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Structural studies on the lipopolysaccharide from a rough strain of *Ochrobactrum anthropi* containing a 2,3-diamino-2,3-dideoxy-D-glucose disaccharide lipid A backbone

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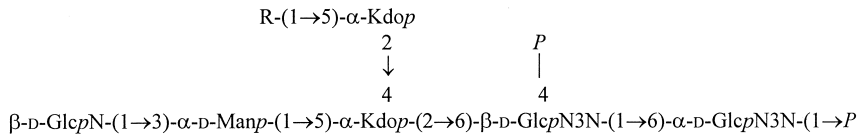
Abstract

A degradation protocol using de-*O*-acylation and subsequent alkaline de-*N*-acylation was applied to the lipopolysaccharide of *Ochrobactrum anthropi* rough strain LMG 3301. Three main oligosaccharide bisphosphates containing core–lipid A backbone structures were obtained after fractionation by anion-exchange HPLC. Using ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional COSY, TOCSY, and NOE spectroscopy (ROESY and NOESY), the following structures were established:



where Kdo is 3-deoxy-D-*manno*-octulosonic acid, D-GlcN3N is 2,3-diamino-2,3-dideoxy-D-glucose and R is H or α-D-Gal*p*A or 4-deoxy-β-L-*threo*-hex-4-enopyranuronic acid, the latter sugar being derived from α-D-Gal*p*A by β-elimination of a substituent attached to O-4. This is the first report on the isolation from a lipopolysaccharide of an oligosaccharide containing

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GlcN3N in the lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*]. Sugar and methylation analysis confirmed the presence of the GalA → Kdo disaccharide and non-stoichiometric substitution of GalA. It is suggested that Glc is the substituent at O-4 in GalA and that in the non-degraded lipopolysaccharide the amino group of GlcN is not acylated.

Keywords: Lipopolysaccharide; Core–lipid A backbone; 2,3-Diamino-2,3-dideoxy-D-glucose; Alkaline degradation; NMR spectroscopy; *Ochrobactrum anthropi*

1. Introduction

Ochrobactrum anthropi is a Gram-negative bacterial species which includes isolates from immunocompromised patients, environmental and hospital water sources, as well as from nematodes, suggesting that the original habitat could be soil (see references cited in ref. [1]). Molecular taxonomy studies place *O. anthropi* within the α-2 subgroup of the class Proteobacteria showing the greatest relatedness with *Brucella*, an important intracellular parasite of animals and humans [2].

Lipopolysaccharide (LPS, endotoxin) of the outer membrane of Gram-negative bacteria is important in pathogenicity, and a link between some properties of the *Brucella* LPS and the ability to multiply intracellularly has been shown [3–5]. Therefore, a molecular comparison of the *Brucella* and *O. anthropi* LPS could contribute to a better understanding of the pathogenicity of these bacteria. The structures of the O-specific polysaccharides and lipid A of the *Brucella* LPS have been characterised [6–8]. Recently, the structure of the O-specific polysaccharide of the reference strain *O. anthropi* LMG 3331 has been elucidated [1]. Now, we report on structural studies of the core and lipid A backbone of the LPS from a rough strain *O. anthropi* LMG 3301 and on the isolation, for the first time, of oligosaccharides with a GlcN3N lipid A backbone structure.

2. Results

The LPS was isolated from dried bacterial cells of rough *O. anthropi* strain LMG 3301 by the phenol–chloroform–light petroleum procedure [9]. Tricine–SDS–PAGE showed that the LPS obtained was similar to those produced by enterobacterial R-strains and revealed a structural heterogeneity with a major compound migrating at the front and two additional bands (Fig. 1).

The LPS was de-*O*-acylated by mild hydrazinolysis and de-*N*-acylated by strong alkaline hydrolysis according to the published protocol [10,11]. Fractionation of the resulting carbohydrates by anion-exchange HPLC on CarboPac PA-1 afforded three main oligosaccharide products designated as OS1, OS2, and OS3.

All oligosaccharides contained 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and phosphate. Sugar analysis using GLC–MS of acetylated methyl glycosides derived after *N*-acetylation, revealed the presence of Man, GlcN, 2,3-diamino-2,3-dideoxyglucose (GlcN3N), and Kdo. In addition, OS2 contained GalA. Man and GlcN were additionally identified by GLC of acetylated alditol acetates.

The ^{1}H NMR spectrum of OS2 (Fig. 2) contained signals for two 3-deoxy groups (H-3) at δ 2.13 and 2.10 (equatorial) and δ 2.01 and 1.83 (axial) typical of α-pyranoid Kdo [10] and six signals in the resonance region of anomeric protons, one of which belongs to H-1 of an α-glycosyl phosphate (δ 5.43, $J_{1,2}$ 3 Hz, $J_{\text{H-1,P}}$ 8 Hz), one to an α-linked pyranose (δ 5.23, $J_{1,2}$ 3.5 Hz), and two to β-linked pyranoses (δ 4.55 and 4.60, $J_{1,2}$ 8–8.5 Hz) with axial H-2. One more doublet at δ 5.30 ($J_{1,2}$ 2 Hz) was assigned to H-1 of a pyranose with equatorial H-2 (Man) and the remaining signal at δ 4.73 ($J_{4,5}$ 1 Hz) to H-5 of GalA (Table 1). Signals for other sugar protons were found in the region δ 2.70–4.32.

The ^{1}H NMR spectrum of OS2 was completely assigned using two-dimensional (2D) gradient selected COSY, double quantum filtered (DQF) COSY (Fig. 2), and TOCSY (Table 1). On the basis of the chemical shifts and coupling constant values, the seven sugar spin systems were ascribed to α-Man*p*, α-Gal*p*A, β-Glc*p*N, α-Glc*p*N3N1*P*, β-Glc*p*N3N4*P*, and two α-Kdo*p* residues (Kdo^{I} and Kdo^{II} enumerated starting from the reducing end of the oligosaccharide, see the structure of OS2 shown below). The assignment of the spin systems of the amino sugars was confirmed by correlation of the protons at the carbons bearing nitrogen (H-2 of GlcN and H-2

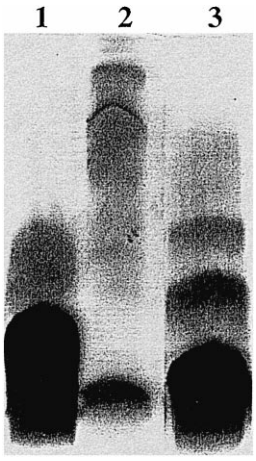


Fig. 1. Silver-stained Tricine–SDS–PAGE of LPSs from (1) *E. coli* F515 (R-type), (2) *O. anthropi* LMG 3331 (S-type), and (3) *O. anthropi* LMG 3301 (R-type).

and H-3 of GlcN3N, δ_H 2.70–3.18) to the corresponding carbons in the region δ_C 53–59 observed in the H-detected $^1H,^{13}C$ HMQC spectrum of OS2. The attachment of the second phosphate group to O-4 of β-GlcN3N followed from a low-field position of the signal for H-4 at δ 3.78, e.g., as compared with δ 3.40 for H-4 in β-GlcN, and from its coupling to phosphorus ($J_{H\text{-}4,P} \sim 10$ Hz).

Linkage and sequence analysis of OS2 was performed using 2D rotating-frame NOE spectroscopy (ROESY). As expected, α-GlcN3N H-1 exhibited no interresidue correlation, and β-GlcN3N H-1 gave a strong cross-peak with α-GlcN3N H-6b at δ 4.55/3.73. This confirmed that the two diamino sugar residues build up the lipid A backbone of the LPS and, therefore, OS2 is a product of the expected type [10,11], containing a core oligosaccharide (or its part) attached to a bisphosphorylated lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*].

Two inter-residue cross-peaks of similar intensity, with Man H-2 and H-3, were observed for GlcN H-1 at δ 4.60/4.32 and 4.60/4.15, respectively, that with the same absolute configurations of both monosaccharides and the β-glycosidic linkage, strongly indicated that Man is 3-substituted by GlcN [12].

Each of Man H-1 and GalA H-1 exhibited two inter-residue correlations, with both H-5 and H-7 of Kdo^I (Man, δ 5.30/4.27 and 5.30/3.72, respectively) and Kdo^{II} (GalA, δ 5.23/4.12 and 5.23/4.15), thus, indicating the α-(1 → 5) linkage between the two monosaccharide pairs (cf. published data for an α-(1 → 5)-linked heptose-Kdo disaccharide [13]). A NOE between Kdo^{II} H-3ax and GalA H-5 observed at δ 1.83/4.73 was consistent with the structure proposed. Such a NOE is characteristic for this linkage and has been repeatedly described for Sug-(1 → 5)-Kdo disaccharides [13,14].

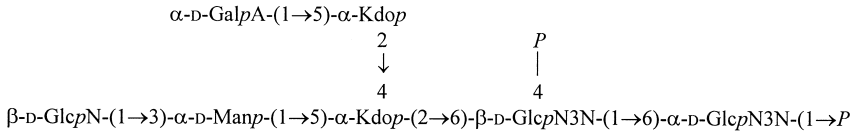
The connectivities between Kdo^I H-3eq,3ax and Kdo^{II} H-6 at δ 2.10/3.71 and 2.01/3.71 proved the α-(2 → 4)-linkage between the two Kdo residues [15]. Finally, the attachment of Kdo^I to β-GlcN3N was confirmed by the characteristic chemical shifts for H-5, H-6a, and H-6b of the latter sugar, δ 3.73, 3.76, and 3.47, respectively [cf., e.g., the corresponding chemical shifts δ 3.70, 3.71, and 3.51 for β-GlcN in the de-*N*,*O*-acylated LPS from *Salmonella minnesota* R595 [10] but δ 3.49, 3.86, and 3.78 in the nonsub-

Table 1
500 MHz ^1H NMR data for OS2 (δ in ppm)

	H-1	H-2	H-3eq/H-3ax	H-4	H-5	H-6a/H-6b	H-7	H-8a/H-8b
β-D-Glc*p*N-(1 →	4.60	2.70	3.45	3.40	3.53	3.93 3.73		
→ 3)-α-D-Man*p*-(1 →	5.30	4.32	4.15	3.89	4.06	3.88 3.88		
α-D-Gal*p*A-(1 →	5.23	3.85	3.98	4.30	4.73			
→ 5)-α-Kdo p^{II}-(2 →			2.13 1.83	4.12	4.12	3.71	4.15	4.00 3.76
→ 5)-α-Kdo p^{I}-(2 → 4 ↑			2.10 2.01	4.14	4.27	3.75	3.72	3.90 3.71
→ 6)-β-D-Glc*p*N3N-(1 →	4.55	2.78	3.16	3.78	3.73	3.76 3.47		
→ 6)-α-D-Glc*p*N3N-(1 →	5.43	2.95	3.18	3.55	4.13	4.30 3.73		

stituted enterobacterial lipid A backbone (Zähringer, unpublished data)].

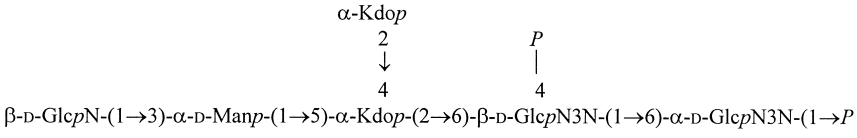
Therefore, on the basis of these data, it was concluded that OS2 has the following structure:



OS2

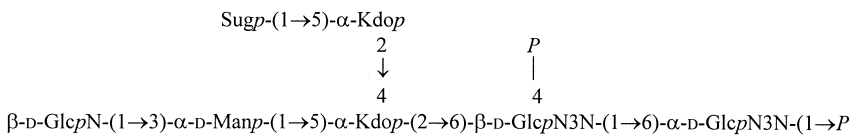
Since substitution of Kdo^{II} at position 5 is not common [16], this was further confirmed by methylation analysis of the GalA-(1 → 5)-Kdo disaccharide released by mild acid degradation of the LPS. The structure of the reduced and methylated disaccharide was identified by the mass spectrum which contained peaks of typical fragment ions at *m/z* 233 (the GalA moiety) and 307 (the reduced Kdo moiety). After methanolysis of the methylated disaccharide and acetylation, GLC–MS analysis revealed methyl 5-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctanoate identified by comparison with the authentic sample, thus strongly demonstrating 5-substitution of Kdo.

Similar NMR studies were carried out with OS1 and OS3 and confirmed that both lack GalA. Comparison of the ^{1}H NMR spectra of OS1 and OS2 demonstrated that the absence of GalA from the former is the only difference between the oligosaccharides. In particular, the following characteristic signals listed above for OS2 had essentially the same chemical shifts in OS1: δ 5.45 (H-1, α-GlcN3N), 5.30 (H-1, Man), 4.64 (H-1, GlcN), 4.54 (H-1, β-Glc3N), 2.15 (H-3eq, Kdo^{II}), 2.10 (H-3eq, Kdo^{I}), 2.02 (H-3ax, Kdo^{I}), 1.80 (H-3ax, Kdo^{II}), while no signals for H-1 and H-5 of GalA were present in the spectrum. Hence, OS1 has the following structure:



OS1

In OS3, instead of the signals for GalA, the ^{1}H NMR spectrum revealed signals for a 4-deoxy-β-L-*threo*-hex-4-enuronic acid (Sug) at δ 5.74 (H-4, $J_{3,4}$ 3 Hz), 5.34 (H-1, $J_{1,2}$ 2.5 Hz), 4.40 (H-3, $J_{2,3}$ 7.5 Hz), and 3.80 (H-2) (cf. published data [17]) together with essentially the same chemical shifts of the characteristic signals listed above for OS1 and OS2. The unsaturated sugar has been reported to derive from a 4-substituted hexuronic acid by β-elimination during strong alkaline treatment [17]. Like GalA in OS2, Sug in OS3 is attached at position 5 of Kdo^{II}, as followed from a 2D NOESY experiment which revealed strong cross-peaks Sug H-1, Kdo^{II} H-5 and H-7 at δ 5.34/4.25 and 5.34/4.04, respectively. The 2D COSY and NOESY patterns displayed by OS2 and OS3 were similar and, therefore, OS3 has the following structure:



OS3

To shed light on the nature of the substituent at O-4 of GalA, sugar and methylation analyses of the native LPS were performed. Neutral sugars derived from LPS by acid hydrolysis were Glc and Man, the latter being detected in only trace amounts. Most likely, this was due to the absence of any acyl group

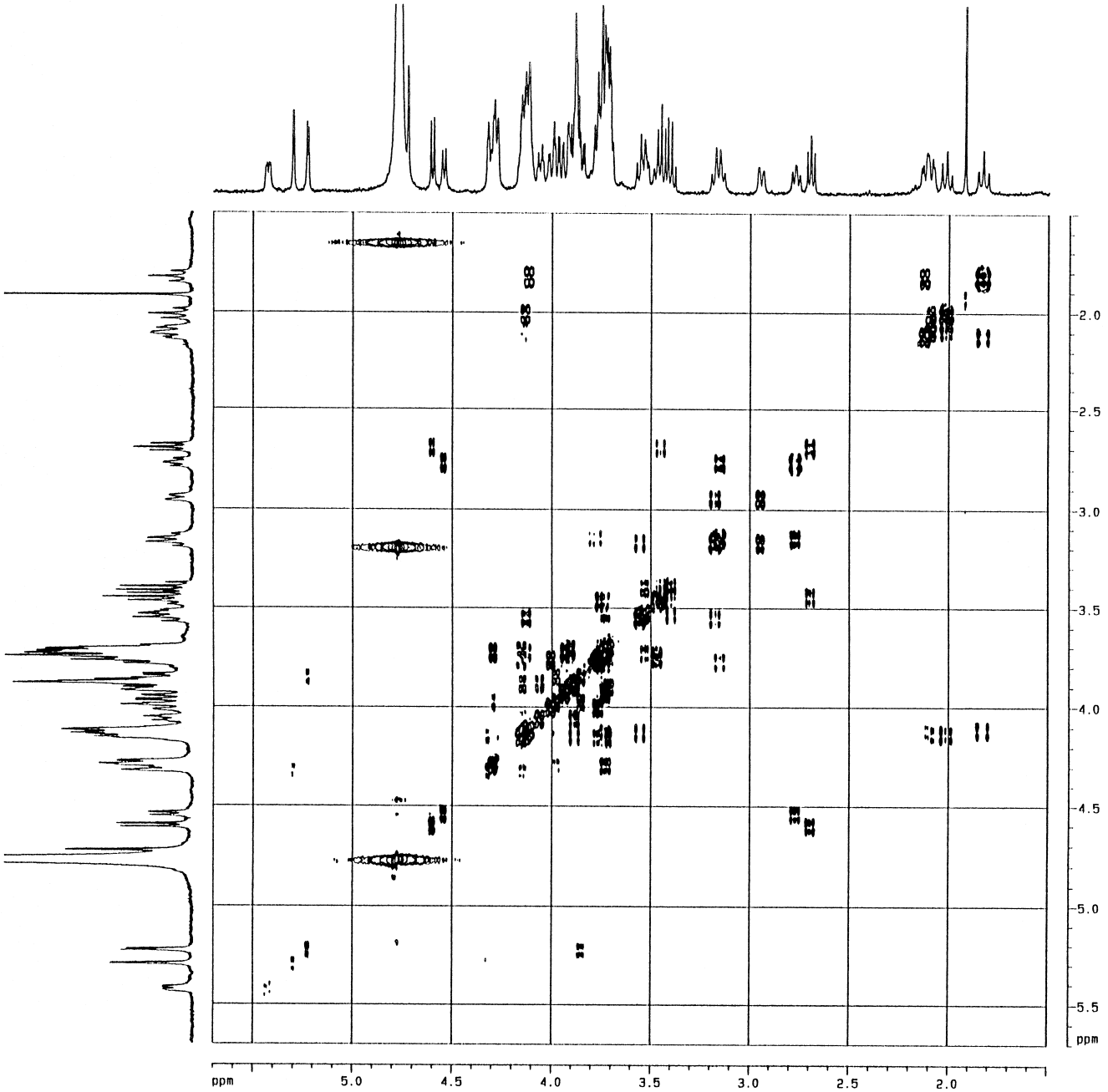


Fig. 2. 500 MHz 2D DQF COSY spectrum of OS2. The corresponding 1D ^{1}H NMR spectrum is displayed along the axes.

at the amino group of GlcN that makes the linkage between GlcN and Man stable to acid hydrolysis [18]. When methanolysis and carboxyl reduction using $NaBD_4$ were performed prior to hydrolysis and the second reduction with $NaBD_4$, almost equal amounts of glucitol-1-*d* and galactitol-1,6,6-d_3 were identified, the latter resulting evidently from GalA. Methylation of LPS followed by hydrolysis and GLC–MS of derived alditol acetates revealed only terminal Glc, since from the other terminal sugar residues (Kdo, GalA, GlcN) suitable derivatives are not obtained by this procedure and GlcN–Man was only detected as a disaccharide. After methanolysis and acetylation GLC–MS analysis indicated the presence of terminal GalA and monosubstituted GalA. These data fitted well with the structures of OS2 and OS3 and, therefore, it can be suggested that in part of the LPS species Glc is attached to GalA at position 4.

3. Discussion

This study is the first successful application of a recently elaborated strong alkaline degradation protocol [10,11] to a LPS containing a GlcN3N disaccharide lipid A backbone, demonstrating that this method is not limited by the enterobacterial-type GlcN disaccharide lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*]. This finding is important in view of the increasing number of bacteria recognised recently to contain a lipid A backbone of the former type [8,19–21].

SDS–PAGE revealed structural heterogeneity in the *O. anthropi* LPS which may be associated with different substitution in Kdo^{II}: this may be unsubstituted (OS1) or substituted with GalA (OS2) or with a longer fragment containing GalA substituted at O-4. The structure of the substituent could not be determined using the strong alkaline protocol because of β-elimination in GalA resulting in a truncated oligosaccharide (OS3). It was suggested that this substituent is a glucose residue, but further studies are necessary to determine the complete structure of the core region of the *O. anthropi* strain LMG 3301 LPS.

Lipid A and the inner core are conserved parts of LPS within the same serogroup and their structures are considered as a marker of the phylogenetic relatedness among Gram-negative bacteria [19]. Therefore, demonstration of a GlcN3N-based lipid A backbone in *O. anthropi* is in keeping with the finding of such lipid A backbone in LPS of other members of the α-subgroup of Proteobacteria [5,8,21], including *Brucella* [5,8].

The inner core of *O. anthropi* includes a Kdo disaccharide but lacks L-*glycero*-D-*manno*-heptose, which is typically linked to Kdo^{I} in most LPS, the structures of which were elucidated. This is the case also for the LPSs of *Brucella* [22] and some *Mycoplana* spp. [21], including *M. dimorpha* which forms a close cluster with *Brucella* and *O. anthropi* [23]. The LPS of all these bacteria contain Man, which in *O. anthropi* occupies the same position as would be expected for heptose; location of Man in *Brucella* and *Mycoplana*, as their core structures as the whole, remains unknown. It is worth noting that the inner core and lipid A backbone structures of the *O. anthropi* LPS resemble much those of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1) [20,24], both bacteria containing the same bisphosphorylated GlcN3N disaccharide in lipid A and the same α-D-Man-(1 → 5)-Kdo^{I} disaccharide and no phosphate in the core.

4. Experimental

Bacterium, growth and isolation of LPS.—*O. anthropi* strain LMG 3301 was from the culture collection of the Laboratory of Microbiology, Gent University, Belgium. The bacterium was grown in a fermenter and harvested by centrifugation as described previously [1]. The sedimented bacterial cells were washed successively with EtOH, acetone (twice) and Et_2O, and then dried in vacuum. LPS was extracted from the dried cells with 2:5:8 phenol–chloroform–light petroleum [9] in a yield of 3% of the bacterial dry mass.

SDS–PAGE.—SDS–PAGE was performed using a discontinuous buffer system with Tris–Tricine buffers [25]. A total of 10 μg of each LPS from *Escherichia coli* strain F515, *O. anthropi* strain LMG 3331, and *O. anthropi* strain LMG 3301 were applied to the gel. The gel was fixed and silver-stained [26].

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were recorded with Bruker DRX 600 and Bruker DRX 500 spectrometers for solutions in D_2O at pD 7 at 300 K using internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) as reference. 2D NMR experiments were performed using standard Bruker software. Mixing times of 70, 250, and 150 ms were used in TOCSY, ROESY, and NOESY experiments, respectively.

Chromatography and GLC–MS.—GPC was performed on a column (120 × 2.5 cm) of Sephadex G-10 in water or a column (60 × 2.5 cm) of Sephadex G-50, and monitored using a Knauer differential refractometer. Anion-exchange HPLC was carried out on a column (250 × 9 mm) of CarboPac PA-1 at 4 mL/min in a gradient of NaOAc (0–0.7 M) in 0.1 M NaOH for 40 min with monitoring using a pulse amperometric detector (Dionex, USA). GLC was performed on a Varian 3700 chromatograph equipped with a fused-silica gel SPB-5 column using a temperature gradient 150 °C (3 min) → 320 °C at 5°C/min. GLC–MS was performed on a Hewlett-Packard 5989A instrument equipped with an HP-5 column under the same chromatographic conditions as in GLC.

De-O-acylation and alkaline degradation of LPS.—LPS (700 mg) was treated with anhydrous hydrazine (0.5 h, 37 °C) [10], and the resulting de-*O*-acylated LPS (566 mg) was de-*N*-acylated with 4 M KOH (16 h, 120 °C). Then the mixture was neutralised with 4 M HCl, fatty acids were extracted with CH_2Cl_2 (twice), the aqueous phase was desalted by GPC on Sephadex G-10, and the product (125 mg)

was fractionated by anion-exchange HPLC. Desalting by GPC of the three major fractions eluted with a retention time of 28.5, 30, and 36 min afforded OS1 (4.8 mg), OS2 (1.8 mg), and OS3 (5.6 mg), respectively.

Sugar and phosphate analyses.—Oligosaccharides (0.3 mg each) were *N*-acetylated with Ac_2O in aq $NaHCO_3$ as described [11], then hydrolysed with 2 M CF_3COOH (4 h, 100 °C) or methanolysed with 2 M HCl in MeOH (16 h, 85 °C). LPS was hydrolysed as above without or with prior methanolysis and carboxyl-reduction with $NaBD_4$ and the products were reduced with $NaBD_4$. Samples were analysed by GLC and GLC–MS after conventional acetylation with Ac_2O in pyridine. Absolute configurations of monosaccharides were determined by GLC of acetylated (+)-2-butyl glycosides [27,28]. Kdo was determined by the thiobarbituric acid reaction according to the modified method [29]. Phosphate was determined by the method of Lowry et al. [30].

Methylation analysis.—LPS was degraded with aq 2% HOAc (3 h, 100 °C), the water-soluble portion was fractionated by GPC on Sephadex G-50, and the low-molecular-mass fraction (a GalA → Kdo disaccharide) was reduced with $NaBH_4$ and methylated [31]. A portion of the methylated material was methanolysed with 2 M HCl in MeOH (85 °C, 4 h) and acetylated (Ac_2O–pyridine, 20 min, 85 °C). LPS was methylated [31], hydrolysed and reduced with $NaBH_4$ or methanolysed as in sugar analysis. Both methylated disaccharide and monosaccharides were analysed by GLC–MS.

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