DOI: 10.1002/ejoc.200800613

Structure and Conformational Studies of the Disaccharides Derived from the Inner Core of the Lipopolysaccharide Isolated from *Sinorhizobium fredii* SMH12

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Keywords: Oligosaccharides / Structure elucidation / Conformation analysis / Rhizobium / Lipopolysaccharide

The structure and conformational behaviour of the core region of the lipopolysaccharide isolated from *Sinorhizobium fredii* SMH12, a wide-ranging host bacterium isolated from nodulated soybean plants growing in Vietnam, have been studied. A mixture of oligosaccharides was isolated from the lipopolysaccharide by mild hydrolysis followed by size-exclusion chromatography. The structures were determined by sugar analysis, ESI-MS/MS and NMR studies. Conformational analysis was performed by molecular dynamics calculations and compared with the NMR spectroscopic data. The results indicate that the core oligosaccharide contains a disaccharide made up of α-D-glucopyranuronic acid (1→4)-linked to D-Kdo. In solution, this disaccharide exists as an equilibrium of three different structures.

Introduction

Sinorhizobium fredii SMH12 is a fast-growing Gram-negative bacterium that is able to establish a nitrogen-fixing symbiosis with plants of the legume family.[1] The symbiosis bacterium–plant results in the formation of root nodules that contain highly differentiated bacterial cells (called bacteroids) in which atmospheric nitrogen is reduced to ammonia, a utilisable nitrogen source for the plants. The rhizobium–plant interaction is host-specific and determined by the exchange of chemical signals between both partners. The host plant root secretes a set of varied flavonoids, a subset of which is able to induce the transcription of nodulating genes in the bacterium. Many of these genes have been shown to be involved in the synthesis and excretion of lipochitooligosaccharides (LCOs or Nod factors), the bacterial nodulation signals secreted by the bacterium that trigger nodule organogenesis in specific regions of the legume root. Thus, although it is known that the development of the nodule is induced by Nod factors, the invasion of the plant cells by rhizobia requires several rhizobial surface polysaccharides that act as signal molecules and/or prevent the plant defence response.[2–4] These polysaccharides are named exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS) and cyclic β-glucans.

The cell wall of Gram-negative bacteria is formed of an inner (the cytoplasmic membrane) and outer membrane with a periplasmic space between them. The cytoplasmic membrane is formed of phospholipids and lipoproteins, whereas the outer membrane is formed of proteins, lipopolysaccharides and phospholipids. Cyclic glucans are located in the periplasmic space. The extracellular matrix of the bacteria (the capsule) contains the acidic exopolysaccharides that are released into the cell's milieu. In the genus *Sinorhizobium*, the outer membrane and the surrounding capsule are composed of a complex array of lipopolysaccharides and capsular polysaccharides.

A lipopolysaccharide is a glycolipid molecule that presents three different regions in its structure: lipid A, the core and the O-chain. In rhizobia, lipid A is made up of amino sugar residues that are *O*- and *N*-fatty acylated. The core oligosaccharide is located between the lipid A and the O-antigen. This oligosaccharide is made up of different sugars and derivatives, including Kdo (3-deoxy-D-*manno*-2-octulosonic acid).

In previous papers we have described the structure and the conformational and dynamic behaviour of the O-antigen of the lipopolysaccharide of *Sinorhizobium fredii* SMH12,[5,6] a wide-ranging host bacterium isolated from nodulated soybean plants growing in Vietnam. The O-antigen has a repeating unit consisting of the trisaccharide →4)-α-D-Gal*p*A-(1→3)-2-*O*-Ac-α-L-Rha*p*-(1→3)-2-*O*-Ac-α-D-Man*p*-(1→ in which the O-6 position of the mannose residue in the repeating unit is unsubstituted, acetylated or methylated in an approximate ratio of 1:1:2. Here we show that *Sinorhizobium fredii* SMH12 produces a lipopolysac-

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charide, the core of which contains a disaccharide made up of α-D-glucopyranuronic acid (1→4)-linked to Kdo that in solution is an equilibrium of three different structures: α-D-Glc*p*A-(1→4)-α-Kdo*p*, α-D-Glc*p*A-(1→4)-β-Kdo*f* and α-D-Glc*p*A-(1→4)-2,7-anhydro-α-Kdo*f*. A combination of NMR methods assisted by molecular dynamics simulations has been employed to deduce its major conformational and dynamic features, and to deduce the absolute configuration of the Kdo moiety.

Results and Discussion

Structural Determination

The isolation and purification of the LPS was performed by following a classical protocol.[7] After incubation the cells were harvested by centrifugation. The surface polysaccharides were extracted from the freeze-dried bacteria cells with a 1:1 hot phenol/water mixture and the two phases were separated. The aqueous phase was treated with enzymes to eliminate other biomolecules present in the mixture. The polysaccharides were fractionated by size-exclusion chromatography (SEC) and the LPS isolated. The three different regions of the LPS can be separated by taking advantage of the lability of the linkage of the Kdo (3-deoxy-D-*manno*-2-octulosonic acid) molecules located between the three regions.[8] Thus, the LPS was subjected to mild hydrolysis and the lipid A was separated by extraction with organic solvents. The aqueous phase, which contains the O-antigen and the core oligosaccharide, was fractionated by SEC. The high molecular weight fraction contains the O-antigen and the low molecular weight fraction contains the putative core oligosaccharide.

The monosaccharide analysis by GC–MS indicated that the oligosaccharide contains glucuronic acid and Kdo in a ratio of 1:1. Glucuronic acid was determined to have a D absolute configuration by GC–MS of its trimethylsilylated 2-butyl glycoside.

The oligosaccharide was analysed by electrospray ionisation mass spectrometry in the positive and negative modes. In the positive mode (Figure 1, a), the mass spectrum shows a set of pseudomolecular ions that could be assigned to the sodium adduct of a disaccharide of glucuronic acid and Kdo, the sodium adduct of the sodium salt, and the sodium adduct of the disodium salt. Another set of pseudomolecular ions at *m*/*z* = 419, 441 and 463 were assigned to the sodium adducts of a disaccharide of glucuronic acid and Kdo that had lost a molecule of water. In the negative mode (Figure 1, b), the mass spectrum only shows the pseudomolecular ions of the disaccharide and the anhydrous disaccharide.

The sequence of sugars in the oligosaccharide can be deduced from collision-activated dissociation (CAD) spectra in the negative mode, obtained on collision of the pseudo-

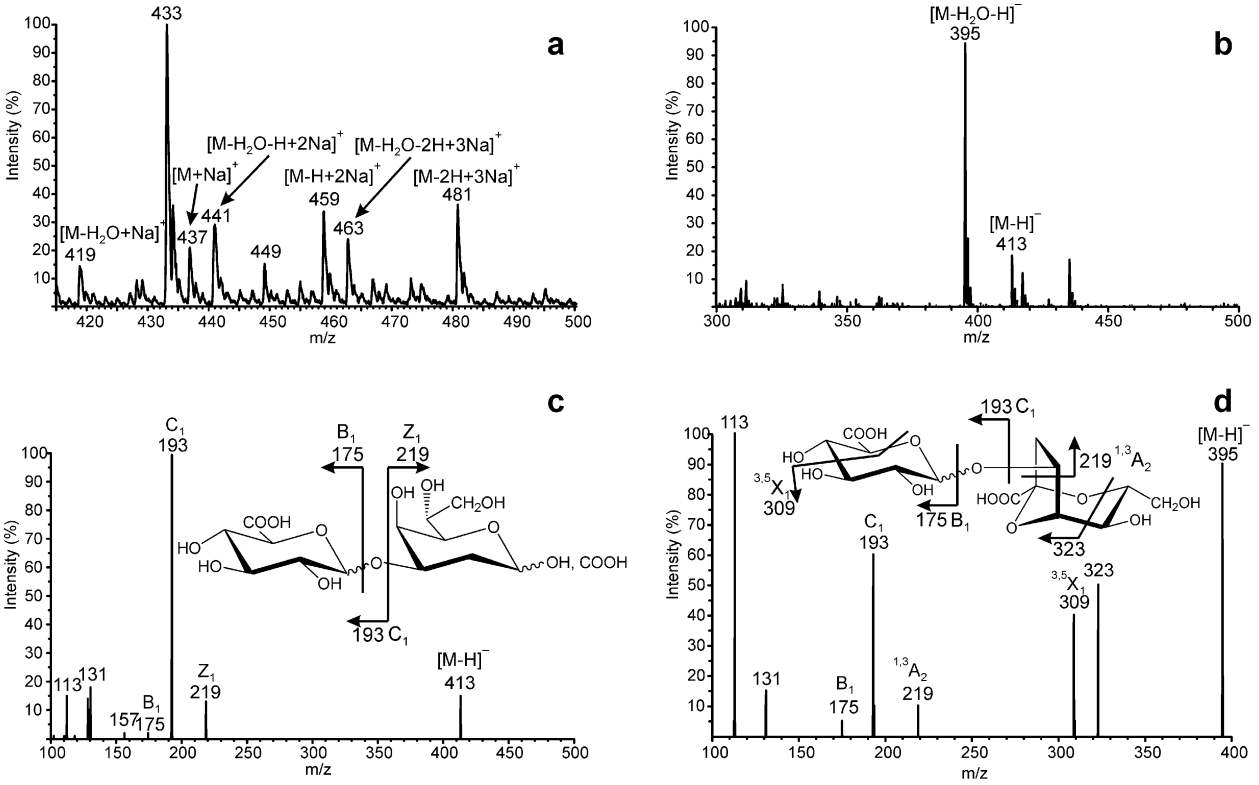


Figure 1. ESI-MS of the low molecular weight fraction obtained from partial hydrolysis of the LPS of *S. fredii* SMH12 acquired in (a) positive and (b) negative modes. CAD-MS/MS spectra from pseudomolecular ions at (c) *m*/*z* = 413 and (d) *m*/*z* = 395 arising from the negative ESI mass spectrum.

molecular ion at m/z = 413 for the disaccharide and at m/z = 395 for the anhydrous disaccharide (Figure 1, c and d). In the spectra we can observe the fragment series B, C and Z arising from the fragmentation of the glycosidic linkage. We can also identify fragments from the series X and A arising from the fragmentation of the ring mainly in the spectrum of the anhydrous oligosaccharide.[9] From the ESI-MS/MS study we can deduce that in the low molecular weight fraction, at least two disaccharides are present.

Their structures were studied by NMR spectroscopy. Assignments of ^{1}H and ^{13}C NMR spectra were made on the basis of DQF-COSY (Figure S1 of the Supporting Information), TOCSY (Figure S2 of the Supporting Information), multiplicity-edited HSQC (Figure 2), HMBC (Figure S3 of the Supporting Information) and ROESY (Figure 3) experiments. All the chemical shifts are given in Table 1.

The ^{1}H NMR spectrum (Figure 2) shows three signals at δ_H = 5.16, 5.01 and 4.92 ppm, which correspond to three anomeric protons from glucuronic acid. In addition to other carbohydrate proton signals, we observe three methylenic spin systems at δ_H = 2.70/2.26, 2.68/2.20, and 2.55/1.93 ppm, which correspond to three different pairs of diastereotopic protons H-3a and H-3b from three different Kdo residues, one of them a pyranose ring and the other two furanose rings. The pyranose/furanose equilibrium shows the same mass spectrum for the two disaccharides, and the other Kdo as a furanose ring corresponds to the

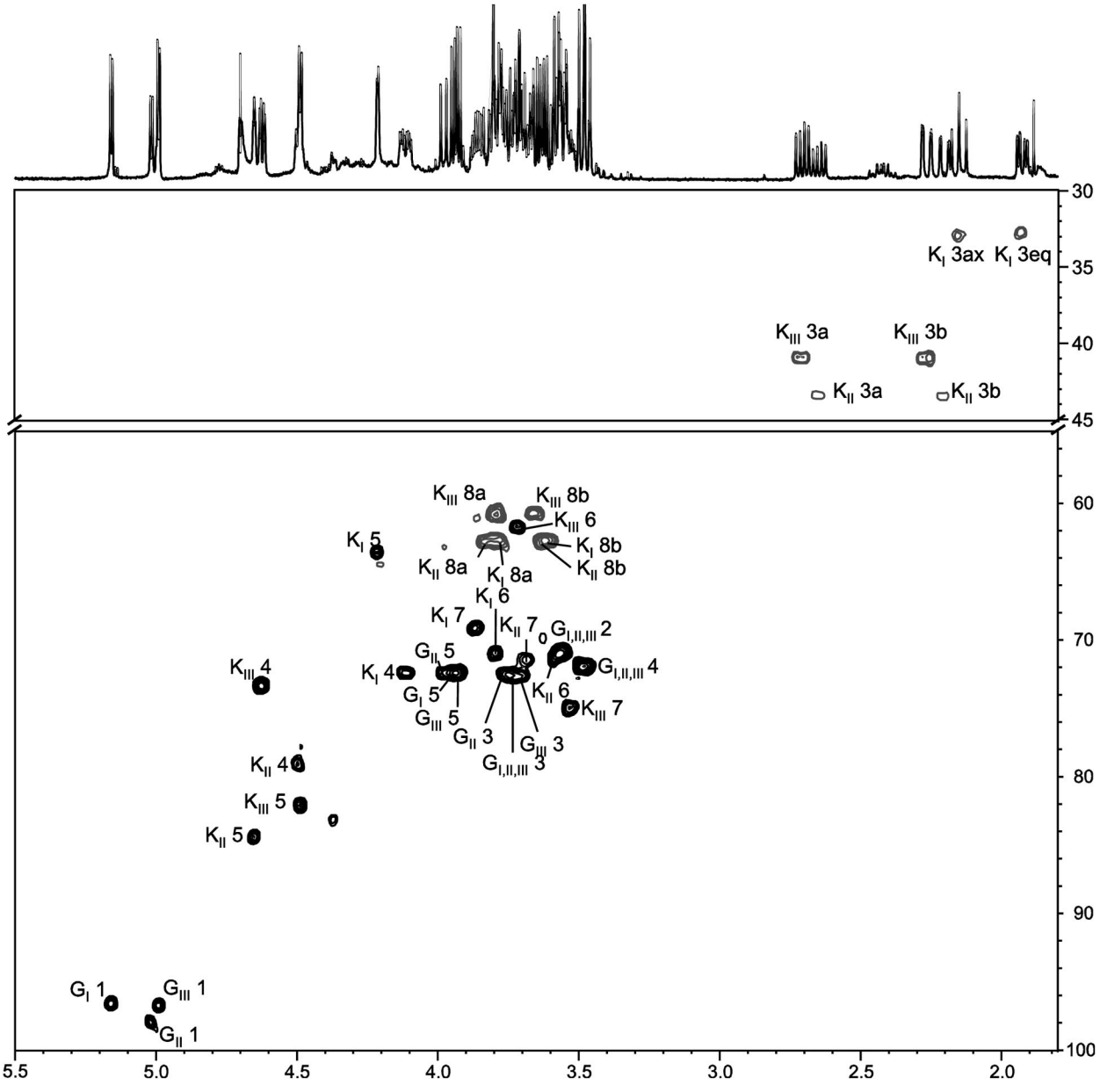


Figure 2. 500 MHz ^{1}H NMR spectrum and multiplicity-edited HSQC spectrum of oligosaccharides isolated from the partial hydrolysis of the LPS of *S. fredii* SMH12.

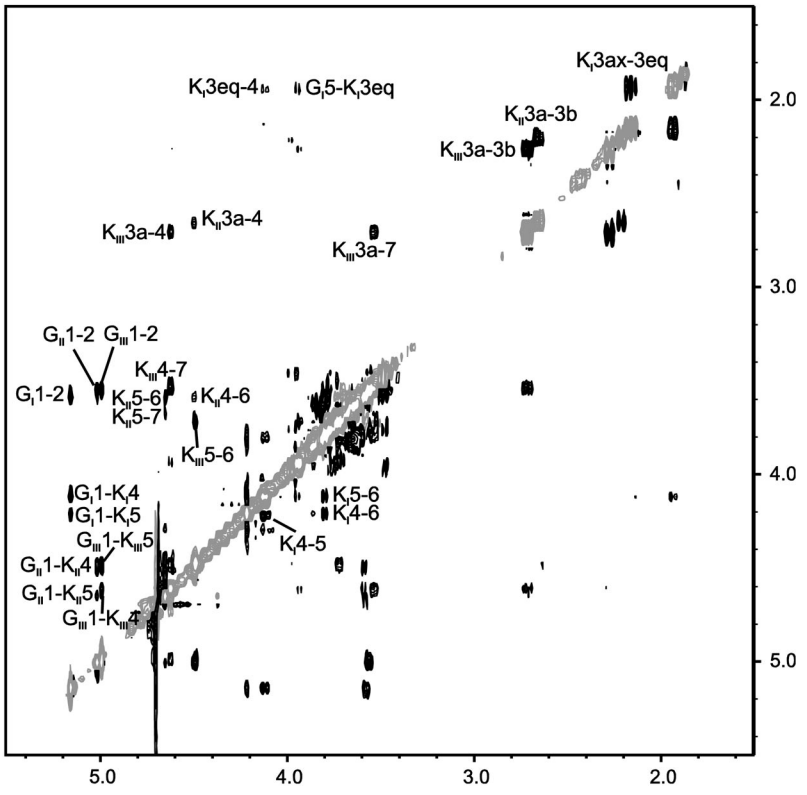


Figure 3. 500 MHz ROESY spectrum (300 ms) of the oligosaccharides isolated from the partial hydrolysis of the LPS of *S. fredii* SMH12.

Table 1. ^{1}H and ^{13}C chemical shifts of the disaccharides detected in the structural determination of the core of the lipopolysaccharide isolated from *S. fredii* SMH12.

Position		δ [ppm]		
		Disaccharide **I**	Disaccharide **II**	Disaccharide **III**
1	^{1}H	–	–	–
	^{13}C	n.d.[a]	n.d.	n.d.
2	^{1}H	–	–	–
	^{13}C	n.d.	n.d.	n.d.
3	^{1}H	2.15/1.93	2.65/2.20	2.70/2.26
	^{13}C	32.9	43.5	41.1
4	^{1}H	4.11	4.49	4.62
	^{13}C	72.7	79.3	73.6
5	^{1}H	4.22	4.65	4.49
	^{13}C	63.9	84.6	82.2
6	^{1}H	3.80	3.59	3.72
	^{13}C	71.2	71.6	62.0
7	^{1}H	3.86	3.68	3.53
	^{13}C	69.4	71.7	75.2
8	^{1}H	3.78/3.61	3.83/3.63	3.79/3.66
	^{13}C	62.9	63.1	61.1
1′	^{1}H	5.16	5.01	4.99
	^{13}C	96.7	98.0	96.8
2′	^{1}H	3.58	3.56	3.55
	^{13}C	71.2	71.1	71.2
3′	^{1}H	3.74	3.76	3.71
	^{13}C	72.8	72.8	72.8
4′	^{1}H	3.47	3.48	3.48
	^{13}C	72.2	72.2	72.2
5′	^{1}H	3.95	3.98	3.93
	^{13}C	72.6	72.6	72.6
6′	^{1}H	–	–	–
	^{13}C	176.5	176.5	176.5

[a] Not determined.

Kdo residue in the oligosaccharide that has lost a water molecule.

The DQF-COSY spectrum shows three cross-peaks for the H-1 and H-2 atoms of the three glucuronic acid residues. One of them is slightly different to the other two peaks, and we assign this cross-peak to H-1/H-2 of the glucuronic acid (G_I) linked to Kdo as a pyranose ring (K_I). This was further confirmed by the ROESY experiment (see below). The other two cross-peaks, practically one signal, were assigned to the glucuronic acid residues linked to Kdo in the furanose ring (G_{II} and G_{III}).

The TOCSY experiment allowed us to assign the three spin systems belonging to the three different glucuronic acid residues and the three Kdo residues of the three disaccharides.

The δ_C values for the three disaccharides were assigned on the basis of the edited HSQC spectrum (Figure 2).

The ^{1}H and ^{13}C chemical shifts, as well as the coupling constants of the glucuronic acid residues, agree with the values described in the literature for terminal non-substituted glucopyranuronic acid residues.[10,11] The small coupling constant $^3J_{1,2}$ (3.8 Hz) and the chemical shifts of H-1 and C-1 indicate the α anomer in the three disaccharides.

The DQF-COSY experiment showed a spin system typical of the diastereotopic protons at C-3 of the Kdo residue in disaccharide **I** and their correlations with H-4. From the δ_H values of H-3 and from the coupling constants $^2J_{3a,3b}$ (13 Hz), $^3J_{3a,4}$ (12 Hz) and $^3J_{3b,4}$ (4.5 Hz), we can identify the axial and equatorial protons and establish that the ring

is in the pyranose form.[12] In addition, the chemical shift difference $\delta_{H3a} - \delta_{H3b}$ of the methylenic protons indicates the α anomery in this disaccharide.[12] The HSQC experiment allowed the assignment of the ^{13}C signals. When compared with spectra in the literature,[12] our spectrum shows a downfield shift of C-4, which indicates the point of substitution. This proposition was confirmed by the cross-peak observed in the ROESY experiment (Figure 3) for the anomeric protons of the glucuronic acid residue and the protons H-4 and H-5 of the α-pyranose Kdo residue. In addition, the HMBC spectrum (Figure S3 of the Supporting Information) shows a correlation between H-1 of the glucuronic acid and C-4 of Kdo.

The DQF-COSY experiment showed that the two diastereotopic protons H-3a and H-3b of disaccharide **II** correlate with H-4. From the δ_H values of H-3a and H-3b and from the vicinal coupling constants $^3J_{3a,4}$ (7.4 Hz) and $^3J_{3b,4}$ (2.0 Hz) we can deduce that the Kdo residue is in the furanose ring and with β anomery.[12] In the HSQC experiment we observed that the signal for C-4 is shifted downfield. Therefore, we propose that the linkage between the glucuronic acid and the Kdo is 1→4. This was confirmed by the correlations observed in the ROESY spectrum for H-1 of the glucuronic acid residue and H-4 and H-5 of the Kdo residue.

As for the above disaccharides, the DQF-COSY experiment allowed the assignment of the diastereotopic protons H-3a and H-3b in disaccharide **III** and showed their correlation with H-4. This spin system is very similar to that described for disaccharide **II** and we propose a furanose ring for the Kdo residue. All the ^{13}C signals were identified in the HSQC spectrum. Signals from C-4 and C-7 were shifted downfield, which indicates substitution at these positions. The linkage between the glucuronic acid and the Kdo as a furanose ring is 1→4, as confirmed by the two cross-peaks of the anomeric proton of the glucuronic acid and H-4 and H-5 of the Kdo residue in the ROESY experiment.

By comparison with the chemical shift values described, and bearing in mind that the Kdo unit has lost a water molecule in this disaccharide, we propose that this Kdo adopts a furanosic form giving rise to a bicycle involving O-5 and O-7. This structure is confirmed by the cross-peaks H-4/7-H and H-3a/7-H in the ROESY spectrum.

McNicholas et al.[13] demonstrated that the furanose anomers in Kdo*f* can be differentiated in the ^1H NMR spectra by the values of $^3J_{3a,4}$ and $^3J_{3b,4}$. Thus, the α anomer mainly adopts a $^2T_3$ conformation giving coupling constants of about 6 and 7 Hz, respectively, whereas the β anomer mainly tends to adopt an E_0 conformation with coupling constants of about 8 and 2 Hz, respectively. Thus, the chemical shifts and the coupling constants of H-3a and H-3b ($^3J_{3a,4}$ = 7.5, $^3J_{3b,4}$ = 2.2 Hz) in this disaccharide indicate that the Kdo is a furanose ring with β anomery, similar to the disaccharide **II**. However, the loss of water and formation of the anhydride is only possible when the Kdo is α-furanose. Therefore, we propose that the formation of the anhydride bridge forces the conformation E_0 of the furanose ring, which was confirmed by the conformational analysis performed by molecular dynamics studies.

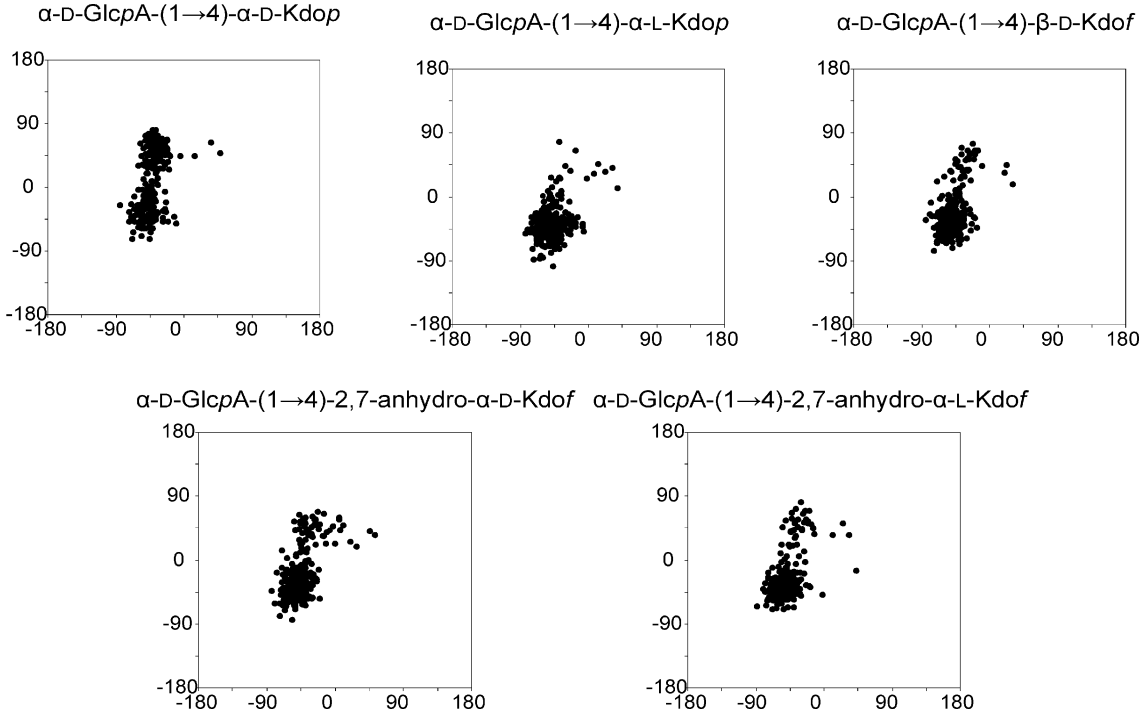


Figure 4. Conformational maps of Φ versus Ψ for the glycosidic linkage α-D-Glc*p*A-(1→4)-Kdo obtained from the disaccharides **I** (α-D-Kdo*p*), **II** (β-D-Kdo*f*) and **III** (2,7-anhydro-α-D-Kdo*f*). In addition, molecular dynamics (MD) calculations were performed on the disaccharides **I** and **III** considering the absolute configuration of the Kdo-moiety as L.

Conformational Study

Figure 4 shows the conformational maps of Φ versus Ψ for the glycosidic linkage α-D-Glc*p*A-(1→4)-Kdo obtained from disaccharides **I** (α-D-Kdo*p*), **II** (β-D-Kdo*f*) and **III** (2,7-anhydro-α-D-Kdo*f*). In addition, molecular dynamics (MD) calculations were performed on disaccharides **I** and **III** with the absolute configuration of the Kdo moiety as L. Disaccharide **II** with L-Kdo was not considered in these calculations given the poorer correlation with the experimental data of the D-Kdo equivalent, which will be seen later. The results were very similar in all cases: a global minimum located at Φ/Ψ values of around –45°/–45° with H-1(GlcA) approaching H-5(Kdo) when Kdo is D or H-3a,b(Kdo) in the other case. Another minimum appears at Φ/Ψ of around –45°/45° (with the exception of disaccharide **I** having L-Kdo), with H-1 and H-5 of the GlcA unit approaching H-3a,b(Kdo) (when Kdo is D).

Molecular modelling data were compared with the experimental NMR spectroscopic data by computing the ROESY cross-peak volumes using a full-matrix relaxation approach in a rigid isotropic motional model.[14] Calculations were made with the NOEPROM program with an external relaxation rate of 0.1 s^{-1}.[5,15] Intramolecular cross-peaks corresponding to fixed distances (i.e., G-1/G-2, K-3eq/K-4, K-4/K-5 and K-4/K-6) were used as references for optimizing the effective correlation time (τ_c) in each case, which results in values of 60–70 ns.

Table 2 shows experimental ROESY cross-peak volumes together with averaged theoretical volumes calculated from the MD trajectories. The good agreements in the case of disaccharide **I**, and especially disaccharide **III**, support the absolute configuration of Kdo as being D. It can be seen that when these disaccharides contain L-Kdo, not only do the computed inter-residual ROEs disagree with the experimental ones, but also calculations predict relatively strong ROE cross-peaks (about 2–4%) between G-1 and K-3eq (K-3b in disaccharide **III**) that do not appear in the NMR spectrum.

The use of a rigid isotropic motional model implies that the results are affected by internal flexibility.[14] This would explain the poorer agreement in the case of disaccharide **II**, which is a result of the flexibility of the furanose ring and a longer exocyclic chain. Disaccharide **III**, however, contains an additional anhydride bond that generates a six-membered ring, the chair conformation of which makes the furanose ring adopt an E^0 conformation. Besides, the exocyclic chain is also fixed by the linkage O-2/O-7, which reduces even more the internal flexibility of this structure.

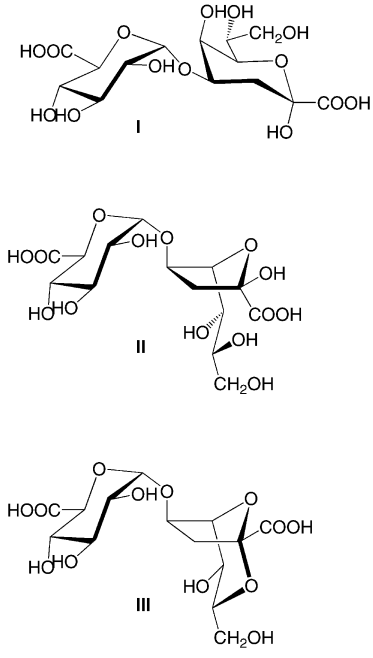
Table 2. Experimental and computed ROE data for a 2D ROESY experiment (300 ms) recorded for the disaccharides α-D-Glc*p*A-(1→4)-Kdo. ROESY values from the MD simulations were calculated by using the MD-computed structures. Inter-residual ROEs are in italics.

Disaccharide	ROE	Exp.[%]	MD [%]	
			D-Kdo	L-Kdo
α-D-Glc*p*A-(1→4)-α-Kdo*p*	G1-G2	2.6	2.6	2.5
	G1-K3eq	n.d.[a]	0.8	3.5
	G1-K4	2.7	3.2	1.9
	G1-K5	0.3	1.7	0.0
	G5-K3eq	0.6	1.1	0.0
	K3eq-K3ax	14.9	17.3	17.8
	K3eq-K4	2.4	2.3	2.4
	K4-K5	2.1	2.4	2.5
	K4-K6	1.5	2.4	2.1
	K5-K6	2.5	2.4	2.3
α-D-Glc*p*A-(1→4)-β-Kdo*f*	G1-G2	2.8	2.7	n.c.[b]
	G1-K4	2.2	2.3	n.c.
	G1-K5	1.4	2.1	n.c.
	G5-K3b	0.7	1.6	n.c.
	K3a-K3b	14.2	17.3	n.c.
	K3a-K4	4.3	3.4	n.c.
	K4-K6	1.1	1.7	n.c.
	K5-K6+K7	1.8	3.0	n.c.
α-D-Glc*p*A-(1→4)-2,7-Anhydro-α-Kdo*f*	G1-G2	2.2	2.5	2.4
	G1-K3b	n.d.	0.0	2.0
	G1-K4	2.7	2.5	2.1
	G1-K5	2.7	2.8	0.0
	G5-K3b	1.0	1.7	0.0
	K3a-K3b	15.3	17.1	16.2
	K3a-K4	3.0	2.9	2.9
	K3a-K7	4.0	3.8	3.5
	K4-K7	2.0	2.7	3.0
	K5-K6	2.3	2.3	2.2

[a] Not detected. [b] Not computed.

Conclusions

The final conclusion is that the core oligosaccharide from the LPS of *Sinorhizobium fredii* SMH12 contains the disaccharide α-D-Glc*p*A-(1→4)-D-Kdo, which in solution exists as an equilibrium of the three structures (Scheme 1).



Scheme 1. Disaccharides derived from the inner core of the lipopolysaccharide isolated from *Sinorhizobium fredii* SMH12.

Experimental Section

Bacterial Culture Conditions and Isolation of Polysaccharides: *S. fredii* SMH12 was routinely grown at 28 °C in TY medium as described by Beringer.[16] For the isolation of bacterial polysaccharides, TY liquid medium (10 L) was inoculated with early-stationary phase cultures of *S. fredii* SMH12 (100 mL) and incubated on an orbital shaker at 160 rev/min for 3 d at 28 °C. After incubation, the cells were harvested by slow-speed centrifugation. Bacterial pellets were washed three times with 0.9% (w/v) NaCl, freeze-dried and stored in sealed bottles at room temperature. The polysaccharide was extracted from the freeze-dried bacterial cells (5 g) with a 1:1 hot phenol/water mixture (100 mL)[7] and the two phases were separated. The aqueous phase was dialysed against water, freeze-dried and redissolved in 10 mM $MgSO_4$ and 50 mM Tris-HCl solution (100 mL, pH 7.0). DNase (1 mg) and RNase (1 mg) were added and the solution was stirred overnight at 5 °C. Proteinase K (2 mg) was added, the solution shaken for 24 h at 37 °C, dialysed and then freeze-dried. The polysaccharide was purified by chromatography on Sephacryl S-500 (60 × 2.6 cm) using 0.05 M EDTA/triethylamine (pH 7.0) as eluent. The carbohydrates were detected using a refractive-index detector and the orcinol/sulfuric acid method on thin-layer chromatography plates. Fractions containing carbohydrates were dialysed and freeze-dried. A 0.1% (v/w) solution of the LPS fraction in 1% (v/v) AcOH was heated at 100 °C for 2.5 h and the lipid A, which precipitated, was separated by centrifugation. The supernatant was extracted with dichloromethane and the aqueous phase containing the O-antigen and core oligosaccharides was fractionated by gel-permeation chromatography on BioGel P-2 (1.6 × 60 cm) (from Bio-Rad) using water as eluent.

Monosaccharide Analysis: Monosaccharides were identified by GLC–MS separation of their trimethylsilylated methyl glycosides obtained as described previously.[17]

The absolute configuration of glucuronic acid was assigned following GLC–MS analysis of its trimethylsilylated (*S*)- and (*R*,*S*)-2-butyl glycosides, prepared as described previously.[18] Derivatives of standard monosaccharides were prepared for comparison.

GLC–MS was performed with a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m × 0.25 mm). The temperature program for separating the trimethylsilylated methyl glycosides and 2-butyl glycosides was isothermal at 150 °C for 2 min followed by a 10 °C/min gradient up to 250 °C. The ionization potential was 70 eV and spectra were recorded in low-resolution mode.

Mass Spectrometry: The disaccharides were analysed by electrospray ionization mass spectrometry (ESI-MS) and collision-activated dissociation (CAD-MS/MS). Experiments were performed with a QTRAP LC/MS/MS system (Applied Biosytems/MDS SCIEX Foster City, USA). Samples were dissolved in methanol/water (1:1, v/v) containing 0.1% formic acid and were continuously infused into the TurboIonSpray source at a flow rate of 5 μL/min using the integral syringe drive. The instrument was operated in the positive and negative modes with the capillary held at a voltage of 5500 and –4500 V, respectively. A declustering potential of ±85 V was used. Nitrogen was used as both curtain and collision gas. The collision energy setting for tandem mass-spectrometry experiments ranged from 25 to 80 V. Spectra were acquired by using the Analyst 1.4.1 software (Applied Biosystems).

NMR Spectroscopy: Samples were deuterium-exchanged several times by freeze-drying from ^2H_2O and then examined in solution (ca. 1 mg/750 μL) in 99.98% ^2H_2O. Spectra were recorded at 303 K on a Bruker AV500 spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Chemical shifts are given in ppm with the H^2HO signal (^{1}H: δ = 4.75 ppm) and external dimethyl sulfoxide (^{13}C: δ = 39.5 ppm) as references. The 2D homonuclear DQF-COSY experiments were performed using the Bruker standard pulse sequence. A data matrix of 256 × 1 K points was used to digitize a spectral width of 4464 Hz; 16 scans were used per increment. Sine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 1 K × 1 K. The TOCSY was acquired by using a data matrix of 256 × 2 K points to digitize a spectral width of 5682 Hz; 8 scans per increment and an isotropic mixing time of 120 ms were used. Cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 256 × 2K. The heteronuclear one-bond H–C correlation experiment was registered in the ^{1}H detection mode via single-quantum coherence (HSQC). A data matrix of 256 × 1 K points was used to digitize a spectral width of 5682 and 22522 Hz in F_2 and F_1, respectively; 32 scans were used per increment. ^{13}C decoupling was achieved by the GARP scheme. Squared-cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 1 K × 1 K. This experiment was slightly modified by the implementation of an editing block in the sequence.[19] The HMBC experiment was performed by using the Bruker standard sequence with 256 increments of 1 K real points to digitize a spectral width of 5682 × 30030 Hz; 64 scans were acquired per increment with a delay of 62.5 ms for evolution of long-range couplings. The ROESY experiment was performed with a mixing time of 300 ms and a relaxation delay between scans of 1.5 s. A data matrix of 256 × 1 K points was used to digitize a spectral width of 5682 Hz; 16 scans were used per increment. Cosine-bell functions were applied in both dimensions.

Molecular Modelling: The key torsional angles were defined as follows:

Φ: H-1(GlcA)–C-1(GlcA)–O-1(GlcA)–C-4(Kdo)

Ψ: C-1(GlcA)–O-1(GlcA)–C-4(Kdo)–H-4(Kdo)

The calculations were performed by using MM3* as integrated in the Macromodel 9.0 package[20] with the GB/SA (generalized Born solvent-accessible surface area) to simulate the water environment.[21]

The starting structure was built by defining the Φ angles as the expected *exo*-anomeric[22] orientation (Φ = –60°), whereas Ψ values were initially set to 0°. These structures were then minimized by using a conjugate gradient protocol until convergence was obtained (energy difference of 0.05 kJ/Å mol).

Then the minimized structure was employed as the starting structure for MD simulations at 303 K using the same force field MM3*. The integration step was 1.5 fs. Snapshots were saved every 40 ps with a total simulation time of 10 ns.

The structures obtained through the MD simulations were then taken to simulate the theoretical ROESY spectra using the program NOEPROM.[23] Basically, all positions of the protons of the MD-sampled disaccharide moieties were considered. Different correlation times were considered to obtained the best fit between the observed and expected ROEs.[24]

Supporting Information (see also the footnote on the first page of this article): 500 MHz DQF-COSY, TOCSY and HMBC spectra of the oligosaccharides isolated from the partial hydrolysis of the LPS derived from *S. fredii* SMH12.

Acknowledgments

We thank Dr. Inmaculada Robina-Ramírez and Dr. Jesús Jiménez-Barbero for the use of the computing software, and Dr. Javier Corzo for the LPS samples. The authors would like to acknowledge the NMR and mass spectrometry services of the General Research Center of the University of Seville (CITIUS). The work was supported by the Ministerio de Ciencia y Tecnología (MCYT) (AGL2002-04188-C06 and AGL2005-07923-C05) and the Junta de Andalucía (Grupo CVI0135). One of the authors (F. J. F. d. C.) was supported by a fellowship from the MCYT.

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Received: June 23, 2008
Published Online: October 21, 2008

