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Targeting bacterial biofilm: a new LecA multivalent ligand with inhibitory activity

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Abstract: Biofilm formation by bacterial pathogens is a hallmark of chronic infections and is associated to increased antibiotic tolerance that makes pathogens difficult to eradicate with conventional antibiotic therapies. Infections caused by *Pseudomonas aeruginosa* are of great concern, especially for immunocompromised and cystic fibrosis patients. *P. aeruginosa* lectins LecA and LecB are virulence factors and play a key role in establishing biofilm; therefore, inhibition of the function of these proteins has potential in dismantling the bacterium from the protective biofilm environment and in restoring the activity of antibiotics. Here we report the NMR characterization of the binding of a galactose-based dendrimer (Gal_{18}) to LecA. Moreover, we demonstrate the activity of Gal_{18} molecule in inhibiting *P. aeruginosa* biofilm formation *in vitro*.

Pseudomonas aeruginosa is an opportunistic human pathogen that can inhabit soil and aqueous environments and is often implicated in a wide range of infections, occurring in different human tissues as well as artificial settings.[1] Due to the extreme adaptability and intrinsic antibiotic resistance of this pathogen, *P. aeruginosa* infections are of great concern for human health, especially for immunocompromised and cystic fibrosis (CF) patients, that suffer of recurrent and often lethal airways infections.[2] *P. aeruginos*a infections can be acute or chronic, depending on the pathogen lifestyle.[3] Indeed, while acute infections are associated to colonization by highly virulent free-living cells, persistent chronic infections are dependent to the adhesion proprieties of the pathogen and to its ability to grow in structured aggregates known as biofilms.[4]

Bacteria in biofilms are encased in a self-made extracellular polymeric matrix composed by exopolysaccharides, DNA and secreted proteins.[4a, 5] The hallmark of biofilm-based chronic infections is an extreme tolerance to antibiotics and an extraordinary capacity of the pathogens to evade the host defenses.[6] This ultimately hampers the identification of the therapy to treat chronic infections and make biofilms very difficult to eradicate.[7] Thus, the identification of strategies to prevent or inhibit *P. aeruginosa* biofilm formation is crucial.[6a, 8]

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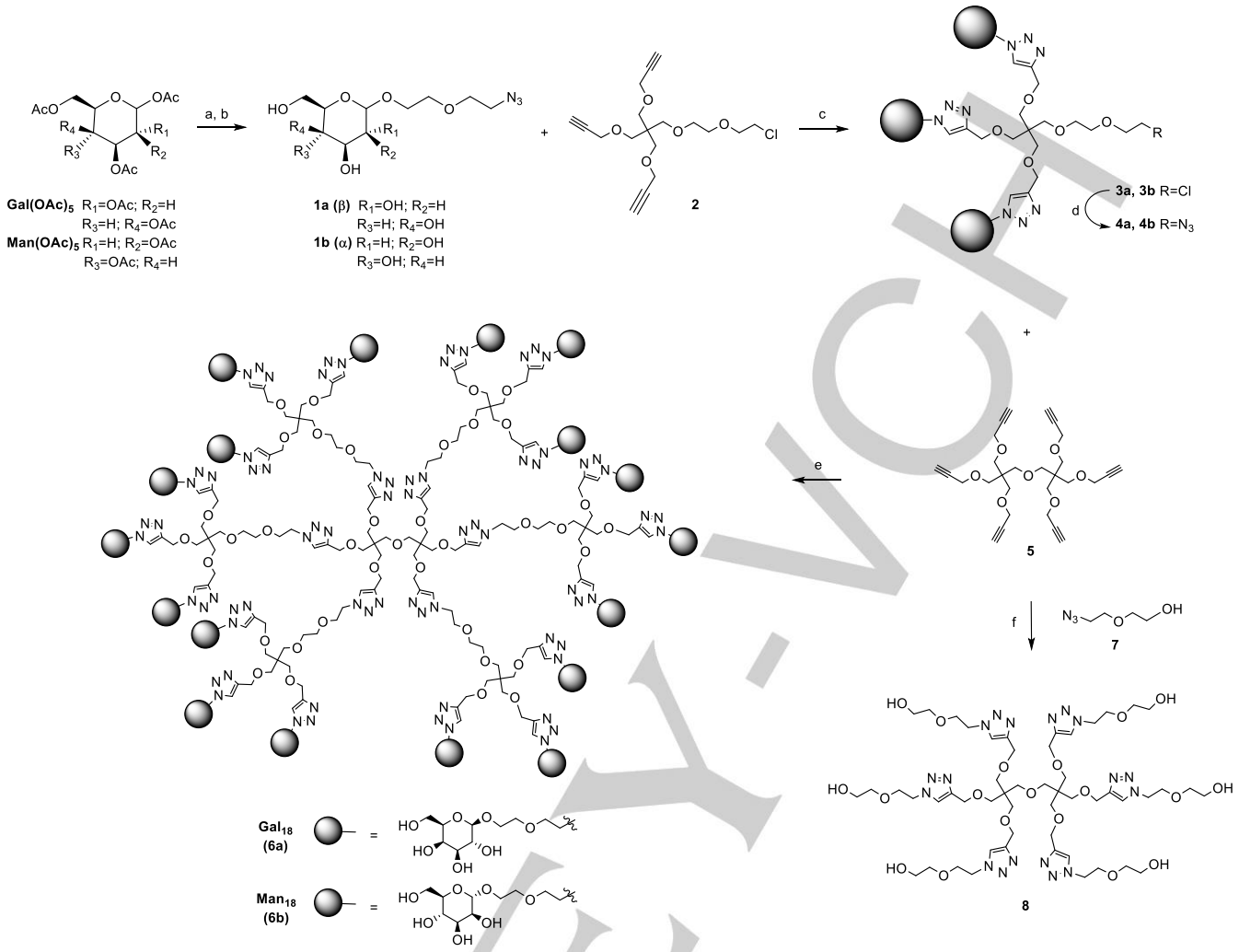
Supporting information for this article is given via a link at the end of the document.

The proteinaceous components of the biofilm matrix include lectins, carbohydrate-binding proteins that exhibit sugar binding specificity.[9] *P. aeruginosa* produces two lectins, LecA and LecB that are involved in biofilm formation[4a, 10] and play a role during infection.[11] Among them, LecA shows specificity for galactose, *N*-acetyl-D-galactosamine and glucose[12] and is involved in *P. aeruginosa* acute and chronic infection.[1a] In acute infections, LecA acts as an adhesion factor mediating the initial interaction with the host cells[13] whereas, in chronic infections, it contributes to biofilm formation possibly by cross-linking a polysaccharide (e.g. LPS) or a glycoprotein and by interacting with galactose and glucose-rich exopolysaccharides in the biofilm matrix.[14]

LecA-mediated biofilm formation involves the galactose binding site of the protein and the presence of galactosides that have high affinities to LecA, such as IPTG (isopropyl-β-D-thiogalactoside) and NPG (*p*-nitrophenyl-α-D-galactoside), reduces biofilm formation *in vitro* and induces dispersion in mature biofilms formed by wild-type *P. aeruginosa* PAO1 strain.[4a, 14] The successful reduction of *P. aeruginosa* counts in CF patients treated with inhalation of galactose and/or fucose solution provides evidence of the efficient competition of the sugars for lectin natural ligands also *in vivo*.[15] During the past decade, several approaches have been adopted to synthesize LecA inhibitors, however, only very few examples have been reported to interfere with *P. aeruginosa* biofilm formation.[16]

The major drawback in exploiting carbohydrates to interfere with lectin biological functions is their low affinity for the natural ligands.[17] Multivalent presentation of ligand epitope is a key principle to achieve high binding affinity in carbohydrate-lectin interactions and an essential condition to interfere with biologically relevant molecular recognition events.[18]

As proof of concept, we synthesized a monodispersed glycodendrimer based on pentaerythritol core and bearing 18 copies of a LecA natural ligand, *i.e.* D-galactose. To this purpose, we adopted a convergent modular strategy (Figure 1) exploiting Cu(I)-catalyzed azide-alkyne cycloaddition conjugation reaction (CuAAC, "click chemistry").[19] Briefly, β-D-galactose pentaacetate was reacted with 2-(2-azidoethoxy) ethanol linker (**7**) in a glycosylation reaction, affording, after deacetylation, compound (**1**). Then the trivalent dendron scaffold (**2**), obtained as reported in literature,[20] was functionalized with compound (**1a**) achieving the intermediate galacto-dendron (**3a**). Finally, after a chloro-azide substitution, the dendron (**4a**) was conjugated with the alkyne hexavalent scaffold (**5**) to afford final dendrimer **Gal_{18} (6a)**. Similarly, hexavalent scaffold (**5**) was functionalized with 2-(2-azidoethoxy) ethanol (**7**) to afford compound (**8**), here used as blank control in NMR binding experiments and biofilm inhibition assays.



Scheme 1. Synthesis of glycodendrimer **Gal_{18} (6a)** and **Man_{18} (6b)**. Reaction conditions: a) 2-(2-azidoethoxy)ethanol (7), BF_3-Et_2O, DCM dry, 0 °C, 70% and 56%; b) NaOMe, MeOH dry, 93% and 85% ; c) $CuSO_4$, TBTA, Na ascorbate, THF:H_2O 1:1, 91% and 98%; d) NaN_3, NaI, H_2O 96% and 93%; e) CuSO4, TBTA, Na ascorbate, THF:H_2O 1:1, 78% and 91%; f) $CuSO_4$, TBTA, Na ascorbate, THF:H_2O 1:1, quant. yield %. TBTA: Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine.

Gal_{18} ability to interact with LecA protein was tested by exploiting STD-NMR (Saturation Transfer Difference Nuclear Magnetic Resonance) spectroscopy,[21] an effective approach allowing the characterization of sugar-protein interactions[22] and the identification of the ligand binding epitope. **Figure 1B** shows the STD-NMR spectrum acquired on a mixture containing **Gal_{18}** 250 μM and LecA 20 μM dissolved in phosphate buffer 10 mM, pH 7.4, 25 °C, in the presence of 1 mM $CaCl_2$, as recognition of galactose is calcium-mediated for several members of the C-type lectin family.[23] The appearance of **Gal_{18}** resonances in this spectrum is an unequivocal demonstration of its binding to the protein.[21] Both the sugar entities and the dendrimer scaffold are involved in the molecular recognition process, suggesting that this specific scaffold could contribute to stabilize the interaction with the target. However, as expected, the presence of D-galactose moieties is an essential requirement for the binding. In fact, STD experiments acquired on a sample containing LecA and the hexavalent dendrimer lacking galactose units (compound **8**) confirmed the absence of binding of the non-functionalized dendrimer core (Figure 1D).

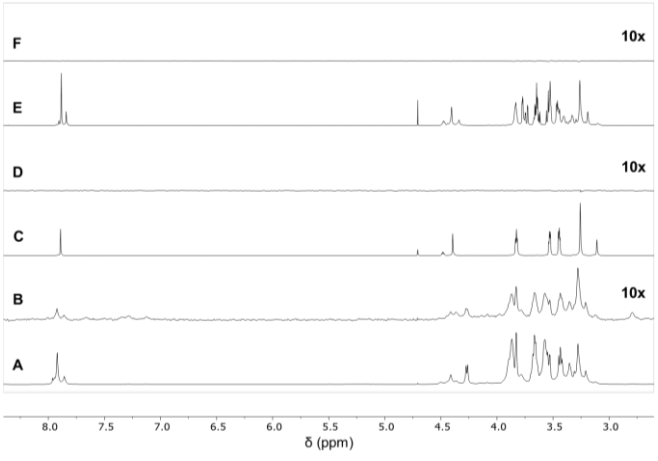


Figure 1. ^1H (**A**) and STD NMR (**B**) spectra recorded on a mixture containing Gal_{18} 250 μM and LecA 20 μM dissolved in PB 10 mM, pH 7.4, $CaCl_2$ 1 mM, 25 °C. ^1H (**C**) and STD NMR (**D**) spectra recorded on a mixture containing compound **8** 250 μM and LecA 20 μM dissolved in PB 10 mM, pH 7.4, $CaCl_2$ 1 mM, 25 °C. ^1H (**E**) and STD NMR (**F**) spectra

recorded on a mixture containing Man_{18} 250 µM and LecA 20 µM dissolved in PB 10 mM, pH 7.4, $CaCl_2$ 1 mM, 25 °C. STD spectra were acquired with 256 scans and 2 s saturation time at 600 MHz.

Similarly, when the same binding experiment was performed in the presence of **Man_{18}**, prepared through the same synthetic pathway described for **Gal_{18}**, but starting from $Man(OAc)_5$ (Scheme 1), no evidence of interaction was found (Figure 1F). This agrees with LecA specificity for D-galactose units.

Moreover, STD NMR experiments were exploited to determine the affinity of **Gal_{18}** for LecA. K_D was determined applying the method reported by Angulo *et al*.[24] A series of STD spectra was recorded dissolved in PB 10 mM, pH 7.4, $CaCl_2$ 1 mM, 25 °C in the presence of LecA 40 µM varying Gal18 concentration. For each ligand concentration (0.2, 0.4, 0.6, 0.8, 1.0 mM), STD-AF values for Gal unit H1 proton were obtained at different saturation times (0.7, 1.1, 1.5, 2.0, 2.5 s) and fit by using the equation $STD\text{-}AF(t_{sat})=STD\text{-}AF_{max}[1-\exp(-k_{sat}t_{sat})]$. The initial slopes, $STD\text{-}AF_0$, were obtained from $STD\text{-}AF_0=STD\text{-}AF_{max}k_{sat}$. $STD\text{-}AF_0$ values were then represented as a function of the ligand concentration, and the mathematical fit to a Langmuir isotherm ($y=B_{max}x/(K_D+x)$) allowed to obtain B_{max} and K_D values of 11,39±0,14 and 40.81±0.57 µM, respectively. Experimental data obtained are reported in Supporting Information.

We next assessed the potential of **Gal_{18}** as inhibitor of *P. aeruginosa* biofilm formation *in vitro* (Figure 2).

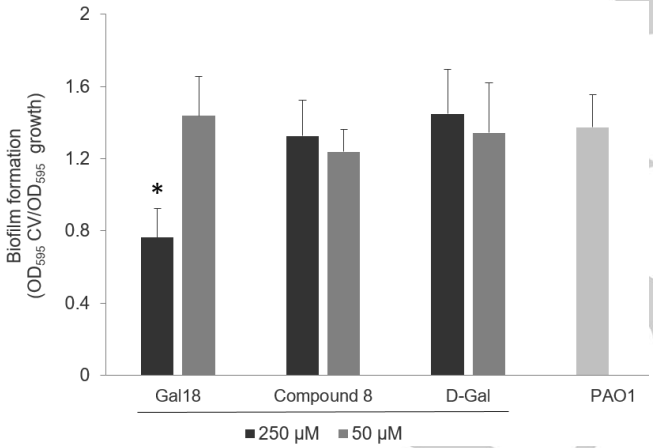


Figure 2. Quantification of *P. aeruginosa* biofilm formation after 24h-treatment with **Gal18**, compound **8**, D-galactose or water in 96-wells microtiter plates. The concentration of each compound was 50 (grey bars) or 250 (black bars) µM. The values are the means ± standard deviation of at least two independent experiments made in triplicate. *p <0.001 versus untreated sample PAO1.

Biofilm was analysed by quantifying the amount of *P. aeruginosa* PAO1 biofilm formed after 24 h of growth in Muller Hinton medium in polystyrene microtiter plates in the presence or absence of 50 or 250 µM **Gal_{18}**, compound **8**, used as blank control, and D-galactose.[4a, 25] The biofilm was quantified by the crystal violet (CV) assay, measuring the amount of CV bound to biofilm in each well of the microtiter plate, normalized by the optical density of the corresponding culture. In this way we can exclude that a decrease in biofilm formation is due to a general effect on bacterial growth resulting in a decreased number of total cells.[26] The addition of **Gal_{18}** to culture media at 250 µM concentration resulted in a significant 1.8-fold decrease in biofilm formation relative to the untreated sample ($p<0.001$), confirming the STD-NMR binding data. It is worth mentioning that, under the same experimental conditions, no biofilm inhibitory effect was observed with compound **8** and the monovalent sugar D-galactose, suggesting that the effect exerted by **Gal_{18}** is due to the multivalent presentation of LecA natural ligand.

In conclusion, we have obtained a galactose-based LecA ligand endowed with biofilm inhibition activity, albeit at micromolar concentration, that represents a very promising lead compound for the development of a novel class of multivalent glycoconjugate inhibitors of LecA-mediated biofilm maturation, taking advantage of a robust and reliable synthetic strategy. Indeed, very few LecA ligands, among the large number of related multivalent lectine ligands, have been reported so far to display biofilm inhibition properties. [16]

Moreover, to the best of our knowledge, this paper reports the first example of STD-NMR experiment exploited for the characterization of LecA interaction with a dendrimer. The experimental approach here described allows discriminating the contribution of the sugar units and the scaffold to the binding to the target protein and can be exploited for the rapid screening of potential LecA ligands and for binding affinity calculation.

Experimental Section

Supporting figures, general procedures and synthetic details including NMR spectra can be found in the ESI.

Chemical Synthesis.
Compound **1**, **2**, **3**, **4** and **5** were prepared as previously described by our group.[27]
Gal18. To a reaction vessel containing compound (**5**) (2.1 mg, 4.35 µmol, 1 eq), TBTA (462 µg, 0.87 µmol, 0.2 eq), $CuSO_4$ (109 µg, 0.44 µmol, 0.1 eq), sodium ascorbate (345 µg, 1.74 µmol, 0.4 eq), compound (**4**) (35.7 mg, 28.72 µmol, 6.6 eq) were added and dissolved in a mixture of H_2O:THF 1:1 (384 µL) reaching a final concentration of 11 mM. Then the reaction was stirred under argon at room temperature in the dark for 5 days, until mass analysis (ESI-TOF) reveals the completion of the reaction. The crude mixture was treated with QuadraSil® MP resin in order to remove copper ions, filtered and purified by RP-18 chromatography (H_2O:MeOH gradient elution). Then the product was further purified by size exclusion chromatography on a Sephadex LH20 column (ø3 cm, H 50 cm, eluent MeOH) obtaining 27 mg of pure compound (**Gal18**) (yield 78 %).^{1}H NMR (600 MHz, D_2O) δ 8.01 (s, 18H, H11), 7.93 (s, 6H, H21), 4.64 – 4.39 (m, 96H, H10, H13, H20, H23), 4.34 (d, $J_{1,2}$ = 7.7 Hz, 18H, H1), 4.03 – 3.91 (m, 54H, H6'', H9), 3.90 (d, J = 3.4 Hz, 18H, H4), 3.85 (s, 12H, H19), 3.79 – 3.57 (m, 150H, H3, H5, H6', H7, H8, H17, H18), 3.50 (m, 30H, H2, H24), 3.42 (m, 4H, H26), 3.37 – 3.30 (m, 36H, H14), 3.27 (s, 12H, H16). 13C NMR (151 MHz, D_2O) δ 144.15 (C12, C22), 125.24 (C11, C21), 102.84 (C1), 75.09 (C5), 72.71 (C3), 70.71 (C2), 70.40 (C26), 69.78 (C8, C18), 69.56 (C24), 68.99 (C16), 68.77 (C9), 68.70 (C19), 68.57 (C4), 68.45 (C7, C17), 68.19 (C14), 63.51 (C23), 60.92, 49.89 (C10), 44.68 (C15, C25). MS (ESI-HRMS) calculated for $C_{316}H_{526}N_{72}O_{163}$: 7937.5084, found after deconvolution 7937.3626
NMR binding studies.
LecA protein was purchased from Sigma-Aldrich (L9895).
NMR spectra were recorded on a Bruker Avance III 600 MHz equipped with a QCI cryo-probe (Bruker, Billerica, MA, USA). A

basic sequence from Bruker library (*stddiffesgp.3*) was employed for STD experiment. A train of Gaussian-shaped pulses of 50 ms each was chosen to selectively saturate the protein envelope; the total saturation time of the protein envelope was adjusted by the number of shaped pulses and set at 2 s. The on- and off-resonance spectra were acquired in an interleaved mode with the same number of scans. The STD NMR spectrum was achieved by subtracting the on-resonance spectrum (selective irradiation at -1.0 ppm) from the off-resonance spectrum (selective irradiation at 40.0 ppm). A blank experiment acquired on a sample containing only Gal_{18} was run under the same experimental conditions to assure the absence of direct irradiation of the ligand. K_D value for the binding of Gal_{18} to LecA was determined applying the method reported by Angulo *et al*.[[24]] To a LecA solution, 40 µM in PB 10 mM, pH 7.4, $CaCl_2$ 1 mM, 25 °C, different concentrations of a solution of Gal_{18} were added. For each concentration, a series of STD spectra was recorded, varying the saturation time. Both ligand concentration and saturation time parameters were optimized. For each Gal_{18} concentration (0.2, 0.4, 0.6, 0.8, 1.0 mM), STD-AF values for Gal unit H1 proton were obtained at different saturation times (0.7, 1.1, 1.5, 2.0 and 2.5 s) and fit by using the equation $STD\text{-}AF(t_{sat})=STD\text{-}AF_{max}[1-\exp(-k_{sat}t_{sat})]$. The initial slopes, $STD\text{-}AF_0$, were obtained from $STD\text{-}AF_0=STD\text{-}AF_{max}k_{sat}$. $STD\text{-}AF_0$ values were then represented as a function of the ligand concentration, and the mathematical fit to a Langmuir isotherm ($y=B_{max}x/(K_D+x)$) allowed to obtain B_{max} and K_D values.

Static biofilm formation assay.

A modified version of the method described in Diggle et al.[14] was employed. Overnight cultures of *P. aeruginosa* PAO1 (OD_{600} 2.5-3.5) were diluted in Muller Hinton medium (1:100) and 100 µl aliquots of bacterial suspension were added into the wells of a 96-well U-bottomed polystyrene microtiter plate in the presence of the tested molecules or water (untreated sample) and diluted in 100 µl of culture medium. The microplate was incubated at 37 °C under static conditions for 24 h to allow biofilm formation. Then, the supernatant (planktonic cells) of each well was moved into a new microplate and the absorbance (OD) at 595 nm was measured using an Enspire microplate absorbance reader (PerkinElmer). Non-adherent bacteria were removed by washing with deionized water and the biofilm was stained with 1.0% crystal violet for 30 min. After washing with deionized water, the crystal violet bound to biofilm was eluted by 33% glacial acetic acid and the OD of the eluent was measured at 595 nm. The biofilms were quantified as the amount of crystal violet bound to biofilms, normalized by the number of planktonic cells expressed in absorbance. *P*-values for testing statistical differences between measurements were calculated by student's t-test.

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Keywords: biofilm inhibitors • functionalized dendrimers • LecA • multivalency • NMR spectroscopy

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