxylic Acid (**19**). A soln. of KOH (3.71 g, 66.2 mmol) and abs. EtOH (230 ml) was added to a soln. of diethyl 4-bromopyridine-2,6-dicarboxylate [16] (20.0 g, 66.2 mmol). After standing for 20 h at r.t. without stirring, the cold mixture was filtered and washed with cold EtOH: 17.8 g (86%). IR (KBr): 1733, 1625 (C=O), 1278 (C–O). ^{1}H-NMR ((D_6)DMSO): 1.35 (*t*, *J* = 7.3, 3 H); 4.37 (*q*, *J* = 7.3); 8.09 (*d*, *J* = 1.6, 1 H); 8.19 (*d*, *J* = 1.6, 1 H).

4-Bromo-6-(hydroxymethyl)pyridine-2-carboxylic Acid (**20**). A mixture of **19** (17.4 g, 55.6 mmol), $NaBH_4$ (6.31 g, 167 mmol), and freshly dried EtOH (400 ml) was stirred for 30 min at r.t. and refluxed for 20 h. After evaporation, the residue was dissolved in H_2O (350 ml), the soln. acidified with 2M HCl (pH 2.5), the mixture filtered, and the filtrate evaporated. After continuous acetone extraction for 24 h and evaporation, the product was crystallized from H_2O: 10.8 g (83%). IR (KBr): 1707 (C=O), 1370, 1220 (C–O). ^{1}H-NMR ((D_6)DMSO): 4.62 (*s*, 2 H); 7.87 (*d*, *J* = 1.5, 1 H); 8.05 (*d*, *J* = 1.5, 2 H).

Ethyl 4-Bromo-6-(bromomethyl)pyridine-2-carboxylate (**21**). A mixture of PBr_5 [16] (8.18 g, 19 mmol) and **20** (2.20 g, 9.5 mmol) was stirred for 1 h at 90°, and the cold mixture was dissolved in $CHCl_3$ (50 ml). After addition of EtOH (75 ml) and refluxing for 15 min, the mixture was evaporated, the residue dissolved in $CHCl_3$ (50 ml), the soln. neutralized with sat. $NaHCO_3$ soln., washed with H_2O (2 × 20 ml), dried (Na_2SO_4), and evaporated, and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:1): 1.85 g (60%). IR (film): 1745, 1718 (C=O), 1304, 1229 (C–O). ^{1}H-NMR ($CDCl_3$): 1.44 (*t*, *J* = 7.3, 3 H); 4.49 (*q*, *J* = 7.3, 2 H); 4.60 (*s*, 2 H); 7.86 (*d*, *J* = 1.5, 1 H); 8.19 (*d*, *J* = 1.5, 1 H).

Trimethyl 6,6′,6″-[(Octahydro-1H-1,4,7-triazonine-1,4,7-triyl)tris(methylene)]tris[4-bromopyridine-2-carboxylate] (**22**). A mixture of **22** (0.37 g, 1.15 mmol), 1,4,7-triazacyclononane (0.50 g, 0.38 mmol), dry K_2CO_3 (0.32 g, 2.30 mmol), and dry MeCN (10 ml) was refluxed for 5 h. After filtration, the product was purified by FC (silica

gel, Et_3N/MeOH/$CHCl_3$ 1:1:8, then petroleum ether (40–60°)/AcOEt/Et_3N 5:2:1): 0.10–0.22 g (33–73%). IR (film): 1748, 1724 (C=O), 1300, 1212 (C–O). ^{1}H-NMR ($CDCl_3$): 2.92 (*s*, 12 H); 3.96 (*s*, 6 H); 4.00 (*s*, 9 H); 7.99 (*d*, J = 1.5, 3 H); 8.16 (*d*, J = 1.5, 3 H). MS: 812 (2, $[M + 2]^+$), 814 (2, $[M + 4]^+$).

*Trimethyl 6,6′,6″-[(Octahydro-1*H*-1,4,7-triazonine-1,4,7-triyl)tris(methylene)]tris[4-(phenylethynyl)pyridine-2-carboxylate]* (**23**). A mixture of **22** (96 mg, 0.112 mmol), bis(triphenylphosphine)palladium(II) chloride (4.7 mg, 7 μmol), CuI (2.6 mg, 14 μmol) in dry Et_3N (1.8 ml), and THF (1.8 ml) was deaerated with N_2. After addition of phenylacetylene (62 μl, 0.560 mmol), the mixture was stirred for 20 h at 50°, filtered, and evaporated. The residue was dissolved in $CHCl_3$ (15 ml), the soln. washed with H_2O (2 × 10 ml), dried (Na_2SO_4), and evaporated, and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3, then petroleum ether (40–60°)/AcOEt/Et_3N 5:3:1): 28–70 mg (28–70%). IR (film): 2216 (C≡C), 1745, 1722 (C=O), 1267 (C=O). ^{1}H-NMR ($CDCl_3$): 2.98 (*s*, 12 H); 3.98 (*s*, 9 H); 4.01 (*s*, 6 H); 7.34–7.43 (*m*, 9 H); 7.52–7.56 (*m*, 6 H); 7.90 (*d*, J = 1.4, 3 H); 8.06 (*d*, J = 1.4, 3 H).

*6,6′,6″-[(Octahydro-1*H*-1,4,7-triazonine-1,4,7-triyl)tris(methylene)]tris[4-(phenylethynyl)pyridine-2-carboxylic Acid]* (**24**). A mixture of **23** (25 mg, 27 μmol) and 0.5M KOH (0.82 ml) in EtOH was stirred for 2.5 h at r.t. After evaporation, the residue was dissolved in H_2O (0.5 ml), the soln. acidified with 2M HCl (pH 1.5), and the product removed by centrifugation and washed with H_2O: 21 mg (93%). UV (H_2O): 303, 288. UV ([Eu^{III} (**11**)], H_2O): 315 (sh), 295. IR (KBr): 2213 (C≡C), 1718, 1605 (C=O), 1387, 1264 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.13 (*s*, 12 H); 4.22 (*s*, 6 H); 7.47 (*t*, J = 6.4, 3 H); 7.49 (*t*, J = 6.4, 6 H); 7.60 (*d*, J = 6.4, 6 H); 7.76 (*s*, 3 H); 7.94 (*s*, 3 H).

Concentration Measurements. The measurement of the total Eu^{III} ion concentration after labeling was performed using a dissociative fluorescence enhancement system [2a] based on the *Wallac-Delfia* enhancement soln. composed of 15 μl of 4,4,4-trifluoro-1-(naphth-2-yl)butane-1,3-dione, 50 μM trioctylphosphine oxide, and 0.1% *Triton X-100* in acetate/phthalate buffer, pH 3.2.

Coupling of Chelates **17** *and* **18** *to Protein.* The activated chelates **17** and **18** were coupled to a model protein (PSA-antibody, clone H50) by incubating the chelate with IgG (1 mg/ml) in borate buffer (500 μl, pH 8.5–9.0) overnight using a 50-fold molar reactant/protein ratio for **17**, and a 20-fold molar ratio for **18**. After the coupling, the protein was purified on a column of *Superdex 200* (prep. grade) by eluting with 50 mM *Tris*-HCl buffer (pH 7.75) containing 0.15M NaCl and 0.05% NaN_3 soln. The fractions corresponding to labeled monomeric IgG were collected. The chelate concentrations in the protein fractions were measured from both absorptions of the conjugated chelate at 330 nm and the total Eu^{III} concentration measured by the dissociative fluorescence enhancement system. The purified protein conjugate and the labeling ratio (chelate per protein) were quantitated by calculating the protein yield or by measuring the absorbance at 280 nm and subtracting the absorption caused by the added chelate.

Luminescence Measurements. The luminescence parameters for free chelates were analyzed in borate buffer, pH 8.5. The luminescence quantum yields (Φ) were measured by a relative method described before [17] using [Ru(bpy)$_3$Cl$_2$] as a standard. In the measurements of luminescence intensities ($\varepsilon \cdot \Phi$), the ligand concentration was maintained at 10 μM, and the lanthanide-ion concentration was 1 μM. For other general considerations, see [3a]. A 0.1% soln. of cetyltrimethylammonium bromide (CTAB) was used in detergent measurements of acetate (pH 6.0), *Tris* (pH 7.75), and borate (pH 8.5) buffers. Results: *Tables 1–3*.

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