28. Development of Luminescent Terbium(III) Chelates for Protein Labelling: Effect of Triplet-State Energy Level

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The synthesis of novel Tb^{III} labels suitable for protein labelling are reported. Their luminescence properties as antibody conjugates were measured and compared to the results of corresponding Tb^{III} chelates of the parent ligand structures. When the lowest triplet-state energy level of the parent donor ligand was over 23000 cm^{-1}, *i.e.*, the energy gap between the $^5D_4$ level of Tb^{III} and the lowest triplet-state energy level of the ligand exceeded 2600 cm^{-1}, the label derivative with a long decay time ($\tau = 1.35-2.93$ ms) and a high luminescence yield ($\varepsilon \cdot \Phi = 3770-4560$) was found to be suitable for bioaffinity assays.

Introduction. – Long-lifetime emitting lanthanide chelates as sensitive labels or probes, and time-resolved fluorometry in detection have already been used in a number of applications in bioaffinity assays [1].

In commercial systems, due to the many requirements of optimal lanthanide chelate labels, compromises have been made, *e.g.*, the detection comprises two separate steps, as a direct measurement of the analyte without any enhancement step is impossible [2]. In this respect, these technologies are not suitable for all applications, such as *in situ* hybridization, immunohistochemistry, homogeneous assays, and fluorescence imaging. In addition to sensitive bioaffinity assays, new highly luminescent and stable lanthanide labels permit the development of multilabel, miniaturized assay devices with simplified protocols and thus make it possible to perform multiparametric assays on sub-microliter volumes of samples. A lot of research effort has been directed to the design and synthesis of optimal labels also suitable for the applications mentioned above. The chelating agents commonly used for the sensitization of Eu^{III} and Tb^{III} ion luminescence include β-diketones [3], multidentate phenol [4], 4-amino-2-hydroxybenzoic acid [5], 7-amino-4-methylquinolin-2(1*H*)-one [6], pyridine [7] [8], bipyridine [9], terpyridine [7b] [9b] [10], and their structural analogues [8b] [11] as polyamine-polycarboxylates, as well as cryptands [12] and calixarenes [12a] [13]. Due to the many strict requirements that lanthanide complexes have to fulfil to be used as labels in bioaffinity assays, only a few viable chelate labels, mainly Eu^{III} complexes, have been developed and tested [3] [4a] [5] [6] [8] [10c] [11b] [12b]. Although numerous highly luminescent parent chelate structures

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have been reported, stable and directly luminescent Tb^{III} labels are more seldomly used; these are primarily based on polyamine-poly(acetic acid) derivatives [4a] [5] [6a] [8b, c].

It has been shown that the energy gap between the triplet-state of the donor ligand and the emitting level of the Tb^{III} ion should be large enough to prevent energy back transfer from the excited Tb^{III} ion to the ligand [14]. A small energy gap decreases the quantum yield and decay time of Tb^{III} complexes, and a clear correlation between luminescence efficiencies and triplet-state energy levels has been observed [11d]. Moreover, even the coupling of Tb^{III} labels to biomolecules, especially to proteins, has a profound effect on the decay time and thus on the quantum yield of Tb^{III} labels [10a, b]. Our studies with the Tb^{III} chelates of 2,2′:6′,2″-terpyridines **1** and **2** [9a] [10a, b] and its thiazole (**3**–**5**), triazole (**6**), and pyrazole (**7**–**9**) [11c, d] analogues encouraged us to prepare modified labelling reagents without changing the aromatic part of the parent ligands **6**–**8** (*i.e.*, reagents with similar modifications as in 2,2′:6′,2″-terpyridine derivatives **10** and **11** [10b]), which have a higher triplet-state energy level than ligands **1** and **2**. Besides the syntheses, we also report luminescence properties of Tb^{III} labelling reagents as antibody conjugates to demonstrate the correlation between the triplet-state energy level of novel Tb^{III} labels, and their decay times and luminescence efficiencies.

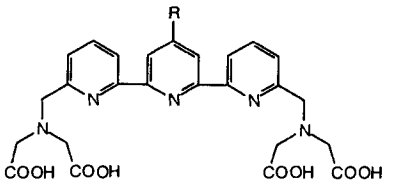
2. Results and Discussion. – *Syntheses.* The α-amino group of L-lysine was protected as a Cu^{II} complex before reaction of the ε-amino group with 4-nitrobenzoyl chloride in a slightly basic solution of 1,4-dioxane and H_2O (*Scheme 1*). After deprotection of compound **12** using disodium dihydrogen ethylenediaminetetraacetate (edta), compound **13** was esterified to methyl ester **14** using $SOCl_2$ in MeOH. The reaction of ester **14** with $BrCH_2COOMe$ generated compound **15**.

Using a two-step one-pot procedure instead of all-in-one [10b], the reaction of compound **15** and dimethyl iminobis(acetate) with 2,2′-(1*H*-pyrazole-1,3-diyl)bis[6-(bromomethyl)pyridine] [11d] (*Scheme 2*), as well as the reaction of ethyl 2-{[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate [10b] and diethyl iminobis(acetate) with 2,6-bis[3-(bromomethyl)-1*H*-pyrazol-1-yl]pyridine [11a] (*Scheme 3*), was successful within a reasonable time when carried out in MeCN instead of $CHCl_3$ [10b]. The nitro group of the intermediates **16**[2]) and **20** was reduced with H_2 over Pd/C giving compounds **17**[2]) and **21**, respectively. After saponification, the Tb^{III} chelates **18**[2]) and **22** were obtained by stirring the tetrakis(acetic acids) with $TbCl_3$ in a slightly acidic solution. These chelates were directly activated with thiophosgene (→ **19**[2]) and **23**, resp.) and 2,4,6-trichloro-1,3,5-triazine (→ **24**).

The 2,2′-(1*H*-1,2,4-triazole-3,5-diyl)bispyridine (**25**) was prepared from pyridine-2-carbonitrile and pyridine-2-carbohydrazide (*Scheme 4*). After protection with benzyl chloride, the terminal pyridine rings of compound **26**[3]) were oxidized with 3-chloroperbenzoic acid to dioxide **27**[3]). The modified *Reissert-Henze* reaction yielded dicarbonitrile **28**[3]), which was hydrolyzed to the corresponding acid with a mixture of sulfuric acid and acetic acid. Esterification to methyl ester **29** was made using $SOCl_2$ in MeOH. Unfortunately, deprotection of the triazole ring occurred during the acid hydrolysis, and, there-

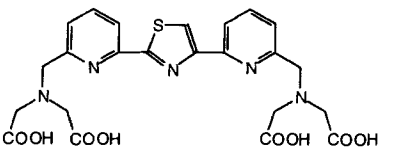
[2]) In fact, the 3-substituted pyrazol-1-yl isomers **16**–**19** are accompanied by their 1-substituted pyrazol-3-yl isomers (ratio *ca.* 1:1) which are not separated.

[3]) The position of the protecting benzyl group at the triazol moiety of **26**–**28** and **30**–**34** is undetermined.



1 R = H

2 R = Ph



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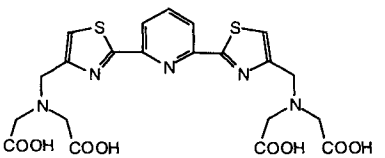

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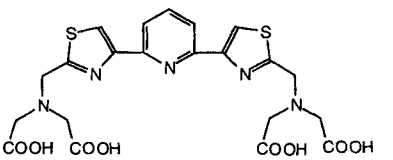

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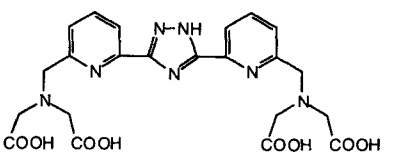

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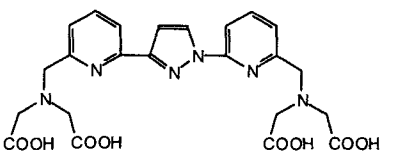

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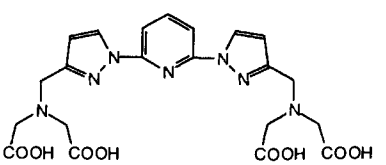

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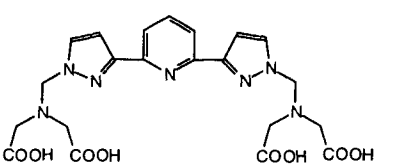

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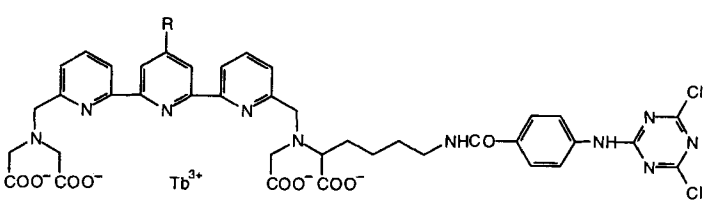
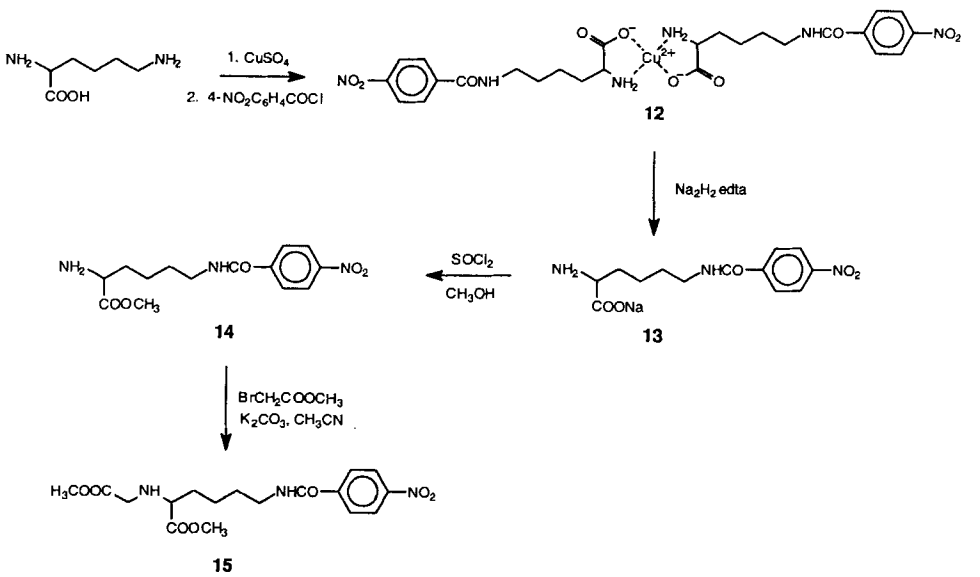


10 R = H

11 R = Ph

Scheme 1



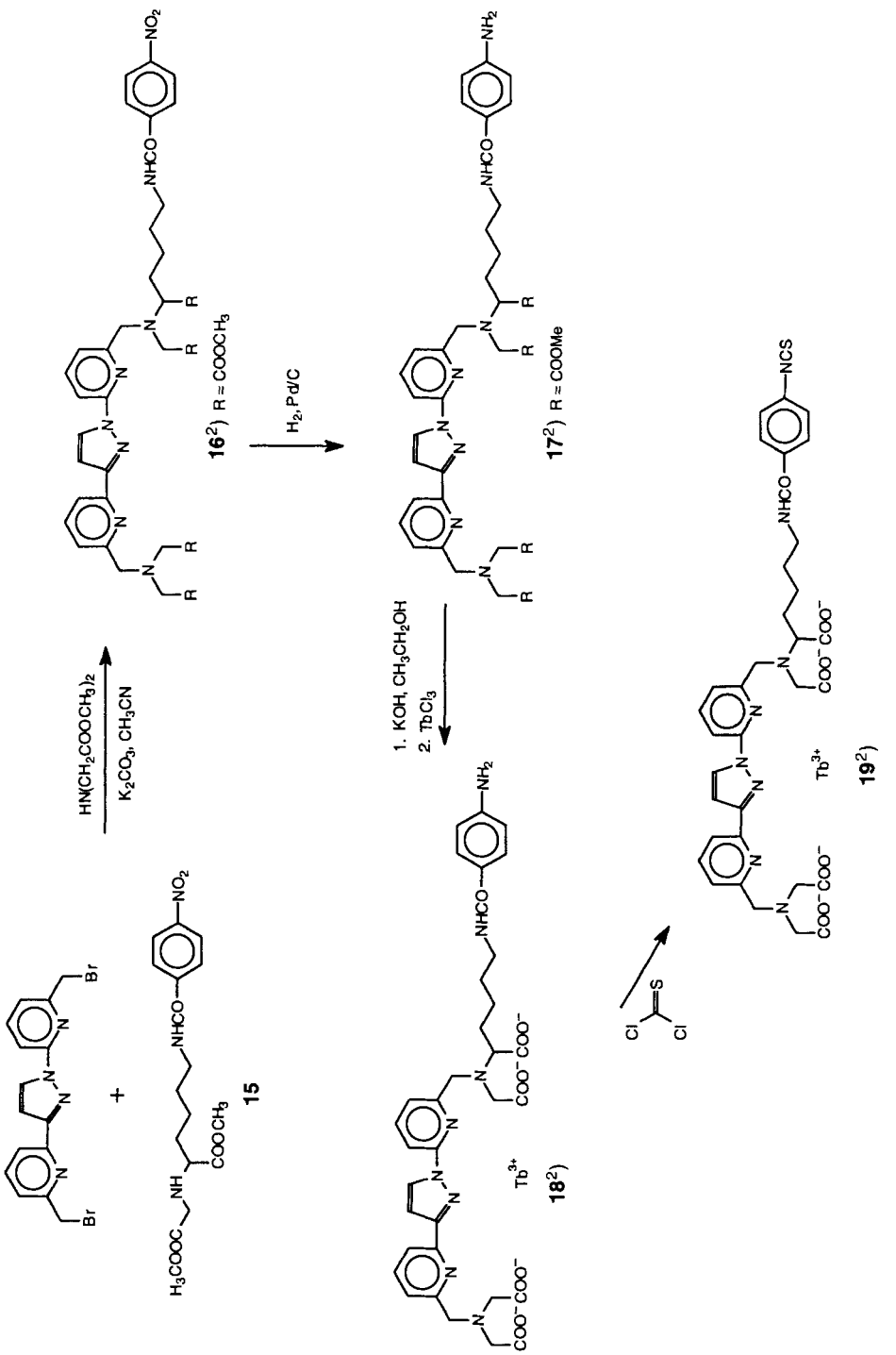
fore, the protection was repeated (→ **30**[3])). After $NaBH_4$ reduction in EtOH, the dialcohol **31**[3]) was transformed into the corresponding dibromide **32**[3]).

The coupling reaction of **32**[3]) with dimethyl iminobis(acetate) and ethyl 2-{[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate (→ **33**[3])) and reduction with H_2 yielded compound **34**[3]) (*Scheme 5*). Deprotection by removal of the benzyl and ester groups as well as reaction with the Tb^{III} ion gave a chelate, which was transformed to the target molecule **35** with thiophosgene.

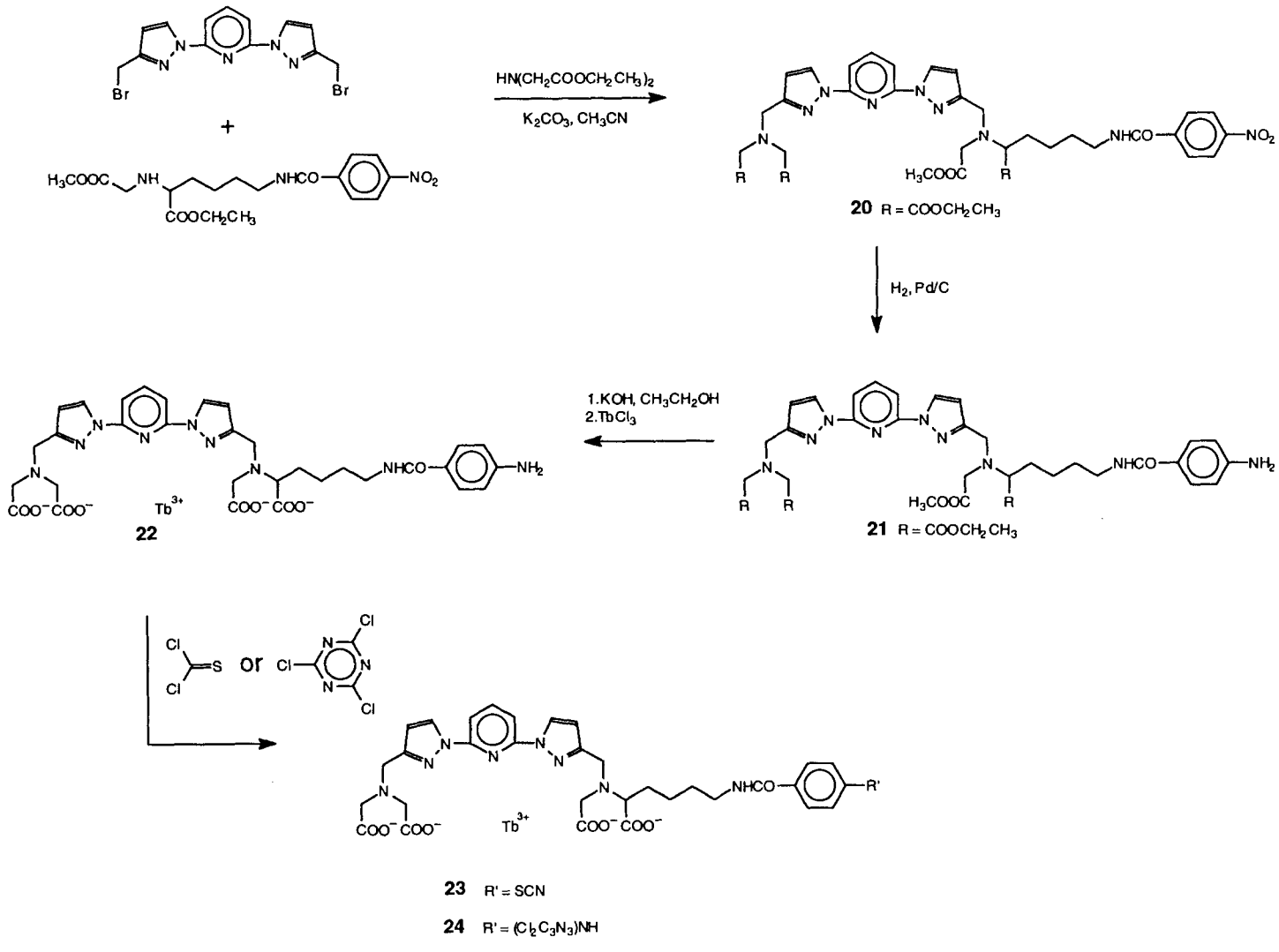
Luminescence. The excitation maxima (λ_{exc}), luminescence decay times (τ), and quantum yields (Φ) [11 d] of the Eu^{III} and Tb^{III} chelates with the parent ligands **1**–**8** as well as the luminescence yields ($\varepsilon \cdot \Phi$) of the Tb^{III} chelates (**1** and **2** in [10 a]; **3**–**5** in [11 c]) and the triplet-state energy levels [11 c, d] of ligands **1**–**8** are presented in *Table 1*. Lanthanide complexes with ligand **9** [15] gave only very low luminescence in borate buffer, mainly because ligand **9** is unstable in aqueous solution. Ligand **9** was prepared using the *Mannich* reaction, which was observed to be reversible with this particular ligand structure. As the triplet-state energy levels indicate, the ligands **6**–**8** whose triplet-state levels are clearly over the values of terpyridines **1** and **2**, produce high quantum yields in the case of the Tb^{III} chelates (12–58%) and almost three-fold decay times compared to terpyridines. As energy backflow from the excited Tb^{III} to the ligand triplet-state in ligands **6**–**8** is insignificant, it is thus presumable that labels based on these ligand structures will be suitable as markers in bioaffinity assays.

The excitation maxima (λ_{exc}), luminescence decay times (τ), and luminescence yields ($\varepsilon \cdot \Phi$) of the Tb^{III} chelates **19**, **23**, **24**, and **35** coupled to antibodies are presented in *Table 2*. For comparison, the corresponding values of the Tb^{III} chelates **10** and **11** are also shown [10 b]. The prepared labels as antibody conjugates have two excitation maxima (due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions) with almost equal intensities (see *Fig. 1*), with the

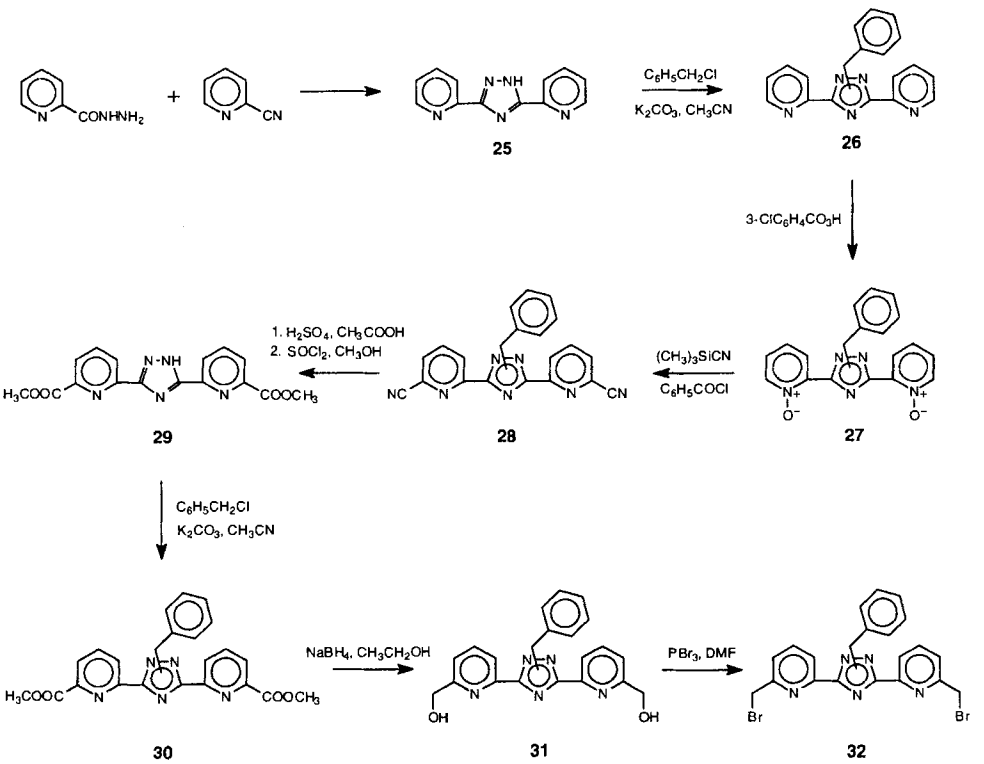
Scheme 2


HN(CH2COOCH3)2
K2CO3, CH3CN
15
16²) R = COOCH3
H2, Pd/C
17²) R = COOMe
1. KOH, CH3CH2OH
2. TbCl3
18²)
Cl
S
Cl
19²)

Scheme 3


HN(CH2COOCH2CH3)2
K2CO3, CH3CN
H2, Pd/C
1.KOH, CH3CH2OH
2.TbCl3
or
20 R = COOCH2CH3
21 R = COOCH2CH3
22
23 R' = SCN
24 R' = (C12C3N3)NH

Scheme 4



exception of chelate **19**. In *Table 2*, the luminescence results are shown using the longest excitation peak at *ca.* 310–330 nm, which is a more convenient excitation wavelength for instrumental reasons relating to the UV transmission of lenses, filters, cuvettes, and glass slides. As *Fig. 1* indicates, chelate **19** is the preferred label in respect of excitation wavelength.

The tested Tb^{III} chelates **19**, **23**, **24**, and **35** behaved quite predictably upon conjugation to proteins, both with regard to the decay times (τ) and luminescence yields ($\varepsilon \cdot \Phi$). The higher was the triplet-state energy level of the (unmodified) parent ligand (see *E* of **1**, **2** and **6**–**8** in *Table 1*), the nearer was the decay time of the Tb^{III} label with a modified ligand (in *Table 2*) to the decay time of the corresponding parent Tb^{III} complex (in *Table 1*). In other words, the energy backflow from the excited Tb^{III} ion to the parent or modified label donor ligand with higher triplet-state energy level is prevented. This phenomenon is reflected in the measured decay times as well as in the luminescence yields. However, the most important observation relates to the question of how much the triplet-state energy level of the parent ligand has to be above the $^5D_4$ level of the Tb^{III} ion to allow the use of the corresponding Tb^{III} label in bioaffinity assays. *Figs. 2* and *3* illustrate the luminescence yields and decay times of the Tb^{III} labels **10**, **11**, **19**, **23**, **24**, and **35** (with modified ligands) coupled to protein in relation to those of the corresponding parent Tb^{III} complexes (with ligands **1**, **2**, **7**, **8**, **8**, and **6**, resp.) *vs.* the triplet-state energy

Scheme 5

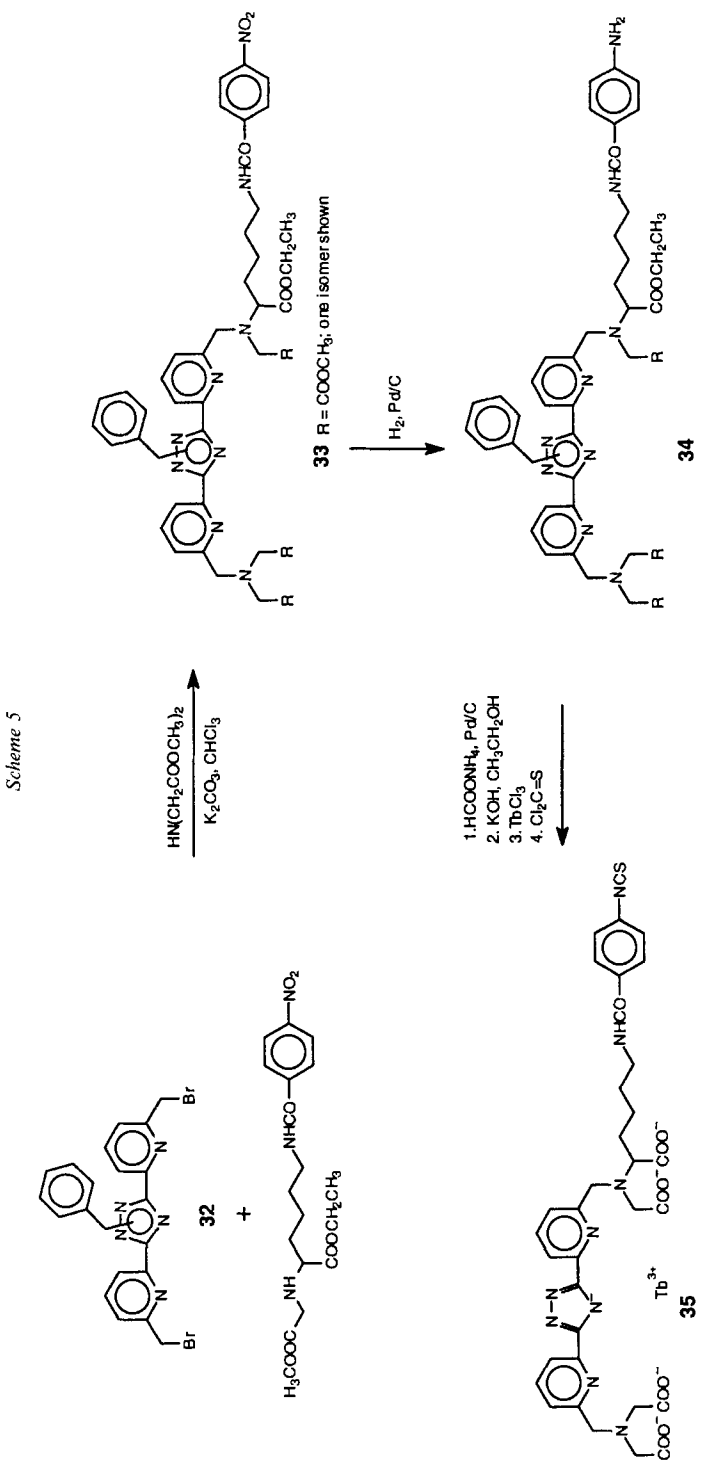

HN(CH2COOCH3)2
K2CO3, CHCl3
32
33 R = COOCH3; one isomer shown
H2, Pd/C
34
1. HCOONH4, Pd/C
2. KOH, CH3CH2OH
3. TbCl3
4. Cl2C=S
35
Tb3+

Table 1. *Triplet-State Energy Levels (E) of Ligands* **1–8** *and Excitation Maxima* (λ_{exc}), *Luminescence Decay Times* (τ), *Quantum Yields* (Φ), *and Luminescence Yields* ($\varepsilon \cdot \Phi$) *of the Europium(III) and Terbium(III) Chelates with Ligands* **1–8**

Ligand	E [cm^{-1}]	$M^{III} = Eu^{III}$ in $[M^{III}L]$			$M^{III} = Tb^{III}$ in $[M^{III}L]$			
		λ_{exc} [nm]	τ [μs]	Φ	λ_{exc} [nm]	τ [μs]	Φ	$\varepsilon \cdot \Phi$
1	22400	333	1310	0.176	333	1100	0.290	3800
2	22250	335	1210	0.160	337	530	0.100	1900
3	20100	330	1110	0.150	290	[a])	0.001	[a])
4	18650	340	1320	0.110	340	[a])	[a])	[a])
5	21550	315	1350	0.067	315	970	0.066	1290
6	23700	280	910	0.006	310	2860	0.120	5420
7	23200	330	1000	0.170	330	2810	0.420	8800
8	25150	320	1440	0.030	320	2990	0.580	5870

[a]) Too low.

Table 2. *Excitation Maxima* (λ_{exc}), *Luminescence Decay Times* (τ), *and Luminescence Yields* ($\varepsilon \cdot \Phi$) *of Terbium(III) Labels* **10**, **11**, **19**, **23**, **24**, *and* **35** *as Antibody Conjugates in 0.05*M *Borate Buffer, pH 8.5*

$[Tb^{III}L]$		λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
10[a])	(**1**[b]))	333	330	1170
11[a])	(**2**[b]))	296	80	600
19	(**7**[b]))	328	1350	3860
23	(**8**[b]))	315	2930	3770
24	(**8**[b]))	310	2660	4050
35	(**6**[b]))	318	2720	4560

[a]) See [10b]. [b]) Parent ligand.

levels of the parent ligands **1**, **2**, and **6–8**. Although different activation groups have been observed to give slightly different luminescence properties [10a] (see also the results of labels **23** and **24**), a quite clear correlation especially as regards decay times is seen. Only the triazole derivatives **35** gave unexpected values. Although a previous observation with

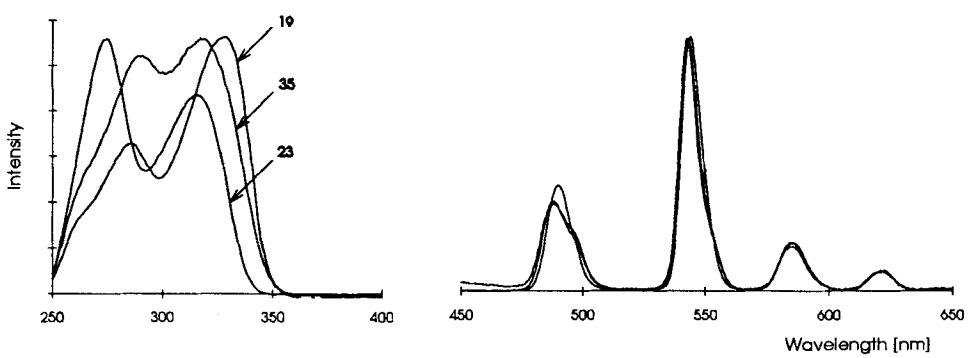


Fig. 1. *Excitation and emission spectra of* Tb^{III} *labels* **19**, **23**, *and* **35** *as antibody conjugates in 0.05*M *borate buffer, pH 8.5*

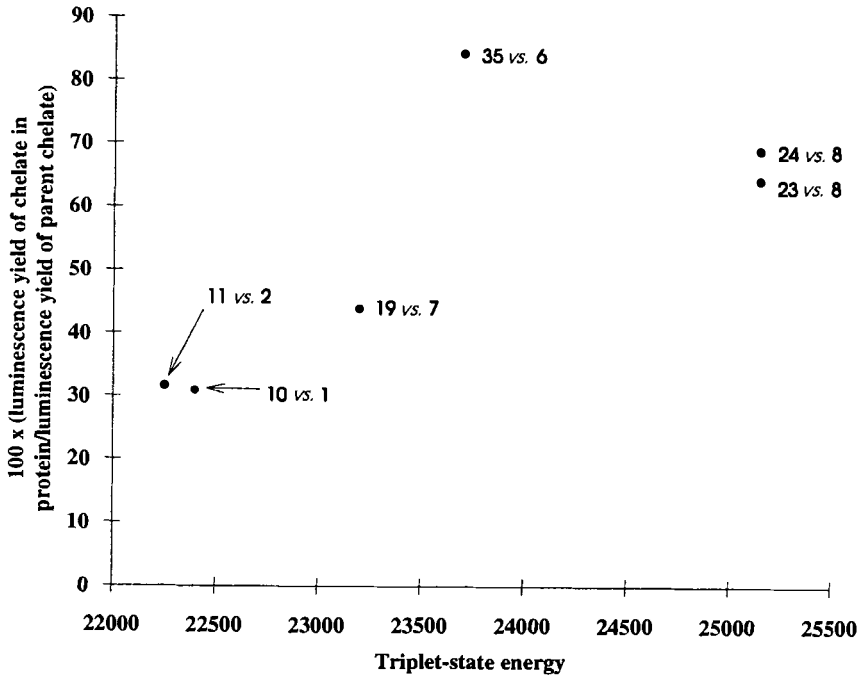


Fig. 2. *The influence of protein coupling of* **10**, **11**, **19**, **23**, **24**, *and* **35** *on luminescence yields* ($\varepsilon \cdot \Phi$) *as a function of the triplet-state energy level* (E) [cm^{-1}] *of the corresponding parent ligands* **1**, **2**, *and* **6**–**8**

the parent ligand structures suggested that the energy gap between the lowest triplet-state energy level of the donor ligand and the $^5D_4$ level of Tb^{III} ion should be over 1850 cm^{-1} [11d], this energy gap is too narrow for Tb^{III} labels to prevent a decrease in the luminescence quantum yield caused by nonradiative processes, *e.g.*, the energy back-transfer. According to the present study, the triplet-state energy level of the donor ligand has to be over 23000 cm^{-1}, *i.e.*, the energy gap should be over 2600 cm^{-1}, so that the corresponding labels posses reasonable decay times and luminescence yields after coupling to biomolecules. In this respect, suitable Tb^{III} labels, based *e.g.* on structures of 2,2′:6′,2″-terpyridine or 2,2′-bipyridine (triplet-state energy level 22400 cm^{-1} for both ligands [11c, d], compared to $^5D_4$ 20400 cm^{-1} of the Tb^{III} ion) may be difficult to prepare without changing the conjugated system over the pyridine rings. This is in accordance with previous observations. In spite of the good luminescence properties of these novel labels, the measured luminescence yields of labeled biomolecules were still below those of the parent Tb^{III} complexes. Especially, the luminescence yields of the labels **19**, **23**, and **24** were about half ($\varepsilon \cdot \Phi$ *ca.* 4000, *Table 2*) of those of the parent Tb^{III} complexes with **7** and **8** ($\varepsilon \cdot \Phi$ *ca.* 6000–9000, *Table 1*). On the other hand, the binding group between the Tb^{III} label and biomolecule has also been shown to have an effect on the luminescence properties of the label [10a, b]. Therefore, in the future, we are going to study the effect of the protein and the binding arm on the luminescence properties of the label with the aim to find new label structures with at least equal luminescence properties but with a more simple preparation on a larger scale.

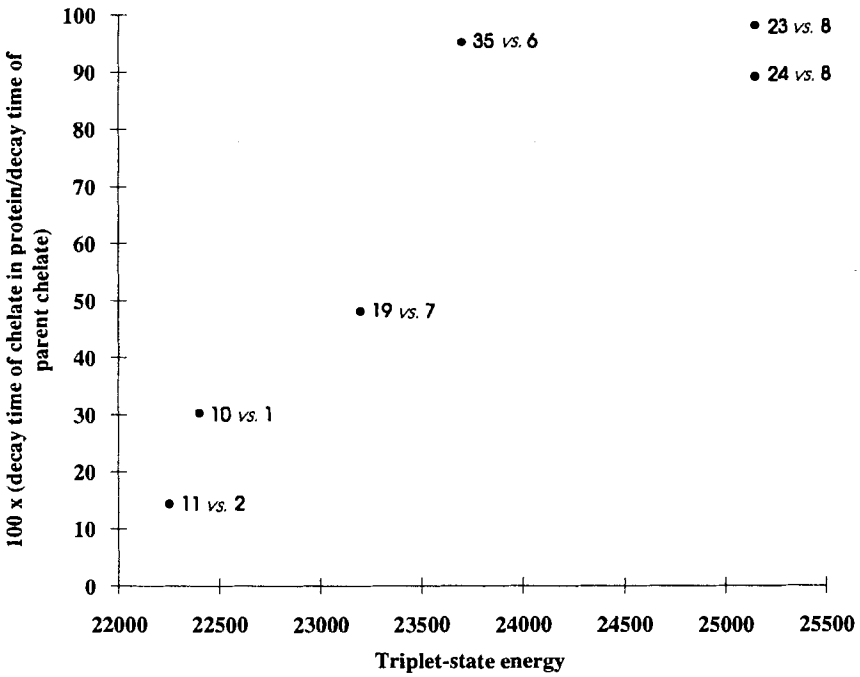


Fig. 3. *The influence of protein coupling of* **10**, **11**, **19**, **23**, **24**, *and* **35** *on decay times* (τ) *as a function of the triplet-state energy level* (E) [cm^{-1}] *of the corresponding parent ligands* **1**, **2**, *and* **6–8**

Experimental Part

General. Flash chromatography = FC. UV Spectra: *Shimadzu-UV-2100* spectrophotometer; λ_{max} in nm. Luminescence spectra: decay times τ in μs and luminescence yields ($\varepsilon \cdot \Phi$) were measured with a *Perkin-Elmer-LS-5* luminescence spectrometer combined with a *Perkin-Elmer-CLS* data station; high-sensitivity fluorometry of terbium, its quantitations, and the measurement of luminescence intensities from plastic surfaces (microtitration strip wells) were performed with a time-resolved *Wallac-1234-Delfia-Research* fluorometer combined with a PC and *Wallac-MultiCalc* program. IR Spectra: *Perkin-Elmer-1600-FTIR*; $\tilde{\nu}$ in cm^{-1}. ^{1}H-NMR Spectra: 400-MHz *Jeol-GX-400* and 200-MHz *Bruker-AC-200* spectrometer; $SiMe_4$ as internal standard, chemical shifts δ in ppm, coupling constants J in Hz.

Bis{2-amino-6-[(4-nitrobenzoyl)amino]hexanoato}copper(II) (**12**). A soln. of $CuSO_4 \cdot 5H_2O$ (12.5 g, 50 mmol) in H_2O (170 me) was added to a soln. of L-lysine hydrochloride (18.3 g, 100 mmol), NaOH (4.0 g, 100 mmol), and H_2O (170 ml). After stirring for 0.5 h at r.t., NaOH (4.0 g, 100 mmol) was added, and the mixture was cooled in an ice bath. A soln. of 4-nitrobenzoyl chloride (37.1 g, 200 mmol) and 1,4-dioxane (250 ml) was added within 5 min, and the mixture was maintained basic with 1M NaOH. After stirring for 0.5 h in an ice bath, the basic mixture was stirred for 14 h at r.t. and then filtered. The precipitate was washed with cold H_2O and EtOH: 31.2 g (96%). IR (KBr): 3412, 3279, 3151 (N–H), 1640, 1620, 1605 (C=O), 1528, 1349 (NO_2).

Sodium 2-Amino-6-[(4-nitrobenzoyl)amino]hexanoate (**13**). A mixture of **12** (31.2 g, 47.8 mmol), Na_2H_2 edta $\cdot$ $2H_2O$ (21.9 g, 58.8 mmol), and H_2O (410 ml) was stirred for 3 h at 80°. The cooled mixture was filtered and the precipitate washed with cold H_2O and EtOH: 19.1 g (63%). IR (KBr): 3422, 3334 (N–H), 1639, 1603 (C=O), 1522, 1352 (NO_2). ^{1}H-NMR (D_2O, (D_6)DMSO): 1.33–1.47 (*m*, 2H); 1.55–1.64 (*m*, 2H); 1.68–1.87 (*m*, 2H); 3.33 (*t*, J=7.0, 2H); 3.42 (*t*, J=5.8, 1H); 8.01 (*d*, J=8.8, 2H); 8.34 (*d*, J=8.8, 2H).

Methyl 2-Amino-6-[(4-nitrobenzoyl)amino]hexanoate (**14**). $SOCl_2$ (12.4 ml, 0.17 mmol) was dropped slowly to cooled MeOH (250 ml). After stirring for 0.5 h at r.t., **13** (13.5 g, 42.5 mmol; co-evaporated twice with toluene (200 ml)) was added and the mixture refluxed for 3 h. After evaporation, the residue was dissolved in $CHCl_3$

(150 ml), neutralized with sat. $NaHCO_3$ soln., dried (Na_2SO_4), and evaporated and the product purified by FC (silica gel, $CHCl_3$, then 5% MeOH/$CHCl_3$): 8.54 g (65%). IR (film): 3380, 3299 (N–H), 1732, 1650 (C=O), 1524, 1347 (NO_2), 1200 (C–O). ^{1}H-NMR ($CDCl_3$): 1.46–1.57 (*m*, 2H); 1.58–1.89 (*m*, 4H); 3.46–3.53 (*m*, 3H); 3.73 (*s*, 3H); 6.64 (*t*, *J* = 5.4, 1H); 7.95 (*d*, *J* = 8.5, 2H); 8.24 (*d*, *J* = 8.5, 2H).

Methyl 2-{[(Methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate (**15**). A mixture of **14** (4.27 g, 13.8 mmol), methyl bromoacetate (1.31 ml, 13.8 mmol), dry K_2CO_3 (9.53 g, 69.0 mmol), and dry MeCN (80 ml) was refluxed for 4 h, filtered, and evaporated. The product was purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 2:5, then 0:1): 4.52 g (86%). IR (film): 3333 (N–H), 1738, 1651 (C=O), 1526, 1348 (NO_2), 1207 (C–O). ^{1}H-NMR ($CDCl_3$): 1.42–1.60 (*m*, 2H); 1.60–1.81 (*m*, 4H); 2.18 (br. *s*, 1H); 3.31 (*dd*, *J* = 5.6, 7.1, 1H); 3.35 (*d*, *J* = 17.4, 1H); 3.44–3.50 (*m*, 2H); 3.47 (*d*, *J* = 17.4, 1H); 3.71 (*s*, 3H); 3.73 (*s*, 3H); 7.00 (*t*, *J* = 5.4, 1H); 7.99 (*d*, *J* = 8.5, 2H); 8.25 (*d*, *J* = 8.5, 2H).

Methyl 2-{N-{6-{3(and 1)-{6-{{Bis[(methoxycarbonyl)methyl]amino}methyl}pyridin-2-yl}-1H-pyrazol-1(and 3)-yl}pyridin-2-ylmethyl}-N-[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate (**16**). Compound **15** (190 mg, 0.5 mmol) was added in two portions within 2 h to a mixture of 2,2′-(1*H*-pyrazol-1,3-diyl)bis[6-(bromomethyl)pyridine] [11d] (204 mg, 0.5 mmol), dry K_2CO_3 (207 mg, 1.5 mmol), and dry MeCN (10 ml). After refluxing for 4 h, dimethyl iminobis(acetate) (81 mg, 0.5 mmol) was added. The mixture was refluxed overnight and filtered, the filtrate evaporated, and the residue dissolved in dry pyridine (4 ml). After addition of Ac_2O (189 mg, 2 mmol), the soln. was stirred for 4 h at r.t. and evaporated and the residue co-evaporated twice with toluene. The product was purified by FC (silica gel, petroleum ether (40–60°)/AcOEt/Et_3N 5:1:1): 150 mg (38%). IR (film): 3349 (N–H), 1732, 1651 (C=O), 1530, 1348 (NO_2), 1204 (C–O). ^{1}H-NMR ($CDCl_3$): 1.55–1.75 (*m*, 4 + 4H); 1.75–1.90 (*m*, 2 + 2H); 3.43–3.51 (*m*, 2 + 2H); 3.55–3.75 (*m*, 3 + 3H); 3.61 (*s*, 3H); 3.62 (*s*, 3H); 3.69 (*s*, 4H); 3.70 (*s*, 4H); 3.71 (*s*, 6H); 3.72 (*s*, 6H); 3.73 (*s*, 3 + 3H); 3.95 (*d*, *J* = 14.6, 1H); 3.99 (*d*, *J* = 14.6, 1H); 4.05 (*d*, *J* = 14.6, 1H); 4.08 (*s*, 2H); 4.09 (*d*, *J* = 14.6, 1H); 4.13 (*s*, 2H); 6.83 (br. *s*, 1H); 6.96 (br. *s*, 1H); 7.06 (*d*, *J* = 2.4, 1H); 7.07 (*d*, *J* = 2.4, 1H); 7.35 (*d*, *J* = 7.8, 1H); 7.40 (*d*, *J* = 7.8, 1H); 7.45 (*d*, *J* = 7.8, 1H); 7.52 (*d*, *J* = 7.8, 1H); 7.59 (*t*, *J* = 7.8, 1H); 7.68 (*t*, *J* = 7.8, 1H); 7.76 (*t*, *J* = 7.8, 1H); 7.82 (*t*, *J* = 7.8, 1H); 7.88–7.98 (*m*, 2 + 2H); 7.90 (*d*, *J* = 8.9, 2H); 7.92 (*d*, *J* = 8.9, 2H); 8.15 (*d*, *J* = 8.9, 2H); 8.18 (*d*, *J* = 8.9, 2H); 8.56 (*d*, *J* = 2.4, 1H); 8.57 (*d*, *J* = 2.4, 1H). It was impossible to assign signals to certain isomers. According to NMR, the isomer ratio was 56:44.

Methyl 6-[(4-Aminobenzoyl)amino]-2-{N-{6-{3(and 1)-{6-{{bis[(methoxycarbonyl)methyl]amino}methyl}pyridin-2-yl}-1H-pyrazol-1 (and 3)-yl}pyridin-2-ylmethyl}-N-[(methoxycarbonyl)methyl]amino}hexanoate (**17**). A mixture of **16** (100 mg, 12 mmol), 10% Pd/C (25 mg), and MeOH (20 ml) was stirred under H_2 (3.4 atm) for 0.5 h. After filtration, the filtrate was evaporated and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt 2:5): 58 mg (60%). IR (film): 3348, 3244 (N–H), 1732, 1633 (C=O), 1180 (C–O). ^{1}H-NMR ($CDCl_3$): 1.53–1.72 (*m*, 4 + 4H); 1.75–1.88 (*m*, 2 + 2H); 3.43–3.50 (*m*, 2 + 2H); 3.50–3.76 (*m*, 3 + 3H); 3.65 (*s*, 3 + 3H); 3.69 (*s*, 4H); 3.70 (*s*, 4H); 3.72 (*s*, 6H); 3.73 (*s*, 6 + 3H); 3.74 (*s*, 3H); 4.03 (*d*, *J* = 14.6, 1 + 1H); 4.09 (*s*, 2H); 4.10 (*d*, *J* = 14.6, 1 + 1H); 4.20 (*s*, 2H); 6.60 (*d*, *J* = 8.5, 2H); 6.61 (*d*, *J* = 8.5, 2H); 7.08 (*d*, *J* = 2.4, 1 + 1H); 7.42 (*d*, *J* = 7.6, 1H); 7.44 (*d*, *J* = 7.6, 1H); 7.59 (*d*, *J* = 8.5, 2H); 7.61 (*d*, *J* = 8.5, 2H); 7.62 (*t*, *J* = 7.6, 1H); 7.67 (*t*, *J* = 7.6, 1H); 7.76 (*t*, *J* = 7.6, 1H); 7.82 (*t*, *J* = 7.6, 1H); 7.90 (*d*, *J* = 7.6, 1H); 7.94 (*d*, *J* = 7.6, 1H); 7.97 (*d*, *J* = 7.6, 1H); 7.98 (*d*, *J* = 7.6, 1H); 8.59 (*d*, *J* = 2.4, 1 + 1H). It was impossible to assign signals to certain isomers.

{6-[(4-Aminobenzoyl)amino]-2-{N-{6-{3(and 1)-{6-{[bis(carboxylatomethyl)amino]methyl}pyridin-2-yl}-1H-pyrazol-1 (and 3)-yl}pyridin-2-ylmethyl}-N-(carboxylatomethyl)amino}hexanoato}terbium(III) (**18**). A mixture of **17** (67 mg, 88 μmol) and 0.5M KOH in EtOH (2.5 ml) was stirred for 1 h at r.t. Some H_2O (0.58 ml) was added, and stirring was continued for 3 h. After evaporation, the residue was dissolved in H_2O (1.2 ml) and the pH adjusted to 6.5 with 5M HCl. A soln. of $TbCl_3$ (36 mg, 97 μmol) in H_2O (0.6 ml) was added within 15 min, and the pH was maintained at 5.0–6.5 with solid $NaHCO_3$. After stirring for 1.5 h at r.t., the pH was adjusted to 8.5 with 1M NaOH. The precipitate was removed by centrifugation, the filtrate triturated with acetone, and the solid material removed by centrifugation and washed with acetone. The solid material was dissolved in H_2O (1.0 ml) and extracted with phenol, and the phenol phase was treated with H_2O (1.0 ml) and Et_2O (10 ml). The H_2O phase was washed with Et_2O (2 × 10 ml) and treated with acetone and the product removed by centrifugation and washed with acetone: 30 mg (40%). UV (H_2O): 331, 320, 287, 279, 263. IR (KBr): 1617 (C=O), 1388 (C–O).

{2-{N-{6-{3(and 1)-{6-{[Bis(carboxylatomethyl)amino]methyl}pyridin-2-yl}-1H-pyrazol-1(and 3)-yl}pyridin-2-ylmethyl}-N-(carboxylatomethyl)amino}-6-[(4-isothiocyanatobenzoyl)amino]hexanoato}terbium(III) (**19**). A soln. of **18** (14 mg, 16 μmol) in H_2O (0.45 ml) was added within 15 min to a mixture of thiophosgene (5.0 μl, 64 μmol), $NaHCO_3$ (6.7 mg, 80 μmol), and $CHCl_3$ (0.45 ml). After stirring for 30 min, the aq. phase was washed with $CHCl_3$ (3 × 1 ml), acetone was added to the aq. soln., and the product removed by centrifugation and washed with acetone: 13 mg (87%). UV (H_2O): 330, 320, 287, 280, 263. IR (KBr): 2099 (S=C=N), 1613 (C=O), 1366 (C–O).

Ethyl 2-{N-{1-{6-{3-{{Bis[(ethoxycarbonyl)methyl]amino}methyl}-1H-pyrazol-1-yl}pyridin-2-yl}-1H-pyrazol-3-ylmethyl}-N-[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate (**20**). Ethyl 2-{[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate [10b] (198 mg, 0.5 mmol) was added in two portions to a soln. of bis[3-(bromomethyl)-1*H*-pyrazol-1-yl]pyridine [11a] (200 mg, 0.5 mmol), dry K_2CO_3 (207 mg, 1.5 mmol), and dry MeCN (25 ml). After refluxing for 9 h, a soln. of dimethyl iminobis(acetate) (94.5 mg, 0.5 mmol) and dry MeCN (4 ml) was added in two portions. After refluxing for 7 h, the mixture was filtered, the filtrate evaporated, and the product purified by FC (silica gel, AcOEt/hexane 3:2): 195 mg (52%). IR (nujol): 1740, 1660, 1655 (C=O), 1530, 1347 (NO_2). ^{1}H-NMR ($CDCl_3$): 1.28 (*t*, *J* = 7.1, 9H); 1.60–1.65 (*m*, 6H); 3.42–3.58 (*m*, 2H + 1H); 3.65 (*s*, 6H); 3.66 (*s*, 3H); 3.95 (*s*, 2H); 4.09 (*s*, 2H); 4.20 (*q*, *J* = 7.1, 6H); 6.20 (*t*, 1H); 6.35 (*d*, *J* = 2.4, 2H); 6.42 (*d*, *J* = 2.5, 2H); 7.66–7.85 (*m*, 3H); 7.95 (*d*, *J* = 10, 2H); 8.15 (*d*, *J* = 10, 2H); 8.36 (*d*, *J* = 2.5, 2H); 8.43 (*d*, *J* = 2.4, 2H).

Ethyl 6-[(4-Aminobenzoyl)amino]-2-{N-{1-{6-{3-{{bis[(ethoxycarbonyl)methyl]amino}methyl}-1H-pyrazol-1-yl}pyridin-2-yl}-1H-pyrazol-3-ylmethyl}-N-[(methoxycarbonyl)methyl]amino}hexanoate (**21**) was synthesized from **20** analogously to **18**. IR (Nujol): 3400 (N–H), 1740, 1650 (C=O). ^{1}H-NMR ($CDCl_3$): 1.30 (*t*, *J* = 7.1, 9H); 1.50–1.85 (*m*, 6H); 3.40–3.60 (*m*, 2H + 1H); 3.66 (*s*, 6H); 3.67 (*s*, 3H); 3.90 (br. *s*, 2H); 4.00 (*s*, 2H); 4.09 (*s*, 2H); 4.20 (*q*, *J* = 7.1, 6H); 6.23 (*t*, 1H); 6.49 (*d*, *J* = 2.4, 2H); 6.49 (*d*, *J* = 2.5, 2H); 7.50–7.90 (*m*, 3H); 7.59 (*d*, *J* = 10, 2H); 7.60 (*d*, *J* = 10, 2H); 8.30 (*d*, *J* = 2.5, 2H); 8.49 (*d*, *J* = 2.4, 2H).

{6-[(4-Aminobenzoyl)amino]-2-{N-{1-{6-{3-{[bis(carboxylatomethyl)amino]methyl}-1H-pyrazol-1-yl}pyridin-2-yl}-1H-pyrazol-3-ylmethyl}-N-(carboxylatomethyl)amino}hexanoato}terbium(III) (**22**). A mixture of **21** (270 mg, 0.36 mmol) and 0.5M KOH in EtOH (16 ml) was stirred for 2h at r.t. Some H_2O (1.6 ml) was added and stirring continued for 2 h. After evaporation, the residue was dissolved in H_2O (6 ml) and the pH adjusted to 6.5 with 5M HCl. A soln. of $TbCl_3$ (134 mg, 0.36 mmol) in H_2O (2.2 ml) was slowly added and the pH maintained at 5.0–6.5 with 2M NaOH. After stirring for 1 h at r.t., the pH was adjusted to 8.5 with 1M NaOH. The precipitate was removed by centrifugation, the filtrate triturated with acetone, and the solid material removed by centrifugation and washed with acetone: 188 mg (60%). UV (H_2O): 309, 276, 270. IR (KBr): 1616 (C=O), 1400 (C–O).

{2-{N-{1-{6-{3-{[Bis(carboxylatomethyl)amino]methyl}-1H-pyrazol-1-yl}-pyridin-2-yl}-1H-pyrazol-3-ylmethyl}-N-(carboxylatomethyl)amino}-6-[(4-isothiocyanatobenzoyl)amino]hexanoato}terbium(III) (**23**) was synthesized from **22** analogously to **19**. Yield 86%. UV (H_2O): 313 (sh), 293, 277, 270 (sh), 253 (sh), 228. IR (KBr): 2077 (S=C=N), 1617 (C=O), 1381 (C–O).

{2-{N-{1-{6-{3-{[Bis(carboxylatomethyl)amino]methyl}-1H-pyrazol-1-yl}-pyridin-2-yl}-1H-pyrazol-3-ylmethyl}-N-(carboxylatomethyl)amino}-6-{{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzoyl}amino}hexanoato}-terbium(III) (**24**) was synthesized from **22** analogously to **20**. Yield 100%. UV (H_2O): 307, 276, 269, 242, 243 (sh). IR (KBr): 1608 (C=O), 1394 (C–O).

2,2'-(1H-1,2,4-triazole-3,5-diyl)bis(pyridine) (**25**). A mixture of pyridine-2-carbonitrile (10.4 g, 0.1 mol) and pyridine-2-carbohydrazide (13.7 g, 0.1 mol) was stirred for 24 h at 140°. The product (13.7 g, 61%) was crystallized from toluene after filtration of insoluble material. IR (KBr): 3328 (N–H), 1590 (arom.). ^{1}H-NMR ((D_6)DMSO): 7.50 (br. *s*, 1H); 8.00–8.25 (*m*, 4H); 8.50–8.80 (*m*, 4H).

2,2'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis(pyridine) (**26**). A mixture of **25** (1.12 g, 5.00 mmol), dry K_2CO_3 (1.38 g, 10.0 mmol), benzyl chloride (0.63 g, 5.0 mmol), and dry MeCN (65 ml) was refluxed for 2.5 h. After filtration, the filtrate was evaporated and the product purified by FC (silica gel, 1% MeOH/CH_2Cl_2): 1.11 g (71%). IR (KBr): 1589 (arom.). ^{1}H-NMR ($CDCl_3$): 6.22 (*s*, 2H); 7.20–7.30 (*m*, 3H); 7.30–7.40 (*m*, 4H); 7.80 (*dt*, *J* = 2.0, 7.8, 1H); 7.83 (*dt*, *J* = 2.0, 7.8, 1H); 8.22 (*d*, *J* = 7.8, 1H); 8.41 (*d*, *J* = 7.8, 1H); 8.67 (*d*, *J* = 4.9, 1H); 8.78 (*d*, *J* = 4.9, 1H).

2,2'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis(pyridine) 1,1'-Dioxide (**27**). Within 8d, 3-chloroperbenzoic acid (50–55%; 10.4 g, *ca.* 30 mmol) was added in small portions to a mixture of **26** (1.05 g, 3.2 mmol) and CH_2Cl_2 (120 ml). After stirring for additional 4d, H_2O (45 ml) was added and the pH adjusted to 10 with solid Na_2CO_3. The H_2O phase was extracted with $CHCl_3$/EtOH 3:1 (4 × 40 ml), the combined org. phase dried (Na_2SO_4) and evaporated, and the product purified by FC (silica gel, 2, 5, and 10% MeOH/$CHCl_3$): 0.71 g (64%). IR (KBr): 1258 (N → O). ^{1}H-NMR ($CDCl_3$): 5.78 (*s*, 2H); 7.16–7.19 (*m*, 5H); 7.20 (*dt*, *J* = 1.2, 7.6, 1H); 7.30–7.36 (*m*, 3H); 7.44 (*dd*, *J* = 2.0, 7.8, 1H); 8.05–8.08 (*m*, 1H); 8.30 (*d*, *J* = 6.3, 1H); 8.40–8.42 (*m*, 1H).

6,6'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis[pyridine-2-carbonitrile] (**28**). Me_3SiCN (2.67 ml, 20 mmol) was added to mixture of **27** (0.69 g, 2.0 mmol) and CH_2Cl_2 (25 ml). After 5 min, benzoyl chloride (1.00 ml, 8.0 mmol) was added and the mixture stirred for 2d. The mixture was then concentrated to 1/2 volume, 10% K_2CO_3 soln. (60 ml) added, and the mixture stirred for 1 h. The H_2O phase was extracted with $CHCl_3$ (3 × 30 ml) and the combined org. phase dried (Na_2SO_4) and evaporated: 0.69 g (95%). IR (KBr): 2239 (C≡N). ^{1}H-NMR ($CDCl_3$): 6.15 (*s*, 2H); 7.26 (*t*, *J* = 7H, 1H); 7.32 (*t*, *J* = 7, 2H); 7.42 (*d*, *J* = 7, 2H); 7.75–7.78 (*m*, 2H); 7.79 (*t*, *J* = 8, 1H); 8.01 (*t*, *J* = 8, 1H); 8.47 (*dd*, *J* = 1, 8, 1H); 8.64 (*dd*, *J* = 1, 8, 1H).

Dimethyl 6,6'-(1H-1,2,4-Triazole-3,5-diyl)bis[pyridine-2-carboxylate] (**29**). A mixture of **28** (3.90 g, 10.7 mmol), AcOH (45 ml), and conc. H_2SO_4 (45 ml) was refluxed for 2 h, poured into ice water (500 ml), filtered, and dried. $SOCl_2$ (2.50 ml, 34.2 mmol) was dropped slowly to cooled MeOH (185 ml). After stirring for 15 min at r.t., the above precipitate was added and the mixture refluxed for 5 h. After concentration to 1/2 volume, $CHCl_3$ (150 ml) was added and the mixture neutralized with sat. $NaHCO_3$ soln. The aq. phase was extracted with $CHCl_3$ (2 × 75 ml) and the org. phase dried (Na_2SO_4) and evaporated: 2.45 g (67%). IR (KBr): 3426 (N–H), 1722, 1311, 1255, 1138 (C=O, C–O). ^{1}H-NMR ($CDCl_3$): 2.64 (br. *s*, 1H); 4.04 (*s*, 6H); 8.03 (*t*, *J* = 7.8, 2H); 8.19 (*d*, *J* = 7.8, 2H); 8.51 (*d*, *J* = 7.8, 2H).

Dimethyl 6,6'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis[pyridine-2-carboxylate] (**30**). A mixture of **29** (2.32 g, 6.84 mmol), dry K_2CO_3 (1.89 g, 13.7 mmol), benzyl chloride (0.79 ml, 6.84 mmol), and dry MeCN (100 ml) was refluxed for 4 h. The mixture was filtered through silica gel, washed with MeCN, and evaporated: 2.22 g (76%). IR (KBr): 1731, 1708, 1315, 1254, 1139 (C=O, C–O). ^{1}H-NMR ($CDCl_3$): 4.03 (*s*, 3H); 4.04 (*s*, 3H); 6.34 (*s*, 2H); 7.20–7.30 (*m*, 3H); 7.47 (*dd*, *J* = 1.5, 7.8, 2H); 7.99 (*t*, *J* = 7.8, 1H); 8.00 (*t*, *J* = 7.8, 1H); 8.17 (*dd*, *J* = 1.0, 7.8, 1H); 8.19 (*dd*, *J* = 1.0, 7.8, 1H); 8.42 (*dd*, *J* = 1.0, 7.8, 1H); 8.62 (*dd*, *J* = 1.0, 7.8, 1H).

6,6'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis[pyridine-3-methanol] (**31**). $NaBH_4$ (0.88 g, 23.3 mmol) was added to a suspension of **30** (2.22 g, 5.17 mmol) and abs. EtOH (45 ml). After stirring for 2 h at r.t., the mixture was refluxed overnight. The soln. was evaporated, sat. $NaHCO_3$ soln. (35 ml) added, the mixture brought to boiling, H_2O (67 ml) added, the cold mixture filtered, and the product (1.93 g, 92%) washed with cold H_2O. IR (KBr): 1625, 1596, 1577 (arom. C–C). ^{1}H-NMR ((D_6)DMSO): 4.66 (*s*, 2H); 4.68 (*s*, 2H); 5.60 (br. *s*, 2H); 6.15 (*s*, 2H); 7.23–7.38 (*m*, 5H); 7.57 (*d*, *J* = 7.8, 1H); 7.63 (*d*, *J* = 7.8, 1H); 7.96 (*t*, *J* = 7.8, 1H); 8.03 (*d*, *J* = 7.8, 1H); 8.05 (*t*, *J* = 7.8, 1H); 8.14 (*d*, *J* = 7.8, 1H).

2,2'-(N-Benzyl-1H-1,2,4-triazole-3,5-diyl)bis[6-(bromomethyl)pyridine] (**32**). A mixture of dry DMF (25 ml) and PBr_3 (0.88 ml, 9.32 mmol) was stirred for 15 min at r.t. Then **31** (1.74 g, 4.66 mmol) was added in small portions, and stirring was continued overnight. After neutralization with sat. $NaHCO_3$ soln. the product (1.88 g, 81%) was filtered and washed with cold H_2O and MeCN. IR (KBr): 1630, 1594, 1573 (arom. C–C). ^{1}H-NMR ($CDCl_3$): 4.54 (*s*, 2H); 4.73 (*s*, 2H); 6.22 (*s*, 2H); 7.20–7.34 (*m*, 3H); 7.36 (*d*, *J* = 7.3, 2H); 7.46 (*d*, *J* = 7.8, 1H); 7.55 (*d*, *J* = 7.8, 1H); 7.83 (*t*, *J* = 7.8, 1H); 7.84 (*t*, *J* = 7.8, 1H); 8.16 (*d*, *J* = 7.8, 1H); 8.31 (*d*, *J* = 7.8, 1H).

Ethyl 2-{N-{6-{N-Benzyl-5-{6-{{bis[(methoxycarbonyl)methyl]amino}methyl}pyridin-2-yl}-1H-1,2,4-triazol-3-yl}pyridin-2-ylmethyl}-N[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzol)amino]hexanoate (**33**). To a soln. of **32** (600 mg, 1.20 mmol) in EtOH-free $CHCl_3$ (5 ml), ethyl 2-{[(methoxycarbonyl)methyl]amino}-6-[(4-nitrobenzoyl)amino]hexanoate (475 mg, 1.20 mmol), dimethyl iminobis(acetate) (193 mg, 1.20 mmol), and dry K_2CO_3 (500 mg, 3.60 mmol) were added. The mixture was refluxed for 7 d and filtered and the filtrate evaporated. The residue was dissolved in pyridine, Ac_2O (0.114 ml, 1.20 mmol) added, and the mixture stirred overnight at r.t. After evaporation, the residue was co-evaporated twice with toluene and the product purified by FC (silica gel, petroleum ether (40–60°)/AcOEt/Et_3N 5:5:1): 94 mg (9%). IR (film): 3306 (N–H), 1738, 1661 (C=O), 1525, 1347 (NO_2), 1203 (C–O). ^{1}H-NMR ($CDCl_3$): 1.30 (*t*, *J* = 7, 3H); 1.52–1.61 (*m*, 2H); 1.63–1.86 (*m*, 4H); 3.41–3.54 (*m*, 2 + 1H); 3.57 (*d*, *J* = 15, 1H); 3.59 (*s*, 3H); 3.61 (*s*, 4H); 3.64 (*d*, *J* = 15, 1H); 3.68 (*s*, 6H); 4.08 (*s*, 2H); 4.12 (*d*, *J* = 12, 1H); 4.18 (*d*, *J* = 12, 1H); 4.19 (*q*, *J* = 7, 2H); 6.13 (*d*, *J* = 12, 1H); 6.18 (*d*, *J* = 12, 1H); 7.18–7.27 (*m*, 5H); 7.62 (*dd*, *J* = 1, 6, 1H); 7.66 (*t*, *J* = 6, 1H); 7.67 (*d*, *J* = 6, 1H); 7.80 (*t*, *J* = 6, 1H); 7.98 (*d*, *J* = 7, 2H); 8.06 (*dd*, *J* = 1, 6, 1H); 8.15 (*d*, *J* = 7, 2H); 8.21 (*d*, *J* = 6, 1H).

Ethyl 6-[(4-Aminobenzoyl)amino]-2-{N-{6-{N-benzyl-5-{6-{{bis[(methoxycarbonyl)methyl]amino}methyl}-pyridin-2-yl}-1H-1,2,4-triazol-3-yl}pyridin-2-ylmethyl}-N-[(methoxycarbonyl)methyl]amino}hexanoate (**34**) was synthesized from **33** analogously to **18**. Yield 90%. IR (film): 3343, 3230 (N–H), 1734, 1628 (C=O), 1182 (C–O). ^{1}H-NMR ($CDCl_3$): 1.28 (*t*, *J* = 7, 3H), 1.52–1.88 (*m*, 6H); 3.39–3.55 (*m*, 2 + 1H); 3.57 (*d*, *J* = 15, 1H); 3.59 (*s*, 3H); 3.63 (*s*, 4H); 3.64 (*d*, *J* = 15, 1H); 3.69 (*s*, 6H); 4.08 (*s*, 2H); 4.16 (*d*, *J* = 12, 1H); 4.19 (*d*, *J* = 12, 1H); 4.20 (*q*, *J* = 7, 2H); 6.17 (*s*, 2H); 6.74 (*d*, *J* = 9, 2H); 7.18–7.31 (*m*, 5H); 7.64–7.74 (*m*, 5H); 7.83 (*t*, *J* = 6, 1H); 8.09 (*d*, *J* = 6, 1H); 8.24 (*d*, *J* = 6, 1H).

*{2-{N-{6-{5-{6-{[Bis(carboxylatomethyl)amino]methyl}pyridin-2-yl}-1,2,4-triazol-*N*-ido-3-yl}pyridin-2-ylmethyl}-N-(carboxylatomethyl)amino}-6-[(4-isothiocyanatobenzoyl)amino]hexanoato}terbium(III)* (**35**). During 13 d, $HCOONH_4$ (72 mg, 1.15 mmol) was added daily to a mixture of **34** (73 mg, 84.6 mmol), 10% Pd/C (60 mg), and MeOH (10 ml) under N_2. The mixture was filtered, the filtrate evaporated, and the residue dissolved in $CHCl_3$ (10 ml), washed with H_2O (5 ml), dried (Na_2SO_4), and evaporated. After purification by FC (neutral alumina, first 2% MeOH in $CHCl_3$, then 3% MeOH in $CHCl_3$), the crude material (18 mg) was dissolved in 0.5M KOH in EtOH (0.5 ml), and the mixture was stirred for 1 h at r.t. Some H_2O (0.1 ml) was added to the mixture and stirring continued for 2 h. After evaporation, the residue was dissolved in H_2O (0.5 ml) and the pH adjusted to 6.5 with 5M HCl. A soln. of $TbCl_3$ (20 mg, 54 μmol) in H_2O (0.3 ml) was added within 15 min and the pH

maintained at 5.0–6.5 with solid $NaHCO_3$. After stirring for 1 h, the pH was adjusted to 8.5 with 1M NaOH and the precipitate removed by centrifugation and washed with acetone. A soln. of amino chelate (33 mg, 36.3 μmol) in H_2O (1 ml) was added within 15 min to a mixture of thiophosgene (12 μl, 145 μmol), $NaHCO_3$ (15 mg, 181 μmol), and $CHCl_3$ (1 ml). After stirring for 1 h, the H_2O phase was washed with $CHCl_3$ (3 × 1 ml), acetone was added to the aq. soln. and the product (36 mg, 45%) removed by centrifugation and washed with acetone. UV (H_2O): 312 (sh), 292, 276 (sh), 228. IR (KBr): 2050 (S=C=N), 1670, 1624 (C=O), 1338 (C–O).

Concentration Measurements. The measurement of the total Tb^{III} ion concentration after labelling was performed using a dissociative fluorescence enhancement system [2a] based on the *Wallac-Delfia* enhancement soln. composed of 15 μM 4,4,4-trifluoro-1-(naphthalen-2-yl)butane-1,3-dione, 50 μM trioctylphosphine oxide, and 0.1% *Triton X-100* in acetate/phthalate buffer, pH 3.2. After stirring for 5–30 min, Tb^{III} enhancement soln. [16] (composed of 10 μM 4-(2,4,6-trimethoxyphenyl)pyridine-2,6-dicarboxylic acid, 0.10% cetyltrimethylammonium bromide and 0.1% *Triton X-100*, pH 6.0) was added, and the Tb^{III} signal was measured after 5–15 min incubation.

Coupling of Chelates **19**, **23**, **24**, *and* **35** *to Protein.* The activated chelates were coupled to a model protein (PSA-antibody, clone H 50) by incubating the chelate with IgG (1 mg/ml) in carbonate buffer (500 μl, pH 9.8) overnight using a 100-fold molar reagent-to-protein ratio for **19**, a 20-fold molar ratio for **24**, and a 30-fold molar ratio for **23** and **35**. 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 labelled monomeric IgG were collected. The chelate concentrations in the protein fractions were measured by the dissociative fluorescence enhancement system. The purified protein conjugates and the labelling ratios (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 chelates.

Luminescence Measurements. The decay times (τ) and luminescence yields ($\varepsilon \cdot \Phi$) for the chelates with the parent ligands **6**–**8** as well as the luminescence parameters for chelate-labelled antibodies were measured in 0.05M borate buffer, pH 8.5; for other general considerations, see [10a]. Results: *Tables 1* and *2*.

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