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# Structure-Dependent Modulation of a Pathogen Response in Plants by Synthetic O-Antigen Polysaccharides

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***Abstract:*** Many phytopathogenic bacteria display lipopolysaccharides (LPS) with the O-chain repeating unit [α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)]$_n$. This trisaccharide unit was synthesized and oligomerized to obtain hexa- and nonasaccharides. The deprotected rhamnans were effective in suppressing the hypersensitive response (HR) and in inducing *PR-1* gene expression in *Arabidopsis thaliana*. Conformational analysis of the oligorhamnans by NMR spectroscopy and molecular dynamics calculations revealed that a coiled structure develops with increasing chain length of the oligosaccharide. This is associated with increasing efficacy in HR suppression and *PR-1* gene expression. We therefore infer that the coiled structure of phytopathogenic bacteria is a plant-recognizable pathogen-associated molecular pattern (PAMP).

## Introduction

The infection of crop plants by phytopathogenic bacteria can either lead to disease, which may cause great economic losses in the crop, or to a rapid activation of plant resistance responses that contain or eliminate the pathogen. During infection, the recognition of bacterial components by plants plays a fundamental role in the outcome of the interaction. Since lipopolysaccharides (LPS)[1] cover almost 80% of the cell surface of Gram-negative bacteria, their role in bacterial interactions with eukaryotic hosts is undoubtedly crucial. LPS are one of a group of general elicitors that can be recognized by plants to trigger defense responses. The mechanism of plant defense activation by LPS is unknown but is suggested to be analogous to the innate immunity system of animals,[2] which is based on the recognition of pathogen-associated molecular patterns (PAMPs),[3] characteristic structures of the pathogen indispensable for its growth within the host.

The O-chain of the LPS from phytopathogenic bacteria generally comprises a rhamnan backbone, with single monosaccharide branches differentiating each serotype.[4] In particular the most frequent backbone consists of a [α-L-Rha-(1 → 3)-α-L-Rha-(1 → 3)-α-L-Rha-(1 → 2)] motif. An efficient synthetic path was developed for this trisaccharide, which was oligomerized to hexa- and nonasaccharides.[5]

The deprotected oligosaccharides were tested for their ability to induce *PR-1* gene expression, a plant immune response, and in suppressing the hypersensitive response (HR) in *Arabidopsis thaliana* caused by an avirulent strain of *Pseudomonas syringae*. These experiments were conducted in order to evaluate the role of O-chain oligosaccharides in plant innate immunity. Information regarding the secondary structure of the synthesized oligosaccharide was obtained through NMR spectroscopy and molecular modeling and correlated to the biological activities.

## Results and Discussion

The synthesis of the three rhamnans was based on the central trisaccharide **1**,[5] which was converted to a glycosyl acceptor by selective removal of the chloroacetyl moiety or to a glycosyl donor by activation as a trichloroacetimidate.[6]

Reaction of the trisaccharide acceptor with the trisaccharide donor gave hexasaccharide **3**, which was further elongated to the nonasaccharide **5** (see Supporting Information). Zemplèn deacylation of the protected oligorhamnans **1**, **3**, and **5** furnished the desired oligosaccharides **2**, **4**, and **6** in good yields (compounds **3** and **5** required higher reaction temperatures than those

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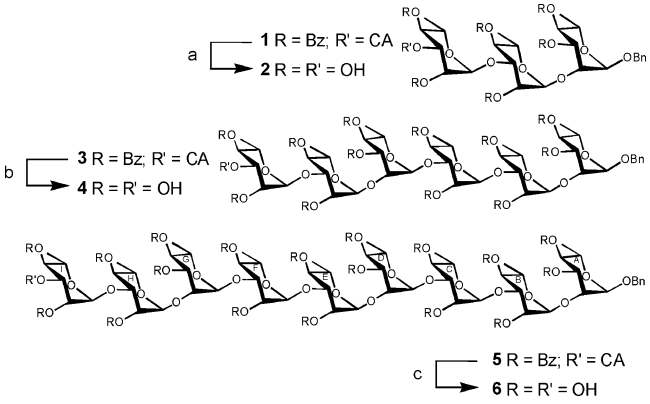


***Figure 1.*** (a) NaOMe, MeOH, rt, 4 h, 74%. (b) NaOMe, MeOH, 40 °C, 16 h, 84%. (c) NaOMe, MeOH, 40 °C, 3 days, 88%; CA = chloroacetyl.

***Table 1.*** Ability of Compounds **2** (Trisaccharide), **4** (Hexasaccharide), and **6** (Nonasaccharide) To Induce *PR-1* Gene Expression in *Arabidopsis thaliana*[a]

| time after treatment (h) | **2** | **4** | **6** |
|---|---|---|---|
| 12 | +1.9 ns | +20.3 *** | +52.6 *** |
| 20 | +4.2 *** | +29.9 *** | +86.8 *** |
| 24 | no change | +28.7 *** | +26.9 *** |

[a] +: fold upregulated compared to water treated tissue. After normalization to 18SrRNA. ns: not significant. *** = $P < 0.001$.

***Table 2.*** Ability of Compounds **2** (Trisaccharide), **4** (Hexasaccharide), and **6** (Nonasaccharide) To Suppress the HR in *Arabidopsis thaliana* Caused by *Pst AvrRPM1*[a]

| compound | **2** | **4** | **6** | control ($H_2O$) |
|---|---|---|---|---|
| 50 $\mu$g/mL | + | ++ | +++ | − |
| 100 $\mu$g/mL | + | +++ | +++ | − |

[a] +++: complete suppression in inoculated area. ++: suppression in 25−80% of inoculated area. +: suppression in <25%. −: no suppression.

for **1** and an unpolar cosolvent because of their reduced solubility in methanol) (Figure 1).

The series of compounds **2**, **4**, and **6** were tested for their ability to induce *PR-1* gene expression (Table 1) and to modulate the hypersensitive response (HR), a programmed cell death of plants triggered by avirulent bacteria (Table 2). The trisaccharide **2**, the hexasaccharide **4** or the nonasaccharide **6** were dissolved in water (50 $\mu$g ml$^{-1}$) and infiltrated into 6 week old leaves of Col-O. A further set of leaves was inoculated with water. The plants were placed in a growth cabinet at 25 °C with 16 h of light. The leaves were harvested 12, 20, and 24 h after inoculation. The changes in *PR-1* gene expression were followed using quantitative real-time RT-PCR analysis (see Supporting Information).

For the HR suppression test, the oligosaccharide **2**, **4**, or **6** was infiltrated into 6 week old leaves of Col-O as aqueous solutions (50 or 100 $\mu$g mL$^{-1}$) and water as a control. After 20 h in a growth cabinet these leaves were inoculated with *Pst/avrRPM1* in the pretreated and the control area, and the elicitation of HR was monitored over 24 h (Table 2). HR can be induced in *Arabidopsis thaliana* accession Columbia (Col-O) after inoculation with *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying the avirulence gene *avrRPM1* (*Pst/avrRPM1*)[7] as described.[8]

The panel of synthetic oligorhamnans showed a pronounced chain-length-dependent induction of the *PR-1* gene expression, and in prevention of the HR in *A. thaliana*. In response to the

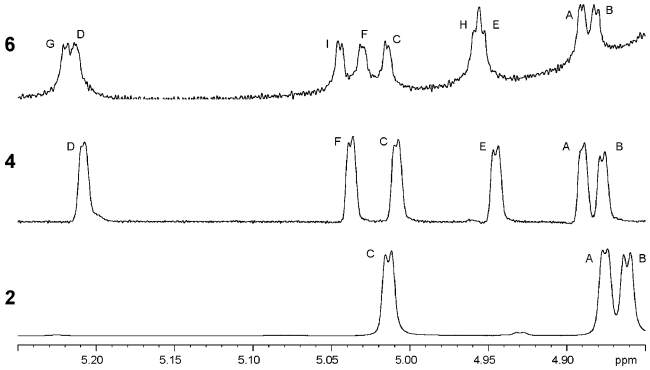


***Figure 2.*** $^1$H NMR comparison of the anomeric region of oligosaccharides **2**, **4**, and **6**.

trisaccharide **2** *PR-1* mRNA increased 4-fold 20 h after treatment, with a decrease at 24 h. This treatment was also only moderately effective in prevention of HR. The hexasaccharide **4** displayed a complete HR suppression at 100 $\mu$g per mL and a partial response at 50 $\mu$g per mL. *PR-1* expression in response to 50 $\mu$g per mL of compound **4** was already increased 20-fold at 12 h after treatment and 29-fold at 20 h, the timespan whereafter the bacteria were introduced into the tissue for the HR suppression test. Nonasaccharide **6** caused complete suppression at both concentrations used and a very marked increase of the *PR-1* gene expression, to 52- and 86-fold at 12 h and 20 h after treatment, respectively. Although it is already established that LPS can prevent the HR response[9,10] and induce plant defense-related genes,[9] nothing is known about the recognition mechanisms of LPS in plants. To our knowledge this is the first demonstration that short oligosaccharides can induce defense related gene expression and prevent HR in a size-dependent manner.

To probe whether the biological activity of the oligomers **2**, **4**, and **6** is based on structural features associated with the growing chain length, we analyzed the oligosaccharides by NMR and both molecular mechanics and dynamics calculations. Comparison of the anomeric regions (Figure 2) revealed distinct H-1 signals for the residues A and B of all compounds, most likely due to the anomeric benzyl moiety.

When one repeating unit was added as in **4**, the new anomeric signals, D, E, and F appeared near the shifts of the O-chain polysaccharide isolated from *Pseudomonas syringae* pv. *Coronafaciens*,[11] which is comprised of the same repeating unit as the synthetic rhamnans, used in this study. This evidence supports that a regular secondary structure is building up together with the chain elongation process. On the basis of the observed proton chemical shifts, this regular conformational motif was present in compounds **4** and **6** but not in oligosaccharide **2**.

Similarly, in compound **6**, the three new residues G, H, and I displayed proton resonances similar to those of the polysaccharide and almost coincident to those of D, E, and F, respectively, suggesting that residues in homologous position in the sequence experience a similar environment.

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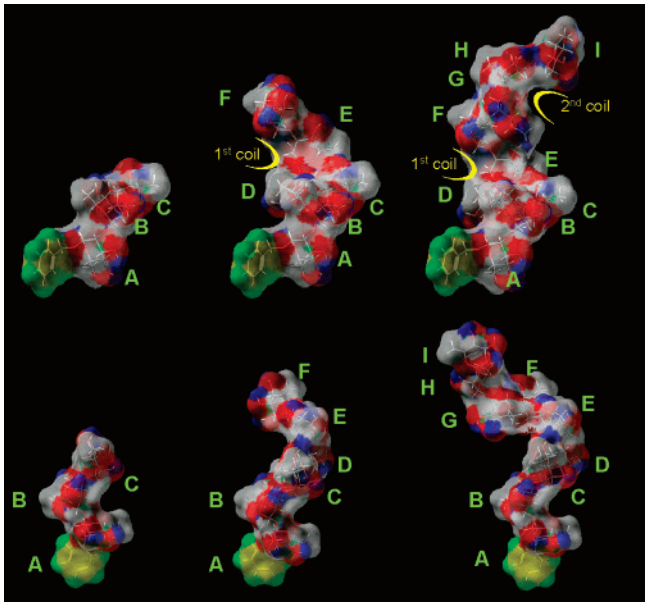


***Figure 3.*** Two different views of the Connelly surface models of oligorhamnans **2**, **4**, and **6** (reported from the left to the right, respectively). On top of the picture, the clefts induced from the rhamnose residues are evidenced.

Further information was obtained by molecular modeling. According to the molecular mechanics approach, the optimal dihedral angles for each glycosidic junction were evaluated using the MM3* force field:[12] the relaxed maps reveal that the rhamnosidic linkage, independent of its connectivity, displays two minima at different energies (Figure S3, Supporting Information).

The dihedral angle values of the lowest minimum were used for the construction of the starting structures for **2**, **4**, and **6** which were submitted to molecular dynamics analysis. The three oligosaccharides were kept in a thermal bath at 303 K (4000 ps for **2** and **4**; 8000 ps for **6**), and ensemble average interproton distances for each molecule were extracted from the simulations and translated into NOE contacts according to a full matrix relaxation approach. The predicted NOEs showed good agreement with those collected experimentally, proving the reliability of the simulation data. Analysis of the dynamics simulation data revealed that each glycosidic angle occupies the preferred values most of the time (Figure S4, Supporting Information) and that the lifetime of the second conformation is short. These results showed that the three oligosaccharides spend most of the time in one main conformational state depicted from the optimal dihedral angles found during the molecular mechanics approach. To gain additional information regarding the overall shape of these molecules, the surface of the three oligosaccharides **2**, **4**, and **6** was visualized (Figure 3) according to the Connelly method.

In agreement with proton NMR data, oligosaccharide **2** does not present a real secondary motif if compared with the other two species; this could be due to the small number of glycidic residues as well as to the presence of the benzyl moiety.

Looking at oligosaccharide **4**, a cleft is defined between residues C and E: residue C apparently starts the cavity that is completed from E; this pattern is repeated in **6** as well, where the cavities are two and are defined by the residues C−E and F−H; in addition, these two conformational motifs display a different orientation with respect to the chain elongation direction.

Application of these results to the conformation of the whole polysaccharide suggests the presence of a helical pattern, although this assumption needs to be confirmed with different experimental approaches.

## Conclusions

The combined NMR and molecular modeling data show that the long synthetic oligomers **4** and **6** adopt a secondary structure defined by the presence of one and two coils, respectively.

The bioactivity of the synthetic oligorhamnans correlates with their chain length. Compound **2** (with no coil) has low biological impact, whereas **4** and **6** are quite effective both in *PR-1* gene expression, a plant immune response, and in HR suppression in *Arabidopsis thaliana* caused by an avirulent strain of *Pseudomonas syringae*.

On the basis of these findings we propose that the coiled structure exposed from dimeric and trimeric oligomers **4** and **6**, of the repeating unit [α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2]$_n$, peculiar for many O-chains of phytopathogenic bacteria, is a PAMP recognized by plants and leads to the elicitation of specific responses including the suppression of HR and *PR-1* gene expression.

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**Supporting Information Available:** Full experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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