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Note

# Structural determination of the O-specific chain of the lipopolysaccharide from the mushrooms pathogenic bacterium *Pseudomonas tolaasii*

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**Abstract**

The complete structure of the O-specific polysaccharide of the lipopolysaccharide isolated from the cultivated mushrooms pathogen *Pseudomonas tolaasii* is described. The structural determination, achieved by chemical and spectroscopical analyses, indicates a novel tetrasaccharide repeating unit built up of two units of 2-acetamido-2,6-di-deoxy-glucopyranose (Quinovosamine, Qui*p*NAc) and two units of 2-acetamido-2-deoxy-gulopyranuronamide (Gul*p*NAcAN), one of which is acetylated at C-3 position:


*Keywords:* Structure elucidation; Lipopolysaccharides; Mushroom pathogenic bacteria; *Pseudomonas tolaasii*

*Pseudomonas tolaasii* is responsible of several diseases on cultivated mushrooms such as *Agaricus bisporus*, *Pleurotus ostreatus*, *Flammulina velutipes* and *Lentinula edodes*.[1] Several studies on *A. bisporus* cultivation have indicated that bacterial diseases and, in particular, brown blotches disease caused by the above bacterium, are the main responsible for crop losses in mushroom growing houses.[1] The taxonomic position of *P. tolaasii* is well defined and this bacterial entity, though related to other pathogens of the cultivated mushrooms such as *P. agarici*, *P. gingeri*, *P. reactans* and *P. fluorescens*,[1–5] is distinguishable for pathogenic, physiological and genetic characteristics.[1,4–8] In this regard, of the practical use is the white line assay which specifically and rapidly discerns *P. tolaasii* from other fluorescent *Pseudomonas* spp. associated to mushrooms.[9] The assay consists in the formation of a white precipitate when virulent *P. tolaasii* strains are grown in close proximity of *P. reactans* strains. The white precipitate is the result of the specific interaction between two diffusible lipodepsipeptides, the tolaasin produced by *P. tolaasii* and the so-called white line inducing principle (WLIP),[10,11] which is produced by virulent strains of *P. reactans*. However, recent findings seems to limit the above mentioned specificity since some other bacteria pathogenic on cultivated mushrooms appear to form the precipitate when grown side by side with *P. reactans* strains.[12]

Lipopolysaccharides (LPSs) are structural components of the Gram-negative outer membranes which appear to play an important role in the interaction of cells of pathogenic bacteria and plant and animal cell hosts.[13–15] LPSs activate host defence systems in either vertebrate and invertebrate inducing the production of antimicrobial peptides or, in mammalian, that of immunoregulatory, infiammatory and cytotoxic molecules. The use of mutants of plant pathogenic bacteria defective or lacking LPSs lead to acknowledge their role in the virulence expression and in the recogni-

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tion process which take place in the first phases of the interaction of the pathogen and plant cells. Furthermore, specific structural behaviours in the LPSs components, such as the O-chain, may have chemotaxonomic value among strains and species of the same genus. It is not excluded that LPSs of bacteria pathogenic on mushrooms, though no information are yet available, may play an important role in the interaction of the pathogens and their hosts as reported above for plant and animal bacterial pathogens. In this paper, the structure of the O-specific polysaccharide of the LPS fraction of the type strain NCPPB 2196 of *P. tolaasii* is reported.

Dried cells were extracted according to the phenol–water method outlined in Section 1 and the LPS fraction was found exclusively in the phenol phase; its compositional analysis showed the presence of 2-acetamido-2-deoxy-guluronic acid and 2-acetamido-2,6-dideoxy-glucose, besides mannose and KDO as minor components most likely belonging to core oligosaccharide. All residues were recognised by comparison with authentic samples.

The LPS material isolated was subjected to vertical gel electrophoresis and it appeared as a simple ladder-like pattern, typical of a regular repetitive lipopolysaccharide species of smooth LPS form. A mild acid hydrolysis allowed the removal of the lipid A moiety by precipitation leaving the polysaccharide in solution which was, in succession, purified by gel filtration. The compositional analysis of the pure O-polysaccharide, via acetylated *O*-methyl glycoside derivatives, confirmed the presence of 2-acetamido-2-deoxy-guluronic acid and 2-acetamido-2,6-di-deoxy-glucose (Qui*p*N). Methylation analysis showed the presence of 3-substituted quinovosamine while the other residue was not visible in this analysis. The $^{1}$H NMR spectrum of the polysaccharide showed in the anomeric region a variety of signals not all attributable to anomeric protons and not integrating for the same area (Fig. 1). Therefore, a detailed 2D NMR analysis (DQF-COSY, TOCSY, NOESY, gHSQC, gHMBC) allowed the complete assignment of all resonances (Table 1). Interestingly, in the HSQC spectrum (Fig. 2), the signal at 5.346 ppm correlated to a carbon at 68.8 ppm, while the signal (integrating for two protons) at 4.916 ppm correlated to a carbon at 66.9 ppm. Thus, all of these resonances accounted for ring protons/carbons and not for anomeric resonances. The signals at 5.060 (spin systems A and A′) and 4.470 (spin systems B and B′), both integrating for two protons, correlated with carbons at 98.0 and 102.5 ppm, respectively, indicative of their anomeric positions. A gHSQC spectrum registered without decoupling during acquisition allowed the measurement of the $^{1}J_{C,H}$ anomeric coupling, thus demonstrating that spin system A and A′ have α configuration whereas the spin system B and B′ have β configuration.

From the signal at 5.060 ppm (**A** and **A**′), by DQF-COSY, TOCSY and HSQC spectra, was possible to assign all resonances within each spin system. In particular, in the COSY spectrum it was possible to find two different H-2 resonances and, from these, two different spin system were generated. Residue **A** had typical proton and carbon signals of a uronic acid substituted at C-4 whereas residue **A**′ had proton and carbon

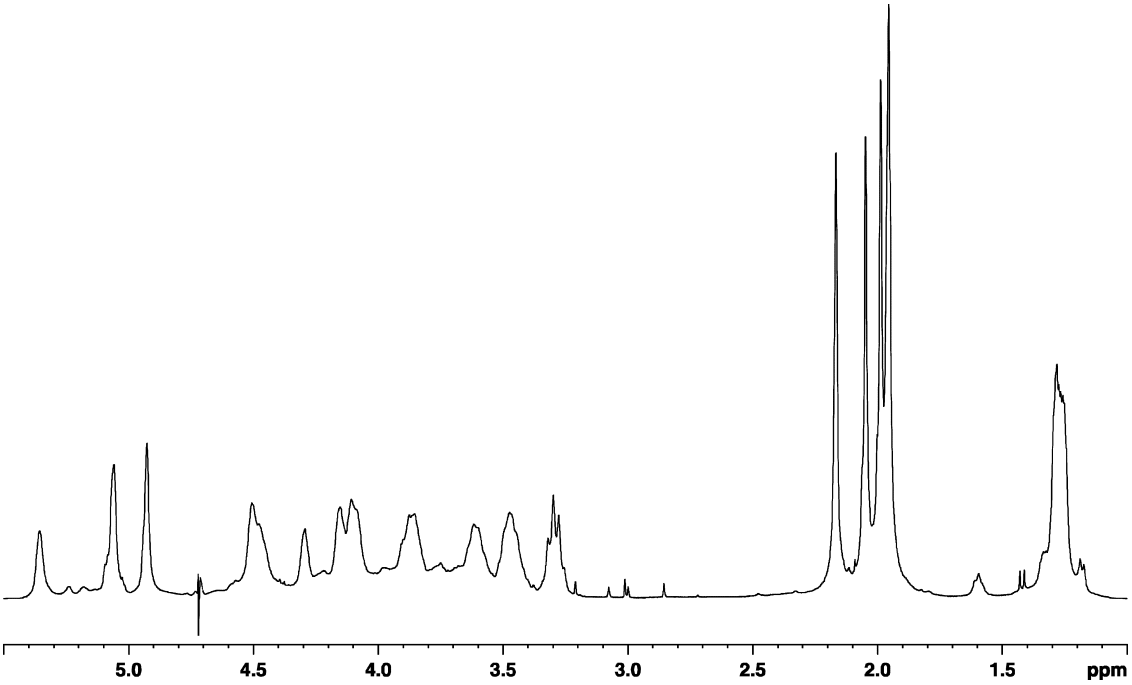


Fig. 1. The $^{1}$H NMR spectrum of the O-chain of the LPS from *P. tolaasii*.

Table 1
$^{1}H$ and $^{13}C$ (*italic*) chemical shifts (ppm) of the O-specific polysaccharide

| Sugar Residue | H1/C1 | H2/C2 | H3/C3 | H4/C4 | H5/C5 | H6/C6 |
|---|---|---|---|---|---|---|
| A | 5.060 | 4.287 | 4.150 | 4.075 | 4.916 | |
| Gul*p*NAcAN | *98.6* | *46.1* | *67.6* | *78.8* | *66.9* | *174.0* |
| A′ | 5.060 | 4.504 | 5.346 | 4.092 | 4.916 | |
| Gul*p*NAc3AcAN | *98.0* | *44.5* | *68.8* | *75.9* | *66.9* | *174.0* |
| B and B′ | 4.470 | 3.852 | 3.606 | 3.279 | 3.457 | 1.253 |
| Qui*p*NAc | *102.5* | *55.8* | *79.5* | *74.2* | *72.3* | *17.3* |

The spectra are measured in $D_2O$ at 303 K. Additional signals for the *N*-acetyl group are at around 2.0/*23.7* (methyl) and *175.6* ppm (carbonyl).

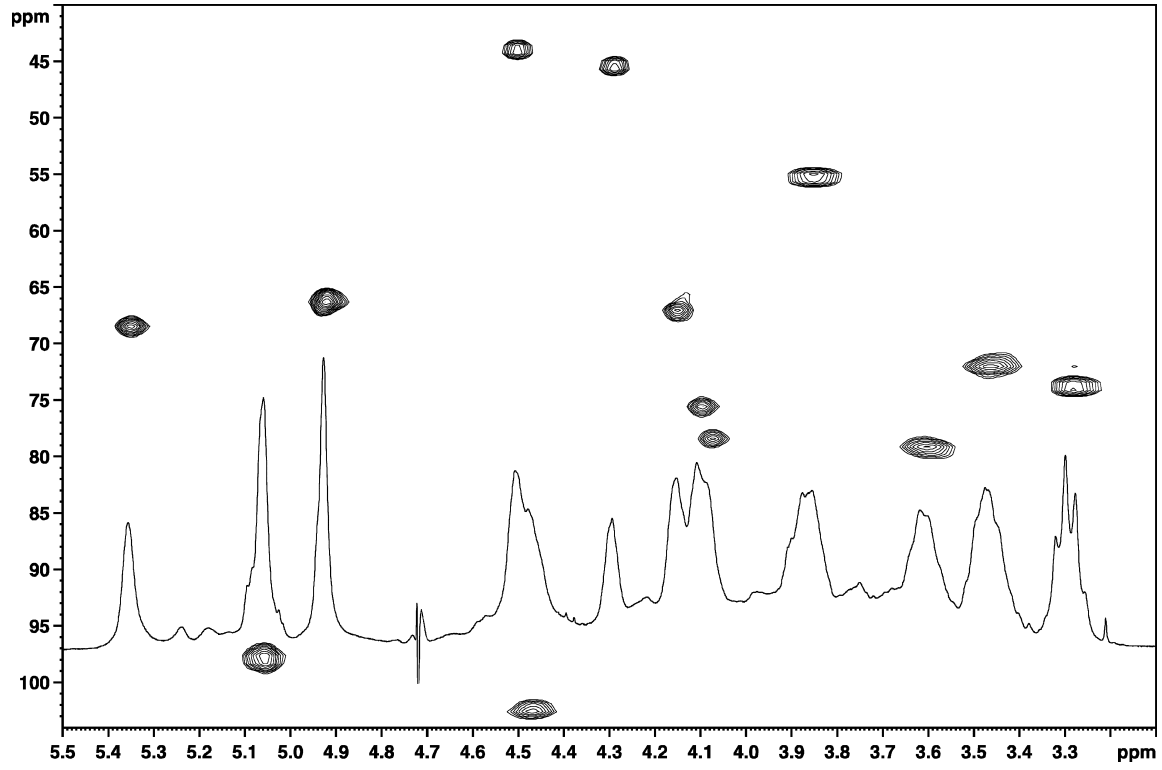


Fig. 2. Zoom of the HSQC spectrum of the O-chain.

resonances for a 4-substituted 3-*O*-acetyl uronic acid. Actually, in this last, H-2 proton, which resonated at 4.504 ppm, was correlated to the signal at 5.346 ppm in the COSY spectrum which, at its turn, correlated to the proton H-4 at 4.092 ppm. Both spin systems ended with resonance at position 5 at 4.916 ppm. The value of the coupling constants and the $^{13}C$ chemical shifts were distinctive of a *gulo*-configurated hexopyranose.[16]

The other two types of spin system (**B** and **B′**) possessed the same resonances and were both characterised by the anomeric signals at 4.470 and 102.5 ppm (Fig. 2). A further indication of the β configuration was the observation of a coupling constant H-1 to H-2, about 8 Hz, and the presence of nuclear Overhauser effect among H-1, H-3 and H-5. The carbon chemical shifts were indicative of a substitution at C3, as found by a downfield shifts of this carbon signal (79.5 ppm). All data identified spin system **B** and **B′** as 3-substituted-β-Qui*p*NAc. The acetyl substitution at C-2 of all residues and at C-3 of residue **A′** was proven by a HMBC spectrum where all H-2 protons, H-3**A′** and the methyl signals around 2 ppm (1.98 and 2.17) ppm were correlated to carbonyl signals at roughly 175.0 ppm.

The sequence of the monosaccharides in the repeating unit was inferred using interresidual NOE data measured by 2D-NOESY and long range scalar connectivities measured by HMBC spectrum (Fig. 3). Actually, in the NOESY spectrum a cross peak was present

between the anomeric proton of residue **B** and **B′** with both H-4 resonances of **A** and **A′**, respectively, demonstrating that Qui*p*NAc is linked to these two residues. On the other side, the signal at 5.060 ppm (H-1**A** and H-1**A′**) was correlated in the same spectrum to the H-3 proton of **B** and **B′** residues. Actually, in the HSQC spectrum, C-4**A**, C-4**A′**, C-3**B** and C-3**B′** signals showed a downfield displacement owed to glycosylation shift. The HMBC spectrum definitely demonstrated these structural assumptions, showing a cross peaks between the H-1/C-1 of **A** and **A′** residues with C-3/H-3 of Qui*p*NAc residues and on the other side, of H-1/C-1 of **B** and **B′** residues with C-4/H-4 of **A** and **A′**. Interestingly, the signals in the $^{1}$H NMR spectrum showed no pD dependence, since this was registered at different pD values, neutral, acid and alkaline, and furthermore, it rapidly gave β elimination of the uronic residue upon treatment in alkali medium, both these proofs evidenced amidation at carboxy group.[17] Actually, many attempts of alkaline hydrolysis resulted in degradation of the polysaccharide with the result of a single disaccharide derivative.

Hence, more selective and milder de-O-acetylation conditions were chosen, the reaction was carried out on the sample with ammonium hydroxide at 4 °C for 4 days and the resulting product was still a polisaccharide with a disaccharide repeating unit as demonstrated by 2D NMR analysis (Table 2). The anomeric region of the $^{1}$H NMR spectrum (Fig. 4) consisted of three signals, at 5.071, 4.937 and 4.469, while the $^{13}$C NMR spectrum showed only two anomeric carbon signals. Actually, in the HSQC spectrum (Fig. 4), the signals at 5.071 and 4.937 ppm correlated to a carbon anomeric signals, whereas the signal at 4.469 ppm correlated to a carbon ring signal at 66.1 ppm and thus it was identified as the H-5 resonance of the uronamide residue. From both anomeric signals it was possible to assign all the ring resonances and thus, to recognise the 2-acetamido-2-deoxy-gulopyranuronamide and 2-acetamido-2,6-di-deoxy-glucopyranose.

The only information missing at this stage on the primary structure of the O-polysaccharide from the LPS fraction of *P. tolaasii* was the absolute configuration of both residues involved in the structure of the tetrasaccharide repetitive unit.

In order to get these informations, we used both the NMR and the Exciton Coupling Method approaches.[18]

A strong methanolysis carried out on the O-polysaccharide chain yielded as products the two O-methyl glycosides derivatives, which were N-acetylated and purified by TLC. The Qui*p*NAc derivative was isolated in good yield and was further *p*-bromobenzoylated to

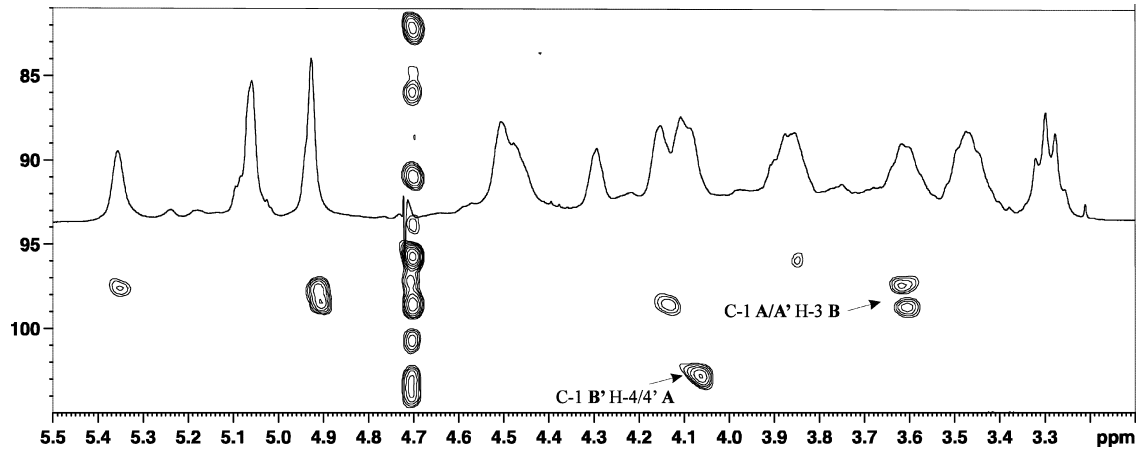


Fig. 3. The relevant HMBC correlations of the anomeric region of the O-chain are showed.

Table 2
$^{1}$H and $^{13}$C (*italic*) chemical shifts (ppm) of the de-O-acetylated O-specific polysaccharide

| Sugar Residue | H1/C1 | H2/C2 | H3/C3 | H4/C4 | H5/C5 | H6/C6 |
|---|---|---|---|---|---|---|
| A | 5.071 | 4.302 | 4.166 | 4.089 | 4.937 | |
| Gul*p*NAcAN | *98.2* | *45.8* | *67.3* | *78.4* | *66.1* | *174.0* |
| B | 4.469 | 3.850 | 3.608 | 3.290 | 3.462 | 1.250 |
| Qui*p*NAc | *102.0* | *55.6* | *79.4* | *73.8* | *72.0* | *17.0* |

The spectra are measured in $D_2O$ at 303 K. Additional signals for the *N*-acetyl group are at 1.995, 2.010/*23.7* (methyl) and *175.6* ppm (carbonyl).

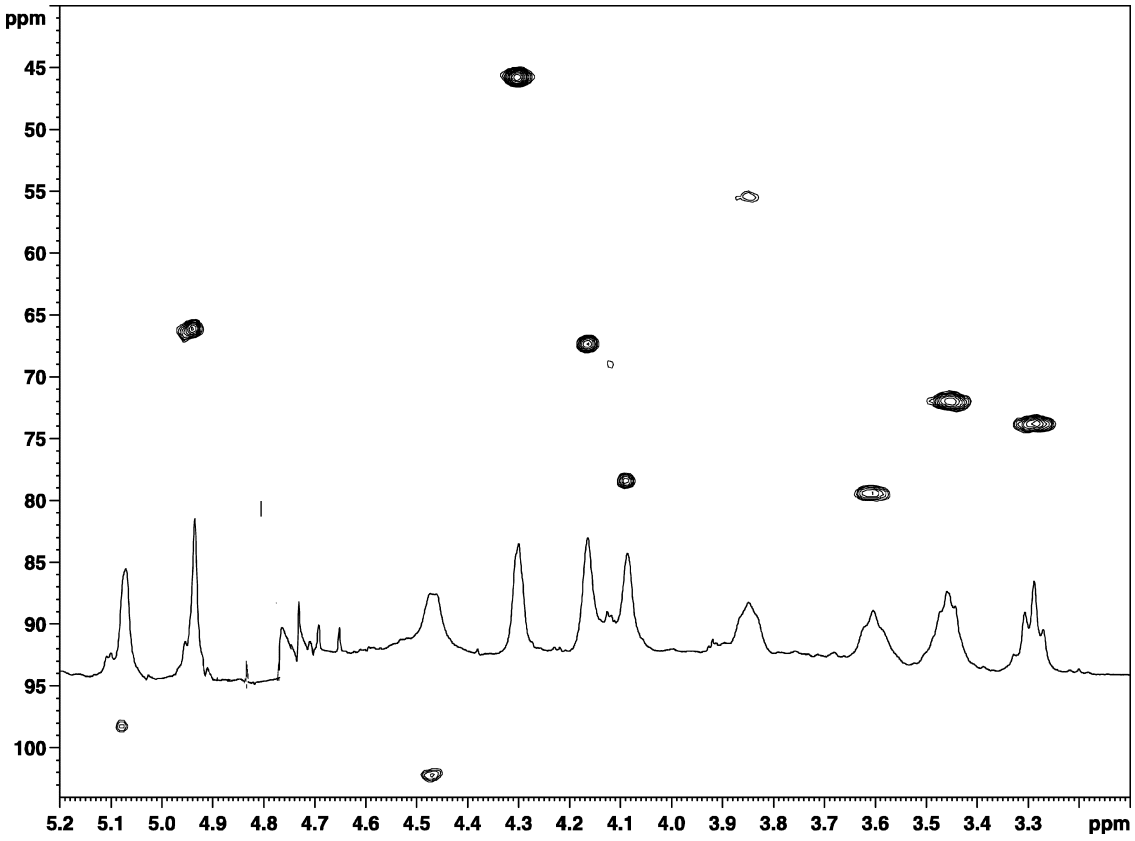


Fig. 4. Zoom of the HSQC and the $^{1}$H NMR spectra of the de-O-acetylated O-chain.

give the *O*-methyl 2-acetamido-2,6-di-deoxy, 3,4-di-*p*-bromobenzyl-glucopyranoside, purified by TLC chromatography.

C.D. spectrum was measured and the D configuration for Qui*p*NAc residue was established from the negative Cotton effect exhibited by the compound, which indicated the counter-clockwise arrangement of the two *p*-bromobenzoate chromophores.

The absolute configuration of both gulopyranose units derivative was established on the basis of $^{13}$C chemical shifts of the de-acetylated derivative. The analysis has been carried out considering the two possible relative configurations for the compound in the disaccharide repeating unit, that is: →4)-α-L-Gul*p*-NAcAN-(1→3)-β-D-Qui*p*NAc-(1→ and →4)-α-D-Gul*p*NAcAN-(1→3)-β-D-Qui*p*NAc-(1→. The $^{13}$C chemical shifts of gulopyranose residue univocally indicate L-configuration in agreement with previous data.[19,20]

Hence, the O-chain of the LPS from the mushrooms associated bacterium *P. tolaasii* consists in a tetrasaccharide repeating unit built up of two units 2-acetamido-2,6-di-deoxy-glucopyranose and two units of 2-acetamido-2-deoxy-gulopyranuronamide, one of which is acetylated at C-3 position, as shown below:

→4)-α-L-Gul*p*NAc3AcAN-(1→3)-β-D-Qui*p*NAc-(1→4)-α-L-Gul*p*NAcAN-(1→3)-β-D-Qui*p*NAc-(1→

To our knowledge this is the second O-polysaccharide chain identified from a mushrooms associated bacterium, since it has been already identified the O-chain from the mushrooms pathogen *P. reactans*.[21]

## 1. Experimental

### 1.1. Growth of *P. tolaasii*, isolation of LPS and OPS

*P. tolaasii* strain NCPPB2192 was maintained lyophilised and routinely subcultured on glycerol nutrient agar slants at 25 °C. Bacterial cells for LPSs extraction were obtained by growing the bacterium in 500 mL Erlenmeyer flasks filled with 200 mL of liquid King B medium amended with 0.5 g L$^{-1}$ of sodium thyoglycolate on a rotary shaker at 150 rpm at 25 °C for 48 h. Cultures were processed as previous reported[21] and dried cells (2.17 g) obtained from 1.5 L culture were extracted according to the hot phenol–water method.[22] Both phases were separately dialyzed against distilled water, freeze-dried and screened by discontinuous SDS PAGE (sodium dodecyl sulphate polyacrylamide electrophoresis),[23] with a 12% gel on a miniprotean gel system from Bio-Rad; the samples where run at constant voltage (150 V) and stained with silver nitrate.[24] The lipopolysaccharide material (125 mg) was recov-

ered exclusively in the phenol phase (5.7% yield). In order to obtain the O-polysaccharide chain, the LPS was hydrolysed with aq. 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant thus obtained (OPS fraction, 100 mg, 80% of LPS) was purified by gel permeation chromatography on a Sephacryl S300-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent and monitored with a Waters differential refractometer.

### 1.2. Compositional and methylation analysis

Monosaccharides were analysed by GLC–MS as acetylated *O*-methyl glycosides derivatives as previously reported.[16,21]

Methylation analysis was attempted according to the procedure described previously.[25] The permethylated polysaccharide was recovered in the organic layer of the water–chloroform extraction, dried and hydrolyzed with 200 μL of 2 M TFA at 120 °C for 2 h, the acid was removed by repeated evaporations with *i*-propanol and the partially methylated monosaccharides were solved in EtOH (200 μL) and reduced with 3 mg of $NaBD_4$ at 25 °C for 16 h, borates were removed by evaporation with MeOH and few drops of glacial acetic acid, acetylation was performed with $Ac_2O$ and pyridine, 150 μL each at 120 °C for 20 min.

GLC–MS was performed on a Hewlett-Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), the temperature program was: 150 °C for 5 min, then 5 °C $min^{-1}$ to 300 °C.

### 1.3. Absolute configuration of quinonovosamine

Fifteen milligrams of O-chain fraction were treated with 1 M methanolic HCl at 80 °C for 20 h. The excess of acid was removed by repeated evaporation with methanol and the methyl glycosides were N-acetylated by addition of dry MeOH (500 μL), dry pyridine (100 μL) and acetic anhydride (50 μL), at room temperature for 30 min, solvents were removed by evaporation and the monosaccharide derivatives purified by preparative TLC irrigated with $CHCl_3$–$CH_3OH$ = 8:2.

The *O*-methyl α-glycopyranoside isolated in these conditions, was *p*-Br-benzoylated in dry pyridine with an 1.5 excess of the corresponding acyl chloride, at 25 °C, for 3 h, ($^1H$ expressed in ppm, measured in $CDCl_3$ at 303 K, H-1 4.99, H-2 5.62, H-3 6.06, H-4 5.77, H-5 4.30, H-6 1.21, aromatic signals were present around 7.5 ppm).

Circular Dicroism spectrum was measured on a JASCO J-715 instrument solving the sample in HPLC grade methanol.

### 1.4. NMR spectroscopy

NMR experiments were carried out on a Bruker DRX 400 MHz equipped with reverse multinuclear probe at 303 K. Chemical shift of spectra recorded in $D_2O$ are expressed in $\delta$ relative to methyl signal of internal acetone (2.225 and 31.4 ppm). Two-dimensional spectra (DQF-COSY, TOCSY and NOESY, gradient-HSQC and HMBC) were measured using standard Bruker software.

For homonuclear experiments, typically 512 FIDs of 1024 complex data points were collected, with 40 scans per FID. In all cases, the spectral width was set to 10 ppm and the carrier placed at the residual HOD peak. A mixing time of 200 ms was used for both NOESY and ROESY experiments. For the HSQC spectrum, 256 FIDS of 1024 complex points were acquired with 50 scans per FID, the GARP sequence was used for $^{13}C$ decoupling during acquisition. Processing and plotting was performed with standard Bruker XWin-NMR 1.3 program.

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