

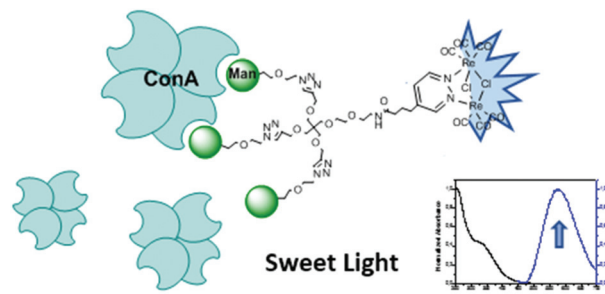
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Glycodendron–rhenium complexes as luminescent probes for lectin sensing

Alessandro Palmioli,* Monica Panigati and Anna Bernardi

Enhanced fluorescence emission of novel Re(I)-glycoprobes resulting from the specific recognition of carbohydrate-binding proteins as a potential tool in bioimaging applications.



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PAPER

Glycodendron–rhenium complexes as luminescent probes for lectin sensing†

Cite this: DOI: 10.1039/c8ob01838c

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Received 30th July 2018,
Accepted 5th September 2018

DOI: 10.1039/c8ob01838c

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The design, synthesis and characterization of novel glycoconjugate luminescent probes based on dinuclear rhenium(i) complexes are reported. A multivalent platform bearing different carbohydrate moieties (Glc, Gal and Man) was used to target carbohydrate-binding proteins (lectins), exploiting the unique photophysical characteristics of a Re(i) luminophore for protein sensing. Our results show that these glycoconjugates, non-luminescent in aqueous medium, are able to specifically bind different lectins (ConA and PNA) with a consequent enhancement of fluorescence emission. These findings suggest the use of Re(i)-based glycoconjugates as switch-on fluorescent probe tools in biological applications.

Introduction

The development of molecular probes capable of tracking and imaging biological key-events or conditions has become a very attractive research field.^{1–11} Due to their unique targeting properties derived from inherent structural diversity, glycoprobes represent a promising molecular tool in sensing, imaging and theragnostic applications.¹² In fact, a number of molecular recognition events between glycans and carbohydrate-binding receptors, called lectins, occur at the cellular surfaces and drive fundamental physiological processes such as cell signaling and immune system responses.¹³ Not least, these interactions play a key role in a number of pathological processes including cancer metastasis, bacterial infection and virus invasion.^{13–16} C-type lectin receptors (CLRs) are a large family of lectins widely found in plants, microorganisms and mammalian cells as multimeric soluble receptors or clustered membrane receptors that share a homologous Ca²⁺-dependent carbohydrate recognition domain (CRD). Microbial lectins usually act as adhesins or virulence factors in host infection.^{17,18} In mammalian cells, endocytic CLRs, such as ASGP-R (asialoglycoprotein receptor), trigger the receptor-mediated endocytosis of soluble ligands,¹⁹ and myeloid CLRs, such as DC-SIGN (Dendritic Cell-Specific Intercellular Adhesion Molecule 3-Grabbing Non-integrin), MR (Mannose Receptor) and MBL (Mannose-Binding Lectin), act as pattern recognition

receptors in innate immunity and contribute to the initiation of immune responses or inflammation processes.^{20,21} For these reasons, C-lectins are considered as promising biomarkers for disease diagnosis, targeted-therapy or sensing molecular tool development.^{22–24}

Recently, we described glycosylated luminescent probes exploiting the unique photophysical features of neutral dinuclear rhenium(i) complexes for bioimaging application.²⁵ In particular, we observed that glyco-Re derivatives show a good solubility in aqueous buffer and the glycosylation does not perturb the optical properties (*i.e.* large Stokes shifts, high quantum yield and long luminescence lifetimes) of the organometallic fluorophore, that is mainly due to the triplet metal-to-ligand charge transfer (³MLCT) character of the emitter.²⁶ Furthermore, glyco-Re-conjugates containing glucose and maltose are efficiently internalized by human cervical adenocarcinoma (HeLa) cells, showing interesting bioimaging properties and negligible cytotoxicity. Interestingly, the most soluble trivalent gluco-conjugate (**1**) (Fig. 1) shows a very poor luminescence quantum yield (<1%) in aqueous

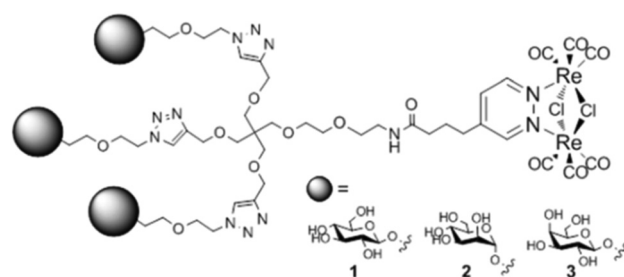


Fig. 1 Structure of multivalent rhenium-complex glycoconjugates, bearing three copies of β -D-glucopyranosidic (**1**), α -D-mannopyranosidic (**2**) or β -D-galactopyranosidic (**3**) moieties.

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† Electronic supplementary information (ESI) available: Full experimental details for the synthesis and characterization of multivalent glycoconjugates. See DOI: 10.1039/c8ob01838c

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buffer and is not internalized by HeLa cells.²⁵ However it is important to note that several members of this class of emitters display an increase of the emission intensity on going from solution to the solid state.^{27–29} This feature is ascribed to the slowing down of the roto-vibrational motions of the dinuclear scaffold, or of some of the substituents of the diazine ligand, in rigid environments. This phenomenon, first reported by Tang and coworkers,^{30,31} is indicated as the Aggregation Induced Emission (AIE) effect. Evidence collected on compound **1** and on this class of dinuclear complexes, prompted us to explore these multivalent glycoconjugates as tracers for membrane glycan-receptors with the idea to exploit their (1) very good water solubility and low cytotoxicity, (2) low background luminescence due to the poor quantum yields in aqueous buffer, and (3) capability as an environment-sensitive emitter probe. Even if the feasibility of this approach has been already demonstrated,^{32–36} for the first time here, multivalent neutral glyco-Re complexes (Fig. 1), differing for the sugar moiety, have been synthesized and characterized in order to develop AIE-active materials able to act as fluorescent sensors after their specific binding to different carbohydrate-binding proteins. The aim is to target specific membrane glycan receptors selectively expressed in certain types of cells, tissue or microorganisms.

Results and discussion

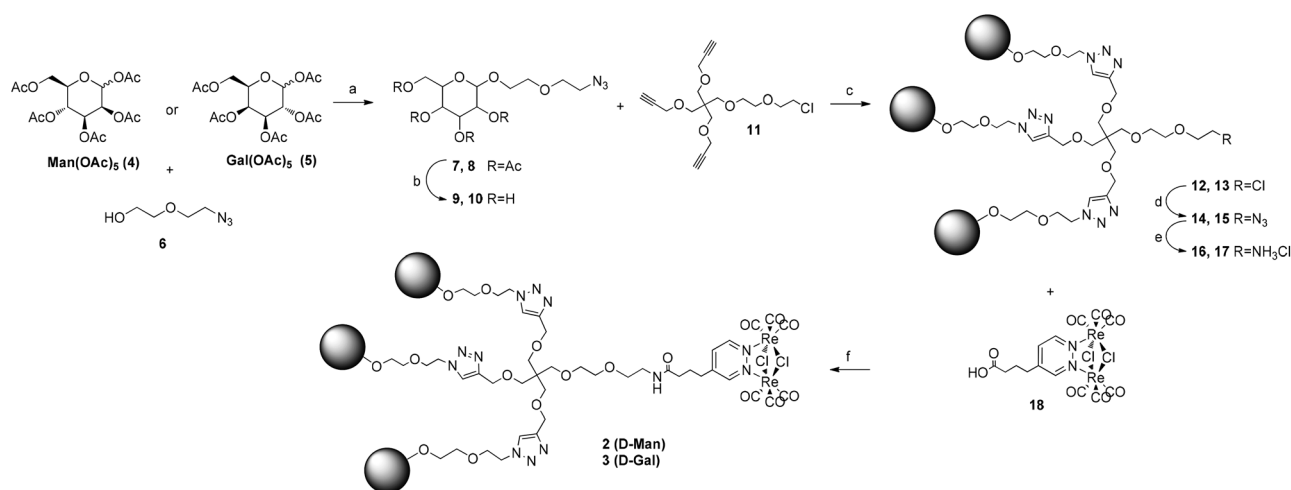
Design and synthesis of multivalent glycoconjugates

We designed compounds **2** and **3** in which D-glucose, the sugar moiety of compound **1**, was replaced by D-mannose and D-galactose moieties, respectively. For this purpose we followed the same strategy recently described^{25,37} and reported in the

Experimental section (Scheme 1). Briefly, the glycodendron was prepared from a trivalent alkyne scaffold (**11**) functionalized with 2-(2-azidoethoxy)ethyl- α -D-mannopyranoside (**9**) and 2-(2-azidoethoxy)ethyl- β -D-galactopyranoside (**10**) derivatives by a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. Then glycoconjugates were prepared by a condensation reaction with the rhenium complex in which the pyridazine ligand is endowed with a COOH coupling group (**18**) using HATU/DIPEA as a coupling agent. Finally, compounds **2** and **3** were isolated after reverse-phase C18 flash chromatography purification. As expected, these glycoconjugates were found to be soluble in aqueous buffer up to mM concentration. All compounds were fully characterized by means of NMR and HR-MS spectrometry.

Fluorescence enhancement

Environment sensing. First, we tested the ability of glycoconjugates to increase their luminescence emission in response to a more hydrophobic environment. For this purpose, we monitored the emission of compound **1** in the presence of an increasing amount of sodium dodecyl sulfate (SDS) in aqueous solution. SDS has been largely employed as a membrane-mimicking agent for membrane protein characterization.³⁸ When dissolved in pure water at 25 °C, it forms small sized and monodisperse micelles with a critical micellar concentration (CMC) of 8.2 mM and an aggregation number of $n = 62$.³⁹ Compound **1** (10 μ M) was incubated for 1 h in the presence of different concentrations of SDS (0, 2.5, 5 or 10 mM), and then emission was recorded under sample excitation at 370 nm. The results are shown in Fig. 2. The intensity of the broad emission band centred at 580 nm due to the Re glycoconjugates (see below) increased upon progressive addition of aqueous SDS, reaching about a five-fold enhancement at



Scheme 1 Synthesis of multivalent glycoconjugates **2** and **3**. Reagents and conditions: (a) BF₃–Et₂O, dry DCM, 0 °C then rt, 4 h, 56% (**7**) and 70% (**8**); (b) 0.1 M NaOMe, dry MeOH, r.t, 1 h, 85% (**9**) and 93% (**10**); (c) TBTA, CuSO₄, Na ascorbate, H₂O : THF 1 : 1, rt, 16 h, 98% (**12**) and 91% (**13**); (d) NaN₃, NaI, H₂O, 60 °C, 4 days, 93% (**14**) and 96% (**15**); (e) H₂, Pd/C, HCl in MeOH, dry MeOH, rt, 83% (**16**) and 96% (**17**); (f) HATU, DIPEA, dry DMA, room temperature, 2 hours, yield 42% (**2**) and 59% (**3**).

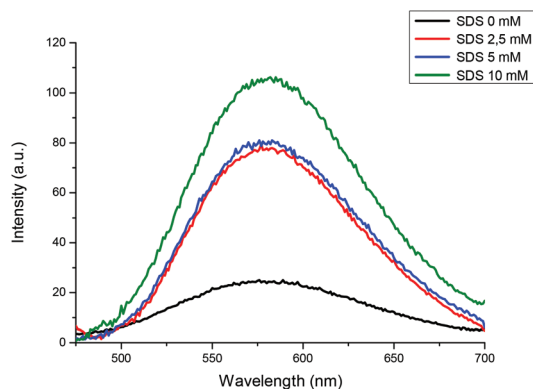


Fig. 2 Changes in the emission spectra of **1** (10 μM) in the presence of different SDS concentrations in $\text{H}_2\text{O}_{\text{mq}}$. The sample was excited at 370 nm.

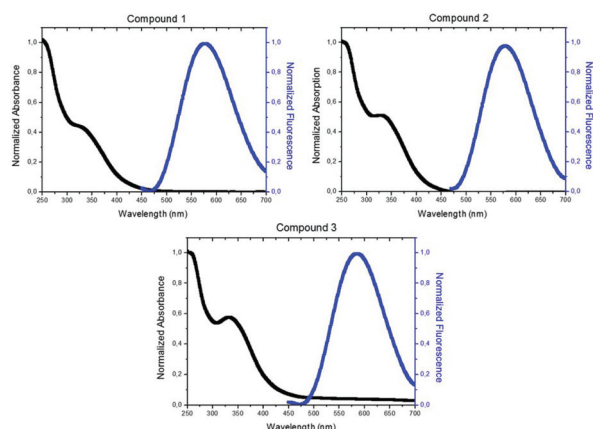


Fig. 3 Normalized absorbance (black traces) and emission (blue traces) spectra of a 10 mM solution of compounds **1–3** in 10 mM Tris buffer pH 7.4 containing 0.1 mM of MnCl_2 and 0.1 mM of CaCl_2 . The compounds were excited at 370 nm and the emission maximum is around 580 nm.

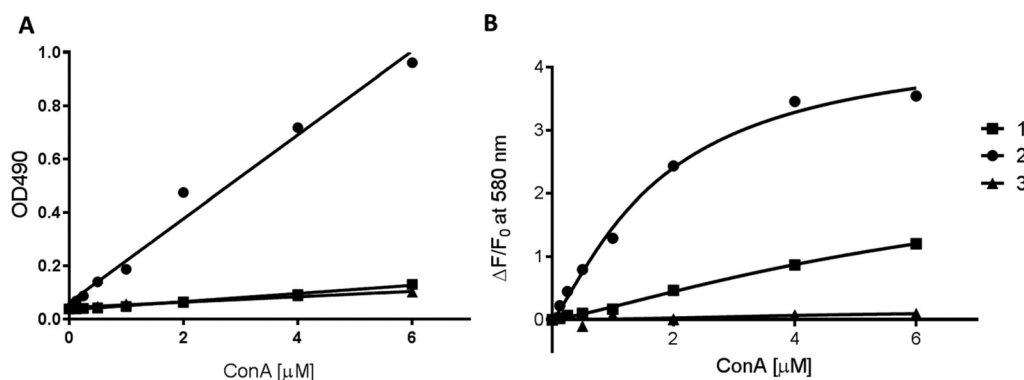


Fig. 4 Turbidimetric (panel A) and fluorescence binding assay (panel B) of compounds **1–3** (5 μM) in the presence of an increasing amount of ConA in 10 mM Tris buffer pH 7.4 containing 0.1 mM CaCl_2 and 0.1 mM MnCl_2 . Fluorescence emission was collected at 580 nm upon excitation at 370 nm.

10 mM concentration of the detergent. This observation suggests that the glycoconjugate was incorporated into aqueous SDS micelles placing the Re-complex in the hydrophobic core.

Lectin sensing. First, the absorption and emission spectra of dilute solutions (10 mM) of glycoconjugates (**1–3**) were recorded in a buffer analysis solution (10 mM Tris-HCl, pH 7.4, 0.1 mM MnCl_2 , 0.1 mM CaCl_2). The absorption spectra display a broad absorption with the maxima centred at 330 nm relative to the $d\pi(\text{Re}) \rightarrow \pi^*(\text{diazine ring})$ $^1\text{MLCT}$ band. Upon excitation at 370 nm at room temperature, all samples show a broad emission with the maximum centred around 580 nm (Fig. 3). As expected, no significant differences were observed between the compounds when compared to the photophysical characterization previously performed in distilled water for compound **1**.²⁵

Then, the ability to sense the presence of specific carbohydrate-binding proteins was evaluated using two well-studied commercial lectins: Concanavalin A (ConA) from *Canavalia ensiformis*, and peanut agglutinin (PNA) from *Arachis hypogaea*. ConA exists as a tetramer at pH 7.4 and selectively recognizes α -D-mannoside and α -D-glucoside residues; PNA also exists as a tetramer but it recognizes β -D-galactoside residues. To investigate the interaction between molecules and lectins, compounds **1–3** (5 μM) were titrated with an increasing concentration of lectins (0, 0.125, 0.25, 0.5, 1, 2, 4 and 6 μM , as a tetramer) in the presence of a catalytic amount of Ca^{2+} and Mn^{2+} required for binding. A turbidimetric assay and fluorescence measurements were performed to investigate the effective and specific carbohydrate-mediated binding to the proteins. As depicted in Fig. 4, compound **2**, containing D-mannose residues, was able to bind ConA in a concentration-dependent manner, inducing protein agglutination. Furthermore, upon binding, an effective enhancement of fluorescence emission was observed. Under the same conditions, only a slight enhancement was observed for compound **1**, containing D-glucose units, and none for compound **3**, endowed with D-galactose residues. Similarly, compound **3**, when titrated

with PNA, caused agglutination of the protein and increased its fluorescence emission up to 2-fold (ESI, Fig. S1†).

Fluorescence data points were fitted with a Hill equation binding model using GraphPad Prism 6 software, where B_{\max} is the maximum specific binding, K_d is the apparent dissociation constant and h is the Hill coefficient. Fitting parameters reported in Table 1 show a favourable binding affinity

Table 1 Data fitting parameters of the Hill plot of compounds **1** and **2** measured as a function of ConA concentration

Ligands	K_d (μM)	Hill coefficient (h)	R^2 ($n = 8$)
Compound (1)	7.270 ± 2.9	1.275 ± 0.13	0.9981
Compound (2)	1.762 ± 0.39	1.266 ± 0.18	0.9937

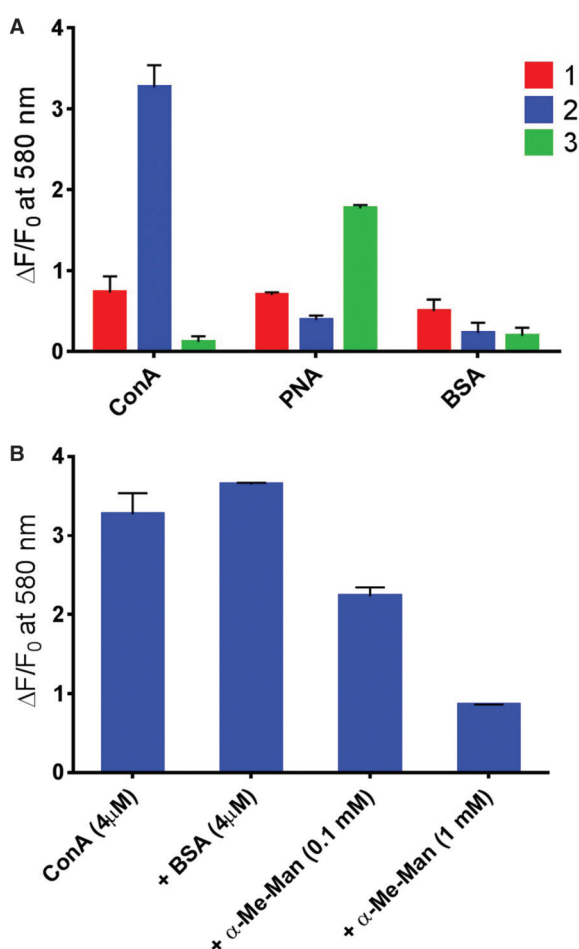


Fig. 5 Panel A: Fluorescence response pattern of compounds **1–3** (5 μM) in the presence of ConA, PNA and BSA (6 μM) in 10 mM Tris buffer pH 7.4 containing 0.1 mM CaCl_2 and 0.1 mM MnCl_2 . Fluorescence enhancements are reported as the intensity difference ($F - F_0$) relative to the untreated intensity F_0 . BSA was used as an aspecific binding protein. Panel B: Response specificity interaction of compound **1** with ConA in the presence of BSA (4 μM) and α -methylmannoside (0.1 mM–1 mM). The results are the mean of three independent determinations. Fluorescence emission was measured at 580 nm upon excitation at 370 nm.

for compound **2** with a Hill coefficient ($h > 1$) that reveals a slight cooperative effect.

$$Y = \frac{B_{\max} X^h}{K_d^h + X^h} \quad (1)$$

These findings are in agreement with the ConA interaction with poly-mannosylated and glucosylated substrates that showed a binding affinity (K_a) in the order of 10^6 M^{-1} (K_d in the μM range).^{40,41}

As depicted in Fig. 5A, compounds **1** and **3** selectively turn-on their fluorescence emission in response to a mannose-binding protein (ConA) and galactose-binding protein (PNA), respectively, whereas treatment with aspecific proteins, such as BSA, had no effect. At the same time, BSA did not interfere with the specific sensing of ConA, as reported in Fig. 5B. On the other hand, the sensing was affected by the presence of an increasing amount of α -D-methylmannoside (K_a $0.82 \times 10^4 \text{ M}^{-1}/K_d$ 0.12 mM)⁴⁰ that selectively competes for the mannose-binding sites. These findings clearly indicate aspecific and cooperative carbohydrate-mediated interactions with the lectins of the glycoprobes, resulting in the enhancement of their fluorescence emission. Several examples of AIE-active lectin probes have been reported in the literature.^{32–36} Although the sensing performances are quite comparable, our detection system, based on neutral binuclear rhenium(i) complexes, presents several favourable characteristics in terms of photophysical properties, such as large Stokes shift, long lifetimes and resistance to photobleaching with respect to organic fluorophores (such as tetraphenylethylene^{33,36} or triazatruxene³⁴). In addition, preventing potential cell damage by UV light, visible light excitation (up to 405 nm) and emission is particularly useful in biological applications.

Conclusion

In conclusion, we designed and synthesized three multivalent glycoprobes based on different glycodendron moieties (glucose, galactose and mannose) conjugated to a neutral dinuclear rhenium(i) fluorophore. Since they are non-luminescent in aqueous solution, we demonstrated that these glycoprobes are sensitive to the presence of membrane-mimicking structures and are able to sense specific carbohydrate-binding proteins, resulting in a significant fluorescence emission enhancement. This turn-on of the emission is mainly due to the specific interaction with the lectins. Indeed, upon binding of proteins mediated by carbohydrates, the Re(i)-fluorophore was placed in more hydrophobic and rigid environments that reduce the nonradiative decay processes of the fluorophore with a consequent enhanced emission. The same phenomenon was observed when compounds were put in a more aspecific hydrophobic environment. These features suggest the possibility to use efficiently dinuclear Re(i)-glycoprobes in lectin sensing, exploiting the optical properties of the Re(i)-complex as a switch-on luminophore, and thus further confirming the

possibility to use organometallic complexes as fluorescent sensors in the fields of biological and medical sciences.

Experimental

Supporting figures and synthetic procedures including NMR and MS spectra can be found in the ESI.†

Materials and methods

Chemicals were purchased from commercial sources and used without further purification, unless otherwise indicated. When anhydrous conditions were required, the reactions were performed in oven-dried glassware under a nitrogen atmosphere. Anhydrous solvents were purchased from Sigma-Aldrich® with a content of water $\leq 0.005\%$. THF was dried over Na/benzophenone and freshly distilled prior to use. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates or RP-C18 silica plates (Merck) with UV detection (254 nm and 365 nm) or using appropriate developing solutions. Flash column chromatography was performed on silica gel 230–400 mesh (Merck), according to the procedure described in the literature. Automated flash chromatography was performed using a Biotage® Isolera™ Prime system. NMR experiments were recorded using a Bruker AVANCE 400 MHz instrument at 298 K. Chemical shifts (δ) are reported in ppm downfield from the residual solvent peak, whereas coupling constants (J) are stated in Hz. The ^1H and ^{13}C -NMR resonances of compounds were assigned by means of COSY and HSQC experiments. Mass spectra were recorded on an Apex II ICR FTMS (ESI ionization-HRMS), ThermoFischer LCQ apparatus (ESI ionization) or Bruker Daltonics Microflex LT (MALDI-TOF apparatus). Specific optical rotation values were measured using a PerkinElmer 241, at 589 nm in a 1 mL cell. Absorbance spectra and fluorescence measurements were recorded using a Varian Cary® 50 UV-Vis and Varian Cary® Eclipse fluorescence spectrophotometer equipped with a multicell holder and temperature controller.

Synthesis

The compound linker (**6**),²⁵ trivalent scaffold (**11**)⁴² and Re-complex (**18**)^{43,44} were prepared as previously described. Compounds **2** and **3** were prepared following the same strategy recently described for compound **1**²⁵ and depicted in Scheme 1.

Compound 2. To a solution of compound **18** (14 mg, 18.1 μmol , 1.2 eq.) and HATU (8.6 mg, 23 μmol , 1.5 eq.) in dry DMA (350 μL), freshly distilled DIPEA (8 μL , 56 μmol , 3 eq.) was added under a nitrogen atmosphere. After 15 min a solution of compound **16** (19 mg, 15 μmol , 1 eq.) in dry DMA (400 μL) was added and the reaction mixture was stirred at r.t. under nitrogen for 1 h. The crude product was directly loaded on a C-18 column and purified by RP-18 chromatography (H_2O :MeOH gradient elution) obtaining a pure compound (**2**) (13 mg, 43% yield). ^1H NMR (400 MHz, MeOD) δ 9.99 (dd, $J_{25, 27} = 2.2$, $J_{26, 27} = 1.0$ Hz, 1H, H-27), 9.89 (dd, $J_{25, 26} = 5.9$, 1 Hz, 1H, H-26), 8.15 (dd, $J_{25, 26} = 5.9$, $J_{25, 27} = 2.2$ Hz, 1H, H-25), 7.97

(s, 3H, H-11), 4.75 (d, $J_{1, 2} = 1.5$ Hz, 3H, H-1), 4.60–4.56 (m, 6H, H-10), 4.54 (s, 6H, H-13), 3.89 (t, $J_{9, 10} = 4.9$ Hz, 6H, H-9), 3.85–3.79 (m, 6H, H-6a, H-7a), 3.78 (dd, $J_{2, 3} = 2.9$, $J_{1, 2} = 1.5$ Hz, 3H, H-2), 3.74–3.49 (m, 27H, H-3, H-4, H-5, H-6b, H-7b, H-8, H-17, H-18, H-19), 3.46 (s, 6H, H-14), 3.42 (s, 2H, H-16), 3.34–3.32 (m, 2H, H-20), 3.01–2.95 (m, 2H, H-21), 2.35 (t, $J_{22, 23} = 7.1$ Hz, 2H, H-23), 2.12–1.99 (m, 2H, H-22). ^{13}C NMR (100 MHz, MeOD) δ 195.75, 195.68, 195.61, 191.79 (ReCO), 174.93 (CONH), 165.08, 163.19 (C26, C27), 151.29 (C24), 146.03 (C12), 133.75 (C25), 125.82 (C11), 101.63 (C1), 74.65 (C5), 72.56 (C3), 72.17 (C17), 72.06 (C2), 71.25 (C18), 71.19 (C8), 70.83 (C16), 70.51 (C19), 70.38 (C9), 70.06 (C14), 68.64 (C4), 67.62 (C7), 65.32 (C13), 62.95 (C6), 51.43 (C10), 46.57 (C15), 40.49 (C20), 35.76 (C23), 32.69 (C21), 26.31 (C22). HRMS (ESI) calculated for $\text{C}_{62}\text{H}_{92}\text{C}_{12}\text{N}_{12}\text{O}_{33}\text{Re}_2$: 1976.43691, found: 1011.21202 $[\text{M} + 2\text{Na}]^{2+}$, deconvoluted: 1976.44519 (error: 4.2 ppm); $[\alpha]_{25}^{\text{D}}$: +16.12 (C = 0.630, MeOH).

Compound 3. To a solution of compound **18** (20 mg, 26 μmol , 1.2 eq.) and HATU (12 mg, 32 μmol , 1.5 eq.) in dry DMA (450 μL), freshly distilled DIPEA (11.2 μL , 64 μmol , 3 eq.) was added under a nitrogen atmosphere. After 15 min a solution of compound **17** (27 mg, 21.4 μmol , 1 eq.) in dry DMA (450 μL) was added and the reaction mixture was stirred at r.t. under nitrogen for 1 h. The crude product was directly loaded on a C-18 column and purified by RP-18 chromatography (H_2O :MeOH gradient elution) obtaining a pure compound (**3**) (25 mg, 59% yield). ^1H NMR (400 MHz, MeOD) δ 9.99 (dd, $J_{25, 27} = 2.2$, $J_{26, 27} = 1.0$ Hz, 1H, H-27), 9.88 (dd, $J_{25, 26} = 5.9$, $J_{26, 27} = 1$ Hz, 1H, H-26), 8.14 (dd, $J_{25, 26} = 5.9$, $J_{25, 27} = 2.2$ Hz, 1H, H-25), 8.04 (s, 3H, H-11), 4.61–4.55 (bt, $J_{9, 10} = 4.8$ Hz, 6H, H-10), 4.53 (s, 6H, H-13), 4.25 (d, $J_{1, 2} = 7.4$ Hz, 3H, H-1), 4.03–3.94 (m, 3H, H-7a), 3.91 (bt, $J_{9, 10} = 4.8$ Hz, 6H, H-9), 3.84 (dd, $J = 3.2$, 0.9 Hz, 3H, H-4), 3.79–3.61 (m, 16H, H-6a, H-7b, H-17, H-18, H-19), 3.58–3.47 (m, 12H, H-2, H-3, H-5, H-6b), 3.46 (s, 6H, H-14), 3.42 (s, 2H, H-16), 3.34 (t, $J_{19, 20} = 5.4$ Hz, 2H, H-20), 3.00–2.93 (m, 2H, H-21), 2.35 (t, $J_{22, 23} = 7.1$ Hz, 2H, H-23), 2.07 (p, $J = 7.2$ Hz, 2H, H-22). ^{13}C NMR (101 MHz, MeOD) δ 191.81, 191.79, 191.75, 191.73, 186.35 (CO), 174.94 (CONH), 165.10, 163.21 (C26, C27), 151.31 (C24), 146.02 (C12), 133.79 (C25), 126.03 (C11), 105.10 (C1), 76.72 (C3), 74.97 (C5), 72.56 (C2), 72.18 (C17), 71.43 (C8, C18), 71.26 (C16), 70.50 (C19), 70.37 (C9), 70.30 (C4), 70.04 (C14), 69.77 (C7), 65.30 (C13), 62.57 (C6), 51.36 (C10), 46.57 (C15), 40.49 (C20), 35.76 (C23), 32.69 (C21), 26.31 (C22). HRMS (ESI) calculated for $\text{C}_{62}\text{H}_{92}\text{C}_{12}\text{N}_{12}\text{O}_{33}\text{Re}_2$: 1976.43691, found: 1011.21000 $[\text{M} + 2\text{Na}]^{2+}$, deconvoluted: 1976.44214 (error: 2.6 ppm); $[\alpha]_{25}^{\text{D}}$: -4.96 (c = 0.675, MeOH).

Solution preparation. Stock solutions (1 mM) of compounds **1**, **2** and **3** were prepared in degassed purified water. A stock solution (40 mM) of sodium dodecyl sulfate (SDS) was prepared in degassed purified water. Stock solutions of ConA (25 μM as a tetramer), PNA (10 μM as a tetramer) and BSA (25 μM as a monomer) were prepared in buffer solution (10 mM Tris-HCl, 0.1 mM CaCl_2 , 0.1 mM MnCl_2 , pH 7.4).

Sensing assay studies. Samples were set by the addition of a fixed aliquot of the tested compound to increasing concen-

trations of SDS solution (2.5 to 10 mM) or protein (0.125 to 6 μ M ConA, PNA or BSA) diluted in buffer solution. The sample was mixed and left incubating for 10 min and then transferred in a quartz semi-micro cuvette (light path 10 mm, Hellma® Analytics) for fluorescence and absorbance measurements. Turbidity assay data were collected by recording the absorbance at 490 nm and fluorescence emission was collected at 580 nm. All measurements were recorded against a blank sample and the results were reported as the mean of three independent determinations.

Conflicts of interest

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