50. Luminescence of Europium(III) Chelates with 4-(Arylethynyl)pyridines as Ligands

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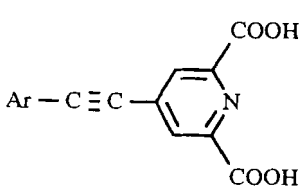
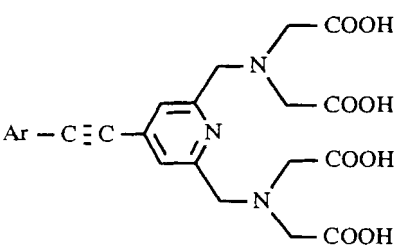
Some spectral properties and luminescence intensities of Eu^{III} chelates with 4-(arylethynyl)pyridine-2,6-dicarboxylic acids **1**–**15** and 2,2′,2″,2‴-{[4-(arylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetic acids) **16**–**26** were measured both in H_2O and EtOH solutions for the purpose of developing suitable labels to be used in time-resolved luminescence-based bioaffinity assays (*Tables 1* and *2*). The substitution at the Ar group has a significant effect upon the observed luminescence intensities, excitation wavelengths, and decay constants of the complexes. Moreover, the changes in the environment cause great variation in those properties of certain Eu^{III} chelates.

Introduction. – In recent years, a research effort was directed to luminescent lanthanide chelates to be used as labels in biological applications such as time-resolved luminescence immunoassays [1]. The attention was focused on stable complexes containing a suitable aromatic ligand or conjugated system capable of transferring the excitation energy to the lanthanide ion by an internal energy-transfer process which causes a strong luminescence of the lanthanide ion.

It was reported that lanthanide cryptates are stable and efficient luminescent species [2]. In addition to them, luminescent polyaminopolycarboxylate chelates proved to be useful labels [3] [4]. In a previous paper, we reported the syntheses as well as some spectral and luminescent properties of the Eu^{III} chelates of the 4-(arylethynyl)pyridine-2,6-dicarboxylic acids **1**–**8** and the 2,2′,2″,2‴-{[4-(arylethynyl)pyridine-2,6-diyl]bis(methylene-



1–**15** Ar: see *Table 1*



16–**26** Ar: see *Table 2*

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nitrilo)}tetrakis(acetic acids) **16–22** [4b]. The Eu^{III} chelate of 2,2′,2″,2‴-{{4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic acid) was used as luminescent label in time-resolved fluorescence microscopy for localization of antigens, mRNA's, and gene sequences at the cell and tissue level [4c]. To further explore features of chelates, we investigated several new structures. Because even minor changes in the environment of a chelate (*e.g.* polarity and H-bonds) may cause great variations in the luminescence intensities, luminescence measurements were carried out both in H_2O and EtOH solutions. The results can be applied, *e.g.*, to the research of labels for homogeneous immunoassays. The formation of a labelled antibody-antigen complex may cause changes in the environment of a lanthanide chelate and, thus, increase or decrease the luminescence of the chelate and thus indicate the amount of labelled material.

Results and Discussion. – The excitation wavelengths (λ_{exc}), relative luminescence intensities (log R) at λ_{exc}, and decay constants (k) of the Eu^{III} chelates with ligands **1–26** in H_2O and EtOH are given in *Tables 1* and *2*.

In fluoroimmunassays, the absorbance maximum of a luminescent probe must exceed 300 nm to diminish the background originating from the sample and plastics. On the

Table 1. *Excitation Wavelengths* (λ_{exc}), *Relative Luminescence Intensities* (log R), *and Decay Constants* (k) *of Europium(III) Chelates of Ligands* **1–15** *in Water and Ethanol*

	Ar	Solvent	λ_{exc} [nm]	log R	$k \cdot 10^3$ [s^{-1}]
1	Ph	H_2O	314	6.98	1.12
2	3-$(NH_2)C_6H_4$	H_2O	308	3.44	1.80
		EtOH	305	6.15	1.47
3	4-$(NH_2)C_6H_4$	H_2O	–	–	–
		EtOH	365	5.26	30.0
4	3-(PhCONH)C_6H_4	H_2O	305	6.55	1.04, 4.77
		EtOH	302	7.27	0.70
5	4-(PhCONH)C_6H_4	H_2O	335	6.66	1.14, 5.06
		EtOH	339	7.38	0.77
6	4-$(Me_2N)C_6H_4$	H_2O	–	–	–
		EtOH	–	–	–
7	4-(MeNH)C_6H_4	H_2O	–	–	–
		EtOH	–	–	–
8	4-[PhCON(Me)]C_6H_4	H_2O	320	6.57	1.06, 4.72
		EtOH	323	6.59	0.73
9	4-(PhCO)C_6H_4	H_2O	318	5.94	1.22, 5.05
		EtOH	318	6.70	0.75
10	4-(OH)C_6H_4	H_2O	335	2.82	1.91, 7.61
		EtOH	335	6.72	0.94
11	4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl	H_2O	325	6.38	1.30, 4.55
12	3,5-dichloro-4-hydroxyphenyl	H_2O	285	3.68	3.71
		EtOH	285	3.34	1.37, 5.72
13	3-carboxy-4-hydroxy-5-methylphenyl	H_2O	332	4.77	3.59
		EtOH	329	7.07	0.86
14	3-carboxy-4-hydroxyphenyl	H_2O	322	3.38	5.32
		EtOH	323	7.07	0.87
15	4-amino-3-carboxyphenyl	H_2O	350	5.54	4.14
		EtOH	356	7.34	0.89

Table 2. *Excitation Wavelengths* (λ_{exc}), *Relative Luminescence Intensities* (log *R*), *and Decay Constants* (*k*) *of Europium(III) Chelates of Ligands* **16–26** *in Water and Ethanol*

	Ar	Solvent	λ_{exc} [nm]	log *R*	$k \cdot 10^3$ [s^{-1}]
16	Ph	H_2O	292	5.89	2.60
17	3-$(NH_2)C_6H_4$	H_2O	295	3.63	2.78
		EtOH	295	3.81	4.08
18	4-$(NH_2)C_6H_4$	H_2O	315	4.07	2.75
		EtOH	320	3.92	4.33
19	3-(PhCONH)C_6H_4	H_2O	300	5.54	2.67
		EtOH	300	5.38	3.00, 5.60
20	4-(PhCONH)C_6H_4	H_2O	328	5.54	2.58
		EtOH	336	5.60	2.67
21	4-$(Me_2N)C_6H_4$	H_2O	310	3.75	3.36
		EtOH	310	3.76	2.78
22	4-(MeNH)C_6H_4	H_2O	300	3.90	3.41
		EtOH	300	4.11	2.59
23	4-(PhCO)C_6H_4	H_2O	314	5.81	2.59
		EtOH	314	5.88	2.00, 4.55
24	3,5-dichloro-4-hydroxyphenyl	H_2O	300	4.60	2.94, 17.5
		EtOH	300	4.56	2.71, 18.6
25	3-carboxy-4-hydroxyphenyl	H_2O	318	4.71	5.66
		EtOH	318	5.58	2.28
26	4-amino-3-carboxyphenyl	H_2O	337	4.13	3.08
		EtOH	350	5.61	2.53

other hand, a high molar absorptivity of the probe indicates a very conjugated aromatic ligand system. The energy of the triplet state of the ligand may be insufficient for transferring the energy from the ligand to the lanthanide ion. The excitation wavelengths of the Eu^{III} chelates with 4-(arylethynyl)pyridine-2,6-dicarboxylic acids **1**–**15** are in the main over 300 nm, while the values for those with the 2,2′,2″,2‴-{[4-(arylethynyl)-pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetic acids) **16**–**26**, having the same substituents, are somewhat lower. The excitation wavelengths of the Eu^{III} chelates of **1**, **15**, **26**, and **3** (in EtOH) are notably higher in comparison with other molecules. The excitation wavelengths both in H_2O and EtOH are in all cases approximately the same, except for the chelate of **26** ($\Delta(\lambda_{exc}(EtOH)\text{-}\lambda_{exc}(H_2O)) = 13$ nm). As anticipated, the position of the substituent in the Ar group shifts the position of the maximum: in *p*-position, λ_{exc} is more red-shifted than in *m*-position (see chelates of **2** *vs.* **3**, **4** *vs.* **5**, **17** *vs.* **18**, and **19** *vs.* **20**). The conjugated aromatic group 4-(PhCO)C_6H_4 in **9** and **23** does not have a significant effect on λ_{exc} which means that the two aromatic rings of Ar are not in the same plane. On the other hand, as a triplet sensitizer, this Ar group has an effect on the luminescence intensity, which is quite good for the chelates of **9** and **23**.

Previous investigations showed that ligands with 4-(arylethynyl)pyridine as chromophoric group give a strong emission and can be used, *e.g.*, as a label in time-resolved spectroscopy [4]. The luminescence properties of Eu^{III} and Tb^{III} chelates of new heteroaromatic complexing agents were reported with the purpose to develop suitable labels for time-resolved luminescence-based bioaffinity assays [3b]. According to those results, chelates of 2,2′,2″,2‴-[(2,2′-bipyridine-6,6′-diyl)bis(methylenenitrilo)]tetrakis-(acetic acid) and 2,2′,2″,2‴-[(2,2,′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo)]tetra-

kis(acetic acid) gave highest luminescence. When compared to those results, the Eu^{III} chelate of 2,2′,2″,2‴-{[4-(phenylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis-(acetic acid) (**16**) has a higher relative luminescence intensity than the corresponding 2,2′-bipyridine derivative and almost the same as the 2,2′:6′,2″-terpyridine derivative, although λ_{exc} is not so high.

The Eu^{III} chelates of **2–5**, **8–10**, and **13–15** demonstrate higher luminescence intensities in EtOH than in H_2O. This may be due to the fact that the luminescence lifetimes of the chelates are longer in EtOH ($k < 1.0 \cdot 10^3\ s^{-1}$) than in H_2O ($k > 1.0 \cdot 10^3\ s^{-1}$), with the exception of compound **2**. The situation is not quite clear with compounds **16–26**. The result is quite surprising, because the pyridine-2,6-dicarboxylic acids and 2,2′,2″,2‴-[(pyridine-2,6-diyl)bis(methylenenitrilo)]tetrakis(acetic acids) form 3:1 and 1:1 complexes with the Eu^{III} ion, respectively. For the study of H_2O coordination numbers, the measurements of decay constants k should be performed also in D_2O. Anyhow, the variation in the decay constants in H_2O *vs.* D_2O seems to be quite small, with an average of *ca.* $0.5 \cdot 10^3\ s^{-1}$ [5]. Using this value, the decay constants of the studied Eu^{III} chelates are mainly in accordance with anticipated values. This means that the Eu^{III} complexes of ligands **1–15** do not contain H_2O molecules coordinated to the Eu^{III} ion, while the complexes of ligands **16–26** should coordinate 2 H_2O molecules. Thus, the changing of the solvent should not affect the luminescence intensities of ligands **1–15**, whereas with ligands **16–26**, the alternation of luminescence should be more evident. The presence of two decay constants with some chelates indicates either the formation of two different chelates in the solutions or two noncoupled decaying states.

The luminescence of the chelate of the *p*-amino-substituted ligands **6** and **7** is too weak to be measured, both in H_2O and EtOH. Neither is the chelate of **3** luminescent in H_2O but gives very high luminescence in EtOH (λ_{exc} 365 nm), although the lifetime is remarkably short (decay constant $30 \cdot 10^3\ s^{-1}$). The chelates of the *m*-amino- and *p*-hydroxy-substituted ligands **2** and **10**, respectively, also show a solvent effect, though to a less significant extent. The reason for these notable solvent effects may be due to a H-bond between the NH_2 or OH groups and the solvent molecules. This is confirmed by comparison with the data obtained for the chelates of the corresponding amido-substituted ligands **8**, **5**, and **4** and alkoxy-substituted ligand **11**, which give strong luminescence in H_2O.

From these results, we conclude that a chelate applicable to homogeneous immunoassays must have either an amino or a hydroxy group in *p*-position, *via* which minor changes in the environment of the chelates (polarity and H-bonds) may cause great variations in the luminescence intensities. Moreover, as a binding site for biological material, we selected a COOH group in *m*-position, as realized in ligands **13–15** and **25** and **26**. But COOH, amino, or OH groups in the ligand are prone to intramolecular H-bonding which can also influence the effect of the environment. According to our results, the chelate of the OH-substituted **14** has a more significant solvent effect than that of the NH_2-substituted **15**. The less pronounced solvent effect of the chelate of **13** *vs.* that of **14** may be due to the Me group in *o*-position to the OH group of **13**, thus hindering H_2O molecules from forming a strong H-bond to this OH group.

With the exception of **25** and **26**, the Eu^{III} chelates of the 2,2′,2″,2‴-{[4-(arylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetic acids) do not exhibit such solvent effects (see *Table 2*): the luminescence intensities in H_2O and EtOH are almost

identical. The higher luminescence in EtOH than in H_2O of the chelates of **15** and **26** differs from that of the chelates of **14** and **15** inasmuch as the NH_2-substituted ligand **26** gives a better solvent effect than the corresponding OH-substituted **25**. Moreover, the excitation maximum of the chelate of **26** is also red-shifted in EtOH. The differences between the chelates of the two series of amino derivatives (**2**, **3**, **6**, and **7** *vs.* **17**, **18**, **21**, and **22**) are remarkable. The chelates of **6** and **7** do not luminesce in H_2O or in EtOH, whereas the corresponding chelates of **21** and **22** show luminescence, although not a very high one. The chelate of the *p*-amino-substituted **3** does not luminesce in H_2O while that of **18** gives a higher luminescence in H_2O than in EtOH. The excitation wavelength for the chelate of **18** in EtOH as well as its luminescent intensity are also reduced compared to that of **3**, while the luminescence lifetime is increased. The major change is observed in the decay constants k ($30 \cdot 10^3$ s^{-1} for chelate of **3** and $4.33 \cdot 10^3$ s^{-1} for that of **18**). Similar effects are observed for the chelates of *m*-amino-substituted ligands **2** and **17** in EtOH.

Although the solvent effect is not particularly significant with the chelates of **25** and **26**, as anticipated by the corresponding values of the chelates of **14** and **15**, the differences are still important enough to be exploited in homogeneous immunoassays. The results encouraged us to prepare derivatives of the ligands **25** and **26** which will be suitable for the labelling of biological material. The results will be reported in a separate publication.

Experimental Part

General. See [3b]. Moreover: Et_3N and THF were dried with Na. IR Spectra: *Perkin-Elmer-180* spectrophotometer; neat samples; $\tilde{\nu}$ in cm^{-1}. Mass Spectra: *VG-7070E* mass spectrometer; *m/z* (rel. intensity). The *4-(arylethynyl)pyridine-2,6-dicarboxylic acids* **1**–**11** and **13**–**15** and *2,2′,2″,2‴-{[4-(phenylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetic acid)* (**16**) were prepared as described previously [4b] [6].

2,6-Dichloro-4-iodophenol. A mixture of I_2 (1.27 g, 5 mmol) and KI (1.66 g, 10 mmol) in H_2O (7.5 ml) was added in small portions to a soln. of 2,6-dichlorophenol (1.63 g, 10 mmol), 60% ethylenediamine in H_2O (0.56 ml), and EtOH (4 ml). After a treatment with $NaHSO_3$, the crude product was filtered and washed with H_2O. Crystallization from EtOH/H_2O yielded pure 2,6-dichloro-4-iodophenol (2.06 g, 72%). M.p. 126.0–126.5° ([7]: 91–92°). MS: 288 (100, M^+), 290 (64, $[M+2]^+$), 292 (11, $[M+4]^+$).

Diethyl 4-Ethynylpyridine-2,6-dicarboxylate. A mixture of diethyl 4-bromopyridine-2,6-dicarboxylate [6a] (3.02 g, 10 mmol), bis(triphenylphosphine)palladium(II) chloride (140 mg, 0.2 mmol), and CuI (76 mg, 0.4 mmol) in dry Et_3N (10 ml) and dry THF (30 ml) was deaerated with N_2. (Trimethylsilyl)acetylene (1.18 g, 12 mmol) was added and the mixture stirred for 2 h at r.t. The mixture was filtered, the filtrate evaporated, the residue dissolved in CH_2Cl_2 (75 ml), the soln. washed with H_2O (2 × 30 ml), dried (Na_2SO_4), and evaporated, and the residue dissolved in EtOH (30 ml) and deaerated with N_2. Dry K_2CO_3 (138 mg, 1.0 mmol) was added to the mixture and stirred for 5 h at r.t. The mixture was evaporated without heating, the residue dissolved in CH_2Cl_2 (60 ml), the soln. washed with 5% $NaHCO_3$ soln. (10 ml) and H_2O (10 ml), dried (Na_2SO_4), and evaporated, and the residue purified by FC (silica gel, petroleum ether (b.p. 50–70°)/AcOEt 5:1): 1.05 g (55%) of diethyl 4-ethynylpyridine-2,6-dicarboxylate. M.p. 153–155°. IR (KBr): 3210 (≡C–H), 2105 (C≡C), 1715, 1335, 1255, 1210 (C=O, C–O). ^{1}H-NMR ($CDCl_3$): 1.46 (*t*, *J* = 7.0, 6 H); 3.48 (*s*, 1 H); 4.50 (*q*, *J* = 7.0, 4 H); 8.30 (*s*, 2 H).

Diethyl 4-[(3′,5′-Dichloro-4′-hydroxyphenyl)ethynyl]pyridine-2,6-dicarboxylate. A mixture of 2,6-dichloro-4-iodophenol (see above; 0.29 g, 1.0 mmol), bis(triphenylphosphine)palladium(II) chloride (14 mg, 0.02 mmol), and CuI (8 mg, 0.04 mmol) in dry Et_3N (3 ml) and dry THF (5 ml) was deaerated with N_2. Diethyl 4-ethynylpyridine-2,6-dicarboxylate (see above; 0.26 g, 1.1 mmol) was added and the mixture stirred for 2 h at 60°. The mixture was 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 residue purified by FC (silica gel, petroleum ether (b.p. 50–70°)/AcOEt 5:1, then 2:1). IR (KBr): 2210 (C≡C), 1735, 1720, 1290, 1260, 1240 (C=O, C–O). ^{1}H-NMR ($CDCl_3$): 1.45 (*t*, *J* = 7.0, 6 H); 4.50 (*q*, *J* = 7.0, 4 H); 7.55 (*s*, 2 H); 8.30 (*s*, 2 H). MS: 407 (100, M^+), 409 (66, $[M+2]^+$), 411 (13, $[M+4]^+$).

Dipotassium Salt of 4-[(3',5'-Dichloro-4'-hydroxyphenyl)ethynyl]pyridine-2,6-dicarboxylic Acid (**12**). A mixture of the diethyl ester described above (0.2 g, 0.5 mmol) and 0.5M KOH in EtOH (10 ml) was stirred for 2 h at r.t. The product (0.18 g, 86%) was filtered and washed with EtOH. IR (KBr): 2200 (C≡C), 1640, 1430, 1350 (C=O, C–O).

*Tetra(*tert*-butyl) Esters of 2,2',2'',2'''-{[4-(Arylethynyl)pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetic Acids)* **17**, **18**, *and* **21–26**. *Method 1.* A mixture of tetra(*tert*-butyl) 2,2',2'',2'''-[(4-bromopyridine-2,6-diyl)-bis(methylenenitrilo)]tetrakis(acetate) [6b] (0.67 g, 1.0 mmol), bis(triphenylphosphine)palladium(II) chloride (14 mg, 0.02 mmol), and CuI (8 mg, 0.04 mmol) in dry Et_3N (10 ml) and dry THF (10 ml) was deaerated with N_2. The arylacetylene [6b] (1.1 mmol) was added and the mixture heated to 45–50°. After stirring for 24 h, the mixture was filtered, the filtrate evaporated, the residue dissolved in $CHCl_3$ (30 ml), the soln. washed wit H_2O (3 × 10 ml) and dried (Na_2SO_4), and the residue purified by FC (silica gel, petroleum ether (b.p. 50–70°)/AcOEt): viscous tetraesters.

Method 2. According to *Method 1* by using tetra(*tert*-butyl) 2,2',2'',2'''-[(4-ethynylpyridine-2,6-diyl)bis-(methylenenitrilo)]tetrakis(acetate) [6c] and 4-iodo-*N*,*N*-dimethylaniline, 4-iodo-*N*-methylaniline [6b], or 2,6-dichloro-4-iodophenol (see above) as suitable starting materials.

*Tetra(*tert*-butyl) Ester of* **17**. *Method 1.* Yield 85%. ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.48 (*s*, 8 H); 4.04 (*s*, 4 H); 6.70 (*dd*, *J* = 2, 8, 1 H); 6.84 (*d*, *J* = 2, 1 H); 6.92 (*d*, *J* = 8, 1 H); 7.14 (*t*, *J* = 8, 1 H); 7.62 (*s*, 2 H).

*Tetra(*tert*-butyl) Ester of* **18**. *Method 1.* Yield 87%. ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.49 (*s*, 8 H); 4.02 (*s*, 4 H); 6.63 (*d*, *J* = 8, 2 H); 7.30 (*d*, *J* = 8, 2 H); 7.57 (*s*, 2 H).

*Tetra(*tert*-butyl) Ester of* **21**. *Method 2.* Yield 90%. ^{1}H-NMR ((D$_6$)DMSO): 1.42 (*s*, 36 H); 2.97 (*s*, 6 H); 3.41 (*s*, 8 H); 3.89 (*s*, 4 H); 6.73 (*d*, *J* = 8.7, 2 H); 7.36 (*d*, *J* = 8.7, 2 H); 7.45 (*s*, 2 H).

*Tetra(*tert*-butyl) Ester of* **22**. *Method 2.* Yield 75%. ^{1}H-NMR ((D$_6$)DMSO): 1.41 (*s*, 36 H); 2.71 (*d*, *J* = 4.7, 3 H); 3.42 (*s*, 8 H); 3.87 (*s*, 4 H); 6.31 (*q*, *J* = 4.7, 1 H); 6.56 (*d*, *J* = 8.9, 2 H); 7.29 (*d*, *J* = 8.9, 2 H); 7.44 (*s*, 2 H).

*Tetra(*tert*-butyl) Ester of* **23**. *Method 1.* Yield 53%. ^{1}H-NMR ((D$_6$)DMSO): 1.41 (*s*, 36 H); 3.44 (*s*, 8 H); 3.92 (*s*, 4 H); 7.59 (*t*, *J* = 8.1, 2 H); 7.60 (*s*, 2 H); 7.71 (*t*, *J* = 8.1, 1 H); 7.75–7.79 (*m*, 4 H); 7.81 (*d*, *J* = 8.1, 2 H).

*Tetra(*tert*-butyl) Ester of* **24**. *Method 2.* Yield 67%. ^{1}H-NMR ((D$_6$)DMSO): 1.42 (*s*, 36 H); 3.35 (*s*, 1 H); 3.43 (*s*, 8 H); 3.89 (*s*, 4 H); 7.52 (*s*, 2 H); 7.60 (*s*, 2 H).

*Tetra(*tert*-butyl) 3'-Methyl Pentaester of* **25**. *Method 1.* Yield 58%. ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.50 (*s*, 8 H); 3.99 (*s*, 7 H); 6.99 (*d*, *J* = 8.8, 1 H); 7.59 (*dd*, *J* = 1.9, 8.8, 1 H); 7.74 (*s*, 2 H); 8.02 (*d*, *J* = 1.9, 1 H); 10.97 (*s*, 1 H).

*Tetra(*tert*-butyl) 3'-Methyl Pentaester of* **26**. *Method 1.* ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.47 (*s*, 3 H); 3.55 (*s*, 8 H); 3.94 (*s*, 4 H); 4.05 (*s*, 2 H); 6.65 (*d*, *J* = 8.2, 1 H); 7.40 (*dd*, *J* = 2.1, 8.2, 1 H); 7.62 (*s*, 2 H); 8.11 (*d*, *J* = 2.1, 1 H).

*Hydrolysis of the Tetra(*tert*-butyl) Esters to* **17**, **18**, *and* **21–24**. A soln. of tetra(*tert*-butyl) ester (0.25 mmol) in CF_3COOH (5 ml) was stirred for 1.5 h at r.t. After evaporation, the residue was triturated with Et_2O and filtered. The yield was usually *ca.* 100%.

2,2',2'',2'''-{{4-[(3'-Aminophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**17**). IR (KBr): 2215 (C≡C), 1730, 1670, 1200 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.62 (*s*, 8 H); 4.11 (*s*, 4 H); 6.74 (*dd*, *J* = 2, 8, 1 H); 6.83 (*d*, *J* = 8, 1 H); 6.86 (*d*, *J* = 2, 1 H); 7.15 (*t*, *J* = 8, 1 H); 7.68 (*s*, 2 H).

2,2',2'',2'''-{{4-[(4'-Aminophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**18**). IR (KBr): 2195 (C≡C), 1730, 1630, 1200 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.57 (*s*, 8 H); 4.05 (*s*, 4 H); 6.60 (*d*, *J* = 7.9, 2 H); 7.30 (*d*, *J* = 7.9, 2 H); 7.36 (*s*, 2 H).

2,2',2'',2'''-{{4-{[4'-(Dimethylamino)phenyl]ethynyl}pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**21**). IR (KBr): 2190 (C≡C), 1730, 1630, 1190 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 2.98 (*s*, 6 H); 3.54 (*s*, 8 H); 4.02 (*s*, 4 H); 6.74 (*d*, *J* = 8.9, 2 H); 7.44 (*d*, *J* = 8.9, 2 H); 7.56 (*s*, 2 H).

2,2',2'',2'''-{{4-{[4'-(Methylamino)phenyl]ethynyl}pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid (**22**). IR (KBr): 2190 (C≡C), 1730, 1630, 1180 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 2.76 (*s*, 3 H); 3.57 (*s*, 8 H); 4.16 (*s*, 4 H); 6.58 (*d*, *J* = 8.9, 2 H); 7.56 (*s*, 2 H); 7.83 (*d*, *J* = 8.9, 2 H).

2,2',2'',2'''-{{4-[(4'-Benzoylphenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**23**). IR (KBr): 2215 (C≡C), 1730, 1655, 1190 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.57 (*s*, 8 H); 4.06 (*s*, 4 H); 7.59 (*t*, *J* = 7.6, 2 H); 7.68 (*s*, 2 H); 7.71 (*t*, *J* = 7.6, 1 H); 7.77 (*d*, *J* = 7.6, 2 H); 7.81 (*s*, 4 H).

2,2',2'',2'''-{{4-[(3',5'-Dichloro-4'-hydroxyphenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**24**). IR (KBr): 2215 (C≡C), 1735, 1630, 1200 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.54 (*s*, 8 H); 4.00 (*s*, 4 H); 7.58 (*s*, 2 H); 7.71 (*s*, 2 H).

2,2',2'',2'''-{{4-[(3'-Carboxy-4'-hydroxyphenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**25**). After treatment of the tetra(*tert*-butyl) 3'-methyl pentaester of **25** with CF_3COOH, the residue

was dissolved in 0.5M KOH in EtOH (5 ml), the soln. stirred for 4 h at r.t., and the product (0.12 g, 64%) filtered and washed with EtOH. IR (KBr): 2195 (C≡C), 1685, 1585, 1405, 1195 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.58 (*s*, 8 H); 4.05 (*s*, 4 H); 7.09 (*d*, *J* = 8.6, 1 H); 7.64 (*s*, 2 H); 7.75 (*dd*, *J* = 2.1, 8.6, 1 H); 7.99 (*d*, *J* = 2.1, 1 H).

2,2′,2″,2‴-{{4-[(4′-Amino-3′-carboxyphenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**26**) was prepared analogously to **25** from the corresponding tetra(*tert*-butyl) 3′-methyl pentaester. IR (KBr): 2200 (C≡C), 1695, 1590, 1400, 1250 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.48 (*s*, 8 H); 3.93 (*s*, 4 H); 6.83 (*d*, *J* = 8.8, 1 H); 7.15 (*dd*, *J* = 2.1, 8.8, 1 H); 7.48 (*s*, 2 H); 7.93 (*d*, *J* = 8.8, 1 H).

2,2′,2″,2‴-{{4-[(3′-Benzamidophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**19**). Benzoyl chloride (53 mg, 0.38 mmol) was added within 0.5 h in small portions to a cold (< 5°) mixture of **17** (121 mg, 0.25 mmol), KOH (77 mg, 1.38 mmol), and H_2O (5 ml). After stirring for 30 min, the mixture was acidified with 2M HCl (pH 1.5). The product (77 mg, 52%) was filtered and washed with H_2O. IR (KBr): 2210 (C≡C), 1720, 1625, 1540 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.52 (*s*, 8 H); 3.98 (*s*, 4 H); 7.37 (*d*, *J* = 7.4, 1 H); 7.46 (*t*, *J* = 8.0, 1 H); 7.51 (*t*, *J* = 7.4, 1 H); 7.55 (*t*, *J* = 8.0, 2 H); 7.6 (*s*, 2 H); 7.87 (*d*, *J* = 7.4, 1 H); 7.98 (*d*, *J* = 8.0, 2 H); 8.13 (*s*, 1 H).

2,2′,2″,2‴-{{4-[(4′-Benzamidophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetic Acid) (**20**) was prepared analogously to **19** from **18**. Yield 33%. IR (KBr): 2205 (C≡C), 1725, 1630, 1515 (C=O, C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.54 (*s*, 8 H); 4.01 (*s*, 4 H); 7.35 (*d*, *J* = 7.5, 2 H); 7.47 (*t*, *J* = 8.0, 1 H); 7.57 (*t*, *J* = 8.0, 2 H); 7.62 (*s*, 2 H); 7.80 (*d*, *J* = 7.5, 2 H); 8.01 (*d*, *J* = 8.0, 2 H).

Luminescence Measurements. For general considerations and the definition of *R*, see [3b]. The luminescence properties of Eu^{III} chelates were measured both in a borate buffer (pH 8.5) and EtOH using 1:3 or 1:1 mixtures of Eu^{III} and the ligands **1**–**15** or the ligands **16**–**26**, resp. The samples for the EtOH measurements were prepared by dilution of a stock soln. of a Eu^{III} chelate in a borate buffer (10^{-3} M, pH 8.5) to EtOH. The concentrations used were 10^{-5} or 10^{-6} M.

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REFERENCES

[1] E. Soini, I. Hemmilä, *Clin. Chem.* **1979,** *25,* 353; I. Hemmilä, Univ. Thesis, Turku, Finland, 1986.

[2] B. Alpha, J.-M. Lehn, G. Mathis, *Angew. Chem. Int. Ed.* **1987,** *26,* 266; B. Alpha, V.Balzani, J.-M.Lehn, S. Perathoner, N. Sabbatini, *Angew. Chem.* **1987,** *99,* 1310; H. Takalo, J. Kankare, *Finn. Chem. Lett.* **1988,** *15,* 95; J.-M. Lehn, J.-B. Regnouf de Vains, *Tetrahedron Lett.* **1989,** *30,* 2209; J.-M. Lehn, M. Pietraszkiewicz, J. Karpiuk, *Helv. Chim. Acta* **1990,** *73,* 106; J.-M. Lehn, C.O. Roth, *ibid.* **1991,** *74,* 572; V. Balzani, J.-M. Lehn, J. van de Loosdrecht, A. Mecati, N. Sabbatini, R. Ziessel, *Angew. Chem. Int. Ed.* **1991,** *30,* 190; O. Prat, E. Lopez, G. Mathis, *Anal. Biochem.* **1991,** *195,* 283.

[3] a) M.P. Bailey, B.F. Rocks, C. Riley, *Analyst* **1984,** *109,* 1449; R.A. Evangelista, A. Pollak, B. Allore, E.F. Templeton, R.C. Morton, E.P. Diamandis, *Clin. Biochem.* **1988,** *21,* 173; S. Dakubu, R. Hale, A. Lu, J. Quick, D. Solas, J. Weinberg, *Clin. Chem.* **1988,** *34,* 2337; A. Canfi, M.P. Bailey, B.F. Rocks, *Analyst* **1989,** *114,* 1405; b) V.-M. Mukkala, J. Kankare, *Helv. Chim. Acta* **1992,** *75,* 1578; V.-M. Mukkala, C. Sund, M. Kwiatkowski, P. Pasanen, M. Högberg, J. Kankare, H. Takalo, *ibid.* **1992,** *75,* 1621.

[4] a) J. Kankare, H. Takalo, P. Pasanen, Eur. Pat. 1990, 0203047 B1; b) J. Kankare, M. Latva, H. Takalo, *Eur. J. Solid State Inorg. Chem.* **1991,** *28,* 183; c) L. Seveus, M. Väisälä, S. Syrjänen, M. Sandberg, A. Kuusisto, R. Harju, J. Salo I. Hemmilä, H. Kojola, E. Soini, *Cytometry* **1992,** *13,* 329.

[5] W.DeW. Horrocks, Jr., D.S. Sudnick, *Acc. Chem. Res.* **1981,** *14,* 384; C.C. Bryden, C.N. Reilley, *Anal. Chem.* **1982,** *54,* 610.

[6] a) H. Takalo, J. Kankare, *Acta Chem. Scand., Ser. B* **1987,** *41,* 219; b) H. Takalo, P. Pasanen, J. Kankare, *ibid.* **1988,** *42,* 373; H. Takalo, J. Kankare, E. Hänninen, *ibid.* **1988,** *42,* 448; c) E. Hänninen, H. Takalo, J. Kankare, *ibid.* **1988,** *42,* 614.

[7] Brazier, Mc Combie, *J. Chem. Soc.* **1912,** *101,* 974.