28. Novel Thiazole-Containing Complexing Agents and Luminescence of Their Europium(III) and Terbium(III) Chelates

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The synthesis of novel thiazole-containing complexing agents and their luminescence properties with Eu^{III} and Tb^{III} ions are reported. One of these terpyridine analogues was also tested as an Eu^{III} labelling reagent, and its luminescence properties as an antibody conjugate were studied.

Introduction. – The important role played by complexes of lanthanide ions is related to their unique luminescence features making them attractive for alternatives as luminescent labels in bioaffinity assays [1]. Lanthanide complexes must fulfill strict requirements to be used as labels in bioaffinity assays: *i*) strong complexation with high thermodynamic stability and kinetic inertness, *ii*) efficient cation emission, *i.e.*, the organic ligand should have a high molecular absorption coefficient, the ligand-to-metal energy transfer should be very efficient, and the nonradiative deactivation of the metal excited state should be minimized. *E.g.*, the metal ion should be efficiently shielded from surrounding H_2O molecules, since they cause vibronic deactivation *via* the O–H oscillators. In addition *iii*) the label complex must be functionalized for the attachment to biomolecules, *i.e.*, the luminescent complex must contain a reactive group(s) to allow its covalent attachment to biomolecules. Finally, *iv*) another important factor is the effect of the complex and the coupling reaction on the affinity and nonspecific binding properties of the used biomolecules. These factors have to be elucidated for each particular biomolecule, especially antibody, to be labelled.

In commercial systems, either a non-luminescent lanthanide chelate is used in the labelling, and the luminescence is developed after the immunoreaction [2], or biomolecules are first labelled with a four-dentate fluorogenic ligand, and excess of lanthanide ions are added after the immunoreaction to saturate the ligand [3]. Stable and directly luminescent chelates are more seldomly used. Besides the use of cryptates [4], only a few applications have emerged, primarily based on polyamine-poly(acetic acid) derivatives [5]. We developed applications employing either 4-(arylethynyl)pyridine [6] [7c] or 2,2′:6′,2″-terpyridine [7] ([Eu^{III}(terpy)] and [Tb^{III}(terpy)][3])) as the chromogenic moiety.

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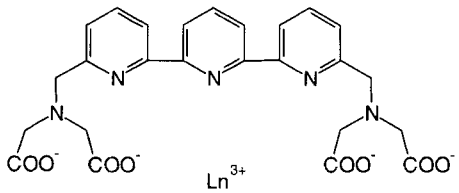
[3]) The abbreviation 'terpy' is used here for 2,2′,2″,2‴-[(2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo)]-tetrakis (acetic acid).



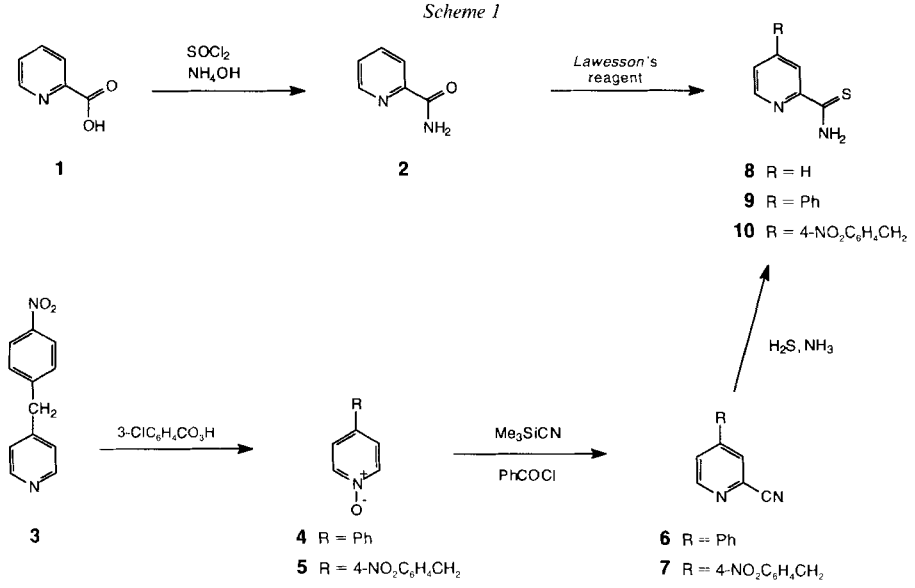
$[Eu^{III}(terpy)]^{3)}$ $Ln^{3+} = Eu^{3+}$
$[Tb^{III}(terpy)]^{3)}$ $Ln^{3+} = Tb^{3+}$

Due to the suitability of 2,2′:6′,2″-terpyridine as an absorbing and energy-transferring moiety, we were also interested in different modifications of terpyridine such as substitution of pyridines [7a], substitution [7b] or deuteration of methyleneiminobis(acetic acids) [8] or replacement of pyridines with five-membered aromatic moieties to get higher luminescence yields or otherwise to improve their properties as labels.

Promising results were obtained with terpyridine analogues in which two pyridine rings were replaced with pyrazole [9] or benzimidazole rings [10]. Also we were interested in various terpyridine analogues of five-membered aromatic N-compounds (*e.g.* thiazoles, triazoles, and oxazoles) [11]. In this article, we report the synthesis of novel thiazole-containing complexing agents and their luminescence properties with Eu^{III} and Tb^{III} ions. One of the complexes was also tested as an Eu^{III} labelling reagent, and its luminescence properties as an antibody conjugate were studied.

Results and Discussion. – *Syntheses.* The three different carbothioamides **8**–**10** (*Scheme 1*) were prepared either from pyridine-2-carboxamide **2** (from **1**) using *Lawesson*'s reagent or from pyridine-2-carbonitriles **6** and **7** using H_2S in abs. EtOH saturated

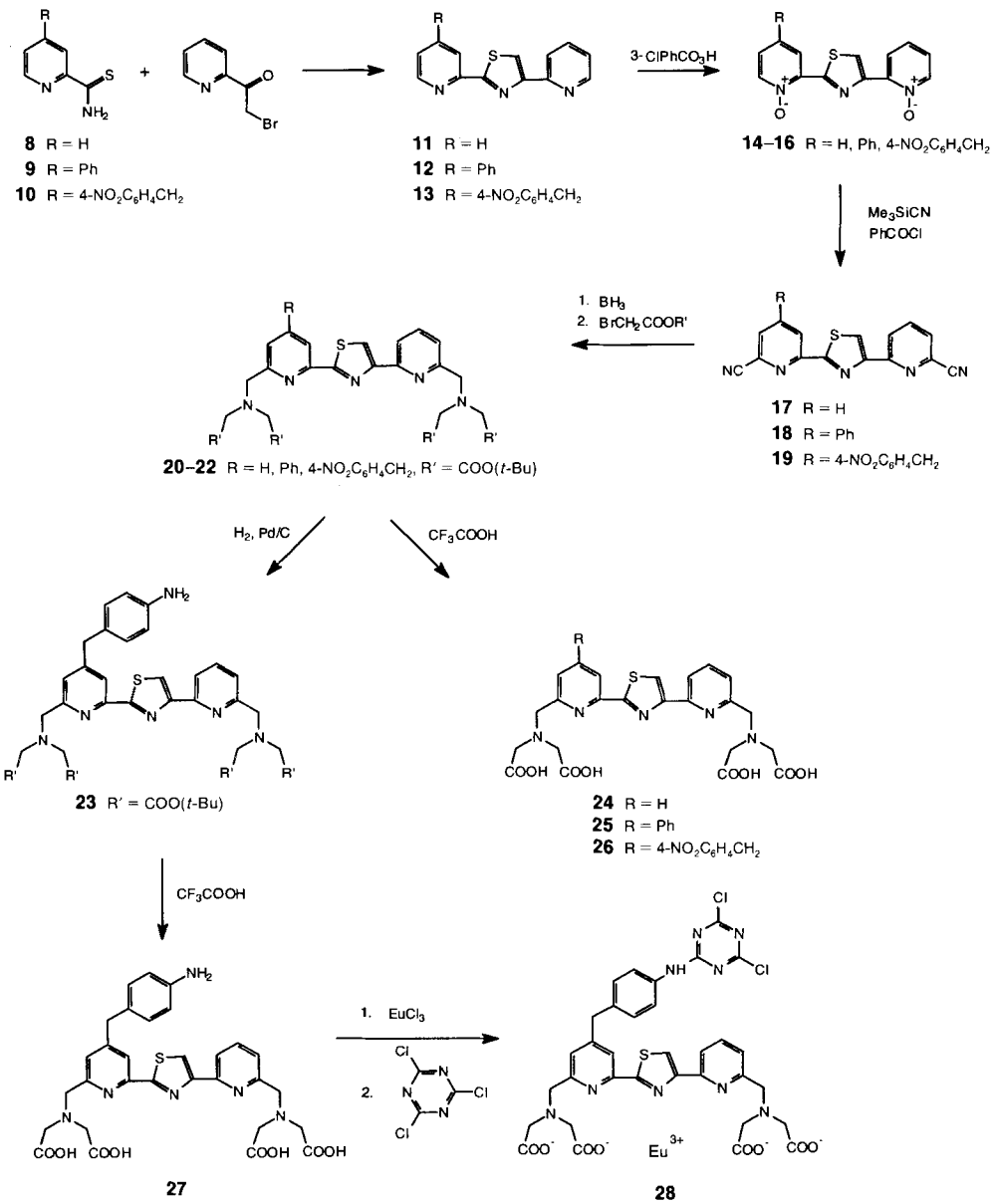
Scheme 1



with NH_3. Compounds **6** and **7** were synthesized from *N*-oxides **4** and **5** (from **3**) using the modified *Reissert-Henze* reaction [12].

The aromatic structures including a thiazole ring between two pyridine moieties (compounds **11–13** in *Scheme 2*) were prepared from carbothioamides **8–10** and 2-bromo-1-(pyridin-2-yl)ethanone [13]. The 2-bromo-1-(pyridin-2-yl)ethanone was prepared by a

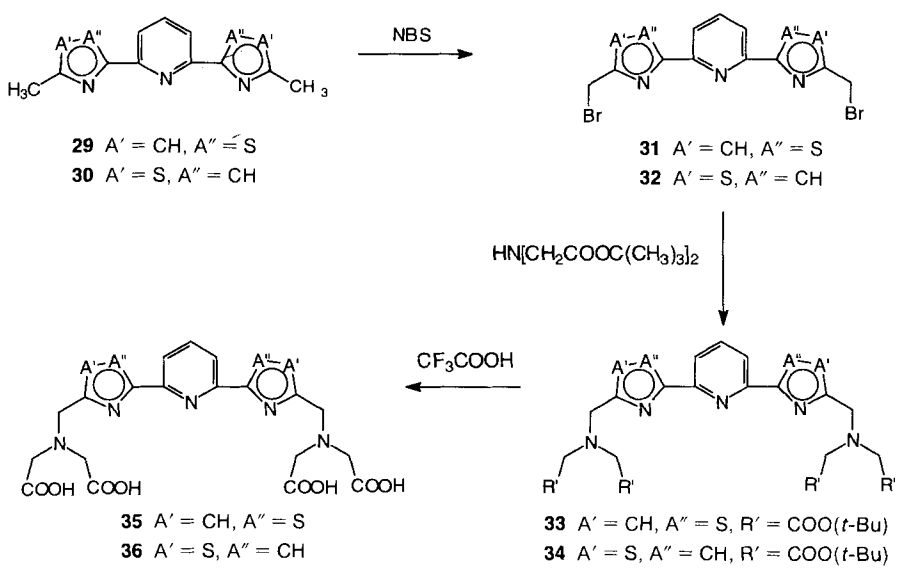
Scheme 2



modification of a literature procedure [14]. In our experiments, the bromination of 1-(pyridin-2-yl)ethanone did not take place at room temperature but needed refluxing until the color of Br_2 disappeared. The terminal pyridine rings were oxidized with 3-chloroperbenzoic acid to dioxides **14**–**16**. Normally, the oxidation of the first pyridine N-atom was relatively fast, but the further reaction of the second N-atom took a considerably longer time, and moreover, the second oxidation required a notable excess of the oxidant. The modified *Reissert-Henze* reaction yielded dicarbonitriles **17**–**19**. Also in this reaction, the monocarbonitriles were formed in a few hours, whereas the formation of dicarbonitriles needed several days. The dicarbonitriles **17**–**19** were reduced with $BH_3 \cdot THF$, and carboxymethylated without further purification with *tert*-butyl bromoacetate to tetraesters **20**–**22**. Using Pd/C as a catalyst, the nitro compound **22** was reduced with H_2 to compound **23**. The esters **20**–**23** were hydrolyzed with CF_3COOH to the target tetrakis(acetic acids) **24**–**27**.

The Eu^{III} chelate of ligand **27** was prepared by stirring the tetrakis(acetic acid) and $EuCl_3$ in a slightly acidic solution, and the chelate was precipitated after the pH adjustment by the addition of acetone (*Scheme 2*). The activation of the amino group by its transformation to (4,6-dichloro-1,3,5-triazin-2-yl)amino derivative **28** was performed with 2,4,6-trichloro-1,3,5-triazine.

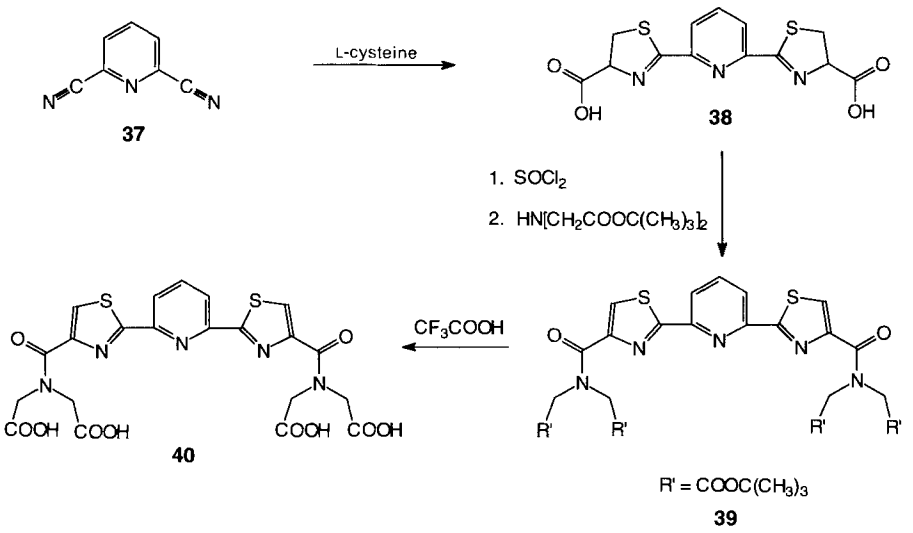
Scheme 3



The dimethyl compounds **29** [15a] and **30** [15b] were brominated to **31** and **32**, respectively with NBS (*N*-bromosuccinimide; *Scheme 3*). The tetra(*tert*-butyl) esters **33** and **34** were prepared from bromides **31** and **32** and di(*tert*-butyl) iminobis(acetate), and hydrolyzed with CF_3COOH to ligands **35** and **36**.

Pyridine-2,6-dicarbonitrile **37** [16] reacted readily with L-cysteine and formed the bis(dihydrothiazole) compound **38** (*Scheme 4*). This was coupled to di(*tert*-butyl)

Scheme 4



iminobis(acetate) by using $SOCl_2$ (→ **39**) and finally hydrolyzed to acid **40**. Surprisingly, the two dihydrothiazole rings of compound **38** were oxidized to corresponding thiazoles (compound **39**) during the reaction.

Luminescence Studies. The excitation maxima (λ_{exc}), luminescence decay times (τ), quantum yields (Φ), luminescence yields ($\varepsilon \cdot \Phi$), and relative luminescence intensities (log R) at λ_{exc} for the measured Eu^{III} and Tb^{III} chelates and for [Eu^{III}(terpy)] and [Tb^{III}(terpy)] as well as the measured triplet-state energy levels (E) are presented in *Tables 1* and *2*.

In the measurements of the luminescence yields and relative luminescence intensities, the ligand-to-lanthanide ratios were 10:1 and 1:1, respectively. The different ratio was to

Table 1. *The Triplet-State Energy Level (E), Excitation Maxima (λ_{exc}), Luminescence Decay Times (τ), Luminescence Yields ($\varepsilon \cdot \Phi$), Relative Luminescence Yields* (log R), *and Quantum Yields (Φ) of Europium(III) Chelates of Prepared Ligands*

Ligand in [Eu^{III}L]	E [cm^{-1}]	Ligand/metal 10:1			Ligand/metal 1:1			
		λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \phi$	λ_{exc} [nm]	τ [μs]	log R	ϕ
terpy[3])	22400	334	1310	2100	333	1370, 200	5.94	0.11
24	20100	342	1110	840	330	890	5.79	0.15
25	20400	342	1070	1420	336	1030	5.95	0.10
26		[a])	[a])	[a])	290	1160	5.10	
27		[a])	[a])	[a])	330	720	4.59	
28[b])		337	1090	790	[a])	[a])	[a])	[a])
35	18700	346	1320	1370	340	1350, 200	5.73	0.11
36	21600	319	1350	550	314	1230, 250	5.05	0.067
40	20900	[a])	[a])	[a])	335	[c])	[c])	0.004

[a]) Not measured.
[b]) In protein; ligand-to-metal ratio 1:1.
[c]) Too low.

Table 2. *The Triplet-State Energy Level (E), Excitation Maxima (λ_{exc}), Luminescence Decay Times (τ), Luminescence Yields ($\varepsilon \cdot \Phi$), Relative Luminescence Yields* (log *R*), *and Quantum Yields (Φ) of Terbium(III) Chelates of Prepared Ligands*

Ligand in [Tb^{III}L]	E [cm^{-1}]	Ligand/metal 10:1			Ligand/metal 1:1			
		λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \phi$	λ_{exc} [nm]	τ [μs]	log *R*	ϕ
terpy[3])	22400	333	1100	3800	333	1320	5.64	0.14
24	20100	[a])	[a])	[a])	290	[a])	[a])	0.001
25	20400	276	1280	19	260	1280	3.45	0.002
26		[b])	[b])	[b])	290	1140, 250	4.11	
27		[b])	[b])	[b])	290	1280	2.87	
35	18700	[a])	[a])	[a])	340	[a])	[a])	0
36	21600	320	970	1290	315	860	4.60	0.066
40	20900	[b])	[b])	[b])	290	[a])	[a])	0

[a]) Too low.
[b]) Not measured.

some extent reflected in the measured parameters, mainly in luminescence yields and relative luminescence intensities (*Tables 1* and *2*). A similar phenomenon was also observed with other ligands; *e.g.* with ligand-to-lanthanide ratios of 1:1 or 10:1, we obtained different luminescence-yield values for [Eu^{III}(terpy)] ($\varepsilon \cdot \Phi = 940$ *vs.* 2100) [7a] [8]. The ligands and their complexes were measured directly, whereas the activated Eu^{III} chelate **28** was measured after coupling to a protein (ratio 1:1). The results were compared to those of corresponding [Eu^{III}(terpy)] and [Tb^{III}(terpy)] [7a].

As with [Eu^{III}(terpy)] and [Tb^{III}(terpy)], the prepared ligands usually exhibited two excitation maxima (due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions) with Eu^{III} and Tb^{III} ions. The Eu^{III} complexes were mainly excited at longer wavelengths ($n \rightarrow \pi^*$ transition), over 330 nm, which is a convenient excitation wavelength for instrumental reasons relating to the UV transmission of lenses, filters, cuvettes, and glass slides. Surprisingly, the position of the S-atom in the two thiazole rings (**35** *vs.* **36**) had a profound effect on the excitation maximum (346 *vs.* 319 nm) of the Eu^{III} complexes. The Tb^{III} complexes were mainly excited at shorter wavelengths ($\pi \rightarrow \pi^*$ transition) than the corresponding Eu^{III} complexes, because λ_{exc} corresponding to the $\pi \rightarrow \pi^*$ transition had a higher ε than $n \rightarrow \pi^*$. Only ligand **36** was measured using $\lambda_{exc} > 300$ nm with Tb^{III}.

With regard to their decay times τ, the Eu^{III} complexes behaved quite predictably as nine-dentate chelates. To calculate the exact number of coordinated H_2O molecules, the measurements should be done also in D_2O [17]. However, based on the estimation of an average decay constant of 0.5 ms^{-1} (decay time of 2000 μs) for Eu^{III} chelates in D_2O [17], the decay times obtained in aqueous media indicated that the ligands **24**–**28** with Eu^{III} contained *ca.* 0.4 coordinated H_2O molecules in the first coordinated sphere. The values for **35** and **36** were almost the same as with [Eu^{III}(terpy)], on the average 0.2 coordinated H_2O molecules. The decay times of Tb^{III} chelates were similar to that of [Tb^{III}(terpy)] with exception of ligand **36**. The unexpectedly short decay times or two decay times may be a result of the low ligand triplet-state level causing an energy back flow from the excited Tb^{III} to the ligand triplet state. The presence of two decay times with some chelates indicates either the formation of two different chelates in the solution or two non-coupled decaying states.

The variations in the measurement system were to some extent reflected in the results. However, ligands **25** and **35** with Eu^{III} seem to give almost as good luminescence as well as quantum yields and relative luminescence intensities as [Eu^{III}(terpy)]. The conjugated label **28** also functions similarly to the basic ligand **24** and can be regarded as a suitable label alternative for bioaffinity assays. Unfortunately, so far we were not able to prepare labels derived from ligands **25** and **35**. It seems that the triplet level of the ligand must be clearly above the following excited state of Eu^{III} ion to yield high luminescence values, *i.e.* 22400 cm^{-1} for terpy *vs.* $^5D_2$ 21500 cm^{-1} of Eu^{III}, 20100 cm^{-1} for **24** and 20400 cm^{-1} for **25** *vs.* $^5D_1$ 19000 cm^{-1}, and 18700 cm^{-1} for **35** *vs.* $^5D_0$ 17300 cm^{-1}. Although the ligand **40** has a high triplet level (20900 cm^{-1}), the luminescence values are still too low for measurements. The low luminescence of an analogous amide was also observed with 2,2′-bipyridine [18]. The effect of a triplet state on luminescence properties is clearly seen in the measured values of Tb^{III} chelates. All thiazole ligands have lower triplet levels than and inferior luminescence to [Tb^{III}(terpy)]. Only ligand **36**, whose triplet level is near to that of terpy, has a moderate luminescence intensity with Tb^{III}, and has the same quantum yield both with Tb^{III} and Eu^{III} ions. As mentioned above, the decay times are unexpectedly short also for [Tb^{III}(terpy)]. In this light, to obtain good Tb^{III} labels, one has to prepare ligands whose triplet level is even higher than that of terpy. Moreover, suitable labels based on the structure of terpy may be difficult to prepare without changing the conjugated system over the three pyridines of terpy. In the future, we will concentrate on the syntheses of terpyridine analogues with new five-membered heteroaromatic rings and with higher triplet levels to find a suitable Tb^{III} label for bioaffinity assays.

According to the present study, suitable alternative Eu^{III} chelates were found from terpyridine analogues in which one or two pyridine rings are replaced with five-membered thiazole rings. For Tb^{III}, new ligand alternatives have to be examined to find a suitable Tb^{III} marker applicable to bioaffinity assays.

Experimental Part

General. Flash chromatography = FC. M.p.: uncorrected. UV Spectra: *Shimadzu-UV-2100* spectrophotometer; λ_{max} in nm. Luminescence spectra: decay times τ in μs and luminescence intensities were measured with a *Perkin-Elmer-LS-5* luminescence spectrometer combined with a *Perkin-Elmer-CLS* data station. IR Spectra: *Perkin-Elmer-1600-FTIR*. ^{1}H-NMR Spectra: 400-MHz *Jeol-GX-400*; $SiMe_4$ as internal standard, chemical shifts δ in ppm, coupling constants J in Hz.

1. *Pyridine-2-carboxamide* (**2**). A mixture of pyridine-2-carboxylic acid (**1**; 24.6 g, 0.20 mol) and $SOCl_2$ (100 ml) was refluxed for 1 h and evaporated. The residue was added in small portions to 25% NH_4OH soln. (500 ml) and the soln. concentrated to 100 ml. The cold mixture was filtered and the product washed with H_2O: 15.6 g (64%). UV (EtOH): 270 (sh), 265, 260 (sh), 217. (KBr): 3417, 3276, 3183 (N–H), 1718, 1660 (C=O). ^{1}H-NMR ($CDCl_3$): 6.25 (br. *s*, 1 H); 7.46 (*ddd*, J = 1.0, 4.4, 7.8, 1 H); 7.87 (*dt*, J = 1.5, 7.8, 1 H); 7.90 (br. *s*, 1 H); 8.22 (br. *d*, J = 7.8, 1 H); 8.59 (br. *d*, J = 4.4, 1 H).

2. *4-(4-Nitrobenzyl)pyridine* N-*Oxide* (**5**). To a cold mixture of 4-(4-nitrobenzyl)pyridine (**3**; 21.4 g, 100 mmol) and CH_2Cl_2 (250 ml), 3-chloroperbenzoic acid (50–55%; 74.7 g, *ca.* 190 mmol) was added. After stirring for 2 h, H_2O (200 ml) was added and the mixture alkalinized with solid Na_2CO_3. The H_2O phase was extracted with EtOH/$CHCl_3$ 1:2 (4 × 150 ml) and the combined org. phase dried (Na_2SO_4) and evaporated: 19.1 g (83%). UV (EtOH): 273. IR (KBr): 1511, 1346 (NO_2), 1229 (N→O). ^{1}H-NMR ($CDCl_3$): 4.08 (*s*, 2 H); 7.09 (*d*, J = 6.8, 2 H); 7.35 (*d*, J = 8.5, 2 H); 8.18 (*d*, J = 6.8, 2 H); 8.21 (*d*, J = 8.5, 2 H).

3. *Carbonitriles* **6** *and* **7**. Me_3SiCN (27 ml, 200 mmol) was added to a mixture of **4** or **5** (50.0 mmol) and CH_2Cl_2 (100 ml). After 5 min, benzoyl chloride (13 ml, 100 mmol) was added and the mixture stirred for 0.5 h. After

addition of H_2O (100 ml) and solid K_2CO_3 (25 g), the mixture was stirred for 0.5 h. The aq. phase was extracted with CH_2Cl_2 (2 × 50 ml) and the combined org. phase dried (Na_2SO_4) and evaporated.

4-Phenylpyridine-2-carbonitrile (**6**). Crystallized from H_2O/EtOH. Yield 81%. UV (EtOH): 264, 230 (sh). IR (KBr): 2234 (C≡N). ^{1}H-NMR ((D$_6$)DMSO): 7.56–7.61 (*m*, 3 H); 7.93 (*dd*, *J* = 1.7, 7.6, 2 H); 8.11 (*dd*, *J* = 2.0, 5.4, 1 H); 8.46 (*d*, *J* = 2.0, 1 H); 8.82 (*d*, *J* = 5.4, 1 H).

4-(4-Nitrobenzyl)pyridine-2-carbonitrile (**7**). Crystallized from toluene. Yield 53%. UV (EtOH): 266. IR (KBr): 2237 (C≡CN), 1513, 1349 (NO_2). ^{1}H-NMR ($CDCl_3$): 4.15 (*s*, 2 H); 7.32 (*d*, *J* = 4.9, 1 H); 7.35 (*d*, *J* = 8.3, 2 H); 7.51 (*s*, 1 H); 8.23 (*d*, *J* = 8.3, 2 H); 8.65 (*d*, *J* = 4.9, 1 H).

4. *Carbothioamides* **8–10**. *Pyridine-2-carbothioamide* (**8**). A mixture of **2** (12.2 g, 0.10 mol), *Lawesson*'s reagent (20.2 g, 0.05 mol), and toluene (100 ml) was stirred at 80–85° for 17 h. H_2O (100 ml) was added, the mixture extracted with Et_2O (3 × 100 ml), the extract dried (Na_2SO_4) and evaporated, and the product crystallized from EtOH: 7.46 g (54%). UV (EtOH): 278 (sh), 271, 266 (sh), 225. IR (KBr): 3349, 3241, 3150 (N–H), 1582 (C=S). ^{1}H-NMR ($CDCl_3$): 7.46 (*ddd*, *J* = 1.0, 4.9, 7.8, 1 H); 7.80 (br. *s*, 1 H); 7.85 (*dt*, *J* = 1.5, 7.8, 1 H); 8.52 (br. *d*, *J* = 4.9, 1 H); 8.71 (br. *d*, *J* = 7.8, 1 H); 9.53 (br. *s*, 1 H).

4-Phenylpyridine-2-carbothioamide (**9**). Abs. EtOH saturated with NH_3 (10 ml) was added to a cold soln. of **6** (1.8 g, 10 mmol) and abs. EtOH (30 ml). The mixture was saturated with H_2S. After stirring overnight, the soln. was concentrated to 10 ml. The cold mixture was filtered and washed with cold EtOH: 1.69 g (79%). UV (EtOH): 322, 241. IR (KBr): 3345, 3252, 3162 (N–H), 1592 (C=S). ^{1}H-NMR ($CDCl_3$): 7.45–7.54 (*m*, 3 H); 7.68 (*dd*, *J* = 1.8, 5.0, 1 H); 7.73 (br. *s*, 1 H); 7.71–7.74 (*m*, 2 H); 8.56 (*dd*, *J* = 0.7, 5.0, 1 H); 8.97 (*dd*, *J* = 0.7, 1.8, 1 H); 9.57 (br. *s*, 1 H).

4-(4-Nitrobenzyl)pyridine-2-carbothioamide (**10**). As described for **9**, with abs. EtOH saturated with NH_3 (20 ml), **7** (4.8 g, 20 mmol), and abs. EtOH (100 ml)/CH_2Cl_2 (80 ml). The crude solid was treated with $CHCl_3$ (100 ml), the mixture filtered, the solid washed with $CHCl_3$, and the filtrate evaporated: 3.3 g (60%). UV (EtOH): 317, 271. IR (KBr): 3388, 3237, 3134 (N–H), 1592 (C=S), 1513, 1345 (NO_2). ^{1}H-NMR ($CDCl_3$): 4.17 (*s*, 2 H); 7.21 (*d*, *J* = 4.9, 1 H); 7.36 (*d*, *J* = 8.8, 2 H); 7.69 (br. *s*, 1 H); 8.20 (*d*, *J* = 8.8, 2 H); 8.45 (*d*, *J* = 4.9, 1 H); 8.60 (*s*, 1 H); 9.51 (br. *s*, 1 H).

5. *Thiazoles* **11–13**. A mixture of **8**, **9**, or **10** (5.00 mmol), 1-bromo-2-(pyridin-2-yl)ethanone [14] (1.00 g, 5.00 mmol) and EtOH (20 ml) was refluxed for 3 h. The cold mixture was filtered and washed with cold EtOH. The mixture of the hydrobromic salt of the product and hot H_2O (40 ml) was alkalinized with solid Na_2CO_3, filtered, and washed with cold H_2O.

2,2'-(Thiazole-2,4-diyl)bis(pyridine) (**11**). Yield 79%. IR (KBr): 1587 (arom.). ^{1}H-NMR ((D$_6$)DMSO): 7.42 (*ddd*, *J* = 1.1, 5.1, 7.8, 1 H); 7.56 (*ddd*, *J* = 1.1, 5.1, 7.8, 1 H); 7.97 (*dt*, *J* = 1.5, 7.8, 1 H); 8.04 (*dt*, *J* = 1.5, 7.8, 1 H); 8.23 (br. *d*, *J* = 7.8, 1 H); 8.29 (br. *d*, *J* = 7.8, 1 H); 8.44 (*s*, 1 H); 8.68 (br. *d*, *J* = 5.1, H); 8.69 (br. *d*, *J* = 5.1, 1 H).

4-Phenyl-2-[4-(pyridin-2-yl)thiazol-2-yl]pyridine (**12**). Yield 79%. UV (EtOH): 320, 282 (sh), 249. IR (KBr): 1588 (arom.). ^{1}H-NMR ($CDCl_3$): 7.25–7.29 (*m*, 1 H); 7.47–7.51 (*m*, 1 H); 7.52–7.58 (*m*, 3 H); 7.76–7.79 (*m*, 2 H); 7.80–7.85 (*dt*, *J* = 2, 8, 1 H); 8.23 (*s*, 1 H); 8.30 (*d*, *J* = 8, 1 H); 8.57 (*d*, 1 H); 8.65–8.69 (*m*, 2 H).

4-(4-Nitrobenzyl)-2-[4-(pyridin-2-yl)thiazol-2-yl]pyridine (**13**). Crystallized from MeOH. Yield 61%. UV (EtOH): 315 (sh), 285, 246. IR (KBr): 1517, 1343 (NO_2). ^{1}H-NMR ($CDCl_3$): 4.18 (*s*, 2 H); 7.12 (*dd*, *J* = 1.5, 5.1, 1 H); 7.26 (*ddd*, *J* = 1.1, 5.1, 7.8, 1 H); 7.40 (*d*, *J* = 8.8, 2 H); 7.80 (*dt*, *J* = 1.5, 7.8, 1 H); 8.19–8.21 (*m*, 3 H); 8.20 (*d*, *J* = 8.8, 2 H); 8.56 (*d*, *J* = 4.8, 1 H); 8.65 (*d*, *J* = 4.8, 1 H).

6. *Dioxides* **14–16**. To a mixture of **11**, **12**, or **13** (10.0 mmol) and CH_2Cl_2 (400 ml), 3-chloroperbenzoic acid (50–55%; 25.9 g, *ca.* 75 mmol) was added in small portions during 24 h. After stirring for 1–4 d, the mixture was washed with 10% Na_2CO_3 soln. (3 × 150 ml) and H_2O (150 ml). The combined H_2O phase was extracted with $CHCl_3$ (150 ml) and the combined org. phase dried (Na_2SO_4) and evaporated.

2,2'-(Thiazole-2,4-diyl)bis(pyridine) N,N'-*Dioxide* (**14**). Yield 100%. UV (EtOH): 324, 249. IR (KBr): 1272 (N→O). ^{1}H-NMR ($CDCl_3$): 7.36–7.52 (*m*, 4 H); 8.43–8.45 (*m*, 2 H); 8.68 (*dd*, *J* = 2.1, 8.3, 1 H); 8.71 (*dd*, *J* = 2.1, 8.3, 1 H); 9.56 (*s*, 1 H).

4-Phenyl-2-[4-(pyridin-2-yl)thiazol-2-yl]pyridine N,N''-*Dioxide* (**15**). Yield 93%. IR (KBr): 1275 (N→O). ^{1}H-NMR ($CDCl_3$): 7.23–7.27 (*m*, 1 H); 7.41–7.46 (*m*, 1 H); 7.48–7.52 (*m*, 1 H); 7.55–7.60 (*m*, 3 H); 7.75 (*d*, *J* = 7, 2 H); 8.41 (*d*, *J* = 6, 1 H); 8.45 (*d*, *J* = 7, 1 H); 8.69 (*dd*, *J* = 2, 8, 1 H); 8.90 (*d*, *J* = 2, 1 H); 9.58 (*s*, 1 H).

4-(4-Nitrobenzyl)-2-[4-(pyridin-2-yl)thiazol-2-yl]pyridine N,N'-*Dioxide* (**16**). Yield 82%. UV (EtOH): 326, 275 (sh), 253. IR (KBr): 1515, 1346 (NO_2), 1264, 1235 (N→O). ^{1}H-NMR ((D$_6$)DMSO): 4.33 (*s*, 2 H); 7.48–7.63 (*m*, 3 H); 7.66 (*d*, *J* = 8.2, 2 H); 8.22 (*d*, *J* = 8.2, 2 H); 8.45 (*d*, *J* = 6.6, 1 H); 8.51 (*d*, *J* = 6.6, 1 H); 8.22 (*d*, *J* = 8.2, 1 H); 8.73 (*s*, 1 H); 9.44 (*s*, 1 H).

7. *Dicarbonitriles* **17–19**. Me_3SiCN (20 ml, 150 mmol) was added to a mixture of **14**, **15**, or **16** (10.0 mmol) and CH_2Cl_2 (110 ml). After 5 min, benzoyl chloride (7.2 ml, 60 mmol) was added, and the mixture was for 5–9 d. The mixture was then concentrated to ½ volume, 10% K_2CO_3 soln. (300 ml) was added and the mixture stirred for 0.5–2 h. The product was filtered and washed with H_2O and cold CH_2Cl_2.

6,6'-(Thiazole-2,4-diyl)bis(pyridine)-2,2'-dicarbonitrile (**17**). Yield 64%. UV (EtOH): 301, 271, 252. IR (KBr): 2238 (C≡N). ^{1}H-NMR ((D$_6$)DMSO): 8.07 (*dd*, *J* = 1.1, 7.8, 1 H); 8.20 (*dd*, *J* = 1.1, 7.8, 1 H); 8.24 (*t*, *J* = 7.8, 1 H); 8.30 (*t*, *J* = 7.8, 1 H); 8.52 (*dd*, *J* = 1.1, 7.8, 1 H); 8.59 (*dd*, *J* = 1.1, 7.8, 1 H); 8.69 (*s*, 1 H). MS: 289 (M^+),

6-[4-(6-Cyanopyridin-2-yl)thiazol-2-yl]-4-phenylpyridine-2-carbonitrile (**18**). Yield 65%. IR (KBr): 2240 (C≡N). ^{1}H-NMR ((D$_6$)DMSO): 7.50 (*t*, *J* = 8, 1 H); 7.58–7.67 (*m*, 2 H); 7.95 (*d*, *J* = 8, 1 H); 8.04–8.08 (*m*, 2 H); 8.24 (*t*, *J* = 8, 1 H); 8.60 (*d*, *J* = 1, 1 H); 8.67 (*d*, *J* = 8, 1 H); 8.70 (*s*, 1 H); 8.83 (*d*, *J* = 1, 1 H).

6-[4-(6-Cyanopyridin-2-yl)thiazol-2-yl]-4-(4-nitrobenzyl)pyridine-2-carbonitrile (**19**). After the addition of 10% K_2CO_3 soln., the mixture was extracted several times with $CHCl_3$ and the extract dried (Na_2SO_4) and evaporated. Yield 69%. UV (EtOH): 328 (sh), 297, 272. IR (KBr): 2237 (C≡N), 1521, 1348 (NO_2). ^{1}H-NMR ((D$_6$)DMSO): 4.38 (*s*, 2 H); 7.67 (*d*, *J* = 8.4, 2 H); 8.06 (*d*, *J* = 7.9, 1 H); 8.16 (*s*, 1 H); 8.21 (*d*, *J* = 8.4, 2 H); 8.24 (*t*, *J* = 7.9, 1 H); 8.49 (*d*, *J* = 7.9, 1 H); 8.55 (*s*, 1 H); 8.68 (*s*, 1 H).

8. *Tetraacetates* **20–22**. Within 10 min, 1M BH_3·THF (52 ml, 52.0 mmol) was added to a suspension of **17**, **18**, or **19** (4.00 mmol) and dry THF (50 ml). After stirring for 24 h, the extra BH_3 was distroyed by adding MeOH, the mixture evaporated, and MeOH saturated with dry HCl (70 ml) added. After stirring for 1 h, the cold mixture was filtered and washed with cold MeOH. A mixture of this material (1.00 mmol), dry (i-Pr)$_2$EtN (2.6 ml, 15.0 mmol), $BrCH_2COO$(*t*-Bu) (0.78 g, 4.00 mmol) and dry MeCN (20 ml) was refluxed for 23 h. After evaporated, the residue was dissolved in $CHCl_3$ (50 ml), the soln. washed with H_2O (3 × 20 ml), dried (Na_2SO_4), and evaporated. The product was purified with FC (silica gel, petroleum ether (b.p. 40–60°)/AcOEt 5:3).

*Tetra(*tert*-butyl) 2,2',2'',2'''-{[6,6'-(Thiazole-2,4-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetate)* (**20**). Yield 26%. ^{1}H-NMR ($CDCl_3$): 1.48 (*s*, 18 H); 1.48 (*s*, 18 H); 3.55 (*s*, 8 H); 4.11 (*s*, 2 H); 4.12 (*s*, 2 H); 7.59 (*d*, *J* = 7.6, 1 H); 7.71 (*d*, *J* = 7.6, 1 H); 7.79 (*t*, *J* = 7.6, 1 H); 7.81 (*t*, *J* = 7.6, 1 H); 8.13 (*d*, *J* = 7.6, 1 H); 8.18 (*d*, *J* = 7.6, 1 H); 8.20 (*s*, 1 H). MS: 753 (M^+).

*Di(*tert*-butyl) 2,2'-{{6-{4-{6-{{Bis[(*tert*-butoxy)carbonylmethyl]amino}methyl}pyridin-2-yl}thiazol-2-yl}-4-phenylpyridin-2-yl}methylimino}bis(acetate)* (**21**). Yield 63%. ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.54 (*s*, 4 H); 3.58 (*s*, 4 H); 3.95 (*s*, 2 H); 4.18 (*s*, 2 H); 7.42–7.94 (*m*, 8 H); 8.11 (*d*, *J* = 2, 1 H); 8.20 (*s*, 1 H); 8.43 (*d*, *J* = 2, 1 H).

*Di(*tert*-butyl) 2,2'-{{6-{4-{6-{{Bis(*tert*-butoxy)carbonylmethyl]amino}methyl}pyridin-2-yl}thiazol-2-yl}-4-(4-nitrobenzyl)pyridin-2-yl}methylimino}bis(acetate)* (**22**). In the first step, after the addition of MeOH saturated with dry HCl and stirring for 1 h, the soln. was evaporated and the residue triturated with cold THF and filtered. The final product **22** was purified by FC (silica gel, petroleum ether (b.p. 40–60°)/AcOEt 5:2). Yield 52%. UV (EtOH): 325 (sh), 292, 251, 232. IR (film): 1743 (C=O), 1521, 1346 (NO_2), 1144 (C–O). ^{1}H-NMR ($CDCl_3$): 1.46 (*s*, 18 H); 1.47 (*s*, 18 H); 3.52 (*s*, 4 H); 3.54 (*s*, 4 H); 4.07 (*s*, 2 H); 4.10 (*s*, 2 H); 4.18 (*s*, 2 H); 7.42 (*d*, *J* = 8.3, 2 H); 7.58 (*s*, 1 H); 7.59 (*d*, *J* = 7.8, 1 H); 7.77 (*t*, *J* = 7.8, 1 H); 8.00 (*s*, 1 H); 8.05 (*d*, *J* = 7.8, 1 H); 8.18 (*s*, 1 H); 8.19 (*d*, *J* = 8.3, 2 H).

9. *Di(*tert*-butyl) 2,2'-{{4-(4-Aminobenzyl)-6-{4-{6-{{bis[(*tert*-butoxy)carbonylmethyl]amino}methyl}-pyridin-2-yl}thiazol-2-yl}pyridin-2-yl}methylimino}bis(acetate)* (**23**). A mixture of **22** (150 mg 0.17 mmol), 10% Pd/C (10 mg), and MeOH (30 ml) was stirred for 5 h under H_2 (690 kPa). After filtration and evaporation, the product was purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 5:3): 40 mg (27%). IR (film): 1738 (C=O), 1144 (C–O). ^{1}H-NMR ($CDCl_3$): 1.46 (*s*, 18 H); 1.47 (*s*, 18 H); 3.54 (*s*, 8 H); 3.94 (*s*, 2 H); 4.06 (*s*, 2 H); 4.10 (*s*, 2 H); 6.64 (*d*, *J* = 8.2, 2 H); 7.03 (*d*, *J* = 8.2, 2 H); 7.49 (*s*, 1 H); 7.57 (*d*, *J* = 7.9, 1 H); 7.77 (*t*, *J* = 7.9, 1 H); 7.99 (*s*, 1 H); 8.09 (*d*, *J* = 7.9, 1 H); 8.15 (*s*, 1 H).

10. *Tetraacids* **24–27**. A soln. of tetraesters **20–23** (0.11 mmol) in CF_3COOH (2 ml) was stirred for 2 h at r.t. After evaporation, the mixture was triturated with Et_2O and filtered.

2,2',2'',2'''-{[6,6'-(Thiazole-2,4-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetic Acid) (**24**). Yield 100%. UV (H_2O): 325 (sh), 294, 247. UV ([Eu^{III}(**24**)], H_2O): 331, 295, 243. IR (KBr): 1733 (C=O), 1198 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.63 (*s*, 4 H); 3.72 (*s*, 4 H); 4.12 (*s*, 2 H); 4.21 (*s*, 2 H); 7.56 (*d*, *J* = 7.6, 1 H); 7.69 (*d*, *J* = 7.6, 1 H); 7.99 (*t*, *J* = 7.6, 1 H); 8.03 (*t*, *J* = 7.6, 1 H); 8.14 (*d*, *J* = 7.6, 1 H); 8.18 (*d*, *J* = 7.6, 1 H); 8.39 (*s*, 1 H).

2,2'-{{6-{4-{6-{[Bis(carboxymethyl)amino]methyl}pyridin-2-yl}thiazol-2-yl}-4-phenylpyridin-2-yl}methylimino}bis(acetic Acid) (**25**). Yield 91%. UV (H_2O): 331 (sh), 252. UV ([Eu^{III}(**25**)]): 334, 288, 251. IR (KBr): 1732

(C=O), 1197 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.66 (*s*, 4 H); 3.70 (*s*, 4 H); 4.18 (*s*, 4 H); 7.54–7.63 (*m*, 4 H); 7.92 (*d*, *J* = 7.6, 2 H); 7.98 (*t*, *J* = 7.8, 1 H); 8.06 (*d*, *J* = 1.5, 1 H); 8.23 (*d*, *J* = 7.8, 1 H); 8.41 (*s*, 1 H); 8.44 (*d*, *J* = 1.5, 1 H).

2,2′-{{6-{4-{6-{[Bis(carboxymethyl)amino]methyl}pyridin-2-yl}thiazol-2-yl}-4-(4-nitrobenzyl)pyridin-2-yl}methylimino}bis(acetic Acid) (**26**). Yield 100%. UV (H$_2$O): 285, 265, 245. UV ([Eu^{III}(**26**)], H$_2$O): 320 (sh), 285. IR (KBr): 1727 (C=O), 1520, 1347 (NO$_2$), 1198 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.53 (*s*, 4 H); 3.56 (*s*, 4 H); 4.04 (*s*, 2 H); 4.07 (*s*, 2 H); 4.30 (*s*, 2 H); 7.21 (*d*, *J* = 7.8, 1 H); 7.57 (*s*, 1 H); 7.60 (*d*, *J* = 8.8, 2 H); 7.94 (*t*, *J* = 7.8, 1 H); 8.07 (*d*, *J* = 7.8, 1 H); 8.09 (*s*, 1 H); 8.17 (*d*, *J* = 8.8, 2 H); 8.33 (*s*, 1 H).

2,2′-{{4-(4-Aminobenzyl)-6-{4-{6-{[bis(carboxymethyl)amino]methyl}pyridin-2-yl}thiazol-2-yl}pyridin-2-yl}methylimino}bis(acetic Acid) (**27**). Yield 100%. UV (H$_2$O): 315, 290. UV ([Eu^{III}(**27**)]): 325, 290. IR (KBr): 3433 (N–H), 1636 (C=O), 1437, 1200 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.56 (*s*, 4 H); 3.59 (*s*, 4 H); 4.00 (*s*, 2 H); 4.04 (*s*, 2 H); 4.09 (*s*, 2 H); 6.81 (*d*, *J* = 8.2, 2 H); 7.12 (*d*, *J* = 7.6, 1 H); 7.53 (*s*, 1 H); 7.54 (*d*, *J* = 8.2, 2 H); 7.95 (*t*, *J* = 7.6, 1 H); 7.99 (*s*, 1 H); 8.06 (*d*, *J* = 7.6, 1 H); 8.33 (*s*, 1 H).

11. *{2,2′-{{6-{4-{6-{[Bis(carboxylatomethyl)amino]methyl}pyridin-2-yl}thiazol-2-yl}-4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzyl}pyridin-2-yl}methylimino}bis(acetato)}europium(III)* (**28**). Tetraacid **27** (25 mg, 40 µmol) was dissolved in H$_2$O (700 µl), and the pH was adjusted to 6.5 with solid NaHCO$_3$. EuCl$_3$ (22 mg, 60 µmol) in H$_2$O (200 µl) was added within 15 min, and the pH was maintained at 5–7. After stirring for 1.5 h, the pH was raised to 8.5 with 1M NaOH, the precipitate filtered off, the filtrate treated with acetone and the precipitate filtered and washed with acetone. A mixture of 2,4,6-trichloro-1,3,5-triazine (2 mg, 10 µmol), acetone (100 µl), and H$_2$O (100 µl) was added to a soln. of the chelate (8 mg, 10 µl), and 0.1M NaOAc (150 µl, pH 4.9). After stirring for 15 min, acetone was added and the precipitate filtered, washed with acetone, and dried. UV (H$_2$O): 331, 287, 250.

12. *2,6-Bis(methylthiazolyl)pyridines* **29** *and* **30**. *2,6-Bis(4-methylthiazol-2-yl)pyridine* (**29**) [15a]. UV (EtOH): 330, 306, 232. IR (KBr): 1569 (arom.). ^{1}H-NMR (CDCl$_3$): 2.54 (*s*, 6 H); 7.03 (*s*, 2 H); 7.86 (*t*, *J* = 7.8, 1 H); 8.15 (*d*, *J* = 7.8, 2 H).

2,6-Bis(2-methylthiazol-4-yl)pyridine (**30**) [15b]. UV (EtOH: 315, 260. IR (KBr): 1587 (arom.). ^{1}H-NMR (CDCl$_3$): 2.80 (*s*, 6 H); 7.83 (*t*, *J* = 7.8, 1 H); 8.02 (*d*, *J* = 7.8, 2 H); 8.03 (*s*, 2 H).

13. *2,6-Bis[(bromomethyl)thiazolyl]pyridines* **31** *and* **32**. A mixture of **29** or **30** (0.63 g, 2.3 mmol), NBS (0.90 g, 5.1 mmol), dibenzoyl peroxide (56 mg, 0.2 mmol), and CCl$_4$ (15 ml) was refluxed overnight. The cooled mixture was filtered, the filtrate evaporated, and the product purified by FC (silica gel, 2% MeOH in CHCl$_3$).

2,6-Bis[4-(bromomethyl)thiazol-2-yl]pyridine (**31**). Yield 82%. UV (EtOH): 328, 302. IR (KBr): 1566 (arom.). ^{1}H-NMR (CDCl$_3$): 4.65 (*s*, 4 H); 7.44 (*s*, 2 H); 7.93 (*t*, *J* = 7.8, 1 H); 8.22 (*d*, *J* = 7.8, 2 H).

2,6-Bis[2-(bromomethyl)thiazol-4-yl]pyridine (**32**). Yield 23%. UV (EtOH): 313, 262. IR (KBr): 1584 (arom.). ^{1}H-NMR (CDCl$_3$): 4.81 (*s*, 4 H); 7.86 (*t*, *J* = 7.8, 1 H); 8.06 (*d*, *J* = 7.8, 2 H); 8.23 (*s*, 2 H).

14. *Tetrakis(acetates)* **33** *and* **34**. A mixture of **31** or **32** (0.82 g, 1.9 mmol), di(*tert*-butyl) iminobis(acetate) (0.93 g, 3.8 mmol), Na$_2$CO$_3$ (0.50 g) and dry MeCN (100 ml) was refluxed overnight. The mixture was filtered and evaporated and the product purified by FC (silica gel, petroleum ether (b.p. 40–60°)/AcOEt 5:1).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[2,2″-(Pyridine-2,6-diyl)bis(thiazole)-4,4′-diyl]bis(methylenenitrilo)}tetrakis(acetate)* (**33**). Yield 31%. UV (EtOH): 329, 299, 230. IR (film): 1755, 1732 (C=O), 1147 (C–O). ^{1}H-NMR (CDCl$_3$): 1.48 (*s*, 36 H); 3.53 (*s*, 8 H); 4.16 (*s*, 4 H); 7.42 (*s*, 2 H); 7.86 (*t*, *J* = 7.8, 1 H); 8.18 (*d*, *J* = 7.8, 2 H).

*Tetra(*tert*-butyl) 2,2′,2″,2‴-{[4,4′-(Pyridine-2,6-diyl)bis(thiazole)-2,2′-diyl]bis(methylenenitrilo)}tetrakis(acetate)* (**34**). Yield 38%. UV (EtOH): 316, 258. IR (film): 1738 (C=O), 1149 (C–O). ^{1}H-NMR (CDCl$_3$): 1.48 (*s*, 36 H); 3.58 (*s*, 8 H); 4.32 (*s*, 4 H); 7.82 (*t*, *J* = 7.8, 1 H); 8.01 (*d*, *J* = 7.8, 2 H); 8.16 (*s*, 2 H).

15. *Tetrakis(acetic Acids)* **35** *and* **36**. A soln. of **33** or **34** (0.22 g, 0.29 mmol) in CF$_3$COOH (5.0 ml) was stirred for 2 h at r.t. After evaporation, the mixture was triturated with Et$_2$O and filtered.

2,2′,2″,2‴-{[2,2′-(Pyridine-2,6-diyl)bis(thiazole)-4,4′-diyl]bis(methylenenitrilo)}tetrakis(acetic Acid) (**35**). Yield 75%. UV (H$_2$O): 323, 287, 230. UV ([Eu^{III}(**35**)], H$_2$O): 341, 310, 278. IR (KBr): 1732 (C=O), 1193 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.85 (*s*, 8 H); 4.33 (*s*, 4 H); 7.90 (*s*, 2 H); 8.18–8.22 (*m*, 3 H).

2,2′,2″,2‴-{[4,4′-(Pyridine-2,6-diyl)bis(thiazole)-2,2′-diyl]bis(methylenenitrilo)}tetrakis(acetic Acid) (**36**). UV (H$_2$O): 309, 256. UV ([Eu^{III}(**36**)], H$_2$O): 316, 254. IR (KBr): 1735 (C=O), 1194 (C–O). ^{1}H-NMR ((D$_6$)DMSO): 3.91 (*s*, 8 H); 4.30 (*s*, 4 H); 7.96–7.99 (*m*, 3 H); 8.45 (*s*, 2 H).

16. *2,2′-(Pyridine-2,6-diyl)bis(4,5-dihydrothiazole)-4,4′-dicarboxylic Acid* (**38**). L-Cysteine hydrochloride (0.66 g, 5.4 mmol) was dissolved in H$_2$O (5.0 ml), and the soln. was neutralized with NaHCO$_3$. A soln. of pyridine-2,6-dicarbonitrile (**37**; 0.24 g, 1.9 mmol) in MeOH (5.0 ml) was added. After evaporation of MeOH, the

product was filtered and washed with acetone: 0.35 g (55%). UV (H_2O): 288. ^{1}H-NMR ((D_6)DMSO): 3.42–3.49 (*m*, 1 H); 3.54–3.62 (*m*, 1 H); 5.04–5.09 (*m*, 1 H); 8.01 (AB_2, J = 7.8, 1 H); 8.12 (AB_2, J = 7.8, 2 H).

17. *Tetra(*tert*-butyl) 2,2′,2″,2‴-{[2,2′-(Pyridine-2,6-diyl)bis(thiazole)-4,4′-diyl]bis(carbonylnitrilo)}tetrakis(acetate)* (**39**). A mixture of **38** (0.35 g, 1.0 mmol) and $SOCl_2$ (5.0 ml) was refluxed for 1 h. After evaporation, the residue was dissolved in dry pyridine (6.0 ml), di(*tert*-butyl) iminobis(acetate) (0.64 g, 2.6 mmol) added, and the mixture refluxed for 2 h. After evaporation, $CHCl_3$ was added and the mixture filtered. The product was purified by FC (silica gel, $CHCl_3$). UV (EtOH): 326, 289. IR (film): 1741 (C=O), 1640 (amide), 1152 (C–O). ^{1}H-NMR ($CDCl_3$): 1.45 (*s*, 18 H); 1.51 (*s*, 18 H); 4.27 (*s*, 4 H); 4.63 (*s*, 4 H); 7.91 (*t*, J = 7.9, 1 H); 8.20 (*d*, J = 7.9, 2 H); 8.33 (*s*, 1 H); 8.33 (*s*, 1 H).

18. *2,2′,2″,2‴-{[2,2′-(Pyridine-2,6-diyl)bis(thiazole)-4,4′-diyl]bis(carbonylnitrilo)}tetrakis(acetic Acid)* (**40**). Analogously to **35**. UV (H_2O): 325, 278. UV ([Eu^{III}(**40**)], H_2O): 325, 278. ^{1}H-NMR ((D_6)DMSO): 4.09 (*s*, 8 H); 4.42 (*s*, 4 H); 8.18 (*t*, J = 7.8, 1 H); 8.30 (*d*, J = 7.8, 2 H); 8.41 (*s*, 2 H).

19. *Concentration Measurements.* The measurement of total Eu^{III} ion concentration before, during, and after labelling were performed using a dissociative fluorescence enhancement system [19] based on the *Wallac-Delfia* enhancement soln. composed of 15 μM 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.

20. *Coupling of the Chelate* **28** *to Protein.* The activated chelate **28** was coupled to a model protein (rabbit anti-mouse IgG; *Dako*, Denmark) by incubating the chelate **28** with IgG (1 mg) in carbonate buffer (500 μl, pH 9.3) overnight using a 20-fold molar reagent-to-protein ratio. After the coupling reaction, the protein was purified on a combined column of *Sephadex G 50* (10 cm) and *Sepharose 6 B* (30 cm) by eluting with 50 mM *Tris*-HCl buffer containing 0.15M NaCl and 0.05% NaN_3 soln. The fractions corresponding to labelled monomeric IgG were collected. The chelate concentrations in the protein fractions were measured from both the absorptions of the conjugated chelate at 330 nm and the total Eu^{3+} ion concentration measured by the dissociative fluorescence enhancement system. The purified protein conjugate and the labelling ratio (chelates per protein) were quantified by calculating the protein yield or by measuring the absorbance at 280 nm and subtracting the absorption caused by the added chelate.

21. *Luminescence Measurements.* The luminescence parameters for free chelates were analyzed in borate buffer, pH 8.5. In the measurements of luminescence intensities ($\varepsilon \cdot \Phi$), the ligand concentration was kept at 10 μM, and the lanthanide-ion concentration was 1 μM; for other general considerations, see [7a]. In the measurements of relative luminescence yields (log R), the concentrations of the ligands and lanthanides were 10 μM or 1 μM (1:1 mixtures). For general considerations and the definition of R, see [20]. The phosphorescence spectra were measured in 5:4 mixtures of glycerol (purified) and 0.05M borate buffer (pH 9.2). Concentration of Gd^{III} (added as perchlorate) was 10 μM and ligand 30 μM. Results: *Tables 1* and *2*.

Authors note: triplet-state energy levels are generally measured from phosphorescence spectra of Gd^{III} chelates not from corresponding Eu^{III} nor Tb^{III} chelates.

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