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Note

The structure of the carbohydrate backbone of the LPS from *Myxococcus xanthus* strain DK1622

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Abstract—Gram-negative rod shaped bacterium *Myxococcus xanthus* DK1622 produces a smooth-type LPS. The structure of the polysaccharide O-chain and the core-lipid A region of the LPS has been determined by chemical and spectroscopic methods. The O-chain was built up of disaccharide repeating units having the following structure:

→ 6)-α-D-Glcp-(1 → 4)-α-D-GalpNAc6OMe*-(1 →

with partially methylated GalNAc residue. The core region consisted of a phosphorylated hexasaccharide, containing one Kdo residue, unsubstituted at O-4, and no heptose residues. The lipid A component consisted of β-GlcN-(1→6)-α-GlcN1P disaccharide, N-acylated with 13-methyl-C14-3OH (*iso*-C15-3OH), C16-3OH, and 15-methyl-C16-3OH (*iso*-C17-3OH) acids. The lipid portion contained O-linked *iso*-C16 acid.

Keywords: LPS; Polysaccharide; Core; Structure; NMR

Myxococcus xanthus is a Gram-negative soil bacterium that can move on a solid surface without the aid of flagella.[1,2] Two genetically and behaviorally distinct systems have been defined for the control of *M. xanthus* gliding motility: adventurous (A)-motility and social (S)-motility, showing different selective advantages on various surfaces.[3] S-Motility requires the cell-surface components type IV pili[4,5] and extracellular matrix fibrils.[6,7] The lipopolysaccharide (LPS) was found to be required for normal *M. xanthus* gliding motility.[8] The *M. xanthus* LPS was found to have a typical structure consisting of three regions: lipid A, which is present in the outer leaflet of the outer membrane; the core oligosaccharide attached to lipid A, and a repeated oligosaccharide of variable length, termed the O-antigen, attached to the core.[9] The precise structure of the *M. xanthus* LPS has not been determined; however, the LPS O-antigen appeared to be methylated during early development.[10,11] Among the LPS O-antigen mutants isolated, those that map to the wzm wzt wbgA operon[8] are the best characterized. The wzm wzt genes code for homologues of the ATP-binding cassette (ABC) transporters, which presumably transfer the O-antigen from the cytoplasm to the periplasm, where it is attached to the lipid A and core components of the LPS. Mutants with the disrupted WbgB gene, participating in O-antigen biosynthesis, as well as other LPS O-antigen mutants, were found to be defective in fruiting body development, sporulation and gliding motility,[12,13] which indicate important role of the LPS in the development of *M. xanthus*.

We now describe the structure of the LPS from *M. xanthus* strain DK1622.

Abbreviations: LPS, lipopolysaccharide; HMBC, heteronuclear multiple bond connectivity; GalNAc, 2-acetamido-2-deoxy-D-galactose

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Mild acid hydrolysis of the LPS from *M. xanthus* led to the release of water-insoluble lipid A. Polysaccharide and core oligosaccharides were isolated from the soluble fraction by gel chromatography.

The polysaccharide composition was analyzed by GC–MS of alditol acetates. Glc, GalN, and 6-*O*-methyl-GalN were identified in a molar ratio of 1:0.5:0.3. The absolute configuration of Glc and GalN was determined to be D by GC–MS of acetylated (*R*)-2-butyl glycosides.

NMR spectra of the polysaccharide (COSY, TOCSY, NOESY, HSQC, and HMBC) contained signals of the spin systems of several monosaccharides, having α-glucopyranose and α-galactopyranose configurations. A detailed analysis of the spectra and the assignment of all signals (Fig. 1, Table 1) led to the conclusion that the polymer contained a disaccharide repeating unit built of α-Glc*p* and α-Gal*p*NAc, where ~50% of the GalNAc residues were methylated at O-6. Partial methylation influenced the position of many signals, giving the spectra an irregular appearance. Identity, relative, and anomeric configurations of the constituent monosaccharides were assigned on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts, which were in agreement with standard values for α-Glc*p* and α-Gal*p*NAc. The sequence of the monosaccharides was determined from NOE data, which showed correlations from anomeric protons to transglycosidic protons: X1:Y4, Y1:X6 (all possible combinations of the monosaccharides from methylated and non-methylated repeating units). HMBC correlations X1:Y4, Y1:X6 between anomeric protons and transglycosidic carbon atoms were also observed. Position of the methyl groups followed from low field shift of C-6 signal of GalNAc* and NOE and HMBC correlations between methyl groups and H-6/C-6 of the GalNAc*. Distribution of methylated residues within the polymer chain has not been determined precisely, but apparently methylation was random. The structure of the polysaccharide repeating units was confirmed by the results of methylation analysis (Scheme 1).

The mixture of oligosaccharides derived from the core part of the LPS, obtained on gel chromatography, was further separated by anion-exchange chromatography to give four fractions. The major fraction contained the core oligosaccharide shown below. Other fractions were its degradation products with lost EtN and/or α-Man-(1→2)-α-Man disaccharide (units I–H) due to partial hydrolysis of phosphodiester linkages. Monosaccharide composition of the core was determined by GC of alditol acetates and mannose, glucose, xylose, and galactosamine were found in the ratio of 2:1:1:1. All monosaccharides had the D-configuration according to GC–MS analysis of their acetylated (*R*)-2-butyl glycosides. NMR analysis showed that the core contained the above mentioned monosaccharides in the pyranose form and additionally a Kdo residue, EtN, and two phosphodiester groups. 2D NMR spectra of the core oligosaccharide were completely interpreted

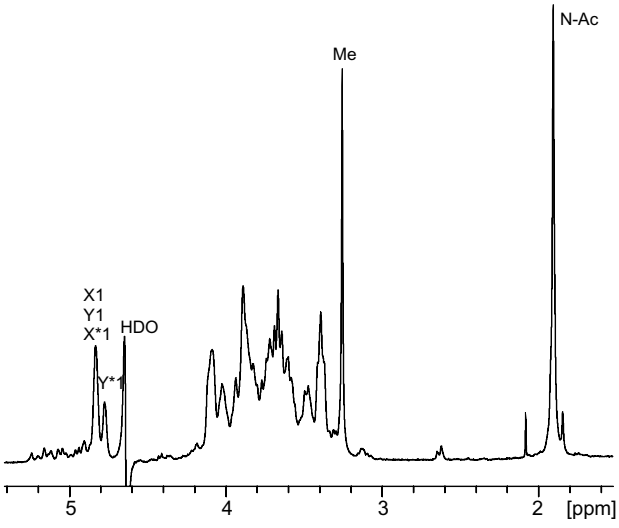


Figure 1. ^{1}H NMR spectrum of the *M. xanthus* O-specific polysaccharide.

→6)-α-D-Glc*p*-(1→4)-α-D-Gal*p*NAc6OMe*-(1→

X **Y** with non-methylated GalNAc

X* **Y*** with 6-*O*-methylated GalNAc

Scheme 1. Proposed structure of the *M. xanthus* strain DK1622 O-specific polysaccharide.

Table 1. NMR spectroscopy data for the *Myxococcus xanthus* DK1622 O-polysaccharide (ppm, $J_{Hn,n+1}$ in brackets, Hz)

Unit	Nucleus	1	2	3	4	5	6
X, Glc	H	4.98 (3.8)	3.55 (10.3)	3.81 (10)	3.53 (10)	4.21	3.66/3.97
	C	101.4	72.7	73.6	70.4	72.1	66.7
Y, GalNAc	H	4.97 (nr)	4.25 (11)	3.99 (nr)	4.03 (s)	4.02	3.86
	C	98.1	50.9	68.3	79.5	72.0	61.3
X*, Glc	H	4.97 (nr)	4.25 (11)	3.99 (nr)	4.09 (s)	4.156	3.77
	C	98.1	50.90	68.3	79.8	70.2	71.6
Y*	H	4.93 (4)	3.54 (10.3)	3.81(10)	3.53 (10)	4.21	3.66/3.97
GalNAc6OMe	C	101.4	70.4	73.6	70.4	72.1	66.7

Table 2. NMR spectroscopy data for the core oligosaccharide and LPS-Hy (δ, ppm)

Residue, compound	Nucleus	1	2 (3ax)	3 (3eq)	4	5	6 (6a)	7 (6b)	8a	8b
α-GlcN A LPS hy	^{1}H	5.32	3.80	3.78	3.69	3.92	3.87	4.08		
	^{13}C	93.7	55.0	71.3	67.5	72.0	68.9			
β-GlcN B LPS Hy	^{1}H	4.55	3.77	3.59	3.43	3.59	3.59	3.63		
	^{13}C	103.3	56.6	74.7	71.5	74.9	63.5			
Kdo C core	^{1}H		2.12	1.92	4.16	4.09	3.91	3.90	3.93	4.06
	^{13}C	175.9	96.9	34.7	66.5	74.9	71.7	68.3	68.1	
Kdo C LPS Hy	^{1}H		1.89	2.06	4.14	4.04	3.68	3.69	3.86	4.23
	^{13}C			35.5	66.6	75.4	72.5	69.6	68.7	
α-GalNAc E core	^{1}H	5.13	4.37	4.13	4.31	4.60	3.97	3.97		
	^{13}C	99.2	49.5	77.1	69.0	69.7	65.2			
α-GalNAc E LPS Hy	^{1}H	5.08	4.37	4.10	4.29	4.51	3.92	3.95		
	^{13}C	99.5	49.5	77.1	68.9	69.6	64.6			
β-Glc G core	^{1}H	4.69	3.53	3.69	3.43	3.45	3.72	3.89		
	^{13}C	102.9	79.9	77.3	70.2	76.3	61.2			
β-Glc G LPS Hy	^{1}H	4.67	3.51	3.69	3.42	3.44	3.72	3.89		
	^{13}C	102.7	80.1	77.3	70.3	76.2	61.2			
β-Xyl F core	^{1}H	4.73	3.27	3.44	3.60	3.23	3.89			
	^{13}C	103.9	74.4	76.5	70.1	65.9				
β-Xyl F LPS Hy	^{1}H	4.71	3.29	3.41	3.62	3.22	3.90			
	^{13}C	103.9	74.4	76.6	70.2	66.0				
α-Man H core	^{1}H	5.66	4.02	4.02	3.73	3.80	3.77	3.87		
	^{13}C	95.4	79.8	70.3	67.4	74.7	61.7			
α-Man H LPS Hy	^{1}H	5.62	3.99	3.99	3.71	3.78	3.77	3.87		
	^{13}C	95.4	79.7	70.4	67.5	74.7	61.7			
α-Man I core	^{1}H	5.06	4.06	3.85	3.67	3.77	3.77	3.87		
	^{13}C	103.0	70.8	71.1	67.5	74.0	61.7			
α-Man I LPS Hy	^{1}H	5.05	4.05	3.83	3.67	3.74	3.77	3.87		
	^{13}C	102.9	70.8	71.2	67.5	74.0	61.7			
EtN	^{1}H	4.10	3.27							
	^{13}C	62.6	40.8							

Signals of the acetate group at E2: 175.4, 2.06/23.1 ppm (^{1}H/^{13}C).

(Table 2). Linkages between monosaccharides were identified on the basis of the following NOE cross-peaks: E1:C5, E1:C7, G1:E3, F1:G2, I1:H2, I1:H1, H1:I5; and the corresponding HMBC correlations

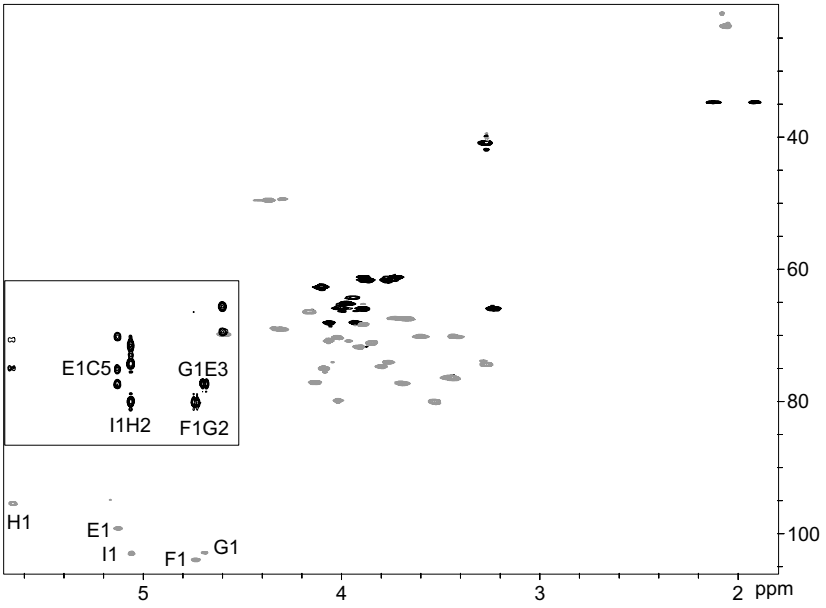


Figure 2. ^{1}H–^{13}C HSQC correlation spectrum of the *M. xanthus* core oligosaccharide. Black signals belong to CH_2 groups and (inside the frame) to HMBC spectrum, gray signals belong to CH and CH_3 groups. Inset shows the fragment of HMBC spectrum with indicated transglycosidic correlation signals.

(Fig. 2). ^{1}H–^{31}P HMQC and HMQC-TOCSY correlation spectra contained two ^{31}P signals at −1.2 and 1.4 ppm, the first of them correlated with the H-1 signal of Man H and H-6 of GalN E, thus indicating that Man H is linked to O-6 of GalN E through a phosphodiester link. The second one correlated with H-1 of EtN residue and H-8 of Kdo, due to the presence of the EtN*P* substituent at O-8 of the Kdo residue. The ESI mass spectrum of the core oligosaccharide contained a single peak at 1263.3 Da as expected for the structure (Scheme 2).

The LPS was O-deacylated with anhydrous hydrazine to give LPS-Hy. Fatty acid analysis of this product showed the presence of 13-methyl-C14-3OH, C16-3OH, and 15-methyl-C16-3OH acids in the ratio ~3:1:5. LPS-Hy was analyzed by NMR spectroscopy and mass spectrometry (Figs. 2–4, Table 2). NMR data showed that it contained the above described core structure linked to O-deacylated lipid A with retained amide-linked acids. The lipid A backbone consisted of a standard β-GlcN-(1→6)-α-GlcN1*P* disaccharide (^{31}P signal at 3.76 ppm, correlating with H-1 of α-GlcN), N-acylated with 3-hydroxy fatty acids. Kdo was α-(2→6)-linked to β-GlcN residue B (determined from HMBC correlation between Kdo C-2 and β-GlcN H-6). In contrast to many other LPS structures, Kdo was not substituted at O-4. The mass spectrum of this product (Fig. 4) contained three peaks with molecular masses of 2202.3 (major, ~50%), 2188.7, and 2174.7 Da, consistent with the structure in Scheme 3, where amino groups of GlcN A and B were acylated with 15-methyl-C16-3OH acids in the major structure and with shorter chain acids in two other variants (ac = fatty acid residue).

F **G** **E** **C**

β-Xyl-(1→2)-β-Glc-(1→3)-α-GalNAc-(1→5)-Kdo

α-Man-(1→2)-α-Man-(1-*P*-6)┘ EtN*P*-8┘

I **H**

Scheme 2. The structure of the core oligosaccharide, isolated after mild acid hydrolysis of the *M. xanthus* strain DK1622 LPS.

The structure of the unusual, for LPS, branched fatty acids was confirmed by NMR spectroscopy of LPS-Hy. Isopropyl end group signals were observed at ($^{1}H/^{13}C$): ω 0.82/23.0; ω-1 1.48/28.5; ω-2 1.14/39.8 ppm. Signals of straight chain C16-3OH acid were also present (ω at 0.85/14.8 ppm). Fatty acid analysis of the whole LPS or liberated lipid A showed the presence of the same acids and additionally of the *iso*-C16 acid, which was O-linked.

Determination of the structure of the LPS of *M. xanthus* may help clarify the role of LPS in the complex social life and motility of this microorganism. The

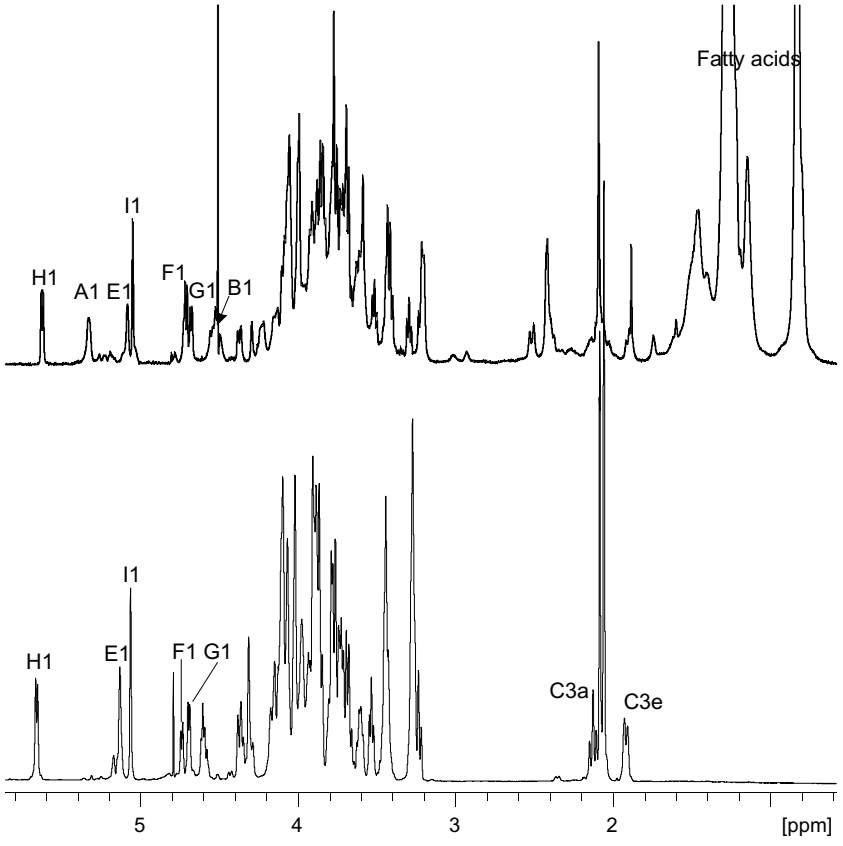


Figure 3. ^{1}H NMR spectra of the core oligosaccharide (lower trace, 25 °C) and of the O-deacylated LPS (upper trace, 60 °C).

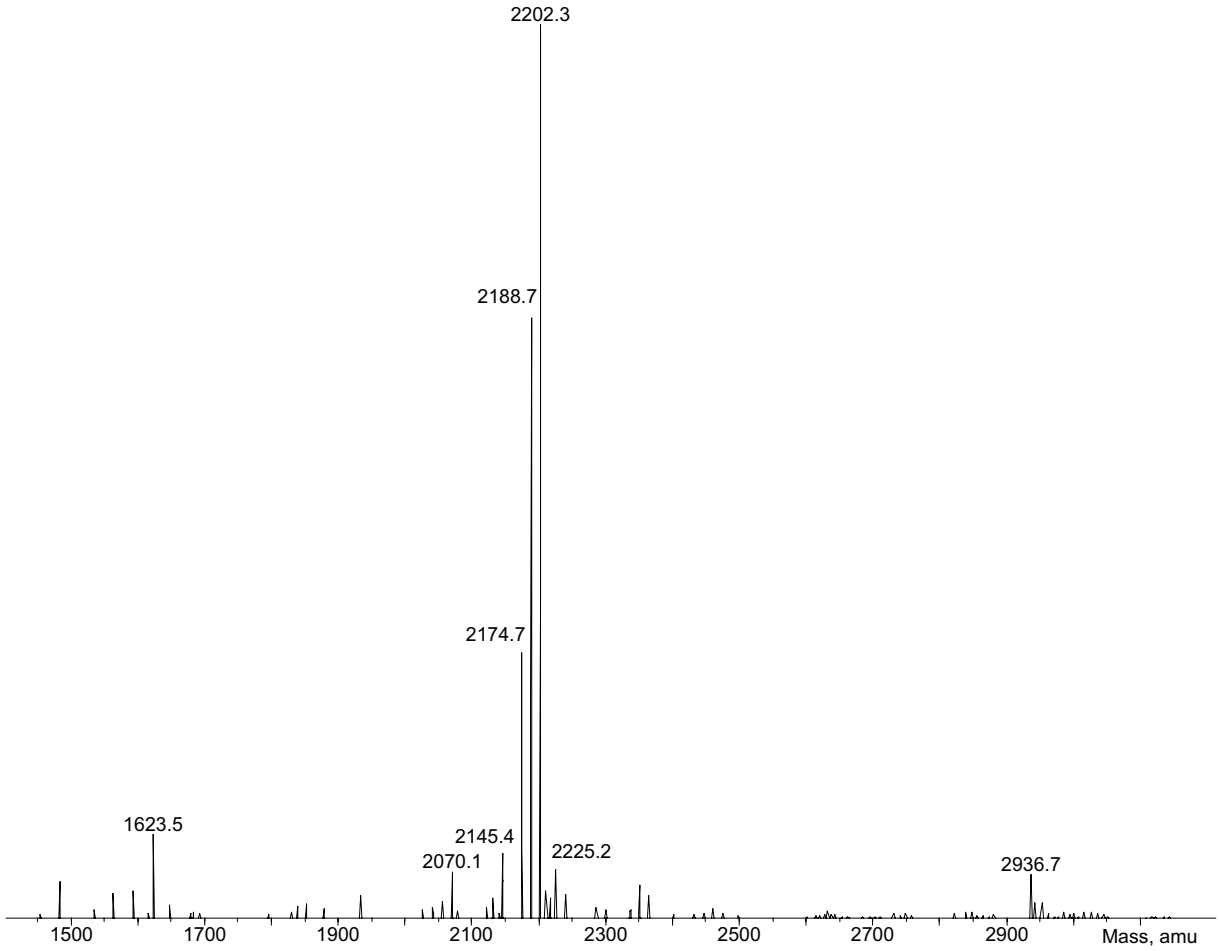
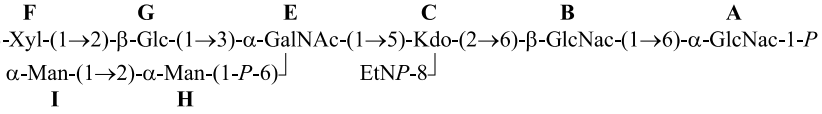


Figure 4. Reconstructed ESI mass spectrum of the *M. xanthus* O-deacylated LPS. Signal at 2202.3 belongs to the structure presented in Scheme 3 with two *iso*-C17-3OH fatty acids.



Scheme 3. The structure of the O-deacylated LPS (LPS-Hy) from *M. xanthus* strain DK1622.

analyzed LPS is a further example of structure incorporating branched fatty acids in the lipid A portion, which were found before in only two microorganisms, *Bacteroides fragilis*[14] and *Porphyromonas gingivalis*,[15] both of them containing the same fatty acids as *M. xanthus*.

1. Experimental

1.1. LPS preparation

Cells of *M. xanthus* DK1622 in 1:1 phenol–water were stirred for 20 min at 80 °C, the suspension was diluted with 200 mL of water and dialyzed against running water until free from phenol (one week). The dialyzed retentate was treated sequentially with RNase, DNase, and proteinase K (37 °C, 2 h each). The digest was cleared by low speed centrifugation (3000*g*) and then subjected to ultracentifugation (105,000*g*, 4 °C, 12 h), the precipitated lipopolysaccharide gel was dissolved in water and freeze-dried to give 40 mg of LPS.

1.2. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 500 MHz spectrometer for samples in D_2O solutions at 60 °C for the polysaccharide and O-deacylated LPS (the last sample contained 5% of fully deuterated

SDS) and at 25 °C for the oligosaccharides with acetone standard (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC and HMBC (optimized for a 8 Hz coupling constant).

1.3. Electrospray mass spectra

These were obtained using a Micromass Quattro spectrometer in 50% MeCN with 0.2% HCOOH at a flow rate of 15 μL/min with direct injection in negative mode.

1.4. Monosaccharide analysis

The polysaccharide, core, or LPS (0.5 mg) were hydrolyzed (0.2 mL of 3 M TFA, p120 °C, 2 h) and evaporated to dryness under a stream of air. The residue was dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with AcOH (0.3 mL), dried, and MeOH (1 mL) was added. The mixture was dried twice with the addition of MeOH, and the residue was acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, and analyzed by GC on a HP1 capillary column (30 m × 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient of 170 (4 min) to 260 °C at 4 °C/min.

1.5. Determination of the absolute configuration of the monosaccharides

The polysaccharide (1 mg) was treated with 10:1 (*S*)-2-butanol-AcCl (0.25 mL, 2 h, 85 °C), dried under a stream of air, acetylated, and analyzed by GC in comparison with authentic standards prepared from the respective monosaccharides with (*S*)- and (*R*)-2-butanol.

1.6. Fatty acid analysis

LPS or LPS-Hy (0.5 mg) was treated with 1 M HCl in MeOH, dried, extracted with water–$CHCl_3$, and the organic layer dried, acetylated with pyridine–Ac_2O (100 °C, 30 min), dried, and analyzed by GC–MS.

1.7. Gel chromatography

It was carried out on Sephadex G-50 (2.5 × 80 cm) or Sephadex G-15 (1.6 × 80 cm) columns using the pyridinium acetate buffer, pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water) as eluent, monitored by a refractive index detector.

1.8. Anion-exchange chromatography

It was performed on a 5 mL Hitrap Q column (Amersham) in water for 10 min, using a linear gradient of 0–1 M NaCl over 60 min with UV detection at 220 nm. Fractions were desalted by gel chromatography on Sephadex G-15 column.

1.9. Preparation of the core

The LPS (25 mg) was hydrolyzed with 2% AcOH (2 mL, 3 h, 100 °C), the lipid precipitate removed by centrifugation at 12,000 rpm, and the soluble products were separated by gel chromatography on a Sephadex G50 column. The polysaccharide and oligosaccharide fractions were obtained. The oligosaccharide (core) fraction was further separated on an anion-exchange column and desalted on Sephadex G-15.

1.10. Methylation analysis

The core oligosaccharide (1 mg) was dissolved in anhyd Me_2SO, dry powdered NaOH (~50 mg) was added, and the mixture was stirred for 1 h, MeI (0.3 mL) was then added, the suspension was stirred for 30 min, the excess of MeI was removed by air stream, the residue was diluted with water (5 mL), and extracted with $CHCl_3$ (5 mL). The organic layer was washed three times with water, dried, and the methylated product was converted into alditol acetates as described for monosaccharide analysis using $NaBD_4$ and further analyzed by GC–MS on a Varian Saturn 2000 ion-trap instrument.

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