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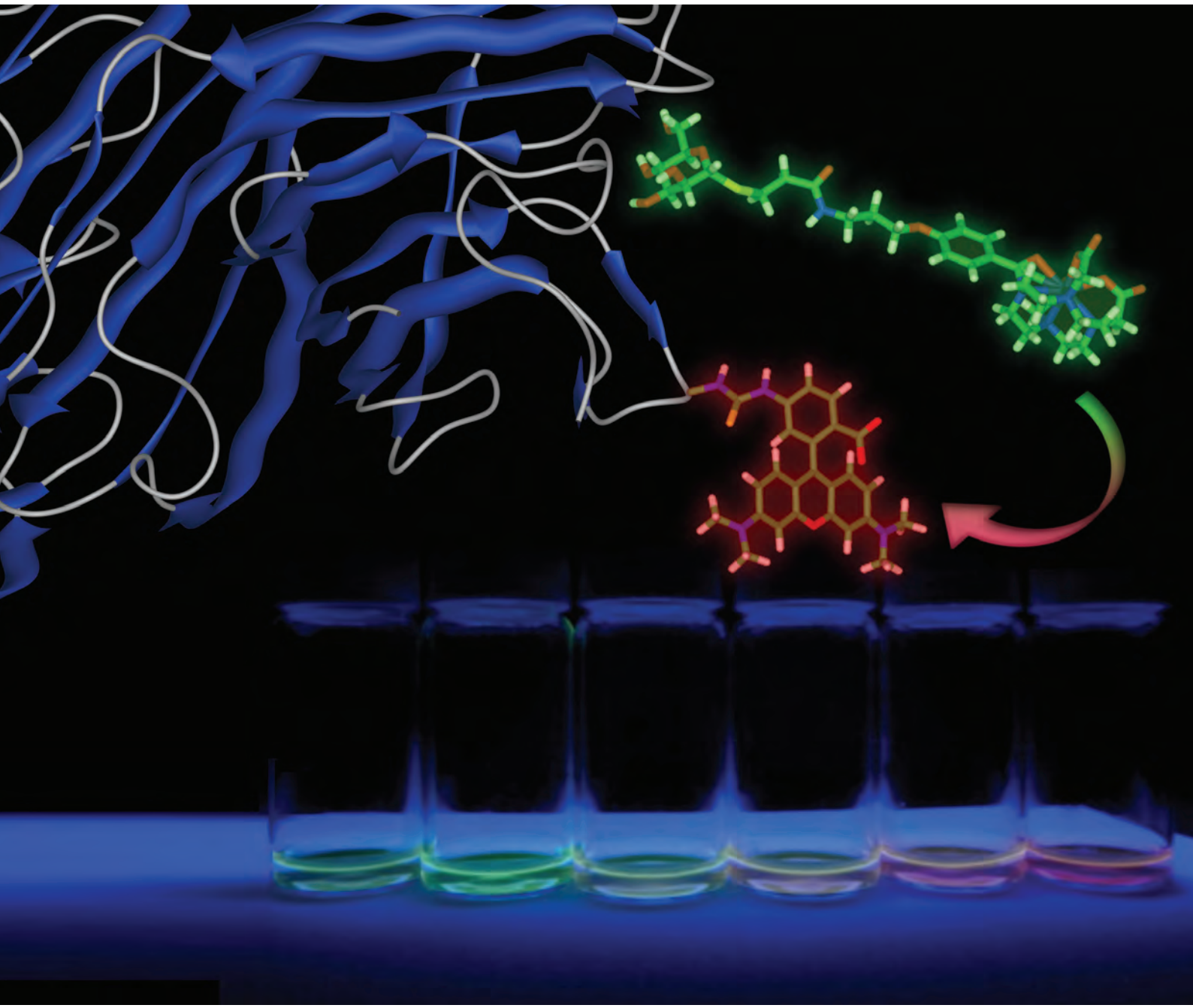

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A highly sensitive luminescent lectin sensor based on an α-D-mannose substituted Tb^{3+} antenna complex†

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Lectin–carbohydrate interactions are the basis of many biological processes and essentially they constitute the language through which intercellular communications are codified. Thus they represent powerful tools in the examination and interpretation of changes that occur on cell surfaces during both physiological and, more importantly, pathological events. The development of optical techniques that exploit the unique properties of luminescent lanthanoid metal complexes in the investigation of lectin–carbohydrate recognition can foster research in the field of ratiometric biosensing and disease detection. Here we report the synthesis of a Tb^{3+}-DO3A complex (**Tb⊂1**) bearing an α-D-mannose residue and the related study of binding affinity with concanavalin A (Con A) labeled with rhodamine-B-isothiocyanate (RITC-Con A). Luminescence spectroscopy and dynamic studies show changes in emission spectra that can be ascribed to a luminescence resonance energy transfer (LRET) from **Tb⊂1** (donor) to RITC-Con A (acceptor). The binding constant value between the two species was found to be one order of magnitude larger than those previously reported for similar types of recognition. To the best of our knowledge this is the first example of the use of a pre-organized luminescent lanthanoid complex in the study of carbohydrate–protein interactions by LRET.

Introduction

The ability to probe the activity and reactivity of biomolecules as they occur within living cells is fundamental to furthering biomedical science. Thus, the detection and simultaneous monitoring of chemical interactions between biological targets have become indispensable in medical diagnosis, targeted therapeutics and molecular biology. In this sense, the interest in lectins, which are proteins containing specific domains for the recognition of sugars, has intensified during the last few years. In fact lectins proved to be extremely valuable tools for the analysis of cell surface sugars, for the assessment of the role of the latter in cell growth and differentiation in both physiological and pathological processes.[1] Thanks to their specific binding with sugars, lectins have been generally used as probes for studying carbohydrates on cell membranes. The nature and the concentration of saccharides or polysaccharides on the different organs and cells can significantly change depending on the cell type and more importantly on whether the cell is healthy or cancerous.[2] Therefore the interaction between lectins and carbohydrates is of paramount importance to quantitatively analyze carbohydrates of interest since it can be used as a diagnostic marker for different types of diseases.[3]

The lectin–carbohydrate binding, which generally relies on the multivalent interactions of the protein with responsive molecules containing several monosaccharide units (glycodendrimers), has been studied among others by microgravimetric,[4] electrochemical[5] and optical methods.[6] In comparison to other methods of investigation, the use of luminescent glycodendrimers as biosensors for the recognition of lectins presents some advantages: luminescence measurements are very sensitive (even single molecule detection is possible), versatile and can be easily performed.[7] Moreover low detection limits would allow the monitoring of biological events as they occur, facilitating the understanding of the origin and growth of several diseases, including cancer.

Glycodendrimers have been synthesized on fluorescent oligothiophenes,[8] Ru(II), Re(II) and Ir(III) metal complexes,[9–11] quantum dots,[12] gold[13] and $NaGdF_4$:Er^{3+},Yb^{3+} up-converting

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†Electronic supplementary information (ESI) available: ^{1}H NMR and ^{13}C NMR spectra of all new compounds are given. See DOI: 10.1039/c3dt33023k

nanoparticles,[14] and applied to monitoring carbohydrate–protein recognition events.

An important issue is the development of new sensors for continuous *in vivo* detection of glucose in diabetic patients. To this aim several fluorescent receptors have been investigated and in many cases lectin concanavalin A (Con A) has been used as the glucose-binding unit. The technique generally used consists of measuring changes in fluorescence (or Förster) resonance energy transfer (FRET), which involves the non-radiative energy transfer from a fluorescent donor molecule to an acceptor molecule in close proximity. This results in a decrease of fluorescence intensity and the lifetime of the donor.[15] On the other hand the use of FRET on biological systems has some limitations that are strictly related to the photophysical properties of fluorescent probes (short lifetime of excited state, poor signal to background limit, small amount of energy transfer above 80 Å).[16]

For these reasons luminescent lanthanoid complexes, such as Eu^{3+} or Tb^{3+} chelates, have been widely used as donors in a simple modification to the standard FRET generally referred to as luminescence (or lanthanoid-based) resonance energy transfer (LRET).[16] The LRET system relies on the same fundamental mechanism, however the unique optical properties of lanthanoid emission (narrow and intense bands, long emission lifetime, absence of luminescence self-quenching processes) offer several potential advantages that can overcome FRET drawbacks. Hamachi and coauthors showed that ratiometric sensing of complicated saccharide derivatives is possible using LRET between an engineered lectin biosensor complexed with Tb^{3+}.[17] To the best of our knowledge, however, no examples of the use of pre-organized lanthanoid antenna complexes in the study of carbohydrate–protein interactions have been reported in the literature. In our opinion, the high values of emission quantum yields of the complexed lanthanoid ion and the excellent spectral overlap with acceptor counterparts could have a strong effect in the resonance energy transfer process, increasing the precision of energy transfer measurements over long distances based on donor lifetime. In addition the remarkable chemical, thermal, kinetic and photophysical stability of lanthanoid metal complexes together with their very low toxicity[18] make them suitable candidates for the development of *in vivo* biosensing technologies.

In this paper we describe (i) the synthesis of the highly luminescent Tb^{3+}-DO3A complex (**Tb⊂1**) bearing an α-D-mannose residue on the side chain; (ii) the complete photophysical characterization of **Tb⊂1** and of rhodamine-B-isothiocyanate-labeled Con A (RITC-Con A) in phosphate buffer solution; and (iii) the study of complex **Tb⊂1** emission in the presence of increasing amounts of RITC-Con A. The results obtained indicate a decrease of the Tb^{3+} ion emission intensity and sensitization of RITC emission due to the binding between Con A and the mannose residue of the Tb^{3+} complex, thus promoting the LRET process in which the terbium complex behaves as the donor and RITC as the acceptor (Fig. 1). The lifetime of the luminescence originating from the $^5D_4$ excited state to the $^7F_6$ level of the Tb^{3+} ion decreases as

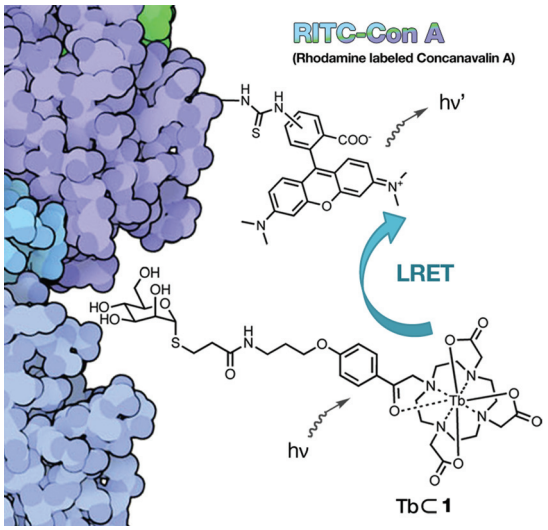


Fig. 1 Schematic representation of luminescence resonance energy transfer (LRET) involving the **Tb⊂1** complex and RITC-Con A.

the concentration of Con A increases. Since in this system both the intensity of the emission and lifetimes are related to the concentration of Con A, the studied complex is behaving as a sensor with respect to Con A.

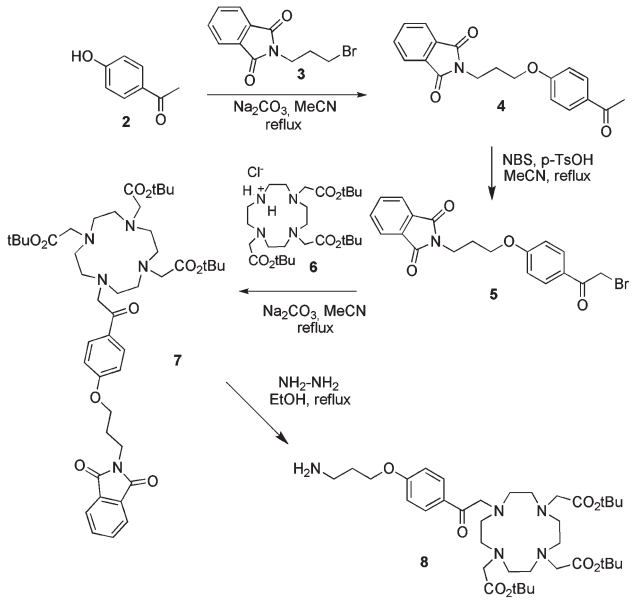
Results and discussion

Design and synthesis of ligands and the complex

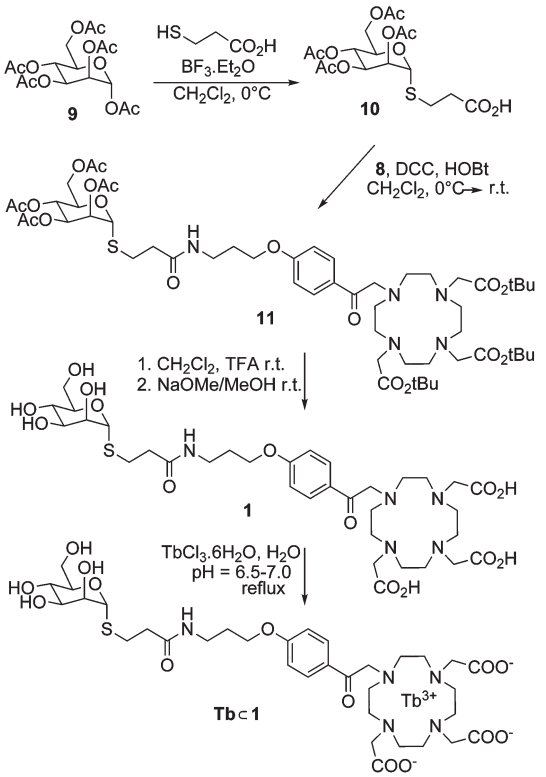
The structure of ligand **1** was designed to perform efficiently the two functions of lectin recognition and sensitization of the metal emission. 1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic acid (DO3A) was selected as the Ln-chelating subunit because of the high thermodynamic and kinetic stabilities which are well assessed for its lanthanoid complexes.[19] An acetophenone was introduced at the 10 position of the macrocycle. The choice of such a chromophore was dictated by its efficiency in the sensitization process that overcomes the low absorption cross section of Tb^{3+} ($\varepsilon < 1\ M^{-1}\ cm^{-1}$).[20] In addition the functionalization at the *para* position of the phenyl ring with a short alkyl chain allows the easy introduction of a primary amino group that can be used to bond a tailored lectin binding residue, *i.e.* α-D-mannose.

The synthesis of the protected ligand **8** bearing the amino group on the lateral chain is shown in Scheme 1.

Reaction of commercially available 4-hydroxyacetophenone **2** with 3-bromopropylphthalimide **3** was carried out in CH_3CN and refluxed for 3 days, in the presence of solid Na_2CO_3 as a base, and afforded 3-(4-acetylphenoxy)-propylphthalimide **4** in 97% yield. Treatment of **4** with *N*-bromosuccinimide (NBS) and *p*-toluenesulphonic acid (*p*-TsOH) in CH_3CN at reflux for 2 h produced 3-[4-(2-bromoacetyl)phenoxy]-propylphthalimide **5**[21] with a 68% yield. Alkylation of DO3A tris(*tert*-butyl) ester hydrochloride **6**[22,23] with **5** carried out in CH_3CN at reflux in the presence of solid Na_2CO_3 as a base afforded **7** in 91%



Scheme 1 Synthesis of ligand **8** bearing the amino group on the lateral chain.



Scheme 2 Synthesis of lanthanoid complex **Tb⊂1**.

yield, after purification of the crude product by column chromatography. Selective deprotection of the phthalimido group of **7** was obtained with hydrazine hydrate in EtOH at reflux for 3 h to afford **8** in 84% yield as a yellow solid foam.

The preparation of related complex **Tb⊂1** has been carried out following the synthesis reported in Scheme 2.

The 3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)propanoic acid **10** was obtained in 69% yield according to a procedure reported for an analogous galactose-derivative,[24] by reaction of pentaacetyl α-D-mannose **9** with 3-mercaptopropionic acid in anhydrous CH_2Cl_2 and in the presence of $BF_3{\cdot}Et_2O$ at 0 °C for 9 h. Reaction of **10** with the DO3A amino derivative **8** was carried out in anhydrous CH_2Cl_2 in the presence of 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC).[25] The temperature was raised from 0 °C to RT overnight and the reactants were continuously stirred. This procedure afforded **11** as a solid yellow foam in 65% yield after purification by column chromatography. Deprotection of **11** was achieved in two steps: it was first treated with CF_3COOH in anhydrous CH_2Cl_2 for 48 h at room temperature to remove the three *tert*-butyl ester protecting groups and then it was reacted with sodium methoxide in CH_3OH for 24 h at RT to remove the four acetate groups on the mannose residue, affording the crude ligand **1**. This product was purified by a short Amberlite XAD 1600T column (from 100% H_2O to 80 : 20 H_2O–acetone) to give 61% yield (over two steps) of the title compound as a white crystalline solid.

The **Tb⊂1** complex was prepared by addition of a solution of $TbCl_3{\cdot}6H_2O$ in H_2O into a water solution of ligand **1** under magnetic stirring and adjusting the pH at 6.5–7.0 with 2% aqueous NaOH. The reaction mixture was then heated at reflux for 4 h. Purification of the residue by a short Amberlite XAD 1600T column eluting first with 100% H_2O, to remove all inorganic salts, and then with 90 : 10 H_2O–MeCN (v/v) afforded the **Tb⊂1** in 83% yield as a white crystalline solid.

Photophysical characterization and LRET

In order to verify the effectiveness of **Tb⊂1** as a sensor for the detection of lectins by means of luminescent energy transfer (LRET), we focused our attention on model plant lectin concanavalin A (Con A) labeled with rhodamine-B-isothiocyanate (RITC-Con A).

Upon excitation at 355 nm, Fig. 2 shows the spectral behaviour of **Tb⊂1** (donor), which emits strong fluorescence at 480, 550, 580 and 625 nm from the $^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$, $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_3$ transitions of the Tb^{3+} ion, respectively. Less intense peaks are observed in the 650 to 700 nm region of the spectrum and are attributed to the $^5D_4 \rightarrow {}^7F_{2,1,0}$ transitions of the Tb^{3+} ion.

The emission spectra of mixed solutions of the lanthanoid complex **Tb⊂1** (1.9 mM) and increasing concentrations of RITC-Con A (from 0.5 to 8.3 μM) in PBS at pH 7.4 were recorded and the results are reported in Fig. 3a as a function of acceptor concentration. The LRET process was evidenced by the decrease in the emission intensities of the peaks at 550 and 480 nm corresponding to the $^5D_4 \rightarrow {}^7F_5$ and $^5D_4 \rightarrow {}^7F_6$ transitions of Tb^{3+} respectively. In addition the occurrence of lanthanoid-based energy transfer phenomena was also supported visually by the color change under UV irradiation of the solutions, from green (typical of Tb^{3+} emission) to reddish (Fig. 3b).

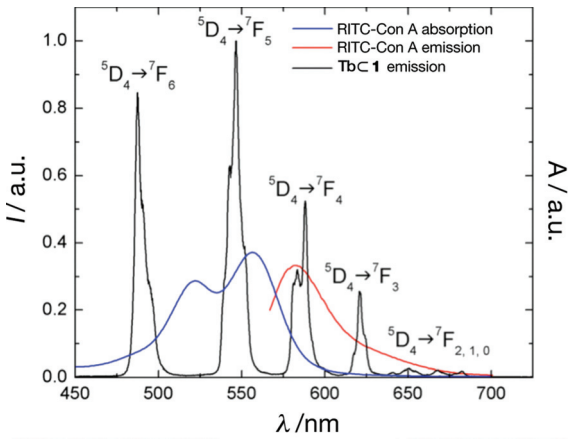


Fig. 2 Absorption and emission spectra (λ_{ex} = 557 nm) of RITC-Con A and the emission spectrum (λ_{ex} = 355 nm) of the **Tb⊂1** complex.

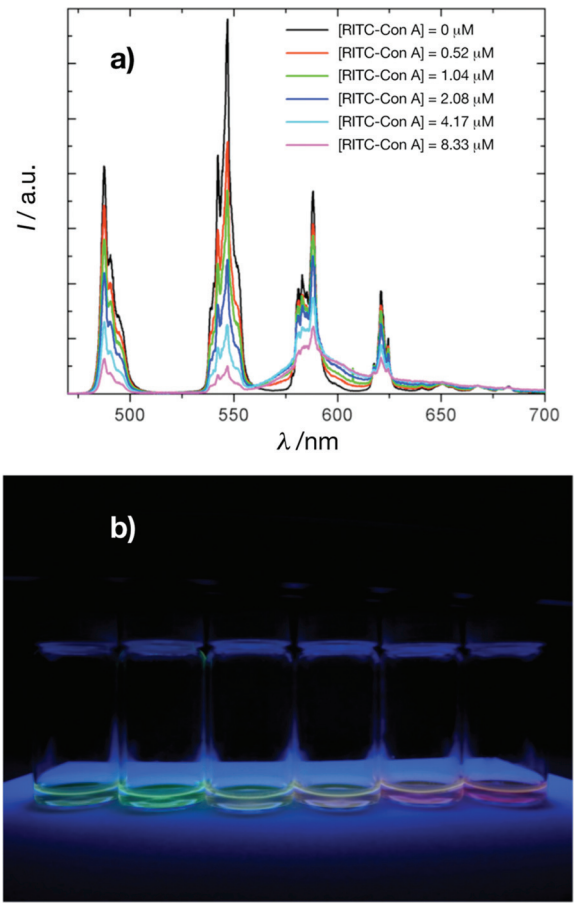


Fig. 3 (a) Emission spectra of solutions containing a fixed concentration of **Tb⊂1** (1.9 mM) and increasing concentration of RITC-Con A (from 0.5 to 8.3 μM); (b) image of the same solutions containing a fixed concentration of **Tb⊂1** (1.9 mM) and increasing concentration of RITC-Con A (from 0.5 to 8.3 μM, from left to right) illuminated with a UV-lamp (325 nm).

The LRET process was confirmed by measuring the luminescence lifetime of the $^5D_4 \rightarrow {}^7F_5$ transition of the Tb^{3+} ion as a function of the acceptor concentration. As shown in Fig. 4

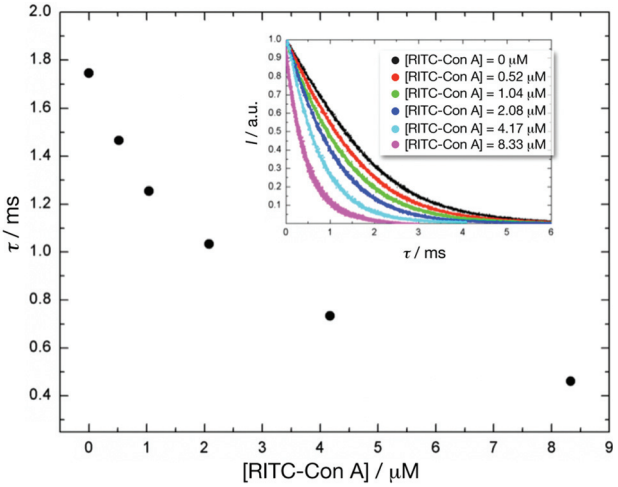


Fig. 4 Lifetime of the $^5D_4$ state of Tb^{3+} measured at 550 nm for different concentrations of RITC-Con A. The inset shows the decay curves used to calculate the lifetime values.

Table 1 Lifetimes, energy transfer rates and efficiencies for the different solutions used in this article

[RITC-Con A]/ μM	[RITC-Con A]/ [**Tb⊂1**] 10^{-3}	τ/ms	k_{ET}/ms^{-1}	E from τ	E from I_{total}
0		1.75			
0.52	0.268	1.47	0.11	0.16	0.16
1.04	0.536	1.25	0.23	0.29	0.23
2.08	1.07	1.03	0.40	0.41	0.32
4.17	2.15	0.73	0.80	0.58	0.46
8.33	4.30	0.46	1.60	0.74	0.59

and Table 1, where the lifetime data at 550 nm are collected, the donor-only signal is a single exponential with a lifetime of 1.75 ms. By adding increasing concentrations of the acceptor the lifetime of the donor decreases to a value of 0.46 ms (Table 1). The decrease in the excited state lifetime of the donor is due to the fact that the acceptor provides an additional relaxation pathway of the donor excited state.

In order to exclude the intervention of non-selective LRET processes in the observed phenomena, we compared the behavior of **Tb⊂1** with that of a similar complex, namely CH_3O-Tb^{3+}·DO3A[26] that does not have mannose residues able to give recognition of the lectin (Fig. 5). The lifetime decay curves of two solutions at the same concentration (1.5×10^{-5} M) of **Tb⊂1** and CH_3O-Tb^{3+}·DO3A, respectively, were measured in the presence and in the absence of a fixed concentration of RITC-Con A (0.5 μM). Due to the overlap of emission spectra of the $^5D_4 \rightarrow {}^7F_4$ transition of the Tb^{3+} ion and RITC-Con A, at this wavelength more noticeable differences are evidenced.

Whereas the decay curve of CH_3O-Tb^{3+}·DO3A maintains the same exponential shape both in the presence and in the absence of the acceptor counterpart, by adding RITC-Con A to **Tb⊂1** the temporal behaviour of the intensity shows a short initial rise time, associated with the population of the

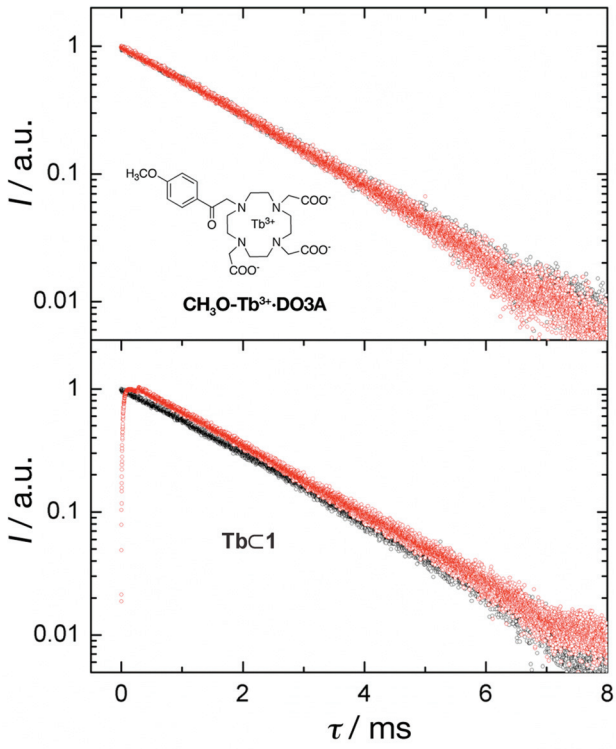


Fig. 5 Comparison of the decay curves of the $^5D_4$ state of Tb^{3+} measured at 588 nm for the CH_3O-Tb^{3+}·DO3A (top) and **Tb⊂1** (bottom) in the presence (red) and absence (black) of RITC-Con A.

RITC-Con A excited level *via* LRET from the Tb^{3+}, followed by an analogous exponential decrease as obtained at 550 nm wavelength. This different behaviour in the lifetime curves between the CH_3O-Tb^{3+}·DO3A (which does not present a rise time or a decrease in the lifetime) and the **Tb⊂1** (which presents the rise time and the decrease in the lifetime) demonstrates that efficient LRET is possible only after recognition of RITC-Con A protein by **Tb⊂1** *via* the mannose molecule which ensures close proximity between donor and acceptor molecules.

The extent of energy transfer may be evaluated from the change in the excited state lifetime of the donor. It is well established that the measured lifetime (τ_D) in the absence of the acceptor can be expressed as

$$\tau_D = \frac{1}{k} = \frac{1}{k_r + k_{nr}} \quad (1)$$

where k_r and k_{nr} are the radiative and non-radiative rates respectively. In the presence of the acceptor the lifetime of the donor may be expressed as

$$\tau_{DA} = \frac{1}{k_{DA}} = \frac{1}{k_r + k_{nr} + k_{ET}} \quad (2)$$

where k_{ET} represents the energy transfer rate. Since the radiative and non-radiative rates may be considered constant, the decrease in the lifetime of the donor in the presence of the acceptor is due to energy transfer. Therefore, the energy transfer rate may be calculated from eqn (1) and (2) and may be expressed as

$$k_{ET} = \frac{1}{\tau_{DA}} - \frac{1}{\tau_D} \quad (3)$$

Table 1 shows the calculated energy transfer rates (k_{ET}), which increase with increasing concentration of the acceptor. The highest transfer rate of 1.60 ms^{-1} is achieved at an acceptor/donor ratio of 4.30×10^{-3} : 1. The LRET efficiencies, E, can be obtained from the measured lifetime values or the luminescence intensities using eqn (4) or (5) respectively:

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (4)$$

$$E = 1 - \frac{I_{DA}}{I_D} \quad (5)$$

where τ_{DA} and τ_D represent the luminescence lifetimes and I_{DA} and I_D represent the luminescence intensities in the presence and absence of the acceptor respectively. As shown in Table 1, the LRET efficiency E is enhanced with increasing acceptor concentration and the efficiencies calculated using the emission intensities are in good agreement with the ones calculated with the lifetime. The high energy transfer efficiency is attributed to the excellent spectral overlap of the donor emission and the acceptor absorption.

From the emission spectra in Fig. 3a, the binding constant between the **Tb⊂1** complex and RITC-Con A was calculated using eqn (6):[27]

$$\frac{[\text{RITC-ConA}]F_0}{\Delta F} = \frac{[\text{RITC-ConA}]F_0}{\Delta F_{max}} + \frac{F_0}{\Delta F_{max} K_a} \quad (6)$$

where $\Delta F = F_0 - F$, $\Delta F_{max} = F_0 - F_{max}$. F_0 and F represent the intensity of the **Tb⊂1** complex before and after the addition of RITC-Con A, respectively. F_{max} is the maximum intensity at 585 nm obtained in the presence of the lectin while K_a is the binding constant (in M^{-1}). Fig. 6 shows the product of the

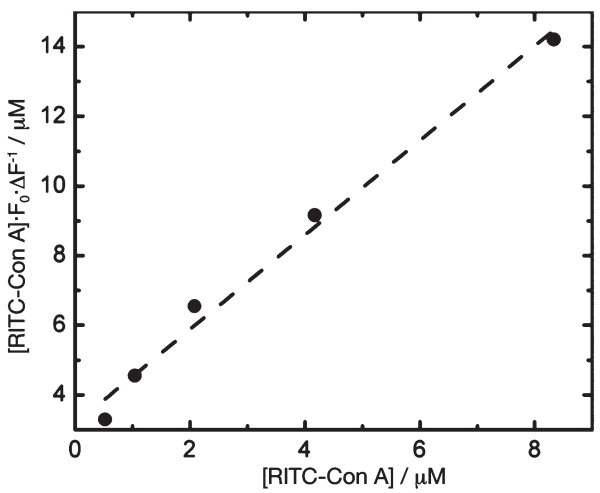


Fig. 6 Product of the concentration of RITC-Con A and $F_0/\Delta F$ as a function of the concentration of RITC-Con A calculated from the spectra.

concentration of RITC-Con A and $F_0/\Delta F$ as a function of the concentration of RITC-Con A, which yields a straight line (R^2 = 0.9852). From the slope and the intercept the binding constant K_a was calculated to be $(4.3 \pm 0.7) \times 10^5$ M^{-1}. This value is one order of magnitude larger than values previously reported,[14,28] which clearly indicates that the **Tb⊂1** is a very efficient donor for energy transfer assays to study the binding-interactions between α-D-mannose and the lectin Con A.

Conclusions

We have synthesized a new luminescent lanthanoid complex, namely **Tb⊂1**, bearing a mannose function on a lateral chain. The binding affinity of such a compound towards concanavalin A labeled with rhodamine-B-isothiocyanate (RITC-Con A) was demonstrated by optical methods. In fact the spectroscopic investigation of mixed solutions of lanthanoid complex **Tb⊂1** and RITC-Con A showed that an effective luminescence resonance energy transfer took place from the excited state of the Tb^{3+} ion to rhodamine, resulting in a decrease of fluorescence intensity and lifetime of the donor. To the best of our knowledge the one presented here is the first example of the use of a pre-organized complex of the lanthanoid ion in the study of carbohydrate–protein interactions by LRET. The binding constant K_a between **Tb⊂1** and RITC-Con A was calculated and its value was found to be one order of magnitude larger than those previously reported for similar compounds. However, since lectin–carbohydrate adhesion generally involves multivalent interactions, we intend to investigate in the future glycodendrimer derivatives of the luminescent lanthanoid complex as optical biosensors in the detection of many biological events.

Experimental section

Materials and methods

All available chemicals and solvents were purchased from commercial sources and were used without any further purification. Thin layer chromatography (TLC) was conducted on plates precoated with silica gel Si 60-F254 (Merck, Darmstadt, Germany). Column chromatography was conducted by using silica gel Si 60, 230–400 mesh, 0.040–0.063 mm (Merck, Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 (400 and 100.6 MHz, respectively); chemical shifts are indicated in parts per million downfield from $SiMe_4$, using the residual proton ($CHCl_3$ = 7.26 ppm, $(CH_3)_2SO$ = 2.50 ppm, HOD = 4.80 ppm) and carbon ($CDCl_3$ = 77.0 ppm, $(CD_3)_2SO$ = 40.45 ppm) solvent resonances as the internal reference. Protons and carbon assignments were achieved by ^{13}C-APT, ^{1}H–^{1}H COSY, and ^{1}H–^{13}C heteronuclear correlation experiments. Coupling constant values J are given in Hz. Low resolution mass spectra were recorded on a Finnigan LCQ Advantage Thermo-spectrometer equipped with an electrospray ion trap. The high resolution mass spectrum of the **Tb⊂1** complex was obtained with an electrospray ion-trap mass spectrometer ICR-FTMS APEX II (Bruker Daltonics) by the Centro Interdipartimentale Grandi Apparecchiature (C.I.G.A.) of the University of Milano. Elemental analyses were carried out by the Departmental Service of Microanalysis (University of Milano).

3-(4-Acetylphenoxy)-propylphthalimide (4). A mixture of 4-hydroxyacetophenone **2** (1.36 g, 10 mmol), 3-bromopropylphthalimide **3** (3.0 g, 11 mmol) and Na_2CO_3 (3.18 g, 30 mmol) in MeCN (30 mL) was refluxed for 72 h under magnetic stirring. After cooling, the mixture was diluted with CH_2Cl_2, filtered through a glass frit and the solvent was removed at reduced pressure. The solid residue was purified by crystallization from MeCN (20 mL) to give the title compound (2.3 g) as a white solid. The mother liquor was concentrated at reduced pressure and the residue purified by chromatography (silica gel, CH_2Cl_2–MeOH 98.5 : 1.5) to give a second portion of the title compound (846 mg) as a white solid (overall yield: 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.22 (quin, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 2.54 (s, 3H, $-COCH_3$), 3.93 (t, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 4.11 (t, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 6.82 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H), 7.73 (m, 2H, Phthal-H), 7.84 (m, 2H, Phthal-H), 7.88 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 26.3 ($COCH_3$), 28.2 ($NCH_2CH_2CH_2O$), 35.4 ($NCH_2CH_2CH_2O$), 65.9 ($NCH_2CH_2CH_2O$), 114.1 (Ar-CH), 123.3 (Phthal-CH), 130.4, 130.5 (Ar-CH), 132.1 (Phthal-C), 134.0 (Phthal-CH), 162.6, 168.4 (Phthal-CO), 196.7 ($COCH_3$); ESI-MS: m/z = 346.2 ([M + Na^+]), calcd for $C_{19}H_{17}NaNO_4$ = 346.1; elemental analysis calcd (%) for $C_{19}H_{17}NO_4$: C 70.58, H 5.30; found: C 70.66, H 5.31.

3-[4-(2-Bromoacetyl)phenoxy]-propylphthalimide (5). *N*-Bromosuccinimide (1.25 g, 7 mmol) was added in one portion to a solution of **4** (2.26 g, 7 mmol) and *p*-toluenesulfonic acid monohydrate (2.0 g, 10.5 mmol) in MeCN (100 mL). The reaction mixture was refluxed under magnetic stirring for 2 h. After cooling, the solvent was removed at reduced pressure and the residue dissolved in CH_2Cl_2 (150 mL). The organic phase was washed with water (100 mL), dried over $MgSO_4$ and after filtration, the solvent was removed at reduced pressure. The solid residue was purified by chromatography (silica gel, CH_2Cl_2–MeOH 98.5 : 1.5) to give the title compound as a white solid (1.91 g, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.22 (quin, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 3.92 (t, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 4.11 (t, ^3J(H,H) = 6.0 Hz, 2H, $NCH_2CH_2CH_2O$), 4.38 (s, 2H, CH_2Br), 6.84 (d, ^3J(H,H) = 7.0 Hz, 2H, Ar-H), 7.72 (m, 2H, Phthal-H), 7.84 (m, 2H, Phthal-H), 7.91 (d, ^3J(H,H) = 7.0 Hz, 2H, Ar-H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 28.2 ($NCH_2CH_2CH_2O$), 30.7 (CH_2Br), 35.3 ($NCH_2CH_2CH_2O$), 66.1 ($NCH_2CH_2CH_2O$), 114.4 (Ar-CH), 123.3 (Phthal-CH), 127.0, 131.3 (Ar-CH), 132.1 (Phthal-C), 134.0 (Phthal-CH), 163.3, 168.4 (Phthal-CO), 189.9 ($COCH_2Br$); ESI-MS m/z = 402.1 ([M + H^+]), calcd for $C_{19}H_{17}BrNO_4$ = 402.0; elemental analysis calcd (%) for $C_{19}H_{16}BrNO_4$: C 56.73, H 4.01, N 3.48; found: C 56.68, H 4.02, N 3.47.

10-[4-(3-Phthalimidopropyloxy)benzoylmethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl)

ester (7). A mixture of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester hydrochloride **6** (0.551 g, 1 mmol), **5** (0.480 g, 1 mmol) and Na_2CO_3 (1.06 g, 10 mmol) in MeCN (20 mL) was refluxed for 30 h under magnetic stirring. After cooling, the mixture was filtered through a glass frit and the solvent was removed at reduced pressure. The residue was dissolved in CH_2Cl_2 (150 mL) and the organic phase was washed with water (100 mL) and dried over $MgSO_4$. After filtration, the solvent was removed at reduced pressure and the residue was purified by chromatography (silica gel, CH_2Cl_2–MeOH 95 : 5) to give the title compound as a white solid (778 mg, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25–1.51 (m, 27H, C(CH_3)$_3$), 2.0–3.5 (m, 24H), 3.92 (m, 4H), 4.08 (t, ^3J(H,H) = 6.0 Hz, 2H, NCH$_2$CH$_2$CH_2O), 6.83 (d, ^3J(H,H) = 8.8 Hz, 2H, Ar-H), 7.73 (m, 2H, Phthal-H), 7.85–7.81 (m, 4H, Phthal-H and Ar-H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 27.8 (C(*C*H$_3$)$_3$), 27.9 (C(*C*H$_3$)$_3$), 28.2 (HNCH$_2$*C*H$_2$CH$_2$O), 35.3 (HN*C*H$_2$*C*H$_2$CH$_2$O), 55.6 (N–C(2)H, N–C(3)H, N–C(5)H, N–C(6)H), 55.9 (N–C(8)H, N–C(9)H, N–C(11)H, N–C(12)H), 59.8 (N(1)*C*H$_2$CO$_2$*t*Bu, N(4)-*C*H$_2$CO$_2$*t*Bu, N(7)*C*H$_2$CO$_2$*t*Bu), 65.8 (HNCH$_2$CH$_2$*C*H$_2$O), 81.8 (*C*-(CH$_3$)$_3$), 81.9 (*C*(CH$_3$)$_3$), 114.2 (Ar-CH), 123.3 (Ar-CH), 128.9, 129.8 (Phthal-CH), 132.1 (Phthal-C), 134.1 (Phthal-CH), 162.9, 168.4 Phthal-CO), 172.8 (*C*O$_2$*t*Bu), 197.8 (Ar-CO); ESI-MS *m*/*z* = 858.5 ([*M* + Na$^+$]), calcd for $C_{45}H_{65}NaN_5O_{10}$ = 858.5; elemental analysis calcd (%) for $C_{45}H_{65}N_5O_{10}$: C 64.65, H 7.84, N 8.38; found: C 64.55, H 7.86, N 3.46.

10-[4-(3-Aminopropyloxy)benzoylmethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester (8). To a solution of **7** (1.55 g, 1.85 mmol) in EtOH (20 mL) was added hydrazine monohydrate (0.9 mL) and the solution refluxed under magnetic stirring for 3 h. After cooling, the solvent was removed at reduced pressure and the residue was dissolved in CH_2Cl_2 (20 mL). The suspension was filtered through a glass frit to remove the phthalhydrazide and the solution was concentrated at reduced pressure. This treatment was repeated three times. After evaporation of the solvent, the title compound was obtained as a yellow foam (1.12 g, 84%). This product was used without further purification. ^{1}H NMR (400 MHz, MeOD, 60 °C): the signals from the cyclen moiety, NCH$_2$CH$_2$N and NCH$_2$CO, appear in the range δ 2.0–3.3 as a set of broad signals overlapped with signals from the aliphatic chain. Selected signals: δ 1.2–1.5 (m, 27H, C(CH_3)$_3$), 3.98 (br s, 2H, N(10)CH_2CO), 4.16 (t, ^3J(H,H) = 6.1 Hz, 2H, NCH$_2$CH$_2$CH_2O), 6.99 (d, ^3J(H,H) = 8.8 Hz, 2H, Ar-H), 7.94 (d, ^3J(H,H) = 8.8 Hz, 2H, Ar-H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 26.9 (C(*C*H$_3$)$_3$), 27.0 (C(*C*H$_3$)$_3$), 30.6 (HNCH$_2$*C*H$_2$CH$_2$O), 37.9 (HN*C*H$_2$*C*H$_2$CH$_2$O), 50.5 (N(10)*C*H$_2$CO), 55.4 (N–C(2)H, N–C(3)-H, N–C(5)H, N–C(6)H), 55.6 (N–C(8)H, N–C(9)H, N–C(11)H, N–C(12)H), 59.6 (N(1)*C*H$_2$CO$_2$*t*Bu, N(4)*C*H$_2$CO$_2$*t*Bu, N(7)-*C*H$_2$CO$_2$*t*Bu), 66.0 (HNCH$_2$CH$_2$*C*H$_2$O), 81.7 (*C*(CH$_3$)$_3$), 114.1 (Ar-CH), 129.1, 129.7 (Ar-CH), 163.4, 173.2 (*C*O$_2$*t*Bu), 198.3 (Ar-CO); ESI-MS *m*/*z* = 728.4 [*M* + Na$^+$], calcd for $C_{37}H_{63}NaN_5O_8$ = 728.4; elemental analysis calcd (%) $C_{37}H_{63}N_5O_8$: C 62.95, H 8.99, N 9.92; found: C 62.89, H 9.01, N 9.90.

3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosylthio)propanoic acid (10). To a solution of pentaacetyl α-D-mannose **9** (3.0 g, 7.69 mmol) in dry dichloromethane (40 mL) was added under an inert atmosphere 3-mercaptopropionic acid (2.7 mL, 31 mmol) and the solution was cooled to 0 °C. After the slow addition of BF_3·Et_2O (3.0 mL, 23.7 mmol), the mixture was stirred at 0 °C for 9 h and then diluted with dichloromethane (20 mL). The mixture was washed with water (3 × 20 mL) and brine (20 mL) and dried over $MgSO_4$. After filtration, the solvent was removed at reduced pressure and the crude was purified by chromatography (silica gel, *n*-hexane–AcOEt 50 : 50, 1% AcOH) to give the title compound (2.3 g, 69%) as a transparent oil. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 1.92 (s, 3H, OCOCH_3), 2.00 (s, 3H, OCOCH_3), 2.01 (s, 3H, OCOCH_3), 2.09 (s, 3H, OCOCH_3), 2.57 (t, ^3J(H,H) = 7.1 Hz, 2H, SCH$_2$CH_2CO$_2$H), 2.79 (t, ^3J(H,H) = 7.1 Hz, 2H, SCH_2CH$_2$CO$_2$H), 4.01 (dd, ^2J(H,H) = 12.0 Hz, ^3J(H,H) = 2.3 Hz, 1H, 6a-H), 4.16 (dd, ^2J(H,H) = 12.0 Hz, ^3J(H,H) = 5.8 Hz, 1H, 6b-H), 4.23–4.27 (m, 1H, 5-H), 5.00 (dd, ^3J(H,H) = 10.0 Hz, ^3J(H,H) = 3.4 Hz, 1H, 3-H), 5.09 (t, ^3J(H,H) = 10.0 Hz, 1H, 4-H), 5.16–5.18 (m, 1H, 2-H), 5.5 (s, 1H, 1-H); ^{13}C NMR (100.6 MHz, DMSO-d$_6$); δ 20.0 (OCO*C*H$_3$), 26.4 (S*C*H$_2$CH$_2$CO$_2$H), 34.7 (SCH$_2$*C*H$_2$CO$_2$H), 62.4 (C-6), 66.1 (C-4), 69.0 (C-5), 69.4 (C-3), 70.4 (C-2), 82.1 (C-1), 169.9 (O*C*OCH$_3$), 170.0 (O*C*OCH$_3$), 170.1 (O*C*OCH$_3$), 170.5 (O*C*OCH$_3$), 173.3 (*C*OOH); ESI-MS *m*/*z* = 459.1 ([*M* + Na$^+$]), calcd for $C_{17}H_{24}NaO_{11}S$ = 459.1; 434.9 ([M − H]$^-$, calcd for $C_{17}H_{23}O_{11}S$ = 435.1; elemental analysis calcd (%) for $C_{17}H_{24}O_{11}S$: C 46.78, H 5.54; found: C 46.84, H 5.55.

10-{4-[3-(3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosylthio)-propanamido)propyloxy]-benzoylmethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester (11). To a solution of **8** (705 mg, 1 mmol) and **10** (452 mg, 1.04 mmol) in dry dichloromethane (30 mL) was added under an inert atmosphere 1-hydroxybenzotriazole (HOBt, 147 mg, 1.09 mmol) and the mixture cooled to 0 °C. After the dropwise addition of a solution of dicyclohexylcarbodiimide (DCC, 225 mg, 1.09 mmol) in dry dichloromethane (10 mL), the mixture was allowed to reach room temperature and further stirred overnight. After filtration of precipitated dicyclohexylurea, the mixture was washed with saturated $NaHCO_3$ (2 × 20 mL) and brine (20 mL) and dried over $MgSO_4$. After filtration, the solvent was removed at reduced pressure and the crude product was purified by chromatography (silica gel, CH_2Cl_2–MeOH 95 : 5) to give the title compound (732 mg, 65%) as a yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$): the signals from the cyclen moiety, NCH$_2$CH$_2$N and NCH$_2$CO, appear in the range δ 2.0–3.5 as a set of broad signals overlapped with signals from the acetate groups and the aliphatic chains. Selected signals: δ 1.10–1.80 (m, 27H, C(CH_3)$_3$), 4.09 (dd, ^2J(H,H) = 12.0 Hz, ^3J(H,H) = 2.3 Hz, 1H, (Man)6a-H), 4.18 (t, ^3J(H,H) = 6.6 Hz, 2H, HNCH$_2$CH$_2$CH_2O), 4.33 (dd, ^3J(H,H) = 12.0 Hz, ^3J(H,H) = 5.3 Hz, 1H, (Man)6b-H), 4.39–4.41 (m, 1H, (Man)5-H), 5.20–5.32 (m, 2H, (Man)3-H, (Man)4-H), 5.33–5.34 (m, 2H, (Man)1-H and (Man)2-H), 6.98 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H), 7.71 (t, ^3J(H,H) = 5.7 Hz, 1H, *H*NCH$_2$CH$_2$CH$_2$O), 7.82 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 20.6 (OCO*C*H$_3$), 20.7 (OCO*C*H$_3$), 20.8 (OCO*C*H$_3$), 20.9 (OCO*C*H$_3$), 27.1 (S*C*H$_2$CH$_2$CONH), 27.8 (C(*C*H$_3$)$_3$), 27.9 (C(*C*H$_3$)$_3$), 28.9

($HNCH_2CH_2CH_2O$), 36.1 (SCH_2CH_2CONH), 36.3 ($HNCH_2$-CH_2CH_2O), 55.6 (N–C(2)H, N–C(3)H, N–C(5)H, N–C(6)H), 55.8 (N–C(8)H, N–C(9)H, N–C(11)H and N–C(12)H), 59.7 (N(1)-CH_2CO_2tBu, N(4)CH_2CO_2tBu, N(7)CH_2CO_2tBu), 62.5 ((Man) C-6), 66.5 ((Man)C-4), 66.8 ($HNCH_2CH_2CH_2O$), 68.8 ((Man)C-5), 69.6 ((Man)C-3), 70.9 ((Man)C-2), 82.2 ($C(CH_3)_3$), 82.4 (C-$(CH_3)_3$), 82.5 ((Man)C-1), 114.7 (CH-Ar), 128.2, 129.9 (CH-Ar), 163.7, 169.7 ($OCOCH_3$), 169.8 ($OCOCH_3$), 169.9 ($OCOCH_3$), 170.8 ($OCOCH_3$), 171.3 (CONH), 172.6 (CO_2tBu), 197.5 (ArCO); ESI-MS m/z = 1146.7 ($[M + Na]^+$), calcd for $C_{54}H_{85}NaN_5O_{18}S$ = 1146.5; elemental analysis calcd (%) for $C_{54}H_{85}N_5O_{18}S$: C 57.69, H 7.62, N 6.23. Found: C 57.61, H 7.62, N 6.22.

10-{4-[3-(3-(α-D-Mannopyranosylthio)-propanamido)propyloxy]-benzoylmethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (1). To a solution of **11** (700 mg, 0.62 mmol) in dry dichloromethane (12 mL) was added TFA (4 mL) and the mixture was left stirring at room temperature. After 48 h the solvent was removed at reduced pressure, the residue was redissolved in dichloromethane (10 mL) and the solvent removed at reduced pressure. This procedure was repeated three times. The residue was then triturated with diethyl ether giving a white powder. After removal of the solvent, the solid was dried under vacuum. ^{1}H NMR analysis showed complete removal of the *tert*-butyl groups. No further purification was undertaken. The solid material was dissolved in dry methanol (20 mL) and after the addition of NaOMe (260 mg, 4.8 mmol) the mixture was left stirring at room temperature for 24 h. The solution was adjusted to pH = 7 (pH paper) with Amberlite IR-120, filtered through a glass frit and the solvent was removed at reduced pressure. The solid residue was purified by a short Amberlite XAD 1600T column (from 100% H_2O to 80 : 20 H_2O–acetone) to give the title compound (300 mg, 61%) as a white crystalline solid. ^{1}H NMR (400 MHz, D_2O, 60 °C): δ 2.37 (quin, 2H, $HNCH_2CH_2CH_2O$), 2.92 (t, ^3J(H,H) = 6.7 Hz, 2H, SCH_2CH_2CONH), 3.17–3.30 (m, 2H, SCH_2CH_2CONH), 3.4–3.6 (br s, 8H, N–C(2)H, N–C(3)H, N–C(5)H and N–C(6)H), 3.72–3.9 (m, 10H, N–C(8)H, N–C(9)H, N–C(11)H, N–C(12)H, $HNCH_2CH_2CH_2O$), 3.95–4.30 (m, 12H, (Man)4-H, (Man)3-H N(4)CH_2CO_2H, (Man)6b-H), N(1)CH_2CO_2H, N(7)CH_2CO_2H, (Man)6a-H, (Man)5-H and (Man)2-H), 4.54 (t, ^3J(H,H) = 6.2 Hz, 2H, $HNCH_2CH_2CH_2O$), 5.62 (s, 1H, (Man)1-H), 7.43 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H), 8.43 (d, ^3J(H,H) = 8.9 Hz, 2H, Ar-H); ^{13}C NMR (100.6 MHz, D_2O, 60 °C): δ 27.4 (SCH_2CH_2CONH), 28.3 ($HNCH_2CH_2CH_2O$), 36.2 (SCH_2CH_2CONH), 36.6 ($HNCH_2CH_2CH_2O$), 48.7 (N–C(2)H or N–C(3)H), 48.9 (N–C(2)H or N–C(3)H), 51.6 (N–C(8)H and N–C(9)H), 54.3 (N(4)-CH_2COOH), 56.9 (N(1)CH_2COOH and N(7)CH_2COOH), 57.6 (N(10)CH_2COOH), 61.3 ((Man)C-6), 66.6 ($HNCH_2CH_2CH_2O$), 67.6 ((Man)C-4 or (Man)C-3), 71.5 ((Man)C-3 or (Man)C-4), 72.1 ((Man)C-2), 73.7 ((Man)C-5), 85.4 ((Man)C-1), 115.2 (CH-Ar), 128.7, 130.9 (CH-Ar), 163.5, 170.7 (CONH), 174.5 (N(4)-CH_2COOH), 174.8 (N(1)CH_2COOH and N(7)CH_2COOH), 197.9 (ArCO); ESI-MS m/z = 810.5 ($[M + Na]^+$), calcd for $C_{34}H_{53}NaN_5O_{14}S$ = 810; elemental analysis calcd (%) for $C_{34}H_{53}N_5O_{14}S$: C 51.83, H 6.78, N 8.89. Found: C 51.89, H 6.80, N 8.88.

10-{4-[3-(3-(α-D-Mannopyranosylthio)propanoyl)aminopropyl-oxy]benzoylmethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid terbium(III) complex (Tb⊂1). A solution of $TbCl_3 \cdot 6H_2O$ (53.4 mg, 0.14 mmol) in water (5 mL) was slowly added to a solution of **1** (100 mg, 0.13 mmol) in water (15 mL) keeping the pH of the solution at 6.5–7.0 by the addition of 2% aqueous sodium hydroxide. The reaction mixture was refluxed for 4 h, cooled to room temperature and concentrated at reduced pressure. The residue was purified by a short Amberlite XAD 1600T column eluting first with 100% H_2O to remove all inorganic salts and then with 90 : 10 H_2O–MeCN to give the title compound (100 mg, 83% yield) as a white crystalline solid. ESI-MS m/z = 966.22294 ($[M + Na]^+$), calcd for $C_{34}H_{50}$-$NaN_5O_{14}STb$ = 966.22206; elemental analysis calcd (%) for $C_{34}H_{50}N_5O_{14}STb$: C 43.27, H 5.34, N 7.42. Found: C 43.30, H 5.35, N 7.41.

Absorption, luminescence spectroscopy and dynamic studies

Concanavalin A-tetramethylrhodamine (RITC-Con A, Molecular Probe®), which contains 4 moles of dye per mole of lectin, was used. The absorption spectrum of RITC-Con A was recorded using a UV-VIS Cary spectrophotometer (Varian 100 Bio). Luminescence from the **Tb⊂1** complex was obtained upon excitation with the 3rd harmonic, 355 nm, of an Nd:YAG Q-switched laser (Quanta Ray, Spectra Physics) with a pulse frequency of 10 Hz and a pulse width of 6 ns. The luminescence and lifetime measurements were performed on samples dissolved in a phosphate buffer saline (PBS) at pH 7.4 in a quartz cuvette (1 cm path length). The emitted light was collected at π/2 with respect to the incident beam and subsequently dispersed by a 1 m Jarrell–Ash Czerny–Turner double monochromator with an optical resolution of ~0.15 nm. A thermoelectrically cooled Hamamatsu R943-02 photomultiplier tube detected the visible emissions. A preamplifier, model SR440 Standard Research Systems, processed the photomultiplier signals and a gated photon-counter model SR400 Standard Research Systems data acquisition system was used as an interface between the computer and the spectroscopic hardware. The signal was recorded under computer control using the Standard Research Systems SR465 software data acquisition/analyzer system. For the lifetime measurements the emitted signal was sent directly from the photomultiplier tube to a Tektronix TDS 520A (500 MHz, 500 Ms s^{-1}) oscilloscope.

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