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Mannose-centered aromatic galactoclusters inhibit the biofilm formation of \textit{Pseudomonas aeruginosa}

Caroline Ligeour, Olivier Vidal, Lucie Dupin, Francesca Casoni, Emilie Gillon, Albert Meyer, Sébastien Vidal, Gérard Vergoten, Jean Marie Lacroix, Eliane Souteyrand, Anne Imbert, Jean-Jacques Vasseur, Yann Chevolot* and François Morvan*

\textit{Pseudomonas aeruginosa} (PA) is a major public health care issue due to its ability to develop antibiotic resistance mainly through adhesion and biofilm formation. Therefore, targeting the bacterial molecular arsenal involved into its adhesion and the formation of its biofilm appears as a promising tool against this pathogen. The galactose-binding LecA (or PA-IL) has been described as one of PA virulence factor involved in these processes. Herein, the affinity of three tetravalent mannose-centered galactoclusters toward LecA was evaluated with different biochemical methods: HIA, ELLA, SPR, ITC and DNA-based glycoarray. Inhibitory potential towards biofilm was then assessed for the two glycoclusters with highest affinity towards LecA (Kd values of 157 and 194 nM from ITC measurements). An inhibition of biofilm formation of 40% was found for these galactoclusters at 10 \textmu M concentration. Applications of these macromolecules in anti-bacterial therapy are therefore possible through an anti-adhesive strategy.

\section*{INTRODUCTION}

\textit{Pseudomonas aeruginosa} (PA) is a Gram-negative, motile, opportunistic bacterium responsible for 50\% of mortality for cystic fibrosis patients.\textsuperscript{1} It is one of the most prevalent bacteria involved in nosocomial infection (10-30%) along with \textit{S. aureus} and \textit{E. coli}.\textsuperscript{2,3} Despite aggressive antibiotic therapy, PA infection in immuno-depressed patients leads to chronic or acute infection leading eventually to death. PA remains a public health issue due to its ability to develop biofilm structure entrapped in host mucins which confer it a particularly high antibiotic resistance. This resistance can be enhanced by a factor of 10 to 1000 compared with the same strain in its planktonic form.\textsuperscript{4,5} Several alternative strategies have been proposed interfering with bacterial attachment to mucins and host tissue, and subsequent biofilm development\textsuperscript{5} such as cell-cell communication,\textsuperscript{6} biofilm dispersion,\textsuperscript{7,8} bacterial adhesion\textsuperscript{9} and virulence factors.\textsuperscript{10} LecA (or PA-IL) is a tetrameric soluble galactose binding lectin identified as one of PA virulence factor.\textsuperscript{10} LecA acts as an adhesin on the host epithelial cell to facilitate PA adhesion.\textsuperscript{11,12} In the meantime, LecA displays several cytotoxic effects\textsuperscript{13} and increases the epithelial barrier permeability to bacterial toxin such as exotoxin A.\textsuperscript{14} Recently, it was demonstrated that LecA promotes cell invasion by PA on host tissue. Finally, its presence is necessary to promote biofilm formation and allow bacterial maintenance in the lungs.\textsuperscript{16}

Inhibition of LecA with synthetic ligands can be achieved with high affinity oligogalactosylated ligands.\textsuperscript{17,18} High binding can be obtained thanks to the so called glycoside cluster effect.\textsuperscript{19,20} The design of multivalent ligands targeting LecA has been recently reviewed.\textsuperscript{21} These authors have underlined the importance of the topology, and the structure of the linker between the core of the cluster and the galactosyl residue. Previous reports have emphasized the benefit of an aromatic aglycon as a mean to further increase the binding of LecA to galactosylated clusters.\textsuperscript{25,31,35,36} It was shown that phenyl \textit{β}-d-galactoside is 57.1 fold more potent than \textit{D}-galactose.\textsuperscript{15} We have recently reported the synthesis and affinity towards LecA of 25 galactocluster-oligonucleotide conjugates exhibiting different linker lengths, rigidities, spatial arrangements and incorporating aromatic aglycons.\textsuperscript{28} For this purpose these conjugates were immobilized on a DNA chip by DNA-Directed Immobilization (DDI)\textsuperscript{37} leading to a glycoarray. Among the 25 glycoclusters evaluated, the \textit{1}\textsubscript{DNA} and the \textit{2}\textsubscript{DNA} (Figure 1) bearing four galactose residues displayed a 565-fold and 844-fold increase of potency respectively, in comparison with the monogalactosylated derivative \textit{4}\textsubscript{DNA} (Figure 1). In contrast,
introduction of the non-aromatic triethylene glycol aglycon in analogue 3DNA led to a much lower increase of potency (~6-fold) in respect with 4DNA demonstrating the dramatic benefit of aromatic aglycon on the binding to LecA of our mannosyl-centered galactoclusters.

For biophysical and biological studies, glycoclusters 1-3 (Figure 1), corresponding to the galactoclusters without the DNA tag, were synthesized in solution at ~100 mg scale (Scheme 1). Their properties against LecA were evaluated using hemagglutination inhibitory assay (HIA), enzyme linked lectin assay (ELLA), surface plasmon resonance (SPR), isothermal titration microcalorimetry (ITC), and glycoarray. Methyl β-D-galactopyranoside (GalOMe) and para-nitrophenyl β-D-galactopyranoside (GalOPNP) were used as monomeric reference for comparison.

Finally, the anti-biofilm properties of glycoclusters 1 and 2 were evaluated. For this purpose biofilm of the wild strain PAO1 and the ΔlecA mutant were grown on abiotic surface in presence or absence of galactoclusters and biofilm development was assessed by fluorescence quantification and confocal scanning laser microscopy (CSLM).

RESULTS AND DISCUSSION

The glycoclusters 1-3 were synthesized in three steps starting from methyl α-D-mannopyranoside 5 (Scheme 1). The hydroxyl groups were phosphorylated using an alkyn phosphoramidite 6a or 6b activated with tetrazole leading to intermediates with phosphitetriester linkages. The phosphitetriesters were then oxidized by means of solid-supported meta-periodate affording the phosphotriester tetraalkyne mannose platform exhibiting pent-4-ynyl 7a or propargyl diethylene glycol 7b groups. Platform 7a was either conjugated by Cu(I)-catalyzed azide alkyn cycloaddition (CuAAC) with galactosyl azide 8a21 bearing an aromatic linker (L2 = AcNPh) or 8b22 bearing a triethylene glycol linker (L2 = EG3) affording galactoclusters 1 and 3 respectively after ammonia treatment. Platform 7b was similarly conjugated using 8a affording galactocluster 2 after ammonia treatment.

The binding of LecA to galactoclusters was probed as the ability of the clusters to inhibit the binding of LecA to rabbit erythrocytes (Hemagglutination inhibition assay, HIA) or to surface bound galactosyl modified polyacrylamide either by Surface Plasmon Resonance (SPR) and Enzyme Linked Lectin Assay (ELLA). In HIA experiments, the minimal inhibitory concentration (MIC) is the minimal concentration of galactocluster inhibiting the hemagglutination of rabbit erythrocytes in presence of the lectin. The lower the MIC, the higher is the binding of the galactocluster to the lectin. SPR
and ELLA were used to determine IC₅₀ values as the concentration of galactocluster inhibiting 50% of LecA binding to the galactosylated surface. The lower the IC₅₀ value as determined by SPR (SPR IC₅₀) and ELLA (ELLA IC₅₀), the higher is the binding of LecA to the galactocluster. GalOMe and GalOPNP were used as reference ligands to determine the impact of the phenyl aglycon on the binding to LecA and the glycoside cluster effect. βₐₐ and βₚₚ are the relative potencies of the galactoclusters with reference to GalOMe and GalOPNP respectively.

In the HI assays, the βₐₐ of galactoclusters 1, 2 and 3 are 128, 513 and 4, respectively (Table 1). Hence, the glycoside cluster effect for glycolcluster 3 remains limited. In contrast, galactoclusters 1 and 2 exhibit a strong increase of potency with a marked benefit for 2 bearing the longest linker between the galactose residue and the mannose core. The calculated potencies in comparison with GalOPNP of 16 and 65 for galactoclusters 1 and 2 respectively clearly showed a strong glycolcluster effect with an increase per residue of 4 and 16 respectively. Therefore, the potency increases are not only related to the presence of the aromatic ring but also to multivalency.

The IC₅₀ values of the three glycolclusters and monomers were determined by ELLA and by SPR (Figure 2 and 3, Table 2). ELLA IC₅₀ values demonstrated the higher binding of 1 and 2 in comparison with GalOMe with βₐₐ of 704 and 3050 respectively, while 3 exhibited a moderate increase (βₐₐ 6.6). The same trend was observed with SPR IC₅₀ values, but with a lower extent (βₐₐ of 15, 54 and 12 respectively, Table 2). In the ELLA IC₅₀ and SPR IC₅₀ measurements, the βₚₚ of glycolcluster 3 was 1.2 and 1.7 respectively, slightly better than for GalOPNP (Table 2). This result suggests that potencies of 3 is similar to GalOPNP. Both ELLA IC₅₀ and SPR IC₅₀ confirmed that glycolclusters 1 and 2 have improved potencies compared to 3 and to the monovalent ligands. The results obtained with HI, ELLA and SPR confirmed that 2 was the best ligand. However, the extent value of these improvements was assay dependent. Indeed, βₚₚ for galactoclusters 1, 2 and 3 were 127, 550 and 1.2 respectively for ELLA IC₅₀ values and 2.0, 7.4 and 1.7 for SPR IC₅₀ values. Hence, in the case of SPR IC₅₀, no clear multivalent cluster effect could be evidenced. Such discrepancy in the extent of the glycoside cluster effect, according to the nature of the assay, has already been reported in literature. Nevertheless, the three experiments showed that glycolcluster 2 exhibited the best affinity for LecA.

### Table 1: Hemagglutination Inhibition Assay (HI): Inhibition of rabbit erythrocyte agglutination by LecA.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Valency</th>
<th>MIC (mM)</th>
<th>βₐₐ</th>
<th>βₚₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalOMe</td>
<td>1</td>
<td>16</td>
<td>1.0</td>
<td>0.13</td>
</tr>
<tr>
<td>GalOPNP</td>
<td>1</td>
<td>2.0</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.125</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.031</td>
<td>516</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>4.0</td>
<td>4.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Table 2: IC₅₀ values of galactosylated ligands determined by Enzyme Linked Lectin Assay (ELLA) and by Surface Plasmon Resonance (SPR).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Valency</th>
<th>ELLA IC₅₀ (µM)</th>
<th>SPR IC₅₀ (µM)</th>
<th>βₐₐ</th>
<th>βₚₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalOMe</td>
<td>1</td>
<td>183</td>
<td>49</td>
<td>1.0</td>
<td>0.14</td>
</tr>
<tr>
<td>GalOPNP</td>
<td>1</td>
<td>33.0</td>
<td>6.7</td>
<td>7.3</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.26</td>
<td>0.91</td>
<td>15</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.06</td>
<td>0.54</td>
<td>54</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>27.6</td>
<td>4.0</td>
<td>12</td>
<td>1.7</td>
</tr>
</tbody>
</table>
binding. Relative potencies of 565, 844 and 5.8 were determined for \(1_{\text{DNA}}\), \(2_{\text{DNA}}\) and \(3_{\text{DNA}}\) respectively. The ELLA\(IC_{50}\) values are in agreement with \(IC_{50\text{Lac}}\) values determined with the glycoarray assay with the same ranking between the different glyoclusters, both highlighted the best affinity of glyocluster \(2\) toward LecA.

Finally, isothermal titration microcalorimetry (ITC) measurements of the interaction between LecA and the three galactoclusters \(1\)–\(3\) were performed (Figure 4) and compared with data obtained previously with the monovalent GalOMe\(^{43}\) (Table 4). A \(\text{ITC } K_d\) value of 11 \(\mu\text{M}\) was measured for glyocluster \(3\), corresponding to a moderate increase of potency of 6.4 fold in respect with GalOMe. The stoichiometry \((n = 0.28)\) is in agreement with four galactose residues linked to LecA monomers. Therefore, our results suggested that the entropic cost upon the interaction is not compensated by enthalpic consideration leading to a similar \(\text{ITC } K_d\) for both the multivalent \(3\) and the monovalent GalOMe. In contrast, galactoclusters \(1\) and \(2\) exhibited a strong increase of potency \(\beta_{\text{Me}}\) of 361 and 446-fold respectively. The stoichiometry of \(1\) and \(2\) was similar \((n = 0.46\) and 0.52, respectively), suggesting that two galactose residues were involved simultaneously with LecA monomers. The entropic cost for both interactions is about 3 to 4 times lower than the one observed for glyocluster \(3\). Both have similar entropic contributions \((-\Delta S)\) similar to the 53 \(\text{kJ/mol}\) observed by Cecioni et al.\(^{21}\) with aromatic monovalent ligands. Surprisingly, despite the presence of more flexible linker such as the diethylene glycol arm, the entropic cost with compound \(2\) was lower than for the more rigid aromatic glyocluster \(1\). A possible reason for this may be due to the hydrophobic nature of the propyl linker of compound \(1\) leading to an increased dehydration entropic cost.

**Table 3:** \(IC_{50\text{Lac}}\) values for DNA-galactoclusters \(1\)-\(4\) determined by DDI glycoarray using lactose as inhibitor.\(^{28}\)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Valency</th>
<th>(IC_{50\text{Lac}} (\mu\text{M}))</th>
<th>(\beta_{\text{DNA}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1_{\text{DNA}})</td>
<td>4</td>
<td>2826</td>
<td>565</td>
</tr>
<tr>
<td>(2_{\text{DNA}})</td>
<td>4</td>
<td>4218</td>
<td>844</td>
</tr>
<tr>
<td>(3_{\text{DNA}})</td>
<td>4</td>
<td>29</td>
<td>5.8</td>
</tr>
<tr>
<td>(4_{\text{DNA}})</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 4:** ITC data for the interaction between LecA and galactoclusters \(1\)-\(3\).

<table>
<thead>
<tr>
<th>Glycoclusters</th>
<th>(n)</th>
<th>(-\Delta H) (kJ/mol)</th>
<th>(-\Delta S) (kJ/mol)</th>
<th>(-\Delta G) (kJ/mol)</th>
<th>(\text{ITC } K_d) ((\mu\text{M}))</th>
<th>(\beta_{\text{Me}})</th>
<th>(\text{array } K_d) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalOMe</td>
<td>0.8</td>
<td>39</td>
<td>15</td>
<td>24</td>
<td>70(^{4})</td>
<td>1</td>
<td>n.a.</td>
</tr>
<tr>
<td>(1)</td>
<td></td>
<td>0.46 ± 0.01</td>
<td>81.4 ± 0.1</td>
<td>43</td>
<td>0.194 ± 0.007</td>
<td>361</td>
<td>0.060</td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td>0.52 ± 0.01</td>
<td>78 ± 3</td>
<td>39</td>
<td>0.157 ± 0.002</td>
<td>446</td>
<td>0.039</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td>0.28 ± 0.02</td>
<td>134 ± 3</td>
<td>105</td>
<td>11 ± 3</td>
<td>6.4</td>
<td>0.395</td>
</tr>
</tbody>
</table>

\(^{a}\)Relative potency \((\beta_{\text{Me}})\) was calculated using the \(K_d\) value of 70 \(\mu\text{M}\) previously reported for GalOMe.\(^{45}\) n.a. = not applicable.
In parallel, dissociation constants of the interaction of the glyoclusters 1<sub>DNA</sub>-3<sub>DNA</sub> with LecA were measured on microarray using isotherm<sup>44</sup> leading to a <sup>array</sup><sub>K<sub>d</sub></sub> value of 60, 39 and 395 nM respectively. These <sup>array</sup><sub>K<sub>d</sub></sub> values were lower than the <sup>ITC</sup><sub>K<sub>d</sub></sub> values (194, 157 and 11000 nM). This difference between the two assays may be due to the fact that <sup>array</sup><sub>K<sub>d</sub></sub> measurements on microarray are done on a heterogeneous phase system while ITC measurements are done in a homogeneous solution phase system. However, here again, the same ranking in affinity of multivalent ligands for LecA was observed. So the <sup>array</sup><sub>K<sub>d</sub></sub> values determined by glycoarray should be taken as relative data like for SPR or HIA.

In the current work, we focused on a simple and reproducible fluorescence-based technique to monitor biofilm development at the bottom of 96 wells microtitration plates. The technique allows rapid screening of glyoclusters as potential inhibitor on PA biofilm development and thus should be a convenient tool for further identification of efficient inhibitors. Anti-biofilm potential of the two best glyoclusters 1 and 2 was then assessed. DAPI labeled biofilm of wild type <i>P. aeruginosa</i> strain (PAO1) was compared to the mutant ΔlecA deficient lecA strain (Figure 5). As expected, lecA mutant biofilm displays a lower fluorescence intensity (Figure 5A and 5B, 25-30%) compared to PAO1 (100%) confirming that ΔlecA mutant is less prone than the wild type to develop a biofilm. Analysis of the three-dimensional structure of the biofilm by CSLM (Figure 6) was quantified using COMSTAT1 program (Figure 7) and confirmed that ΔlecA mutant was strongly impaired in biofilm formation. Indeed, ΔlecA biofilm are thinner (average thickness of 0.1 vs 4.6 µm) than for PAO1 biofilm, contain less biomass (0.05 vs 0.91 µm<sup>3</sup>/µm<sup>2</sup>) and exhibit a strongly lower surface coverage (1.2% for ΔlecA vs 51.0% of surface colonized for PAO1 and occupying only 5.0 × 10<sup>3</sup> µm<sup>2</sup> vs 85.5 x 10<sup>3</sup> µm<sup>2</sup> for PAO1). Consequently ΔlecA presents a more irregular biofilm structure than the wild type does (roughness coefficient of 1.94 vs 0.69 for PAO1).

Figure 4: Titration curves obtained from the titration of LecA (from 50 to 270 µM depending on the ligand affinity) with glyoclusters 1-3 (175 µM) at 25 °C. Lower panels: the total heat released as a function of total ligand concentration for the titration shown in the upper panels.
Figure 5. Fluorescence-based quantification of PA biofilm development on permanox chamber slide with or without galactoccluster inhibitor, [A] 1; [B] 2. [C] Dispersion of bacteria from 24 h old PA biofilm by 10 µM galactoccluster inhibitor, 1 and 2. Bars represent mean values ±SEM of n=8 independent experiments and biofilm fluorescence is normalized to PAO1 (100%). The asterisk(s) indicates that the Mann-Whitney calculated P value is less than 0.05 for comparison to PAO1 wild type.* p<0.05 ** p<0.01, *** p<0.0001.

Galactoclusters designed to target LecA are supposed to decrease biofilm formation of PAO1 and should reach biofilm development similar (surface coverage and thickness) of the ΔlecA mutant. Indeed, concentration higher than 10 µM of glycocluster 1 (Figure 5A) and above 5 µM for glycocluster 2 (Figure 5B) inhibited biofilm development with an average of 40%. Analysis of the three-dimensional structure of the biofilm developed by PAO1 in the presence of 10 µM of glycoclusters 1 and 2 (Figure 6C and 6D) was also performed. Images analysis showed that biofilms of PAO1 grown in presence of each inhibitor contained less biomass (0.62 and 0.37 µm^3/µm^2 respectively for 1 and 2 vs 0.91 µm^3/µm^2 for PAO1) and were thinner (average thickness of 2.1 and 1.3 µm respectively for 1 and 2 vs 4.6 µm for PAO1; maximum thickness of 5.3 and 5.8 µm respectively for 1 and 2 vs 8.2 µm for PAO1) than without the glycoclusters (Figure 7). Moreover, they displayed a lower surface coverage (38% and 16% of surface colonized respectively for 1 and 2 vs 51% for PAO1, occupying 62.2×10^3 µm^2 and 50.1×10^3 µm^2 respectively for 1 and 2 vs 85.5×10^3 µm^2 for PAO1). Together, these results demonstrated the efficiency of both glycoclusters 1 and 2 to reduce biofilm development of the wild type PAO1 strain. Comparatively, at the same concentration (10 µM) galactocluster 2 appears more efficient to inhibit biofilm growth compared to compound 1 confirming its better affinity toward LecA observed through HIA, ELLA, SPR, glycoarray and ITC techniques.

Addition of galactoclusters, 1 and 2 (10 µM) to a 24 h grown biofilm of PAO1 (Figure 5C) resulted in an increase of bacterial dispersion compared to the wild type (* p < 0.05) as well as the ΔlecA mutant strain. This experience demonstrates that galactoccluster/LecA interactions did not only reduce biofilm growth on abiotic surface but also increased the bacterial release from an established biofilm.
Biofilm inhibition studies showed that compound 2 displayed a twice higher efficiency than glycocluster 1 (minimum biofilm inhibitory concentration (MBIC) = 5 vs 10 µM) and is consequently the best inhibitor evaluated in the present assay. The diverse techniques (HIA, ELLA, SPR, ITC) used in this paper to monitor in vitro lectin/galactoclusters interactions have already indicated that glycocluster 2 displayed the best affinity towards LecA. The biofilm inhibition assay was in agreement with the in vitro results and validated that the galactoclusters 1 or 2 can inhibit PA biofilm formation. Therefore, there are good candidates for anti-adhesive therapy.

CONCLUSION

PA colonization of host tissue and biofilm formation provides to the bacteria a selective advantage against antibiotic therapy. LecA is a virulence factor suspected to be involved in PA adhesion. Inhibition of LecA with multivalent galactosylated molecules is forecasted as a mean to inhibit PA-adhesion.

In our strategy, glycoclusters conjugated to a DNA sequence are rapidly synthesized, at a microgram scale, on solid support combining the phosphoramidite chemistry and Cu(I) catalyzed azide alkene cycloaddition. Their screening is performed using a DNA directed immobilization-glycoarray that allows a rapid determination of their binding towards LecA using only a minute amount of them (~1 µg). This strategy is suitable to avoid the preparation of a mass of glycoclusters at tens of milligram to select the good one. Only the “hit” ones are synthesized in solution at milligram scale for further evaluations. Herein, we presented the binding evaluation by glycoarray of two selected mannose centered galactoclusters to LecA which displayed high affinity to LecA and one of low affinity as control. The selected galactoclusters without the DNA sequence were synthesized in solution at hundreds of milligram scale. Then their binding was determined using four different techniques (HIA, ELLA, SPR and ITC) and compared with data obtained on microarray. The galactoclusters 1 and 2 bearing a phenyl aglycon were found to display a much better affinity for LecA than the galactocluster 3 with a triethylene glycol aglycon. This result confirmed the known "aromatic benefit" of aromatic galactosides towards LecA binding with Kₐ values below 200 nM found for 1 and 2. The difference of structure between 1 and 2 corresponds to a propyl and a diethylene glycol-methylene linking the galactoside-phenyl-triazole to the mannose core. This moderate modification is not involved in the interaction with the CRD. However it has a significant effect on the affinity with LecA since 2 was found better than 1. This result confirmed high affinity of a glycocluster to a lectin is a subtle adjustment of its topology. The same trend was observed by Pieters who showed that digalactosides differing from only four methylenes displayed difference of Kd values of almost four times (28 vs 130 nM). The best galactoclusters reported in the literature display Kₐ values for LecA of 28 nM and 82 nM for digalactosides and 79 nM, 90 nM, 100 nM, 176 nM for tetragalactosides. The galactoclusters 1 and 2 with Kd values of 194 nM and 157 nM respectively are in the same range. Finally, galactoclusters 1 and 2 showed some inhibition of biofilm formation of PA with 40% of inhibition at 10 µM and 5 µM concentration respectively where ΔlecA

![Table](image)

Figure 7. COMSTAT analysis of the CSLM Images of DAPI-Stained Biofilms of PA developed for 24 h on permanox chamber slides. (■) PAO1, (□) ΔlecA, (■) PAO1+1 (10 µM), (□) PAO1+2 (10 µM).
mutant displayed 70% of inhibition. The fact that ΔlecA mutant of PA was still able to form some biofilm confirmed that LecA should not be the only lectin involved in the biofilm formation. Our data confirmed that LecA is a pertinent target to limit the growth of PA should improve the therapeutic effect.

Experimental

Materials and methods: All reagents were commercial and used without further purification. Acetonitrile was distilled over CaH2. Phosphorylations were performed under an argon atmosphere. NMR spectra were recorded at 293 K using a 300 MHz, 400 MHz or 600 MHz spectrometer (Bruker). Shifts are referenced relative to deuterated solvent residual peaks. MALDI-TOF mass spectra were recorded on a Voyager mass spectrometer equipped with a nitrogen laser. High-resolution (HR-ESI-QToF) mass spectra were recorded using a Q-Tof Micromass spectrometer. Thin-layer chromatography (TLC) was performed with C18 flash columns. TLC plates were inspected by UV light (λ = 254 nm) followed by heating. Reverse phase chromatography was performed with a C18 column.

Synthesis

General procedure for phosphorylation: A solution of methyl α-β-mannopyranoside 5 (50 mg, 0.26 mmol, 1 eq) in anhydrous DMF/CH2Cl2 (1:1.5, v/v) was stirred for 1.5 h with molecular sieves (3 Å). Then, the alkyne phosphoramide 6a or 6b was added and a solution of tetrazole (0.4 M in anhydrous CH2Cl2, 6.4 mL, 2.60 mmol, 10 eq). The mixture was stirred at 30 °C for 2 h and the reaction was quenched with H2O (0.4 mL). After 15 min, AAO(OH2) resin was added (1.0 g, 2.50 mmol, 9.6 eq) and the mixture was stirred for 2 h. After filtration of the resin, the DMF was evaporated. The residue was dissolvod in dichloromethane (40 mL), the solution was washed with an aqueous saturated solution of NaHCO3 (60 mL) and brine (60 mL). The organic layer was dried (Na2SO4), filtered and concentrated to afford the desired tetraalkyne mannoside derivatives 7a-7b.

1- Methyl-2,3,4,6-tetra-O-pentynylphosphoryl-α-L-mannopyranoside 7a: Obtained as a pale yellow oil (208 mg, 81%). 1H NMR (300 MHz, D2O) δ 4.98 (d, J=2.1 Hz, 1H, H-1), 4.87-4.57 (m, 3H, H-2, H-5, 6-), 4.37-4.12 (m, 16H, OCH2CH2CN, POCH2CH2), 3.94-3.89 (m, 1H, H-6), 3.45 (s, 4H, OCH3, H-3), 3.40 (m, 1H, H-4), 2.88-2.78 (m, 8H, CH2CN), 2.39-2.34 (m, 4H, CH2CH2CH2), 2.08-1.90 (m, 8H, POCH2CH2J), 1.73-1.64 (m, 4H, CH2CCN). 13C NMR (100 MHz, CDCl3) δ -1.65 to -3.01 (m, P). 18F NMR (162 MHz, CDCl3) δ 115.5 (CN), 98.3 (C-1), 81.5 (OCH2CH2), 68.5 (CH2CH2, C-2, C-5, C-6), 65.6 (C-3, C-4), 60.9 (2s, POCH2), 55.7 (OCH2), 27.7 (POCH2CH2), 18.7 (CH2CN), 13.1 (CH2CH2CH2). MALDI-TOF MS average m/z calcld for C38H74N2O6P3 [M+H]⁺ = 910.77 found 910.63. HR-ESI-QToF MS: isotopic m/z calcld for C38H74N2O6P3 [M+H]⁺ = 910.7778.

Methyl-2,3,4,6-tetra-O-propargyldiethyleneglycol phosphotriester-α-β-mannopyranoside 7b: Obtained as a colourless oil (279 mg, 87%). 1H NMR (400 MHz, CDCl3) δ 4.93 (d, J=2.4 Hz, 1H, H-1), 4.84-4.79 (m, 1H, H-6), 4.73-4.59 (m, 2H, H-2, H-5), 4.37-4.18 (m, 16H, POCH2CH2CN, POCH2CH2), 4.17-4.12 (m, 8H, OCH2CH2), 3.86-3.80 (m, 1H, H-6), 3.68 (m, 8H, POCH2CH2), 3.63 (s, 17H, OCH2CH2O, H-3), 3.61-3.57 (m, 1H, H-4), 3.38 (s, 3H, OCH3), 2.82-2.74 (m, 8H, CH2CN), 2.46 (m, 4H, OCH2CH2). 13C NMR (162 MHz, CDCl3) δ -1.67 to -3.11 (m, P). 13C NMR (100 MHz, CDCl3) δ 117.1 (CN) 98.3 (C-1), 97.6 (OCH2CH2), 74.9 (CH2CH2, C-2, C-5, C-6), 70.2-69.7 (2m, POCH2CH2, C-3, C-4), 69.1 (OCH2CH2O), 67.8-62.5 (5m, POCH2), 58.3 (OCH2CH2), 55.7 (OCH3), 19.5 (CH2CN). MALDI-TOF MS average m/z calcld for C38H74N2O6P3 [M+H]⁺ = 1231.97 found 1231.19. HR-ESI-QToF MS isotopic m/z calcld for C38H74N2O6P3 [M+H]⁺ = 1231.3297 found 1231.3307.

General procedure for 1,3-dipolar cycloaddition and deacetylation of carbohydrate: The alkyne-functionalized compounds 7a or 7b (1.0 eq) and the azido-tetraacylgalactose derivatives 8a or 8b (4 to 4.8 eq) were dissolved in dioxane with triethylammonium acetate buffer (175 µL, 0.1 M, pH 7.7) and nanopowder copper (2 mg). The resulting mixture was stirred overnight at 70 °C. Metallic copper was filtered and the solution was diluted with CH2Cl2 (15 mL), and washed with brine (3x15 mL). The organic layer was dried (Na2SO4), filtered, and concentrated to dryness. The resulting product was dissolved in acetone (5 mL) and concentrated ammonia solution (30%) was added (20 mL). The mixture was stirred 1 h at room temperature. After evaporation, the crude product was dissolved in milliQ water, and the solution was passed through a column filled with DOWEX-50W X8 resin (Na⁺ form). After concentration, the residue was purified by C18 flash column chromatography (40 g (H2O/CH2CN/triethylammonium acetate buffer 0.1 M pH 7.7, 97:0.3 to 47:50:3 v:v:v). The fractions containing pure glyoclusters 1-3 were combined, evaporated and coevaporated with water several times to eliminate the buffer. Pure glyoclusters 1-3 were dissolved in water (2 mL) and passed through a column filled with DOWEX-50W X8 resin (Na⁺ form). After evaporation, the residue was dissolved in the minimum amount of water and lyophilized.

Glyocluster 1: Obtained as a pale white solid (141 mg, 64%) from 7a (100 mg, 0.1 mmol, 1 eq), 8a (211 mg, 0.4 mmol, 4 eq), dioxane (2.0 mL). 1H NMR (300 MHz, D2O) δ 7.82-7.72 (4s, 4H, H-triaz), 7.34-7.27 (m, 8H, H-ar), 7.04-6.99 (m, 8H, H-ar), 5.30-5.19 (4s, 8H, C(O)CH2-N-triaz), 4.92-4.89 (5H, 5H, H-1 gal, H-1 man), 4.86-4.84 (m, 2H, H-2 man, H-3 man), 4.74-4.72 (m, 2H, H-4 man, H-5 man), 3.95-3.93 (m, 10H, H-6 man, OCH2CH2), 3.85-3.68 (m, 24H, H-2 gal, H-3 gal, H-4 gal, H-5 gal, H-6 gal, OCH2CH2).
2.0 Hz, 4H, H-4 gal), 3.89-3.74 (m, 50H, H-2 gal, H-3 gal, H-5 gal), 6.79 (OCH$_3$CH$_2$), 64.7, 64.1 (C-2 man, C-3 man, C-4 man, C-5 man, C-6 man), 60.1 (C$_3$ gal), 51.3 (C(O)CH$_2$N-triaz, OCH$_2$), 29.5 (CH$_3$CH$_2$), 28.8 (CH$_3$-triaz). MALDI-ToF MS average m/z calc for C$_{51}$H$_{121}$N$_{12}$O$_{25}$P$_2$ [M-H] = 2194.76 found 2194.84 HR-ESI-QToF MS isotopic m/z calc for C$_{51}$H$_{121}$N$_{12}$O$_{25}$P$_2$ [M+2H]$^{2+}$ = 1098.3090 found 1098.3064.

**Glycocluster 2:** Obtained as a pale white solid (190 mg, 95% yield) from 7b (100 mg, 0.082 mmol, 1 eq.), 8a (204 mg, 0.4 mmol, 4.8 eq.), dioxane (2.8 mL). H NMR (600 MHz, D$_2$O) δ 8.19-8.15 (m, 4H, H-triaz), 7.46-7.44 (m, 8H, H-ar), 7.17-7.15 (m, 8H, H-ar), 5.45-5.43 (m, 8H, C(O)N-triaz), 4.99 (m, 1H, H-1 man), 4.74 (d, J = 2.4 Hz, 8H, OCH$_2$-triaz), 4.45-4.32 (m, 3H, H-2 man, H-3 man, H-5 man), 4.17-4.10 (m, 50H, H-2 gal, H-3 gal, H-5 gal, H-6 gal), 3.39 (s, 3H, OCH$_3$). 13C NMR (150 MHz, D$_2$O) δ 165.3 (C=O), 153.6 (C$_3$-triaz), 130.8 (C$_2$-triaz), 124.1 (CH-triaz), 122.5 (C$_3$-triaz), 100.4 (C-1 gal), 98.3 (C-1 man), 74.78, 72.0, 70.0 (3s, 3C, C-2 gal, C-3 gal, C-4 gal, C-5 gal), 67.9 (OCH$_3$CH$_2$), 64.7, 64.1 (C-2 man, C-3 man, C-4 man, C-5 man, C-6 man), 60.1 (C$_3$ gal), 51.3 (C(O)CH$_2$N-triaz, OCH$_2$), 29.5 (CH$_3$CH$_2$), 28.8 (CH$_3$-triaz). HPLC Rt = 11.25 min. MALDI-ToF MS average m/z calc for C$_{51}$H$_{121}$N$_{12}$O$_{25}$P$_2$ [M-H] = 2194.76 found 2194.84 HR-ESI-QToF MS isotopic m/z calc for C$_{51}$H$_{121}$N$_{12}$O$_{25}$P$_2$ [M+2H]$^{2+}$ = 1098.3090 found 1098.3064.

**Glycocluster 3:** Obtained as a pale white solid (66 mg, 62%) from 7a (50 mg, 0.050 mmol, 1 eq.), 8a (101 mg, 0.200 mmol, 4 eq.), dioxane (1.5 mL). H NMR (600 MHz, D$_2$O) δ 8.00-7.92 (m, 4H, H-triaz), 5.01 (m, 1H, H-1 man), 4.62-4.64 (m, 8H, CH$_2$N-triaz), 4.48 (dd, J=1.8 Hz, J=7.8 Hz, 3H, H-2 man, H-3 man, H-5 man), 4.45 (d, J=7.4 Hz, 8H, OCH$_2$-triaz), 4.14-4.12 (m, 4H, H-6 man, OCH$_2$CH$_2$N-triaz), 3.91-3.88 (m, 5H, H-6 man, H-4 gal), 3.85-3.77 (m, 20H, H$_2$GalOCH$_2$, POCH$_2$CH$_2$, H-6 gal), 3.76-3.67 (m, 32H, H-2 gal, H-3 gal, H-5 gal, OCH$_2$CH$_2$O), 3.61-3.56 (m, 5H, H-3 gal, H-4 man), 3.47 (s, 3H, OCH$_3$), 2.91-2.78 (m, 8H, CH$_3$CH$_2$). 1H NMR (150 MHz, D$_2$O) δ 103.7 (C-1 gal, C-1 man), 76.0 (POCH$_2$CH$_2$), 75.9, 73.6, 71.6 (3s, 3C, C-2 gal, C-3 gal, C-5 gal), 70.6, 70.5, 70.4, 70.3, 70.2 (C-2 man, C-3 man, C-4 man, C-5 man, C-6 man, OCH$_2$CH$_2$), 70.0 (OCH$_2$CH$_2$N-triaz), 69.5 (C-4 gal, GalOCH$_2$), 61.8 (C$_3$ gal), 51.0 (CH$_2$N-triaz), 44.0 (CH$_2$CH$_2$C-triaz), 30.4 (CH$_3$CH$_2$C-triaz). MALDI-ToF MS average m/z calc for C$_{51}$H$_{121}$O$_{27}$P$_2$ [M-H]$^{-}$ = 2126.80 found 2126.54. HR-ESI-QToF MS isotopic m/z calc for C$_{51}$H$_{121}$O$_{27}$P$_2$ [M+2H]$^{2+}$ = 1064.3709 found 1064.3835.

**Hemagglutination inhibition assays (HIA):** Hemagglutination inhibition assays (HIA) were performed in U-shaped 96-well microtitre plates. Rabbit erythrocytes were purchased from Biomerieux and used without further washing. Erythrocytes were diluted to a 8% solution in NaCl (100 mM). Recombinant LeCA was produced in *Escherichia coli* and purified as described previously. LeCA solutions of 3 μM were prepared in tris(hydroxymethyl)aminomethane (TRIS-HCl) 20 mM, NaCl 100 mM, and CaCl$_2$ 100 mM. The hemagglutination unit (HU) was first obtained by the addition of the 4% erythrocyte solution (50 μL) to aliquots (50 μL) of sequential (twice) lectin dilutions. The mixture was incubated at 25 °C for 30 min. The HU was measured as the minimum lectin concentration required to observe hemagglutination. For the following lectin-inhibition assays, lectin concentrations of 4 HU were used. For LeCA, this concentration was found to be 3 μM. Subsequent inhibition assays were then carried out by the addition of lectin solution (25 μL, at the required concentration) to sequential dilutions (50 μL) of glycoclusters, monomer molecules, and controls. These solutions were incubated at 37 °C for 30 min, then 8% erythrocyte solution (25 μL) was added, followed by an additional incubation at 37 °C for 1 h. The minimum inhibitory concentration for each molecule was determined for each duplicate.

**Determination of lectin concentration by enzyme-linked lectin assay (ELLA):** 96-Well microtiter plates (NuncMaxisorb) were coated with α-PAAGal (PAAG-polyacrylamide) for LeCA (Lectinity Holding, Inc.): 100 μL of 5 μg·mL$^{-1}$ in carbonate buffer, pH 9.6 for 1 h at 37 °C, then blocking at 37 °C for 1 h with 100 μL per well of 3% (w/v) bovine serum albumin (BSA) in phosphate buffer solution (PBS). Biotinylated LeCA solutions (75 μL) were diluted (1:2) starting from 30 μg·mL$^{-1}$. After 1 h incubation at 37 °C and three washes with T-PBS (PBS that contained 0.05% Tween 20), horseradish peroxidase (HRP)-streptavidin conjugate (100 μL; dilution 2:8000; Boehringer-Mannheim) was added and left for 1 h at 37 °C. Coloration was developed by using 100 μL per well of 0.05% phosphate/citrate buffer that contained o-phenylenediamine dihydrochloride (0.4 μg·mL$^{-1}$) and urea hydrogen peroxide (0.4 mg·mL$^{-1}$) (OPD kit, Sigma-Aldrich) for 15 min and stopped with sulfuric acid (50 μL, 30%). Absorbance was then read at 490 nm using a microtiter plate reader (BioRad 480). The concentration of biotinylated lectins was determined by plotting the relative absorbance versus lectin concentration. The concentration that led to the highest response in the linear area was selected as the standard lectin concentration for the subsequent inhibition experiments. The final concentrations were 0.5 μg·mL$^{-1}$ for LeCA.

**Isothermal titration microcalorimetry (ITC):** Recombinant lyophilized LeCA was dissolved in buffer (100 mM TRIS-HCl, 6 μM CaCl$_2$, pH 7.5) and degassed. Protein concentration (between 50 and 270 μM depending on the ligand affinity) was checked by measurement of optical density by using a theoretical molar extinction coefficient of 28000. Glycoclusters were dissolved directly into the same buffer, degassed, and placed in the injection syringe (concentration: 175 μM). ITC was performed using a VP-ITC Microcalorimeter from MicroCal Incorporated. LeCA was placed into the 1.4478 mL sample cell,
at 25 °C. Titration was performed with 10 µL injections of carbohydrate ligands every 300 s. Data were fitted using the “one-site model” using MicroCal Origin 7 software according to standard procedures. Fitted data yielded the stoichiometry (n), the association constant (K_a), and the enthalpy of binding (ΔH). Other thermodynamic parameters (i.e., changes in free energy ΔG and entropy ΔS) were calculated from the equation ΔG = ΔH - TΔS = -RTlnK_a in which T is the absolute temperature and R = 8.314 J.mol⁻¹.K⁻¹. Two or three independent titrations were performed for each ligand tested.

Surface plasmon resonance (SPR): SPR inhibition experiments were performed using a Biacore 3000 instrument at 25 °C. Measurements were carried out on two channels with two immobilized sugars: α-L-fucose (channel 1) and α-D-galactose (channel 2). Immobilization of sugars was performed at 25 °C using running buffer (HBS) at 5 mL/min. Immobilization on each channel (CM5 Chip) was performed independently as follows. First, the channel was activated by injecting a fresh mixture of EDC/NHS (35 µL, 420 s). Then a solution of streptavidin (100 mg/mL in AcONa pH 5 buffer) was injected (50 µL, 600 s). The remaining reactive species were quenched by injecting ethanolamine (1M, 35 µL, 420 s) into the solution. Finally, a solution of the desired biotinylated-polyacrylamide-sugar (Lectivity, 200 mg/mL) was coated onto the surface (50 µL, 600 s) through streptavidin-biotin interaction. This procedure led to 804 RU (resonance units) (fucoside) and 796 RU (galactoside) of immobilized sugars on channels 1 and 2, respectively. Inhibition experiments were performed with the galactosylated channel 2 and plots represent subtracted data (channel 2-channel 1). The running buffer for LecA experiments was HEPES 10 mM, NaCl 150 mM, CaCl₂ 10 mM, Tween 20 (0.005%), pH 7.4. Inhibition studies consisted of the injection (150 µL, 10 µL/min, dissociation 120 s) of incubated (>1 h, RT) mixtures of LecA (5 µM) and various concentrations of inhibitor (two-fold cascade dilutions). For each inhibition assay, LecA (5 µM) without inhibitor was injected to observe the full adhesion of the lectin onto the sugar-coated surface (0% inhibition). The CMS chip was fully regenerated by successive injections of p-galactose (2x30 µL, 100 mM in running buffer). Binding was measured as RU over time after blank subtraction, and data were then evaluated using the BiAevaluation Software version 4.1. For IC₅₀ evaluation, the response (Rₑₛ centre) was considered to be the amount of lectin bound to the carbohydrate-coated surface at equilibrium in the presence of a defined concentration of inhibitor. Inhibition curves were obtained by plotting the percentage of inhibition against the inhibitor concentration (on a logarithmic scale) by using Origin 7.0 software (OriginLab Corp.). and IC₅₀ values were extracted from sigmoidal fit of the inhibition curve.

Fabrication of Microarray: Microstructured borosilicate glass slides (Nexterion Glass D, Schott Germany) were fabricated using standard photolithography and wet etching process detailed elsewhere. Microstructured slides featured 40 square wells (3 mm width, 60±1 µm depth). The resulting fabricated slides were functionalized according to the protocol reported in. The slides were washed in freshly prepared piranha rinsed in DI water and dried under dry nitrogen at 150 °C for 2h. After return to room temperature, tert-buty11-(dimethylamino)silylundecanoate in dry pentane was allowed to react with glass slide surfaces (RT). After pentane evaporation, the slides were heated at 150 °C overnight and finally washed in THF and water. The tert-buty1 ester function was converted into NHS ester.

Amino modified oligonucleotides were purchased from Eurogentec. Spotting of 0.3 nL of the various oligonucleotides at 25 µM in PBS 1X (pH 8.5) at the bottom of each reactor (64 spots per well). The substitution reaction was performed overnight at room temperature in a water saturated atmosphere, and then, water was allowed slowly to evaporate. Washing of the slides was performed with SDS (0.1%) at 70 °C for 30 min and deionized water briefly. All slides were blocked with BSA 4% solution in PBS 1X (pH 7.4, 37 °C, 2h) and washed successively in PBS-Tween 20 (0.05%), PBS 1X (pH 7.4) and DI water before being dried by centrifugation.

**Lectin labeling:** Alexa647 labeling of LecA lectin: LecA lectin was labeled with Alexa Fluor® 647 Microscale Protein Labeling Kit (A30009) from Invitrogen. Labeled-lectin concentration and the dye to lectin ratio were estimated by optical density read out with a dual beam spectrometer (Safas) equipped with a microcuvette (Hellma, 5 µL, 1 mm optical path). The absorbance at 281 nm and 650 nm were measured. LecA concentration was estimated to be 11.58 µM with a degree of labeling of 0.51 dyes for tetrameric LecA.

**“In solution” biological recognition:** The methodologies for K_d and IC₅₀ value determination have been previously reported. The determination by glycoarray: Galactocluster oligonucleotide conjugates 1DNA or 2DNA (1 µM final concentration) were diluted in PBS-0.02% Tween₂₀-2% BSA solution. CaCl₂ (1 µg/mL final concentration) was added. LecA at the desired final concentration was then added. Two µL of each solution (corresponding to the desired LecA concentration) were poured in the corresponding microwells. The slide was incubated (3h, 37 °C) in a water vapor saturated chamber and finally washed in PBS-Tween 20 (0.02%, 5min, 4 °C) and dried. A Microarray scanner, GenePix 4100A software package (Axon Instruments; λ_ex 532/635 nm and λ_em 575/670 nm) was used for fluorescent imaging of both fluorophore (Cy3 and Alexa 647). The average of the mean fluorescence signal was calculated from eight spots. The resulting Langmuir Isotherms were linearized using Scatchard plot to give the K_d values at the ordinate intercept.

**Biofilm Inhibition**

**Quantification of biofilm inhibition:** PA strains, wild type PAO1 (kindly provided by Dr. Reuben Ramphal, University of Florida Gainsville, FL, USA) and lecA mutant (Two-Allele Library,
Three-dimensional organization of the biofilms was analyzed by confocal laser scanning microscopy (CLSM) and image using DAPI associated fluorescence (ex. 350 nm/em. 460 nm) using Zeiss confocal microscope (LSM780). Biofilms were observed on a Zeiss confocal microscope (LSM780). Biofilms were observed using Zeiss confocal microscope (LSM780) with an objective 40x 1.3NA Oil Plan-Apochromat DIC. The DAPI die was excited with a 405 nm laser diode and the emission was collected between 410-500nm on GaAsP detector.

Structural Analysis of the Biofilm

Structural analysis was performed using the COMSTAT program. The image stacks obtained for each CLSM analysis were examined for the following structural features: volume of the biofilm divided by substrate area (µm³/µm²) indicating total biomass of bacteria; average thickness (µm) of the biofilm as well as the maximum thickness (µm); roughness coefficient (adimensional), a measure of heterogeneity of the biofilm surface reflecting important variations of biofilm thickness; substrate coverage (%) and surface colonized area (x10²µm²), a reflection of the efficiency with which the bacteria colonizes the surface.

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Notes and references