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The linkage between O-specific caryan and core region in the lipopolysaccharide of *Burkholderia caryophylli* is furnished by a primer monosaccharide

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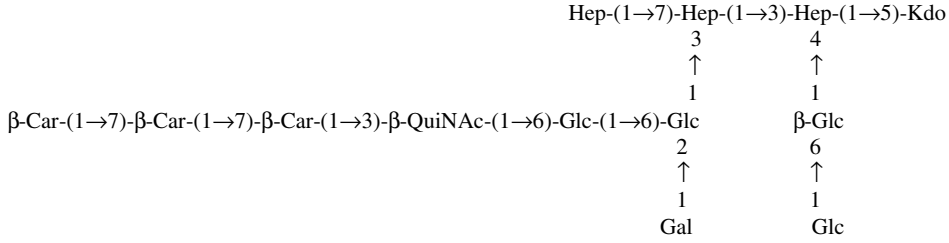
Abstract—From the lipopolysaccharide (LPS) fraction of the plant-pathogenic bacterium *Burkholderia caryophylli*, the linkage between O-specific caryan and core region was characterised. The LPS fraction was first treated with 48% aqueous HF at 4 °C and successively with 1% acetic acid at 100 °C. A main oligosaccharide representing the carbohydrate backbone of the core region and a portion of the caryan (three unit of caryose) was isolated by high-performance anion-exchange chromatography. Compositional and methylation analyses, matrix-assisted laser desorption/ionisation mass spectrometry and 2D NMR spectroscopy identified the structure:



The above residues are α-linked pyranose rings, if not stated otherwise. Hep is L-*glycero*-D-*manno*-heptose, Car is 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose and Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid. This finding indicates that QuiNAc residue is the primer monosaccharide, which connects the core oligosaccharide to caryan O-chain.

Keywords: *Burkholderia caryophylly*; Lipopolysaccharide; Primer; Core region; O-Chain

1. Introduction

Burkholderia caryophylli is a phytopathogenic Gram-negative bacterium responsible for the wilting of carnation[1] and it shares the presence of lipopolysaccharides (LPS) in its cell wall with other Gram-negative species. Lipopolysaccharides are amphiphilic macromolecules composed, in most cases, of a hydrophilic hetero-polysaccharide (formed by a core oligosaccharide and O-specific polysaccharide or O-chain) covalently linked to a lipophilic moiety termed lipid A, which anchors the macromolecule in the outer membrane. LPS not containing an O-chain are termed rough (R) LPS or lipooligosaccharides (LOS). LOS may occur in both

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wild and laboratory strains possessing mutations in the genes encoding the O-specific polysaccharide biosynthesis or transfer. LPS of plant-pathogenic bacteria have been shown to play a role in phytopathogenicity.[2] In order to contribute to the structure–function relationship of the LPS from *B. caryophylli*, we have carried out its full structural characterisation.

The LPS from *B. caryophylli* possesses several characteristic features, among which is the occurrence of two different O-specific polysaccharides. Each one is built up of a unique monosaccharide residue to result in two linear homo-polysaccharides, one of which is composed of 3,6,10-trideoxy-4-*C*-(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulo*-decose (caryophyllose, α-(1→7)-linked, caryophyllan) and the other of 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose (caryose, β-(1→7)-linked, caryan).[3–6] The caryan is acetylated in non-stoichiometric amounts, leading to a very unusual block pattern with repeating units of 19 residues,[7] while the caryophyllan is randomly acetylated and no chemical repeating unit could be found.[8]

Also, the core region and lipid A moieties of LPS from *B. caryophylli* possess important and novel structural features. The core region possesses two different Hep-α-(1→5)-Kdo moieties as a novel element in the inner part, and the lipid A bears a residue of 4-amino-4-deoxy-L-arabinopyranose solely at the glycosidic phosphate.[10] We now report the linkage between caryan and core region.

2. Results and discussion

After dephosphorylation of the LPS with 48% HF, which destroyed the greater part of the O-specific polysaccharides, and hydrolysis in 1% aqueous acetic acid, an oligosaccharide (Fig. 1) was isolated by high-performance anion-exchange chromatography (HPAEC). Its compositional analysis identified D-Glc, D-Gal, L,D-Hep, 2-amino-2,6-dideoxy-D-glucose (Quinovosamine, QuiN), 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose (Caryose, Car) and a low quantity of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Methylation analysis of the oligosaccharide yielded the derivatives of terminal Glc, terminal Gal, terminal Hep, terminal Car, 6-substituted Glc, 3-substituted QuiN, 7-substituted Car, 2,6-disubstituted Glc, 3,4-disubstituted Hep and 3,7-disubstituted Hep.

The negative ion MALDI-TOF mass spectrum (Fig. 2) of the oligosaccharide showed a major molecular ion at *m/z* 2466.6 [$(M+H)^+$], which was consistent with a molecule consisting of five hexose, three heptose, one Kdo, one 6-deoxy-hexosamine and three caryose residues. Together with this major ion identifying an oligosaccharide containing one reducing Kdo (**Kdo_{RED}**), an ion in minor intensity was present consistent with an oligosaccharide possessing one Kdo in lactone or *anhydro* form (Δ*m/z* 18). Other ions were present (Δ*m/z* 218)

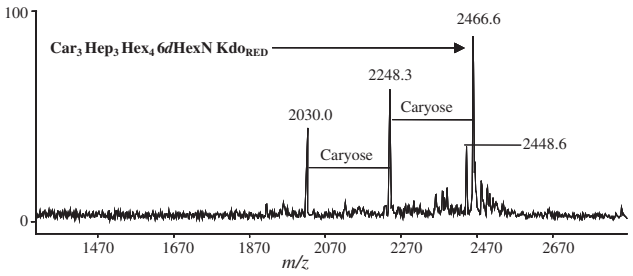

Figure 2. The negative ion MALDI-TOF- mass spectra (linear mode) of the O-chain-core-region from LPS of *B. caryophylli*. The assignment of the main ion peaks is shown.

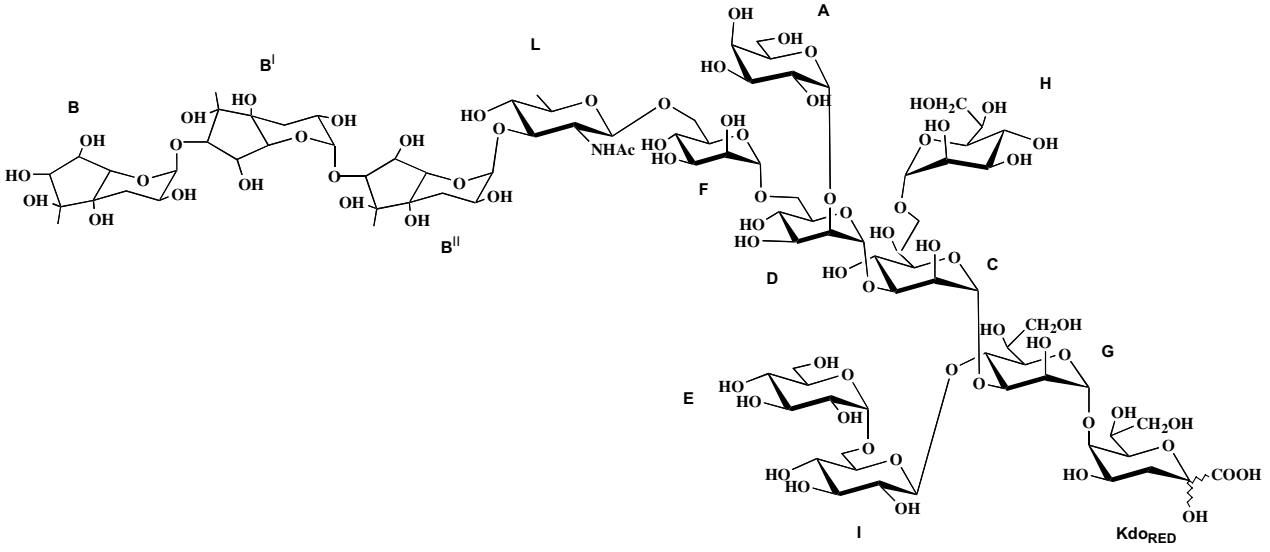

Figure 1. The structures of the oligosaccharide representing the O-chain-core-region of the LPS of *B. caryophylli*.

identifying oligosaccharides lacking one and two caryose units.

The 1D ^{1}H NMR spectrum of the isolated oligosaccharide revealed a certain heterogeneity, probably due to Kdo present as pyranose, furanose, *anhydro* and lactone forms, and in agreement with this, Kdo methylene signals were considerably spread. The anomeric region of the ^{1}H NMR spectrum (Fig. 3) contained several spin systems (**A**–**L**), identified as three heptose, five hexose, one 6-deoxy-hexosamine and three caryose residues. The complete structure of the oligosaccharide was established by a combination of 2D NMR spectroscopy. Chemical shifts were assigned utilising DQF-COSY, TOCSY, NOESY, ROESY, *g*-HSQC and *g*-HMBC experiments (Table 1). The identification of each residue was possible by the complete assignment of all resonances and the determination of the $^3J_{H,H}$ vicinal coupling constants. Anomeric configurations were assigned on the basis of the chemical shifts of H-1, C-1, C-5 and $J_{1,2}$ values, which were determined from the DQF-COSY experiment.

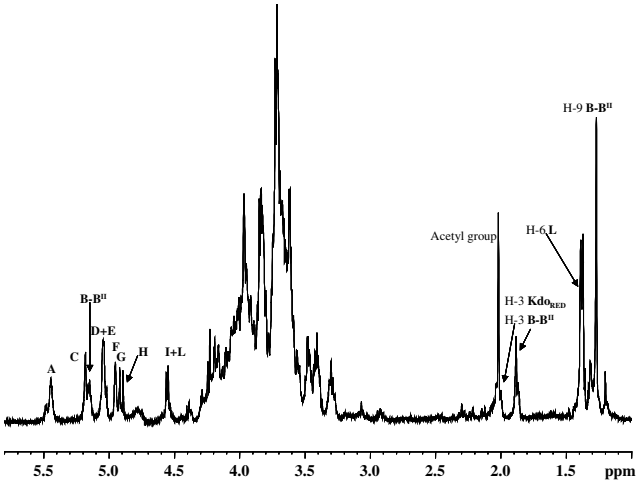

Figure 3. ^{1}H NMR spectrum of the O-chain-core-region fraction. The spectrum was recorded at 600 MHz and 30 °C. Spin systems are labelled as shown in Figure 1.

Spin systems **G**, **C** and **H** were recognised as *manno*-configured α-heptose residues, since they possessed low $^3J_{H\text{-}1,H\text{-}2}$ and $^3J_{H\text{-}2,H\text{-}3}$ values, which are diagnostic for an equatorially oriented H-2. By TOCSY and ROESY spectra, from H-2 it was possible to assign all other cross-peaks up to H-7s.

All three hexoses (**D**, **E** and **F**) possessed large ring proton $^3J_{H,H}$ values, which are typical for a *gluco*-configuration, while spin system **A** possessed the α-*galacto*-configuration with low $^3J_{H\text{-}3,H\text{-}4}$ and $^3J_{H\text{-}4,H\text{-}5}$ values (3 and 1 Hz, respectively). The latter impaired any magnetisation transfer in the TOCSY experiment over H-4 and, therefore, the H-5 resonance was deduced by a NOE connectivity with H-4 in the NOESY experiment. Spin systems **A**, **D**, **E** and **F** possessed the α-anomeric configuration ($^3J_{H\text{-}1,H\text{-}2} < 4$ Hz). Spin systems **I** and **L**

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of sugar residues of the O-chain-core-region of LPS from *B. caryophylli*

Units	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8/C-8	H-9/C-9
A	5.446	3.631	3.820	4.099	3.970	3.717			
t-Gal	98.1	70.1	70.5	68.4	71.7	61.7			
B	5.181	4.090	1.888/2.067	—	4.271	4.211	3.988	—	1.269
t-Car	97.3	65.3	30.6	79.0	75.5	78.5	78.0	78.8	16.0
B^{I}	5.179	4.119	1.862/2.015	—	4.200	4.193	3.919	—	1.269
7-Car	97.3	65.7	30.0	78.9	75.1	78.5	88.5	—	16.0
B^{II}	5.177	4.119	1.874/2.064	—	4.200	4.193	3.921	—	1.269
7-Car	102.3	65.0	30.5	78.9	75.1	78.5	88.5	—	16.0
C	5.190	4.070	4.174	3.814	3.788	4.158	3.713		
3,7-Hep	102.3	68.6	79.9	67.1	69.9	68.3	70.3		
D	5.046	3.904	3.850	3.691	3.936	4.067/3.983			
2,6-Glc	98.7	80.4	71.6	69.8	68.8	67.1			
E	5.045	3.785	3.843	3.698	3.850	3.730/3.830			
t-Glc	98.4	69.9	71.6	70.2	67.9	61.4			
F	4.949	3.548	3.726	3.407	3.667	3.820/3.740			
6-Glc	98.6	71.9	69.9	70.2	72.8	67.5			
G	4.922	4.033	4.111	4.130	3.831	3.976	3.703/3.728		
3,4-Hep	100.9	69.6	75.8	75.6	71.6	70.6	63.8		
H	4.891	3.989	3.840	3.891	3.656	4.022	3.656/3.703		
t-Hep	100.9	69.9	71.8	67.1	70.9	69.7	63.8		
I	4.554	3.293	3.465	3.406	3.625	3.620/3.870			
6-Glc	103.5	74.0	76.4	70.2	75.0	67.4			
L	4.553	3.742	3.440	3.460	3.609	1.375			
3-QuiNAc	102.9	55.7	85.0	71.5	76.3	17.1			
Kdo$_{RED}$	—	—	1.832/2.011	4.003	4.186	n.d.	n.d.	n.d.	
	n.d.	n.d.	34.0	66.2	71.2	n.d.	n.d.	n.d.	

Chemical shifts are expressed relative to acetone (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm; at 30 °C): monosaccharides are as shown in Figure 1. The resonances of the acetamido group were at 2.012/23.0 and 175.0 ppm; n.d, not determined. **Kdo$_{RED}$** is reducing Kdo.

possessed large $^{3}J_{H,H}$ values identifying the β-*gluco*-configuration. In the TOCSY spectrum, starting from the H-1 proton signals, all ring proton correlations could be identified, in case of **L** up to the methyl proton signals. Residue **I** was identified as β-glucose while **L** was identified as β-quinovosamine, since in the HSQC experiment its H-2 resonance correlated with a nitrogen bearing carbon at 56.7 ppm. The presence of acetamido groups at C-2 of **L** was verified by a HMBC experiment in which both H-2 **L** and the methyl signal at 2.10 ppm correlated with a carbonyl signal at 175.6 ppm.

The assignment of the anomeric orientation of the identified above residues was further supported by the NOESY experiment that yielded *intra*-residue NOE connectivities from H-1 to H-3 and to H-5 for β-anomers, and *intra*-residue NOE connectivities from H-1 to H-2 for α-anomers. All the hexose and heptose residues were present as pyranose rings as established by either ^{13}C chemical shift values or to the occurrence of long-range correlations between C-1/H-1 and H-5/C-5 in the $^{1}H,^{13}C$-HMBC spectrum.

Caryose (**B**–**B**$^{\mathbf{II}}$) resonances were completely assigned in comparison with published data.[7] Briefly, the caryose monosaccharide furnishes two unconnected spin systems, that is, H-1 to H-3 and H-5 to H-7, and the signal of the methyl group (H-9) that is not connected by spin–spin coupling to any of the other ^{1}H signals. Each of these spin systems was assigned using COSY and TOCSY spectra and the separate spin systems were connected through NOE contacts and long range $^{1}H,^{13}C$ coupling constants. The small coupling constant $^{3}J_{H\text{-}1,H\text{-}2} < 4$ Hz indicated a synclinal orientation of the H-1 and H-2 protons, that is, the β-configuration of caryose glycosidic linkage.

The ^{13}C NMR chemical shifts could be assigned by an HSQC experiment, using the interpreted ^{1}H NMR spectrum. Low-field shifted signals indicated substitutions at O-7 of residues **B**$^{\mathbf{I}}$–**B**$^{\mathbf{II}}$, at O-3 and O-7 of **C**, at O-2 and O-6 of **D**, O-6 of **F**, at O-3 and O-4 of **G**, O-6 of **I** and O-3 of **L**, whereas **A**, **B**, **E** and **H** were present as non-substituted residues.

The sequence of the monosaccharide residues was determined using NOE data of the NOESY spectrum (Fig. 4), where all crucial NOE contacts between anomeric protons and those geminal to glycosylated carbons were detectable. Accordingly, a strong NOE contact between H-1 of **C** and H-3 of **G** and H-1 of **H** and H-7 of **C** proved the close proximity of heptose residues in the oligosaccharide skeleton. Moreover, H-1 proton signal of **G** residue showed a strong NOE contact with H-5 of Kdo, thus demonstrating a Hep-(1→7)-Hep-(1→3)-Hep-(1→5)-Kdo skeleton. A NOE connectivity between H-1 of Glc **I** and H-4 of Hep **G** demonstrated that β-glucose was attached to O-4 of heptose **G**, and, moreover, H-$6_{a,b}$ of Glc **I** gave a strong NOE signal to H-1 of **E** residue indicating that this latter residue was attached to O-6 of **I** residue. Heptose **C** was substituted at O-3 by a 2,6-disubstituted α-glucose, residue **D**. This was inferred from the identified NOE contacts between H-1 of **D** and H-3 (strong) of **C**. The H-1 protons of Glc **F** and Gal **A** showed NOE contacts to H-$6_{a,b}$ and H-2 of **D**, respectively, indicating that **F** and **A** residue are linked at O-6 and O-2 of **D**, respectively. A further NOE contact was found between **A** and **D** anomeric protons, which unequivocally proved the (1→2)-linkage between these two residues. A NOE contact between H-1 **L** and H-$6_{a,b}$ of **F** demonstrated that glucose **F** was substituted at O-6 by QuiNAc **L**.

This latter residue was substituted at O-3 by a caryose trisaccharide. Actually, the anomeric proton signal of caryose **B**$^{\mathbf{II}}$ gave a NOE with H-3 of QuiNAc **L**. Caryose

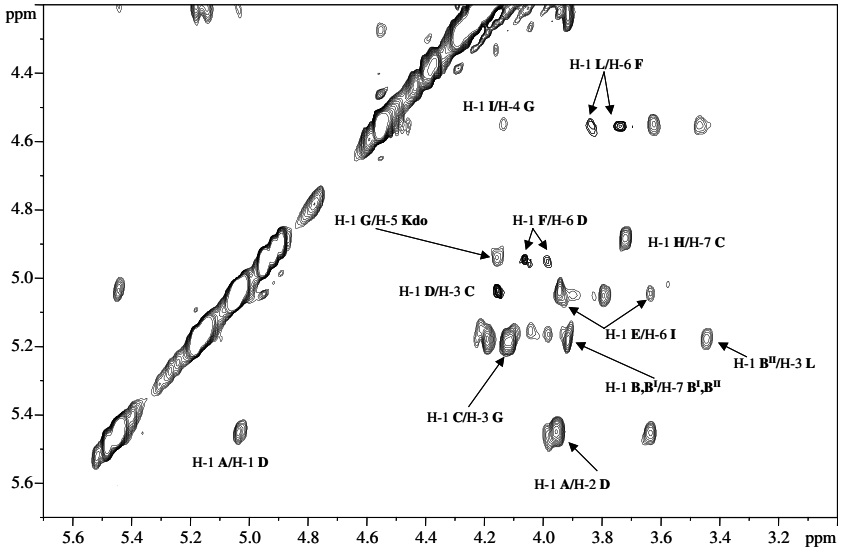

Figure 4. Section of the NOESY spectrum of the O-chain-core-region. Spin systems are labelled as shown in Figure 1.

$\mathbf{B^{II}}$ was substituted at O-7 by caryose $\mathbf{B^{I}}$, as identified by a NOE contact of its H-1 with H-7 of $\mathbf{B^{I}}$. Finally, caryose **B** was attached at O-7 of the caryose $\mathbf{B^{I}}$ as revealed by a NOE contact of its H-1 with H-7 of caryose $\mathbf{B^{I}}$.

The HMBC spectrum confirmed the proposed structure of the oligosaccharide, containing all long-range correlations required to demonstrate the proximity of the residues. Together with *intra*-residual connectivities, the *inter*-residual ones between H-1/C-1 of heptose **G** and C-5/H-5 of Kdo, H-1/C-1 of **I** and C-4/H-4 of **G**, H-1/C-1 of **E** and C-6/H-6 of **I**, H-1/C-1 of **C** and C-3/H-3 of **G**, H-1/C-1 of heptose **H** and C-7/H-7 of **C**, H-1/C-1 of **D** and C-3/H-3 of **C**, H-1/C-1 of **A** and C-2/H-2 of **D**, H-1/C-1 of **F** and C-6/H-6 of **D**, H-1/C-1 of **L** and C-6/H-6 of **F**, H-1/C-1 of $\mathbf{B^{II}}$ and C-7/H-7 of $\mathbf{B^{I}}$ and H-1/C-1 of $\mathbf{B^{I}}$ and C-7/H-7 of **B** were identified.

In summary, by methylation analysis, MALDI-TOF mass spectrometry and NMR spectroscopy we have established the structure of the carbohydrate backbone of the core-O-chain region of the LPS from *B. caryophylli* as depicted in Figure 1.

It is well established that aqueous hydrofluoric acid hydrolyses the glycosidic linkage of deoxy-sugars,[9] however, in the case of caryan an incomplete hydrolysis of the polymer was observed, which may be due to an inaccessibility of a R- or SR-LPS assembled in micelles, thus impairing the action of the acid on the inner part of the molecule. This hypothesis was confirmed by a different treatment of LPS, that is, if a partial de-acylation is carried on the molecule before HF treatment, all the 6-deoxy-residues are hydrolysed by aqueous HF.[9] The partial de-acylation obviously prevents an effective association of LPS in micelles.

Monosaccharide primers (to which the first monosaccharide of the O-chain is transferred) in O-chain biosynthesis have been found in several LPS, for example, of *Serratia marcescens*,[11] *Klebsiella pneumoniae*[12] (β-GlcNAc), *Salmonella enterica* sv. *typhimurium*[13] (β-Gal) and *Vibrio cholerae* H11 (β-sedoheptulose).[14] In the case of the O-chain-core region of LPS fraction from *B. caryophylli*, the identification of a single β-QuiNAc residue at the non-reducing terminus of the core region suggests that this residue may serve as a primer in caryan O-chain biosynthesis. Interestingly, data obtained from methylation analysis of the intact LPS indicated the presence of additional 3-substituted rhamnose. Thus, it may be hypothesised that this residue serves as a primer for the second O-chain, namely caryophyllan. Remarkably, both monosaccharide primers are 6-deoxy-residues. A main difference to the LPS from *S. marcescens*, *K. pneumoniae* and *S. enterica* sv. *typhimurium* is that there the primer monosaccharide can also be present in the O-chain while in *B. caryophylli* LPS it is not (as in *V. cholerae* H11).

3. Experimental

3.1. Bacteria and bacterial LPS

Burkholderia caryophylli strain NCPP 2151 was cultivated as described.[3,4] The LPS were obtained from lyophilised bacteria by the phenol/water extraction as described[3,4] (240 mg, yield: 6% of the bacterial dry mass).

3.2. Isolation of the O-chain-core region oligosaccharide

The LPS fraction (240 mg) was treated with 48% aqueous HF (4 °C, 48 h) to degrade the O-specific polysaccharides, followed by extensive dialysis against water and lyophilisation (53 mg, 21% of the LPS). Then the sample was hydrolysed in 1% acetic acid (100 °C, 3 h) and the precipitate (lipid A) was removed by centrifugation (4000*g*, 4 °C, 30 min). The supernatant was separated by gel-permeation chromatography on a column (100 × 1.5 cm) of Sephadex G-50 (Pharmacia). Three fractions were obtained, the first of which eluted in the void volume and contained a small aliquot of non-reacted polymer, the second fraction contained oligosaccharide molecules whereas the third fraction only contained caryophyllose and caryose monosaccharide derivatives. The second fraction (10.5 mg) was further purified using HPAEC on a column (4 × 250 mm) of CarboPac PA100 (Dionex) that was eluted at 1 ml min^{-1} with a linear gradient of 4–20% 1 M sodium acetate in 0.1 M NaOH over 45 min. Several fractions were obtained still containing caryose oligosaccharides, and one fraction (1.5 mg) that yielded the oligosaccharide fraction representing the complete carbohydrate backbone of the core region, substituted by three caryose residues.

3.3. General and analytical methods

Determination of caryose, neutral sugars including the determination of the absolute configuration of the heptose residues, absolute configuration of the hexoses and GLC and GLC–MS were carried out as described.[15–18] Methylation of the complete core region was carried out as described,[19] and the sample was hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with NaB^2H_4, acetylated and analysed by GLC–MS.

3.4. NMR spectroscopy

For structural assignments of oligosaccharide fraction, 1D and 2D ^{1}H NMR spectra were recorded in 0.5 ml of D_2O with a Bruker DRX 600 spectrometer. Measurements were achieved at 30 °C, relative to internal acetone [δ ^{1}H 2.225, δ ^{13}C 31.45].

Nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame Overhauser enhancement spectroscopy (ROESY) were measured using data sets ($t_1 \times t_2$) of 4096×1024 points, and 32 scans were acquired. A mixing time of 200 ms was employed. Double quantum-filtered phase-sensitive COSY experiment was performed with 0.258 s acquisition time using data sets of 4096×1024 points and 64 scans were acquired. The total correlation spectroscopy experiment (TOCSY) was performed with a spinlock time of 80 ms, using data sets ($t_1 \times t_2$) of 4096×1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096×2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first order basis from 2D phase-sensitive double quantum-filtered correlation spectroscopy (DQF-COSY).[20,21] The intensities of NOE signals were classified as strong, medium and weak using cross-peaks from intra-ring proton–proton contacts for calibration. The heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments spectrum were measured in the ^{1}H-detected mode with proton decoupling in the ^{13}C domain, using data sets of 2048×512 points, and 64 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.[22] ^{1}H,^{13}C HMBC was optimised for 6 Hz coupling. In all the heteronuclear experiments the data matrix was extended to 2048×1024 points using forward linear prediction extrapolation.[23]

3.5. Mass spectrometry

MALDI-TOF analysis was conducted in linear mode using a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated through 24 kV. Mass spectra reported are the result of 256 laser shots. The sample was analysed in negative polarity in 2,5 dihydroxybenzoic acid (DHB) 50 mg/ml TFA 0.1%–acetonitrile (80/20).

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