Minocycline-Based Europium(III) Chelate Complexes: Synthesis, Luminescent Properties, and Labeling to Streptavidin

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Dedicated to Professor *Jean-Claude Bünzli* on the occasion of his 65th birthday

Two chelate ligands for europium(III) having minocycline (=(4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxonaphthacene-2-carboxamide; **5**) as a VIS-light-absorbing group were synthesized as possible VIS-light-excitable stable Eu^{3+} complexes for protein labeling. The 9-amino derivative **7** of minocycline was treated with H_6TTHA (=triethylenetetraminehexaacetic acid=3,6,9,12-tetrakis(carboxymethyl)-3,6,9,12-tetraazatetradecanedioic acid) or H_5DTPA (=diethylenetriaminepentaacetic acid=*N*,*N*-bis{2-[bis(carboxymethyl)amino]ethyl}glycine) to link the polycarboxylic acids to minocycline. One of the Eu^{3+} chelates, [Eu^{3+}(minocycline-TTHA)] (**13**), is moderately luminescent in H_2O by excitation at 395 nm, whereas [Eu^{3+}(minocycline-DTPA)] (**9**) was not luminescent by excitation at the same wavelength. The luminescence and the excitation spectra of [Eu^{3+}(minocycline-TTHA)] (**13**) showed that, different from other luminescent Eu^{III} chelate complexes, the emission at 615 nm is caused *via* direct excitation of the Eu^{3+} ion, and the chelate ligand is not involved in the excitation of Eu^{3+}. However, the ligand seems to act for the prevention of quenching of the Eu^{3+} emission by H_2O. The fact that the excitation spectrum of [Eu^{3+}(minocycline-TTHA)] is almost identical with the absorption spectrum of Eu^{3+} aqua ion supports such an excitation mechanism. The high stability of the complexes of [Eu^{3+}(minocycline-DTPA)] (**9**) and [Eu^{3+}(minocycline-TTHA)] (**13**) was confirmed by UV-absorption semi-quantitative titrations of H_4(minocycline-DTPA) (**8**) and H_5(minocycline-TTHA) (**12**) with Eu^{3+}. The titrations suggested also that an 1:1 ligand Eu^{3+} complex is formed from **12**, whereas an 1:2 complex was formed from **8** minocycline-DTPA. The H_5(minocycline-TTHA) (**12**) was successfully conjugated to streptavidin (SA) (*Scheme 5*), and thus the applicability of the corresponding Eu^{3+} complex to label a protein was established.

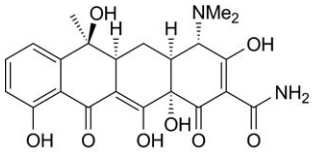
Introduction. – Time-resolved luminescence measurement of lanthanide chelate labels is recognized as a sensitive detection principle suitable for the determination of biological components in fluids and tissues, where the complex matrices of biological samples cause substantial background fluorescence and interfere with the analysis with normal non-time-resolved fluorescence detection [1–5]. Although a number of luminescent lanthanide chelate complexes (especially those of Eu^{3+}, Tb^{3+}, Sm^{3+}, and Dy^{3+}) have been synthesized [6–18], only a few complexes are satisfactory for the practical use in biological analysis, such as in immunoassay, DNA-hybridization assay, ligand–receptor-binding assay, cell and tissue imaging, microarray, and many other biological analyses. Efforts to improve the luminescence properties still continue such as to develop stronger luminescence, higher labeling efficiency while maintaining the

original biological functions of the labeled proteins or nucleic acids, and higher water solubility and stability of the chelate complex in biological matrices. A recent trend in the research is also directed to multicolor labeling and lanthanide complexes emitting in the near-infrared region (complexes of Nd^{3+}, Er^{3+}, and Yb^{3+}) [19] for biological analysis as well as for light-source materials.

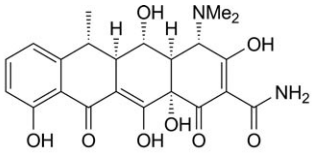
Lanthanide chelates are usually excited with UV light of the wavelength specific to the ligand aromatic groups, and emit VIS light at the wavelength specific to the central lanthanide ion (Eu^{3+} 615 nm and Tb^{3+} 545 nm). The excitation light (usually in the range 300–340 nm) is absorbed by the aromatic moiety of the ligand, and the energy is transferred from the ligand to the lanthanide ion, and the lanthanide ion finally emits VIS light at the wavelength specific to the lanthanide energy level. Owing to such an emission mechanism, the excitation and emission wavelengths are separated very much from each other, leading to a lower fluorescence background level at the emission wavelength, and thus a high detectability is obtained. In addition, the long lifetimes of lanthanide complexes (often more than 1 ms) afford time-resolved measurements very effective avoiding undesired short-lived background fluorescence caused by the matrix materials and cuvette materials. However, in some applications, the UV-light excitation damages biological samples, and excitation with VIS light is desired. Lanthanide chelate complexes excitable with VIS light are strongly sought in the current research; however, so far, only a few complexes have been reported, and even though these are luminescent with ca. 400 nm excitation, the luminescence is significantly strong only in organic solvents. For biological applications, lanthanide luminescence in H_2O is necessary, but such complexes seem so far not easy to realize, due to the strong quenching effect of H_2O. VIS-Light excitation is also desired because various laser and diode light sources are available for the VIS region, and it is expected that more compact instruments will be possible with such light sources, compared to the Xe lamp currently used as a UV-light source.

We noted a recent report that addition of tetracycline (**1**) to Eu^{3+} gives moderately strong luminescence in H_2O with excitation at 405 nm, and the [Eu^{3+}(tetracycline)] complex has been used for several analyses [20–25]. As far as we know, the [Eu^{3+}(tetracycline)] complex and analogous [Eu^{3+}(doxycycline)] complexes [26][27] are the only Eu^{3+} complexes which give moderate luminescence in H_2O by excitation at 405 nm. However, considering the corresponding ligand structure, it seems that the stability of these metal-chelate structures in water and the luminescence intensities can be improved to allow a wider range of bio-applications. In addition, these ligands have not been used to label proteins and nucleic acids, since no group is attached to them allowing such a labeling. We expected that the luminescence in H_2O would be enhanced by attaching a more strongly and stable chelating group to the tetracycline moiety to satisfy the usual coordination number of 9 or 10 of Eu^{3+}. In the present study, an attempt was made to link tetracycline to H_4EDTA (=ethylenediaminetetraacetic acid = *N*,*N*′-ethane-1,2-diylbis[*N*-(carboxyethyl)glycine]) or H_6TTHA (triethylenetetraminehexaacetic acid = 3,6,9,12-tetrakis(carboxymethyl)-3,6,9,12-tetraazatetradecanedioic acid). However, the amide N-atom of tetracycline was not reactive enough for such a linking, and the target ligands were not obtained in practical yields. Therefore, minocycline (=4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxonaphthacene-2-carboxamide; **5**) was used in-

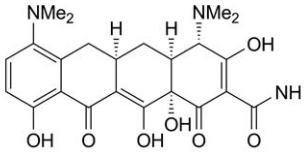
stead of tetracycline, since amination of the 9-position of minocycline was known in the literature. The multidentate chelate ligands H_5DTPA and H_6TTHA were then attached successfully to the amino group of 9-aminominocycline (**7**). Such multidentate chelate structures were expected to enhance the complex stability of the metal chelate in



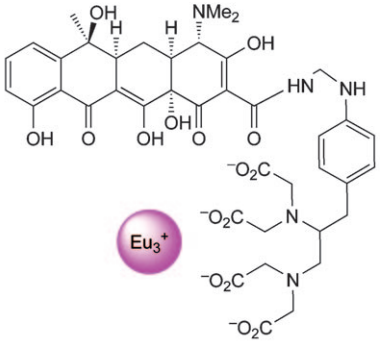
Tetracycline (**1**)



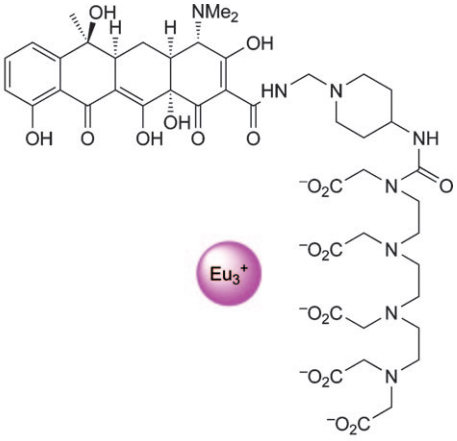
Doxycycline



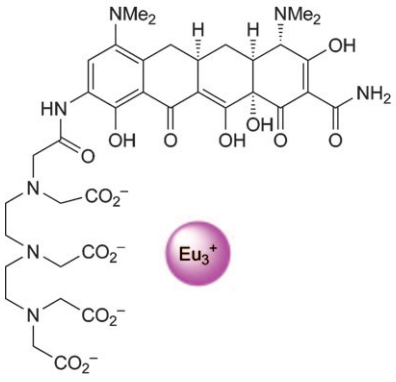
Minocycline (**5**)



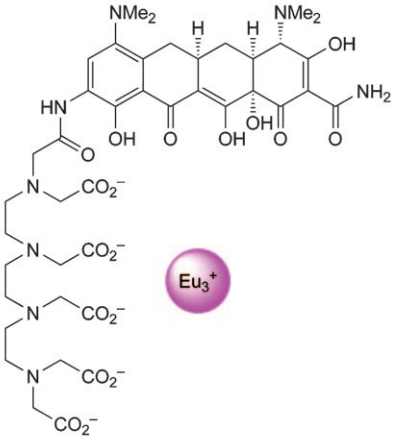
[Eu^{3+}(tetracycline-EDTA)]



[Eu^{3+}(tetracycline-TTHA)]



9 [Eu^{3+}(minocycline-DTPA)]

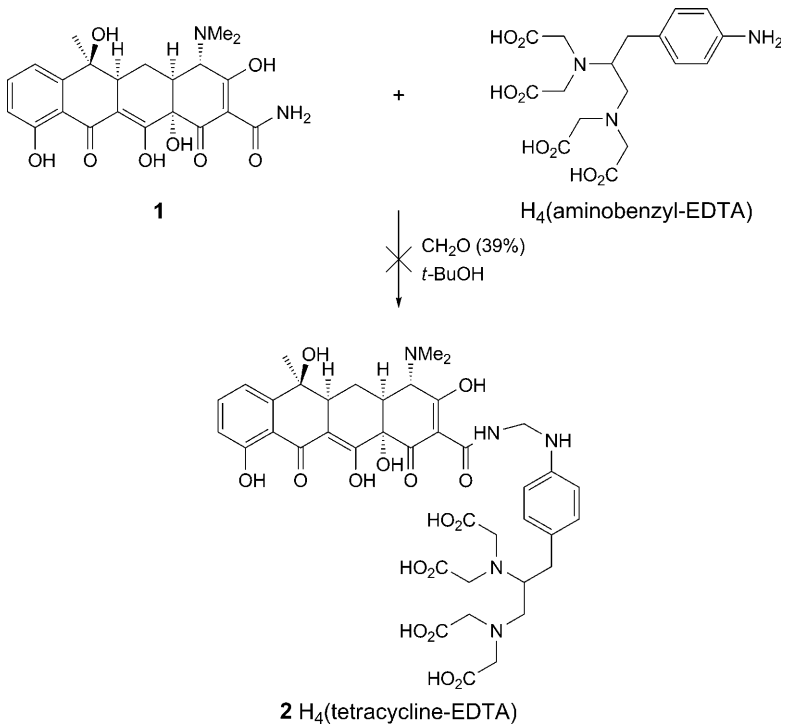


13 [Eu^{3+}(minocycline-TTHA)]

various buffers including phosphate and carbonate. In these buffers, the stability of lanthanide chelate complexes decreases due to the high coordinating affinity of the buffer materials to lanthanide ions [28][29]. High complex stability would exclude H_2O molecules from the coordination sphere of the lanthanide ion, and thus enhance the lanthanide emission.

Results and Discussion. – *Attempt to Synthesize the Ligands H_4(tetracycline-EDTA)* (**2**) *and H_5(tetracycline-TTHA)* (**4**). Tetracycline hydrochloride (**1**·HCl; 6.25 mg, 13 μmol) was treated with H_4(aminobenzyl-EDTA) (5.17 mg, 13 μmol) and formaldehyde (11 μl, 136 μmol) in *t*-BuOH (8 ml) at room temperature for 30 min with stirring, and after a further reaction for 15 min under reflux, the hot solution was filtered and the filtrate cooled to give a yellow precipitate (*Scheme 1*). The proceeding of the reaction was monitored by reversed-phase HPLC and ESI-MS, and two new peaks were found in the MS. However, these peaks did not correspond to **2**, and even on varying several factors of the reaction conditions including the relative amount of the reactants and different solvents and reaction times, the target compound **2** was not obtained.

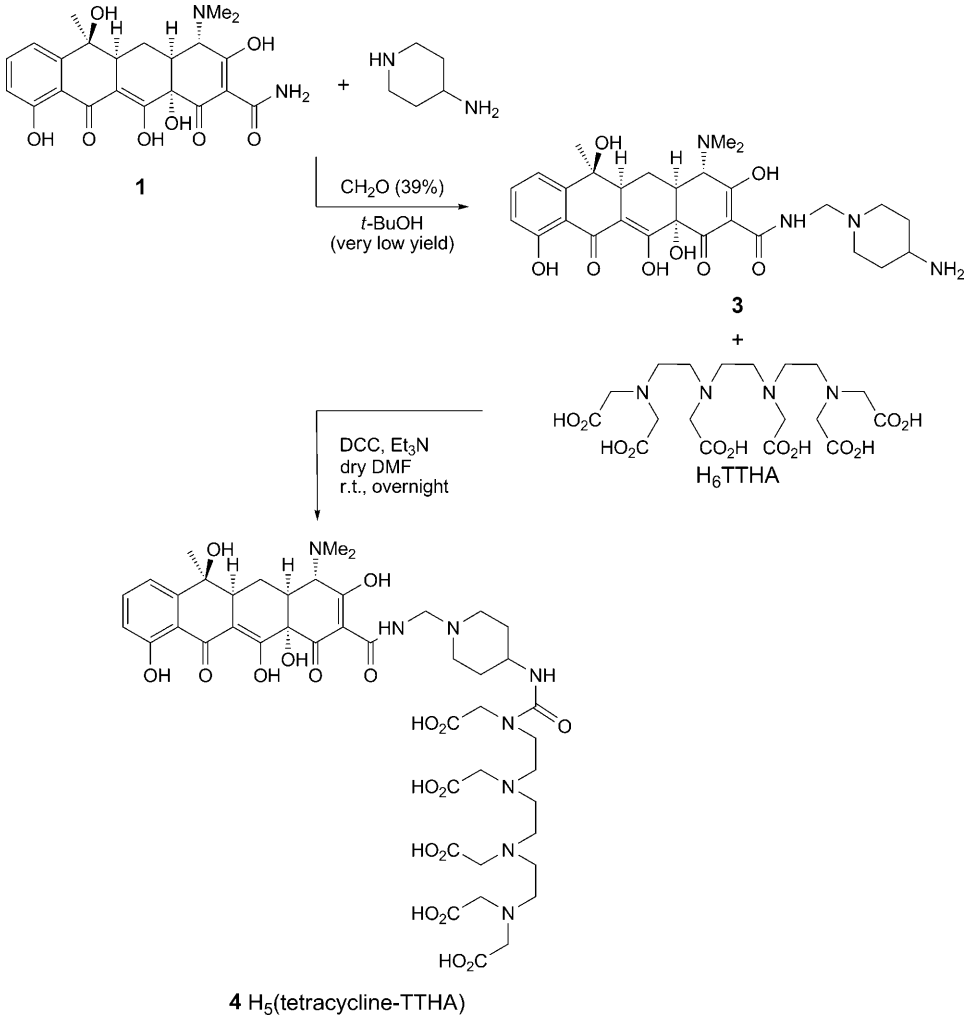
Scheme 1



It seemed that the direct introduction of the H_4(aminobenzyl-EDTA) to the amide group at the 2-position of tetracycline *via* the reaction of *Scheme 1* is difficult. In the literature, no such reaction of an arenamine with the tetracycline amide N-atom is

reported. However, since the morpholine N-atom is reported to react with the minocycline amide N-atom in the presence of formaldehyde [30], the cyclic piperidin-4-amine was selected as a linker to attach H_6TTHA to tetracycline to give H_5(tetracycline-TTHA) (**4**), as shown in *Scheme 2*. Reversed-phase HPLC monitoring of the reaction solution for the first step in *Scheme 2* showed, however, only the peak of tetracycline, and although the ESI-MS of the separated product showed a small peak of the target compound **3** in addition to the main peak of tetracycline, the reaction was not pursued any further because the yield of **3** seemed negligibly low.

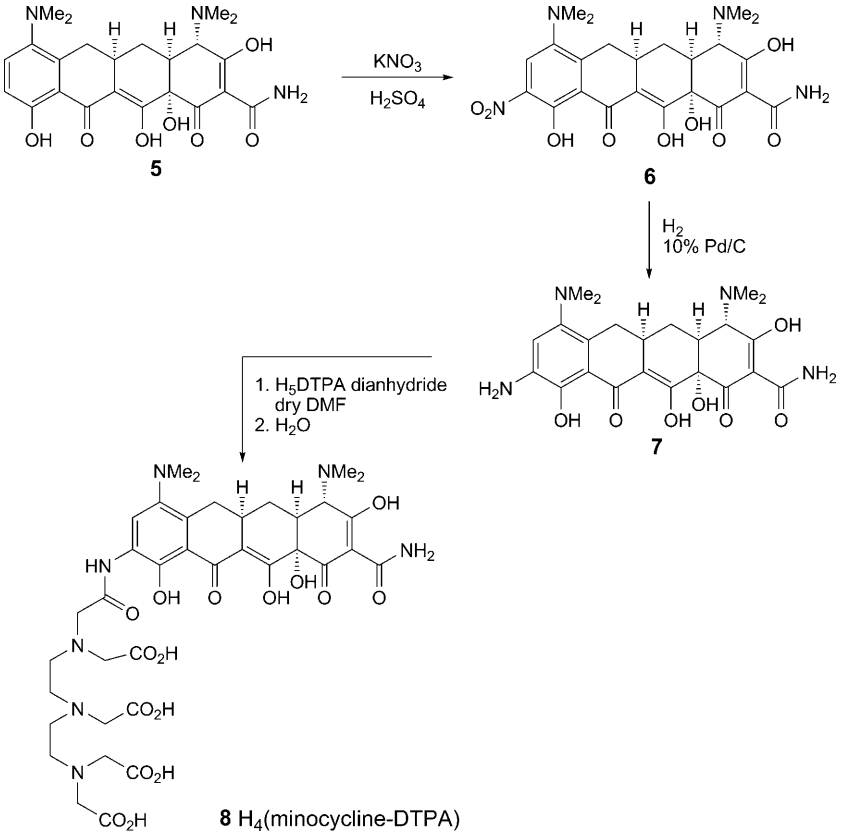
Scheme 2



Ligands H_4(minocycline-DTPA) (**8**) *and H_5(minocycline-TTHA)* (**12**). From all of the above unsuccessful attempts to link H_4EDTA or H_6TTHA to the amide N-atom of

tetracycline, we concluded that the amide group at the 2-position of tetracycline is not suitable to link amino groups of other molecules, and we moved to minocycline (**5**) instead of tetracycline (**1**) since amination at the 9-position of **5** had been reported [31], and H_5DTPA (= diethylenetriaminepentaacetic acid = *N*,*N*-bis{2-[bis(carboxymethyl)amino]ethyl}glycine) and H_6TTHA were used as the chelating groups. The overall reaction scheme to H_4(minocycline-DTPA) (**8**) is shown in *Scheme 3*.

Scheme 3

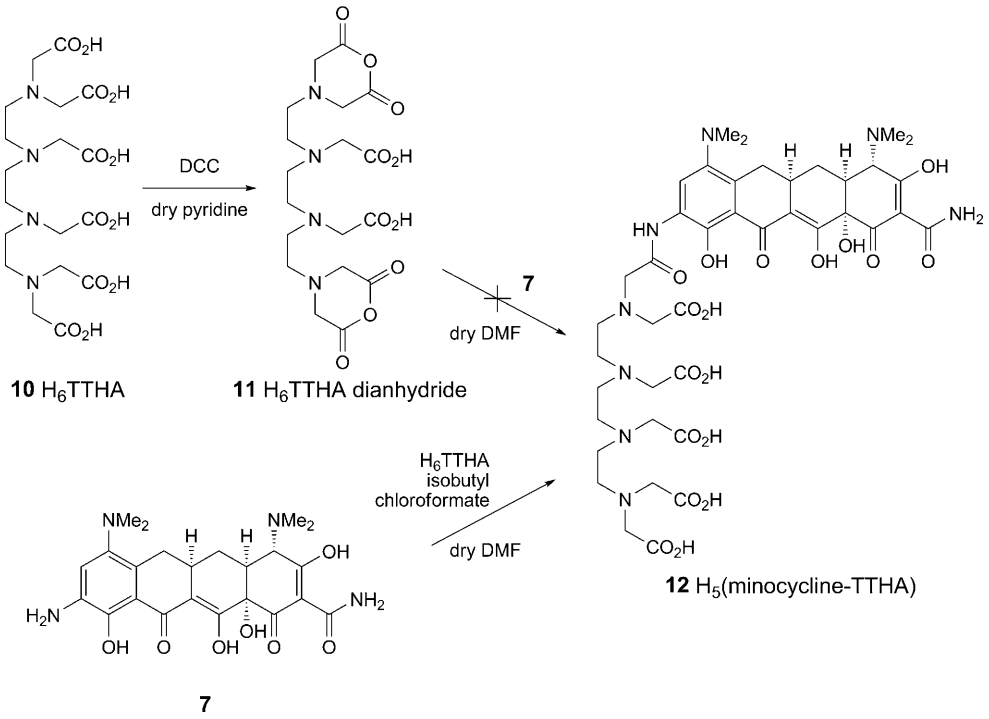


The first and second steps of the synthesis of **8** (**5** → **6** → **7**) were carried out according to [31], and the product of each step was confirmed by ESI-MS. The yields were 76.6 and 32.7%, respectively. The reaction of 9-aminominocycline (**7**) with H_5DTPA dianhydride proceeded easily and gave the final product H_4(minocycline-DTPA) (**8**) in 64.3% yield. The product was confirmed by ESI-MS after reversed-phase HPLC purification. H_4(minocycline-DTPA) was then treated with $EuCl_3 \cdot 6\,H_2O$ in *Tris* · HCl buffer (pH 6.5) to obtain the metal complex [Eu^{3+}(minocycline-DTPA)] (**9**). The ligand-to-metal 1:1 complex was confirmed by ESI-MS. The complex formation reaction was also carried out in Et_4N(OAc) buffer (pH 5.0 and 6.0), $NaHCO_3$ buffer (pH 6.5), and 0.1M NaOH; however, only *Tris* · HCl buffer gave the peaks correspond-

ing to the metal complex in the ESI-MS. In the reaction of 9-aminominocycline (**7**) with the DTPA carboxyl group (*Scheme 3*), several solvents (0.1M *Tris*·HCl (pH 8.0), $Et_4N(OAc)$ buffer (pH 5.0 and 6.0), $NaHCO_3$ buffer (pH 6.5), and 0.1M NaOH were compared, and the final products were analyzed with ESI-MS; however, only 0.1M *Tris*·HCl (pH 8.0) gave a product which showed the ESI-MS peak of $[\mathbf{8}+H]^+$.

Compound **7** was similarly treated with H_6TTHA dianhydride **11** (from H_6TTHA (**10**) with DCC in dry pyridine) to obtain H_5(minocycline-TTHA) (**12**) (*Scheme 4*). However, different from our expectation, the reversed-phase HPLC of the reaction solution of **11** with **7** showed only one peak, which however, was identified as the starting compound 9-aminominocycline (**7**). The reaction conditions were as follows: To a solution of **11** (5.90 μmol) in dry DMF (2 ml), dry Et_3N (3 μl) was added. A dry DMF solution (2 ml) containing **7** (2.0 mg) was added to the DMF solution of **11**, and the solution was stirred under Ar at room temperature for 2 h. Then $Et_4N(OAc)$ buffer (0.1M, pH 6.5; 2 ml) was added, and the solution was further stirred at room temperature for 30 min. After evaporation of the solvent, a white powder was obtained. However, reversed-phase HPLC analysis of the white compound showed only the peak of **7**.

Scheme 4



Since H_6TTHA dianhydride **11** did not react with **7**, unmodified H_6TTHA (**10**) itself was treated with **7** in the next attempt. The reactions described in the *Exper. Part* gave the final product **12** after reversed-phase HPLC purification in 86.1% yield. The

product was confirmed by ESI-MS. The ligand **12** was treated with $EuCl_3 \cdot 6\ H_2O$, and the [Eu^{3+}(minocycline-TTHA)] complex **13** was obtained, which was confirmed by ESI-MS.

These syntheses showed that the 2-amide group of tetracycline (**1**) is much less reactive compared to the 9-amino group of 9-aminominocycline (**7**).

Photophysical Properties of the Minocycline-Based Eu^{3+} Complexes **9** *and* **13**. The UV absorption spectra of **9** and **13** are shown in *Fig. 1*. Both spectra are very similar to each other, and suggest that the spectra are dominated by the minocycline moiety and are not appreciably affected by the aminopolycarboxy moiety. The absorption maxima and the molar extinction coefficients are listed in the *Table*, together with those of free minocycline, free H_4(minocycline-DTPA) (**8**), and free H_5(minocycline-TTHA) (**12**). The absorption bands of **8** or **12** at ca. 355 nm (*Fig. 2*) are significantly shifted to longer wavelengths (*ca.* 395 nm) on metal-complex formation for both **8** and **12** (*Fig. 1*),

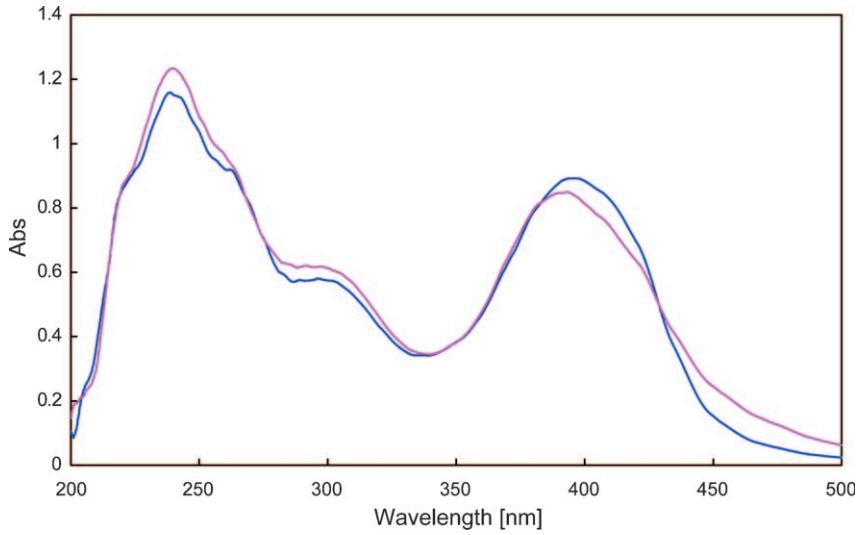


Fig. 1. *UV Absorption spectra of [Eu^{3+}(minocycline-DTPA)]* (**9**; dark blue) *and [Eu^{3+}(minocycline-TTHA)]* (**13**; purple). Both solns. were 100 μM in 0.1M *Tris* · HCl (pH 8.0).

Table. *Properties of the UV-Absorption for the Free Ligands* **8** *and* **12** *and Eu^{3+} Complexes*

	λ_{max} [nm] (ε [$cm^{-1}\,M^{-1}$])	Buffer
Minocycline	375 ($1.40 \cdot 10^4$), 273 ($1.56 \cdot 10^4$), 244 ($1.95 \cdot 10^4$)	20 mM · *Tris* · HCl, pH 8.5
[Eu^{3+}(minocycline)] (1 : 1)[a])	387 ($1.17 \cdot 10^4$), 241 ($1.52 \cdot 10^4$)	20 mM *Tris* · HCl, pH 8.5
H_4(minocycline-DTPA) (**8**)	351 ($1.10 \cdot 10^4$), 240 ($1.51 \cdot 10^4$)	0.1M *Tris* · HCl, pH 7.0
[Eu^{3+}(minocycline-DTPA)] (**9**; 1 : 2)[a])	397 ($1.39 \cdot 10^4$), 240 ($1.50 \cdot 10^4$)	0.1M *Tris* · HCl, pH 7.0
H_5(minocycline-TTHA) (**12**)	351 ($9.80 \cdot 10^3$), 240 ($1.50 \cdot 10^4$)	0.1M *Tris* · HCl, pH 7.0
[Eu^{3+}(minocycline-TTHA)] (**13**; 1 : 1)[a])	395 ($1.20 \cdot 10^4$), 240 ($1.50 \cdot 10^4$)	0.1M *Tris* · HCl, pH 7.0

[a]) Ratio Eu^{3+}/ligand.

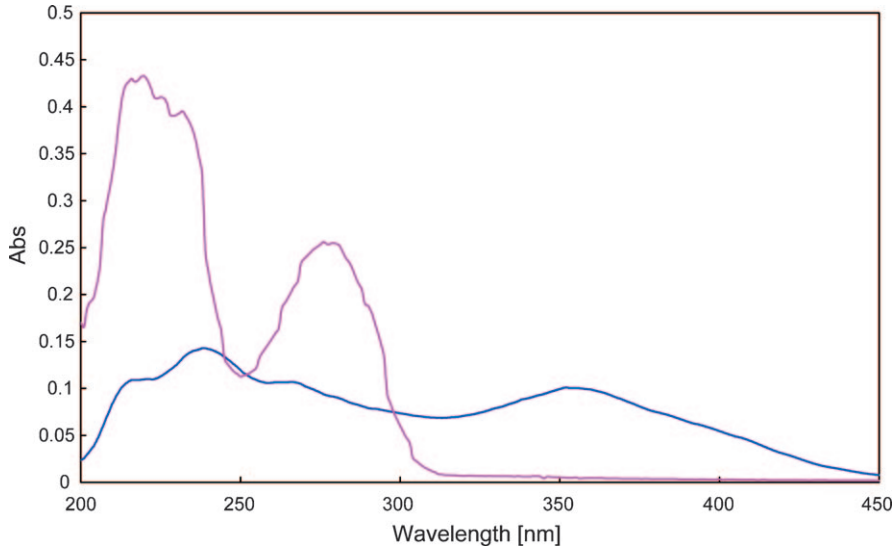


Fig. 2. *UV Absorption Spectra of SA* (purple) *and H_5(minocycline-TTHA)* (**12**; dark blue) *in 50 mM AcONa* (pH 3.5) *adjusted to pH 7.0 with 1M* Tris · *HCl* (pH 9.0)

whereas the absorption maximum of the free ligands at around 240 nm is not significantly shifted on metal-complex formation.

The excitation and emission spectra of **9** and **13** are shown in *Fig. 3.* Although complex **9** is not luminescent, **13** gives moderate luminescence with excitation at

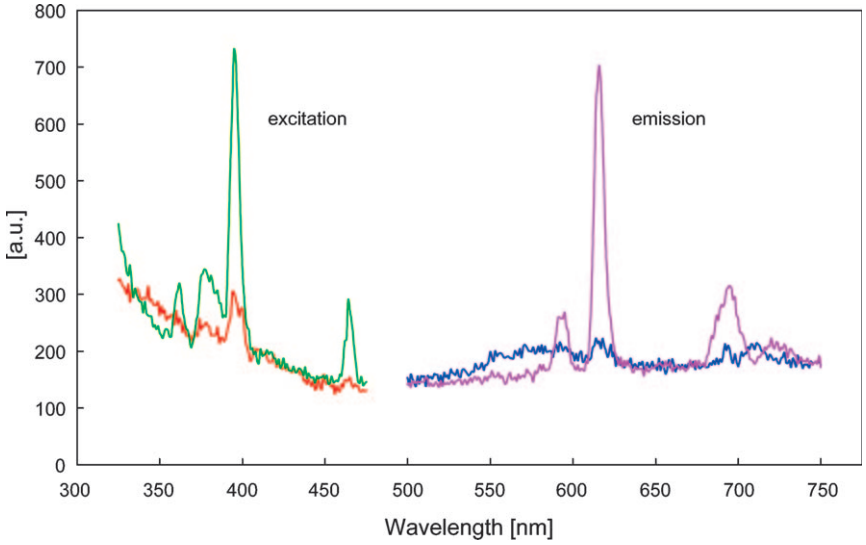


Fig. 3. *Excitation and emission spectra of [Eu^{3+}(minocycline-DTPA)]* (**9**; red and dark blue lines) *and of [Eu^{3+}(minocycline-TTHA)]* (**13**; green and purple lines). Both solns. were 100 μM in 0.1M *Tris* · HCl (pH 8.0), and were excited at 395 nm and observed at 616 nm.

395 nm. The excitation spectrum of **13** is very unusual, since its spectral pattern in the range 350 to 450 nm consists of several sharp peaks somewhat similar to the absorption peaks of lanthanide metal ion. We recognized that the excitation spectrum is very similar to the reported absorption spectrum of Eu^{3+} aqua ion [10][32]. The excitation spectra of **9** and **13** in *Fig. 3* also show that in the wavelength range below 350 nm, both complexes are excited strongly by the ligand absorption *via* the ligand-to-metal intramolecular energy transfer, similarly to many other luminescent Eu^{3+} complexes. Apparently the broad and strong absorption band around 400 nm of **9** and **13** in *Fig. 1* are not effective for luminescence excitation.

At first we thought that the metal ion of **13** was dissociated from the ligand, and thus the excitation and emission spectra of Eu^{3+} aqua ion was observed in the spectra of *Fig. 1*. To check this point, a 100 μM $EuCl_3$ solution in *Tris*·HCl buffer at pH 8.0 was prepared, and the emission spectrum was measured. However, the solution was not luminescent. Hence, the metal ion of **13** was not dissociated in the solution of *Fig. 3*. The absorption spectrum of **13** in *Fig. 1* is definitely different from that of the free ligand, and the spectrum is not an overlap of the spectra of free ligand and Eu^{3+} ion, which supports that the structure of the metal-coordinated complex is retained in the solution under the spectral-measurement conditions. As for the excitation and emission mechanism of **13**, the minocycline moiety of **13** is not effective for energy transfer to Eu^{3+}, and the emission occurs *via* direct absorption by the metal ion at its strongest absorption line at 395 nm [10][33]. For comparison, emission was examined for *Tris*·HCl buffer (pH 8.0) solutions containing 100 μM minocycline and $EuCl_3 \cdot 6\,H_2O$ at a molar ratio of 1:1 and 1:0.5; however, the solutions were not luminescent. Minocycline seems different from tetracycline and doxycycline in the energy-level diagram. The latter ligands absorb excitation light at *ca.* 400 nm and transfer the energy from the ligand triplet state to the ^5D_n level of Eu^{3+}. The central Eu^{3+} ion finally emits luminescence *via* the $^5D_0 \rightarrow {}^7F_2$ transition [20–25]. Free minocycline cannot transfer the absorbed energy at *ca.* 395 nm to Eu^{3+}, presumably because of the unsuitable triplet-state-energy level. The excitation line at 395 nm in *Fig. 3* is the transition of $^7F_0 \rightarrow {}^5L_6$ [10][33], and the energy is emitted as the 616 nm line of the $^5D_0 \rightarrow {}^7F_2$ transition of Eu^{3+}.

To examine the complex stability of **9** and **13** in solution, and the stoichiometry of the metal-complex formation, both ligands **8** and **12** were titrated with Eu^{3+} by monitoring the absorption spectra. To a 0.1 mM solution of **8** in 0.1M *Tris*·HCl (pH 7.0), a solution of $EuCl_3 \cdot 6\,H_2O$ at suitable concentration was added, so that the molar ratio of the ligand and Eu^{3+} was varied stepwise from 1:0 to 1:20, and the absorption spectra were measured. As the spectra in *Fig. 4* and the titration curve (expressed as the absorbance change at 397 nm) in *Fig. 5* show, the complex formation was completed after 2 equiv. of Eu^{3+} were added. Similar measurements were carried out for **12**, and the spectra and the titration curve are shown in *Figs. 6* and *7*.

For ligand H_5(minocycline-TTHA) (**12**), the complex formation was completed at a 1:1 ratio, suggesting a 1:1 complex **13** is formed. However, for ligand H_4(minocycline-DPTA) (**8**), the Eu^{3+} complex **9** seems to be a 1:2 complex, and such a difference of the metal-to-ligand ratio between **13** and **9** suggests that complex **13** would be more stable than **9**. This difference may also explain the difference of the luminescence properties of the two metal complexes. It is also highly probable that the metal complexes stably

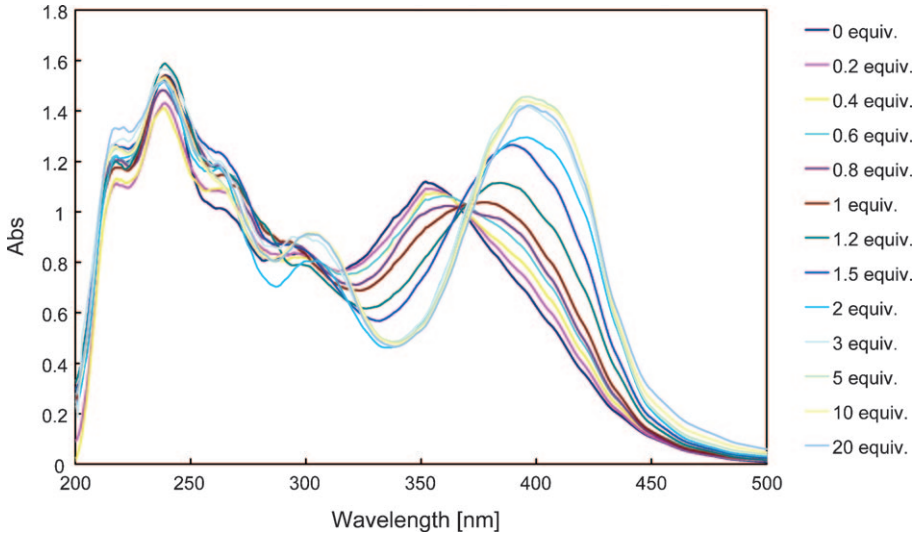


Fig. 4. *Spectral change in the titration of H_4(minocycline-DTPA)* (**8**) *with $EuCl_3$*

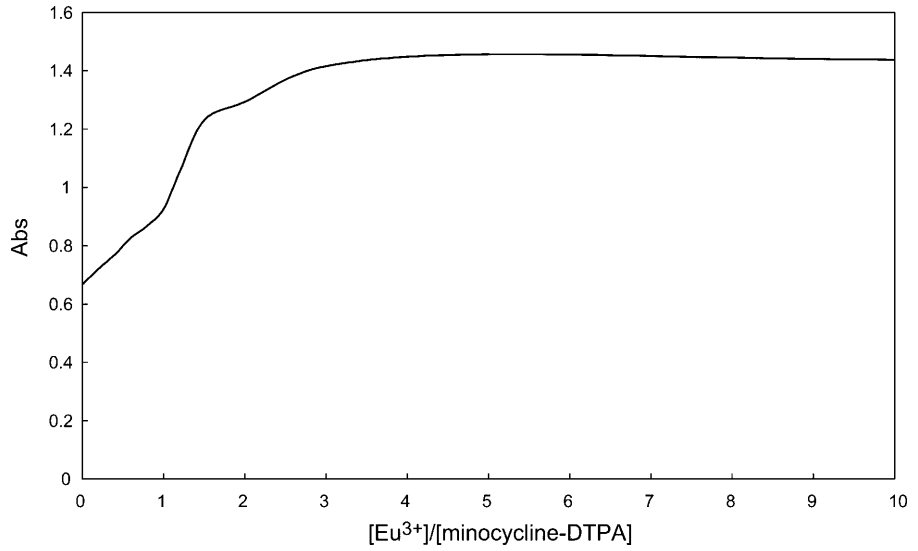


Fig. 5. *Titration curve of H_4(minocycline-DTPA)* (**8**) *with $EuCl_3$, observed at the absorption at 397 nm*

exist and are not dissociated in the solutions of *Figs. 1* and *3*, since the final spectra of the titration solutions (*Figs. 4* and *6*) are identical to the spectra in *Fig. 1*. The excitation spectrum such as that of **13** having several line-like peaks is very rare, compared to those of other luminescent Eu^{3+} complexes. The latter exhibit excitation spectra having broad absorption bands almost identical to the absorption spectra of the aromatic ligands. Measurements of absorption or emission spectra of a simple aqueous Eu^{3+}

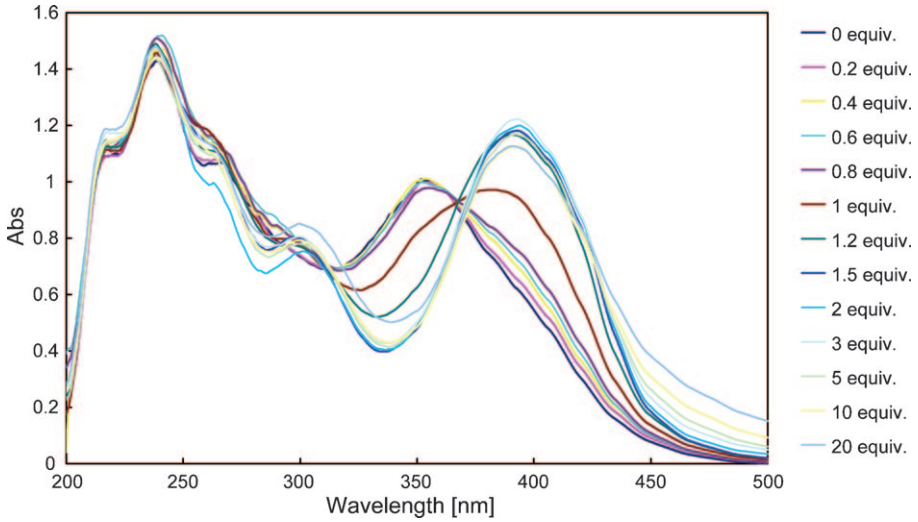


Fig. 6. *Spectral change in the titration of H_5(minocycline-TTHA)* (**12**) *with $EuCl_3$*

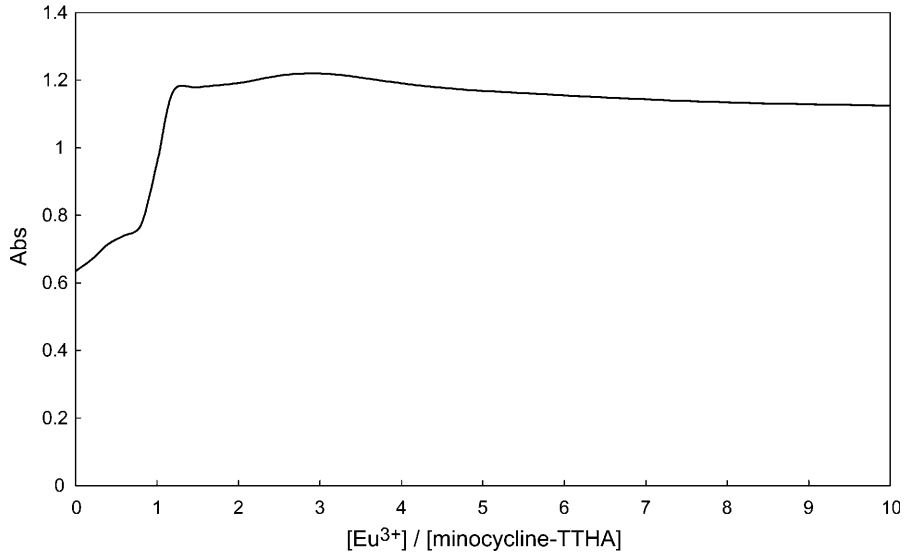


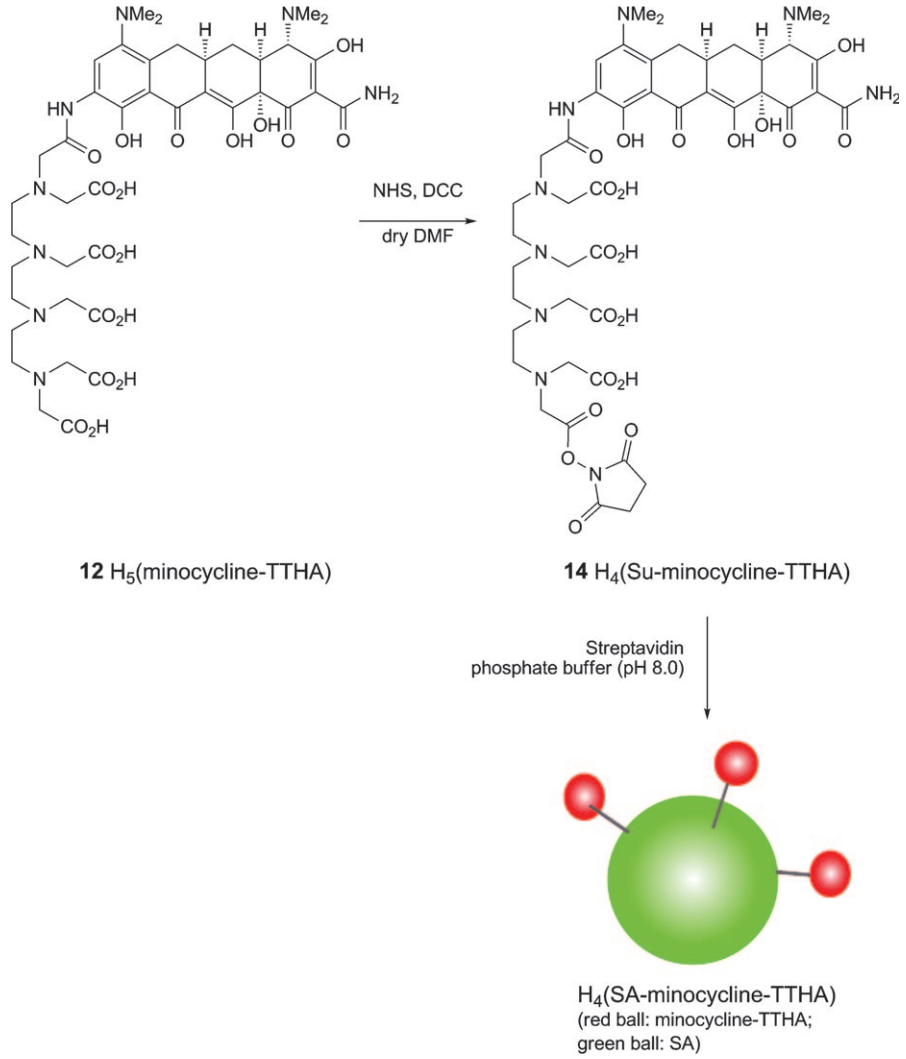
Fig. 7. *Titration curve of H_5(minocycline-TTHA)* (**12**) *with $EuCl_3$, observed at the absorption at 391 nm*

solution need higher concentrations than those used in the present experiments, and the solutions must be acidic to avoid precipitation of lanthanide hydroxide. Thus, obviously the ligand in **13** acts as inhibitor preventing precipitation and as protection or against quenching of the emission of the metal complex by H_2O.

Conjugation of **13** *to Streptavidin.* To test the suitability of Eu^{3+} complex **13** for the labeling of proteins, streptavidin (SA) was conjugated with ligand **12** according to

Scheme 5. Streptavidin was selected as a typical protein to see whether the new labeling reagent can be labeled to proteins without any inconvenience, since our past experience showed that for some labeling reagents for Eu^{3+}, the conjugation reaction causes precipitation of the protein. If SA can be conjugated successfully to H_5(minocycline-TTHA) (**12**), it means that the reagent can be used in many bio-analytical applications taking advantage of the selective and strong binding of the conjugated SA to biotin. Thus, one of the carboxy groups of compound **12** was succinimidylated (ester formation with *N*-hydroxysuccinimide (NHS)) to give **14**, which was treated with SA in 0.1M phosphate buffer (pH 8.0) at room temperature for 6 h. The details of the purification of the conjugated streptavidin, H_4(SA-minocycline-TTHA), is described in the *Exper. Part*.

Scheme 5



Luminescence Properties of the Labeled SA. The absorption spectrum of the H_4(SA-minocycline-TTHA) (*Fig. 8*) consists of the absorption bands almost identical to those of SA and free H_5(minocycline-TTHA) (**12**; *Fig. 2*). The time-resolved luminescence spectrum of [Eu^{3+}(SA-minocycline-TTHA)] was measured after the addition of 2 equiv. of $EuCl_3 \cdot 6\,H_2O$ and 1 h reaction at room temperature. The concentration of H_4(SA-minocycline-TTHA) was determined with the UV spectrum, assuming that the molar extinction coefficient of the conjugated H_4(SA-minocycline-TTHA) is not changed from that of free H_5(minocycline-TTHA). The luminescence spectrum of the labeled SA, *i.e.*, of complex [Eu^{3+}(SA-minocycline-TTHA)] is shown in *Fig. 9*, together with that of [Eu^{3+}(minocycline-TTHA)] at the same concentration of the minocycline-TTHA moiety. Both spectra were measured with the excitation wavelength 395 nm and the luminescence-peak wavelength 615 nm. It should be noted that the luminescence of [Eu^{3+}(minocycline-TTHA)] is 10-fold increased on conjugation to SA, in spite of the loss of one of the carboxy groups needed for the conjugation.

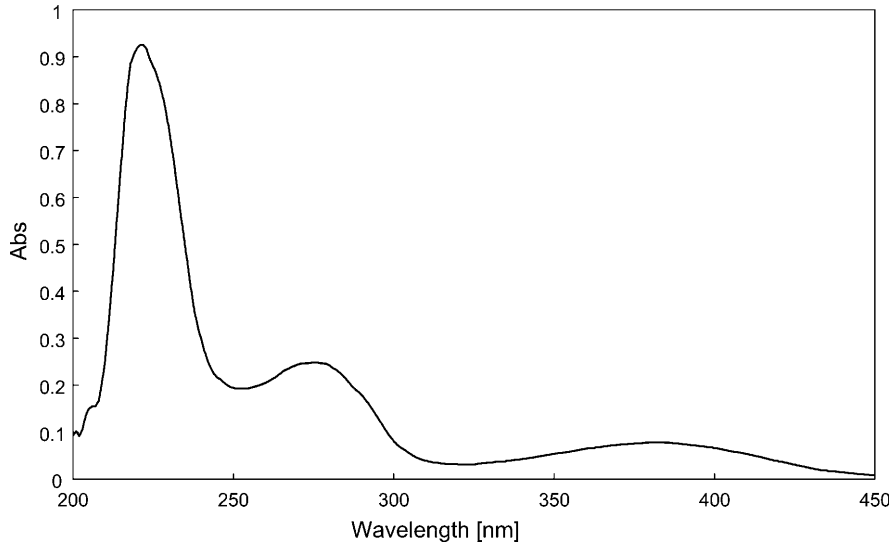


Fig. 8. *UV Spectrum of H_4(SA-minocycline-TTHA) in 50 mM AcONa* (pH 3.5) *adjusted to pH 7.0 with 1M* Tris · *HCl* (pH 9.0)

Conclusions. – As a candidate for a VIS-light-excitable lanthanide complex, [Eu^{3+}(minocycline-DTPA)] (**9**) and [Eu^{3+}(minocycline-TTHA)] (**13**) were synthesized, and the luminescence properties and suitability to label SA were studied. While **9** was not luminescent in H_2O, **13** could be excited at 395 nm in H_2O to emit luminescence at 615 nm. The luminescence intensity of **13** is moderate, and the excitation spectrum of the complex suggests that the luminescence occurs not *via* the conventional energy transfer from the ligand to the central Eu^{3+} ion but by direct absorption of the excitation light by the Eu^{3+} ion ($^7F_0 \rightarrow {}^5L_6$) and emission at 615 nm as $^5D_0 \rightarrow {}^7F_2$. The effect of the ligand of **13** seems to consist of the protection of the metal ion from

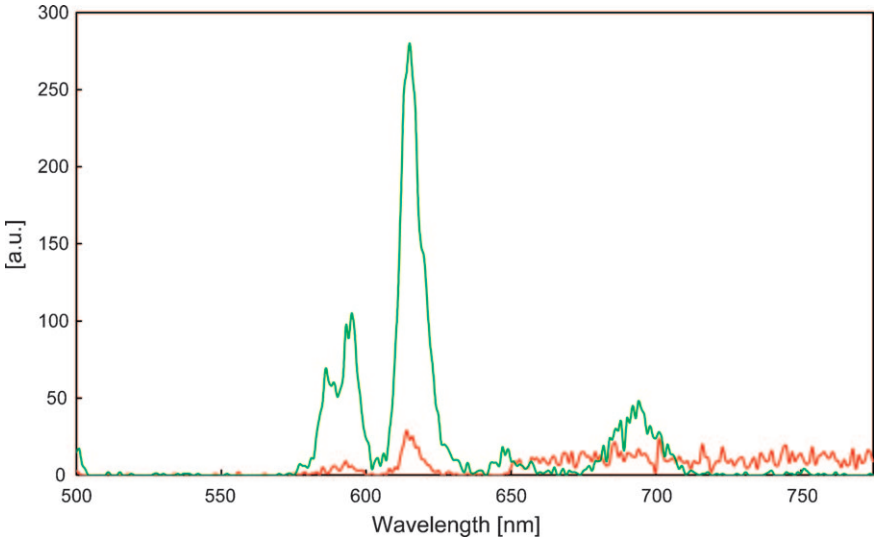


Fig. 9. *Time-resolved luminescence spectra of [Eu^{3+}(SA-minocycline-TTHA)]* (green) *and [Eu^{3+}(minocycline-TTHA)]* (red). Both solns. were 19.4 μM for the minocycline-TTHA moiety in 50 mM AcONa (pH 3.5) adjusted to pH 7.0 with 1M *Tris*·HCl (pH 9.0).

quenching by H_2O, since none of the simple aqueous solutions of $EuCl_3$, a 1:1 mixture of $EuCl_3$ and H_6TTHA, or a 1:1 mixture of $EuCl_3$ and minocycline at the same Eu^{3+} concentration level did show any luminescence. The minocycline-DTPA ligand of **9** does not exert such a preventing function on H_2O quenching. It seems that the triplet-state energy of minocycline is not suitable for energy transfer to Eu^{3+}. Since the molecular absorption coefficient of Eu^{3+} is not large, the luminescence of **13** is not strong; however, observation of the luminescence at such a low concentration was not known previously, and complex **13** is noteworthy in this regard. The conjugation of **13** to SA showed that a protein can be labeled with **13** without troubles under usual reaction conditions.

Experimental Part

General. All reactions with air- or humidity-sensitive materials were carried out under Ar by using standard *Schlenk* technique or a glove box. All the reagents were used as received without purification. HPLC: *Waters-XTerra-MS-C18* column (ϕ 2.5 μm 4.6 × 50 mm); mobile phase: MeCN containing 0.01% of CF_3COOH (*A*) and H_2O containing 0.01% of CF_3COOH (*B*); flow rate 1.0 ml/min; gradient 0 min *A*/*B* 5:95, 5 min *A*/*B* 5:95, and 50 min *A*/*B* 50:50. UV/VIS Spectra: *Jasco-Ubest-V-570* UV/VIS spectrophotometer. ESI-MS: *Finnigan-Thermo-Quest-LCQ-Deca* spectrometer. Luminescence excitation and emission spectra: *Perkin-Elmer-LS-50B* luminescence spectrometer.

*(4*S*,4a*S*,5a*R*,12*S*)-4,7-Bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-9-nitro-1,11-dioxonaphthacene-2-carboxamide* (**6**). Minocycline hydrochloride (**5**·HCl; 405 mg, 0.821 mmol) was dissolved in conc. H_2SO_4 (100 ml), and while the soln. was cooled with ice-water, KNO_3 (98.4 mg, 0.974 mmol) was added. After the soln. was stirred for 1.5 h at 0° (reversed-phase HPLC (see *General*) and ESI-MS monitoring), Et_2O (800 ml) cooled with ice-water was added dropwise. The formed precipitate was filtered, washed with Et_2O, and vacuum dried at 40°: **6** (76.6%). ESI-MS: 503.1 ($[M+H]^+$).

*(4*S*,4a*S*,5a*R*,12a*S*)-9-Amino-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxonaphthacene-2-carboxamide* (**7**). To the soln. of **6** (250 mg, 0.358 mmol) in 2M H_2SO_4 (10 ml) and 2-methoxyethanol (15 ml), 10% Pd/C (50 mg) was added, and the mixture was stirred for 1.5 h under 2.72 atm of H_2. The soln. was filtered and the filtrate concentrated. A small amount of MeOH was added to the residue, and the MeOH soln. of the product was dropwise added to a mixture of i-PrOH (70 ml) and Et_2O (50 ml). The precipitate formed was collected by centrifugation, washed with Et_2O, and vacuum dried at 40°. The product was purified by reversed-phase HPLC and then vacuum-dried: **7** (32.7%). ESI-MS: 473.1 ($[M+H]^+$).

H_4(minocycline-DTPA) (**8**). The dianhydride of H_5DTPA (27 mg, 74.3 μmol) was dissolved in dry DMF (2 ml), to which molecular-sieves-dried Et_3N (36 μl, 247.5 μmol) was added. Then **7** (25.2 mg, 49.5 μmol) in dry DMF (3 ml) was added, and the soln. was stirred for 2 h at r.t. under Ar. To this soln. 0.1M Et_4N(OAc) (buffer pH 6.5; 2 ml) was added, and the soln. was stirred for 30 min at r.t. After the solvent was evaporated, the product was purified by reversed-phase HPLC and dried under reduced pressure: **8** (64.3%). ESI-MS: 848.3 ($[M+H]^+$).

[Eu^{3+}(minocycline-DTPA)] (**9**). To a soln. of **8** (3 mg, 3.54 μmol) in 0.1M *Tris*·HCl (pH 8.0; 2 ml), a soln. (1 ml) of 0.1M *Tris*·HCl (pH 8.0) containing $EuCl_3 \cdot 6\,H_2O$ (1.95 mg, 5.31 μmol) was added, and the soln. was stirred for 30 min at r.t. After adjusting the pH to 9.0 with 0.1M NaOH, the precipitate was removed by filtration, and acetone (50 ml) was added to the filtrate to precipitate the metal complex. The precipitate was collected by centrifugation, washed with acetone, and vacuum-dried: **9**. ESI-MS: 998.2 ($[M+H]^+$).

H_2(TTHA Dianhydride) (**11**). H_6TTHA (665 mg, 1.35 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC; 555 mg, 2.7 mmol) were stirred in dry pyridine (20 ml) for 48 h at 40°. The precipitate was collected by filtration, washed with Et_2O, and vacuum-dried: **11** (almost 100%).

H_5(minocycline-TTHA) (**12**). To a soln. of H_6TTHA (265.2 mg, 537 μmol) in dry DMF (5 ml), molecular-sieves-dried Et_3N (1.5 ml) was added. The soln. was stirred overnight at r.t. under Ar. While the soln. was cooled with ice-water, a mixture of dry DMF (300 μl) and isobutyl chloroformate (=2-methylpropyl carbonochloridate) (76.7 μl, 537 μmol) was gradually added. After the soln. was stirred for 15 min at 0° under Ar, dry DMF (1 ml) containing **7** (27.3 mg, 53.7 μmol) was added, and the soln. was stirred for 5 h at r.t. At this stage, reversed-phase HPLC and ESI-MS monitoring showed that **12** was produced as the major product. The solvent was evaporated and the product purified by reversed-phase HPLC and vacuum-dried: **12** (86.1%). ESI-MS: 949.3 ($[M+H]^+$).

[Eu^{3+}(minocycline-TTHA)] (**13**). As described for **9**, with 0.1M *Tris*·HCl (pH 8.0; 2 ml) containing **12** (2.5 mg, 2.63 μmol) and a soln. (1 ml) of 0.1M *Tris*·HCl (pH 8.0) containing $EuCl_3 \cdot 6\,H_2O$ (1.93 mg, 5.26 μmol): **13** (1.7 mg). ESI-MS: 1099.3 ($[M+H]^+$).

Succinimidyl Derivative **14** *of H_5(minocycline-TTHA)*. H_5(minocycline-TTHA) (**12**), *N*-hydroxysuccinimide (NHS), and DCC were vacuum-dried for 24 h before use. To a soln. of dried **12** (4.6 mg, 4.85 μmol) in dry DMF (1 ml), a soln. of dry DMF (1 ml) containing NHS (0.56 mg, 4.85 μmol) and DCC (1 mg, 4.85 μmol) was added. The soln. was stirred for 24 h at r.t. under Ar. After an undissolved precipitate was removed by centrifugation, the filtrate was concentrated: H_4(Su-minocycline-TTHA) (**14**). Since **11** is easily hydrolyzed, it was immediately used for the following conjugation to streptavidin (SA).

Conjugation of H_4(minocycline-TTHA) (**14**) *to SA*. Compound **14** was dissolved in 0.1M phosphate buffer (pH 8.0; 700 μl), and to this soln., 0.1M phosphate buffer (pH 8.0; 200 μl) containing SA at the concentration of 5 mg/ml was added. The soln. was stirred for 6 h to give a soln. of crude H_4(SA-minocycline-TTHA). Since the product soln. contained unreacted **14**, the target product was purified by column chromatographies (*Sephadex G25* and iminobiotin immobilized column) as follows. *Sephadex G25* was equilibrated with 0.1M *Tris*·HCl/0.1M NaCl buffer (pH 8.0). The crude H_4(SA-minocycline-TTHA) soln. was eluted with 0.1M *Tris*·HCl/0.1M NaCl buffer (pH 8.0) (500 μl fractions, UV monitoring). The product fractions were further purified with a iminobiotin immobilized column. The column was equilibrated with 0.1M *Tris*·HCl/0.1M NaCl buffer (pH 8.0), and the *Sephadex-G25*-purified product was eluted with the same buffer as the one used for equilibration (500 μl fractions). After the elution of unreacted **14** UV monitoring, the column was washed, and the H_4(SA-minocycline-TTHA) on the column was eluted with AcONa buffer (pH 3.5; 50 ml). Each 250 μl fraction was neutralized by the

addition of 1M *Tris*·HCl (pH 9.0; 20 µl). The purified H_4(SA-minocycline-TTHA) was confirmed by its UV spectra having absorptions of both SA and H_5(minocycline-TTHA) (*Figs. 2* and *8*).

Titrations of H_4(minocycline-DTPA) (**8**) *and H_5(minocycline-TTHA)* (**12**) *with Eu^{3+}.* The titrations were carried out by using 0.1 mM ligand solns. in 0.1M *Tris*·HCl (pH 7.0). Solns. of $EuCl_3 \cdot 6\,H_2O$ with appropriate concentrations in 0.1M *Tris*·HCl (pH 7.0) were added to the ligand solns., and the UV spectra were measured after stirring for 20 min at r.t. For construction of the titration curves, absorbance at 397 and 391 nm were used for **8** and **12**, resp.

Measurement of the Time-Resolved Spectra of [Eu^{3+}(SA-minocycline-TTHA)] and [Eu^{3+}(minocycline-TTHA)] (**13**). The luminescence spectrum of [Eu^{3+}(SA-minocycline-TTHA)] was measured with a time-resolved mode, to remove the background fluorescence of the SA moiety. The concentration of the purified H_4(SA-minocycline-TTHA) was determined by UV absorption, assuming that the molecular absorption coefficient is not changed by conjugation to SA. For the luminescence-spectrum measurement, 2 equiv. of $EuCl_3 \cdot 6\,H_2O$ were added to the H_4(SA-minocycline-TTHA) soln., and after the soln. was stirred for 1 h at r.t., the spectrum was measured under the following conditions: window time 0.5 ms, delay time after flash 0.1 ms, number of flash 200, and concentration of the ligand 19.4 µM in 50 mM AcONa/1M *Tris*·HCl buffer at pH 7.0.

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