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Characterization of the cell surface glycolipid from *Spirochaeta aurantia*

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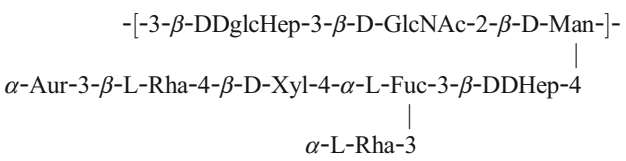
Abstract *Spirochaeta aurantia* is a free-living saprophytic spirochete that grows easily in simple laboratory media, and thus can be used as a model for the investigation of surface carbohydrate structures in spirochetae, which are normally not available in sufficient amounts. Freeze-substitution electron microscopy indicated the presence of a capsule-like material projecting from the surface of *S. aurantia*. Extraction of cells gave two major glycolipids, the one with a higher molecular mass glycolipid was designated large glycolipid A (LGLA). LGLA contained small amount of branched and unsaturated *O*-linked fatty acids, L-rhamnose, L-fucose, D-xylose, D-mannose, D-glucosamine, D-glycero-D-gluco-heptose (DDglcHep), D-glycero-D-manno-heptose (DDHep), and a novel branched tetradeoxydecose monosaccharide, which we proposed to call aurantose (Aur). The carbohydrate structure of LGLA was extremely complex and consisted of the repeating units built of 11 monosaccharides, arrangement of nine of them was determined as:



which wasdeduced from the NMR and chemical data on the LGLA and its fragments, obtained by various degradations. Tentative position of two remaining sugars is proposed. LGLA was negative for gelation of Limulus amebocyte lysate, did not contain lipid A, and was unable to activate any known Toll-like receptors.

Keywords Glycolipid · Spirochaete · *Spirochaeta aurantia* · TLR · Structure · NMR

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Introduction

Spirochetes are phylogenetically coherent phylum but show considerable structural, physiological and genomic diversity. With inner and outer membranes and a periplasmic space containing peptidoglycan, spirochetes resemble typical Gram-negative cells though with the unique feature that their flagella are located in the periplasm [1]. One of the major characteristics of the Gram-negative cell is the presence of lipopolysaccharide (LPS, endotoxin), consisting of *O*-antigenic polysaccharide, oligosaccharide core, and a glycolipid "lipid A" [2]. Recognition of LPS by the innate immune system involves at least three receptor molecules: CD14, TLR4 and MD-2. "LPS-sensing apparatus" also includes heat shock protein, chemokine receptor 4

(CXCR4) [3, 4]. Although spirochetes may be considered Gram-negative bacteria, LPS has only been detected in *Leptospira*, the pathogen causing leptospirosis [5]. *Treponema pallidum* subsp. *pallidum* str. Nichols and the *Borrelia burgdorferi* strain B31, pathogens causing syphilis and Lyme disease, respectively, lack LPS as shown chemically and through analysis of their complete genome sequences [6]. Two monosaccharide glycolipids were identified in *B. burgdorferi*, suggesting that within spirochetes there may be a diversity of molecules able to constitute membranes [7, 8]. Glycolipids isolated from oral treponemes, *T. denticola*, *T. pectinovorum*, *T. maltophilium* and *T. brennaborense* are similar to smooth or rough LPS but most do not contain characteristic LPS components Kdo and 3-hydroxy fatty acids [9–11].

Spirochaeta aurantia is a saprophytic spirochete found in aquatic ecosystems that has been implicated in global nitrogen cycling [1, 12, 13]. In contrast to other spirochetes, *S. aurantia* is easily and inexpensively propagated, facilitating characterization of polysaccharide structures that in other spirochetes may be limited by the cost and difficulty of obtaining sufficient biomass. We have isolated two large glycolipids (LGL) from *S. aurantia*. LGLB is the smaller of the two, with a mass and carbohydrate structure that, while anchored on a glycerolipid instead of lipid A, resembles that of Ra LPS [14]. The present study investigates the biological nature and structure of the larger *S. aurantia* glycolipid, LGLA.

Results and discussion

The *S. aurantia* cell envelope

The envelope structure of *S. aurantia* was investigated using freeze substitution microscopy (Fig. 1). A clearly defined inner membrane surrounds the cytoplasm, and a periplasmic space can be seen separating the inner membrane and outer sheath. The outer sheath bulges out to accommodate the periplasmic flagella and appears slightly thicker than the inner membrane. External to the outer sheath is an amorphous fluffy layer, distinguishable from the surrounding background and unequally distributed around the surface, suggesting the present of a surface molecule external to the outer sheath, which varies in size, such as capsule or smooth LPS.

Isolation of the glycolipid LGLA

The method of Darveau and Hancock gives high yields of both smooth and rough forms of LPS [15]. Extraction of stationary phase *S. aurantia* cells gave a white powdery substance in a yield of 15.6% based upon the cell dry weight. This high yield is due to the surface to volume ratio

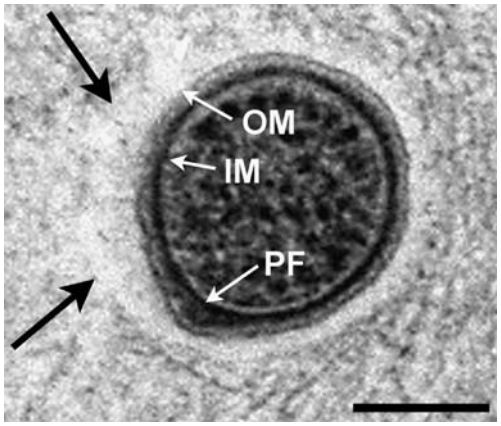

Fig. 1 A cross-section of *S. aurantia* visualized by freeze substitution electron microscopy. Scale bar corresponds to 0.125 μM. The inner membrane (*IM*, *white arrow*) and outer membrane (*OM*, *white arrow*) encase the electron-dense periplasm containing the periplasmic flagella, PF (*white arrow*). LGLA capsule-like material extends from the outer membrane OM, and the interface between the extracellular milieu and distal polysaccharide repeats is indicated by *black arrows*

of *S. aurantia* is 13.6 μm^{-1}, approximately 3.5 times that of *Escherichia coli* or *Salmonella enterica* sv. *typhimurium* (3.9 μm^{-1}). The term large glycolipid (LGL) was coined to describe the isolated material.

Sephacryl-S300 gel chromatography of the crude LGL extract resolved three distinct fractions, discernable after samples collected were analysed for total sugar and hexosamine content (Fig. 2). The first fraction eluted in the void volume of the column; was high in carbohydrate content, but low in hexosamine; and was identified previously as glycogen [16]. A low molecular weight fraction (LGLB) eluted near the end of the chromatogram and contained sugars and hexosamines. The biological and structural characterization of LGLB has been described [14].

A high molecular weight fraction (LGLA) on SDS-PAGE with silver staining showed two slowly migrating high molecular weight bands and faint banding pattern similar to that observed for the crude LGL (Fig. 3). The presence of banding pattern suggested that LGLA may contain repeating units analogous to those found in LPS *O*-antigen. Large distance between bands suggested large size of the repeating unit of the polysaccharide.

Anti-LGLA polyclonal IgY was generated and Western blot analysis of whole cell lysates and isolated outer membranes showed that *S. aurantia* outer membrane was enriched in LGLA, corroborating findings by freeze fracture transmission electron microscopy.

Structural analysis of the LGLA polysaccharide repeat subunit

LGLA was analyzed for monosaccharide and fatty acid composition. Monosaccharide analysis (GC-MS of alditol

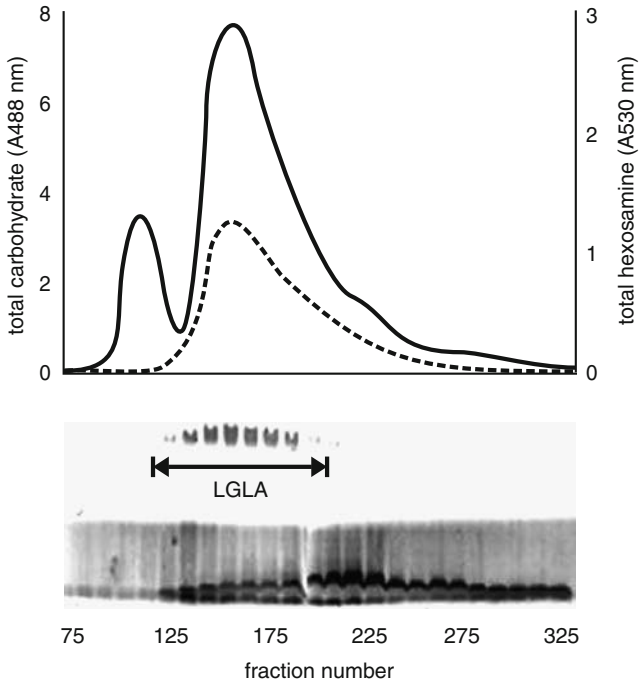

Fig. 2 Column chromatography and initial compositional analysis of *S. aurantia* LGL. **a** Sephacryl S-300 HR fractionation of *S. aurantia* LGL. Total carbohydrate (*solid line*) was quantitated by measuring the absorbance at 488 nm and hexosamine (*dotted line*) content by measuring the absorbance at 530 nm. **b** Tricine SDS-PAGE and silver stain of every tenth fraction, showing the early eluting, high molecular weight LGLA and the late eluting, low molecular weight LGLB. LGLA was obtained by pooling of fractions 140–190

acetates), and subsequently of TMS of 2-(*S*)-butyl glycosides showed the presence of L-rhamnose, L-fucose, D-xylose, D-mannose, D-glucosamine, D-glycero-D-gluco-heptose (DDglcHep) and D-glycero-D-manno-heptose (DDHep) in approx. 2:2:2:1:1:1:1 molar ratio. An unidentified sugar peak of the intensity comparable to heptose peaks was present before hexosamine-heptose region in GLC. Application of the standard thiobarbituric acid assay [17] to detect Kdo in *S. aurantia* LGLA gave a weak absorption at 520 nm (orange) while controls using *S. typhimurium* LPS or pure Kdo yielded a strong absorption at 550 nm (pink).

FAME analysis revealed the presence of C14:0, C14:1, C16:0, and C16:1 straight chain acids fatty acids in about 1% each by mass. Fatty acids could be removed from LGLA after mild hydrazine treatment indicating ester linkage.

NMR analysis of the whole LGLA was not possible due to very bad spectral quality. In order to improve the spectra, LGLA was deacylated by anhydrous hydrazine treatment and the product separated by anion-exchange chromatography on Hitrap Q column (Fig. 4a).

Several fractions thus obtained showed very similar NMR spectra and all had the same monosaccharide composition as the whole LGLA. Main fraction (LGLA-Hy) was analyzed by NMR in detail (Fig. 4b). The spectra in D_2O contained some sharp anomeric signals, but some other anomeric signals were broad and of low intensity. In order to improve the spectra, NMR was run at 60°C in 5% SDS. Still only part of the constituents were visible in NMR; no signals attributable to DDHep, DDglcHep, GlcNAc, and Man could be identified in the spectra. Assignment of the 2D NMR spectra (COSY, TOCSY, NOESY, HSQC, HMBC) led to the identification of the pentasaccharide fragment, containing two rhamnose residues A and C, fucose residue F, xylopyranose residue D, and a novel monosaccharide with ten carbon skeleton, which we proposed to call aurantose (Tables 1 and 2, Fig. 5). Connection between monosaccharides was determined from NOE and HMBC data. The following NOE correlations were identified: B1:C2,3; C1:D3,4,5; D1:F4; A1:F3. Together with HMBC correlations B1:C3; C1:4; D1:F4; A1:F3 these results led to the structure of a pentasaccharide side-chain fragment of LGLA-Hy (Fig. 5). H-1 of the residue F gave weak interresidual NOE correlation to the unidentified proton and no HMBC correlations, thus it was not possible to identify the position

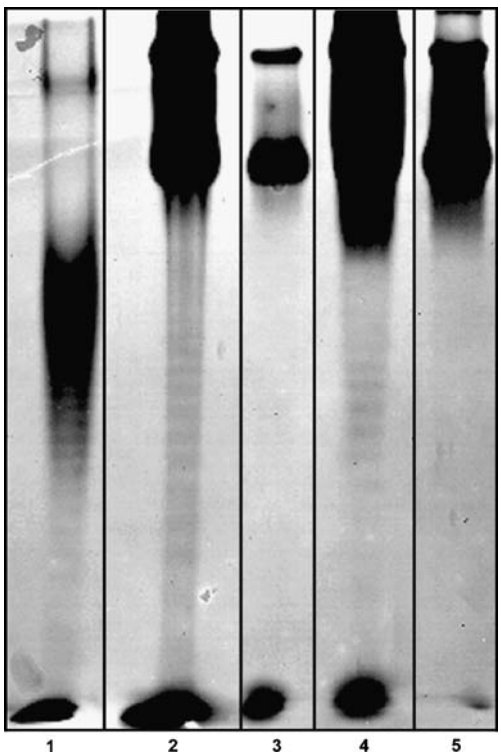

Fig. 3 SDS-PAGE of *S. aurantia* LGL, LGLA and *S. typhimurium* LPS. Silver stained 8% SDS-PAGE of material isolated from *S. aurantia* in comparison to LPS from wild type *S. typhimurium*. The *S. aurantia* sample was loaded ten-fold in excess of the other samples in order to allow visualization of the faint banding pattern. *Lane 1* wild type *S. typhimurium* LPS, 20 μg; *lane 2*, *S. aurantia* LGL, 200 μg; *lane 3*, *S. aurantia* LGL 20 μg; *lane 4*, *S. aurantia* LGLA, 200 μg; *lane 5*, *S. aurantia* LGLA, 20 μg

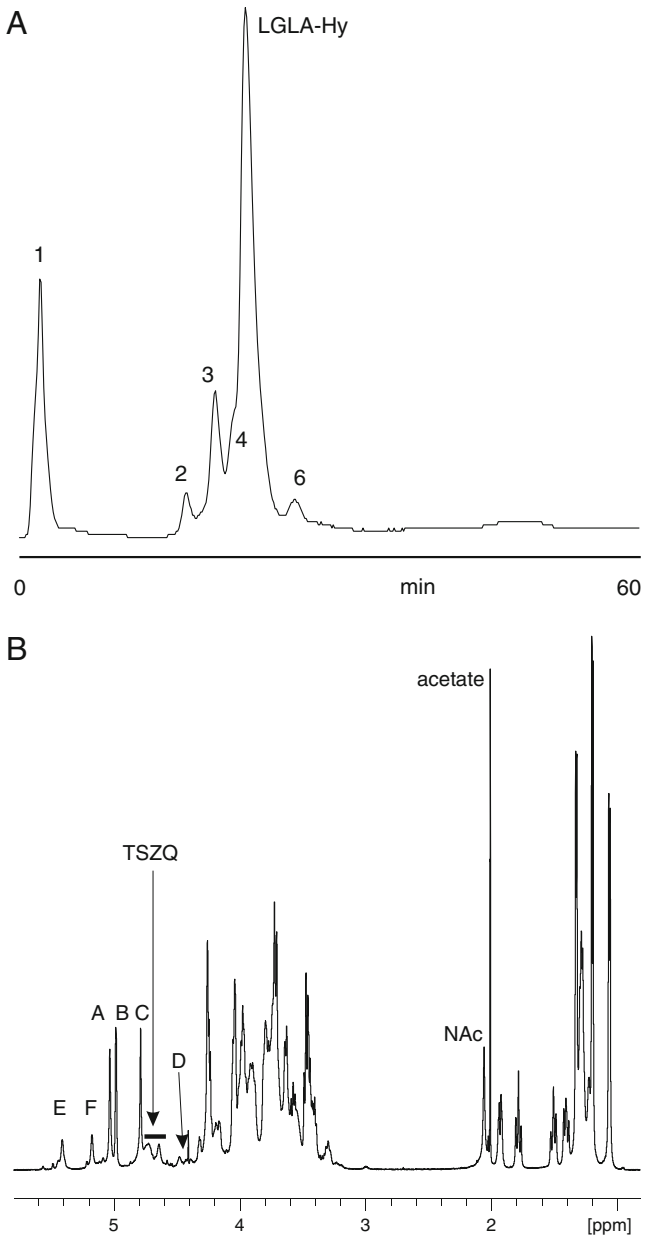

Fig. 4 Anion-exchange separation of the polymeric product, obtained after hydrazine treatment of LGLA (**a**) and ^{1}H NMR spectrum of the purified LGLA-Hy in D_2O with deuterated SDS at 60°C (**b**). *Labels* indicate H-1 signals of the monosaccharides. Signal of xylose residue D is suppressed because of the water suppression. Position of the signals of the residues TSZQ is given tentatively based on their positions in the spectra of the oligosaccharide **3** (OS3)

of attachment of the residue F at this stage; it was however found from the other experiments (see below).

Additionally, the signals of the residue of α-xylofuranose (residue E) were present in the spectra of LGLA-Hy. From NMR data (low field position of all ^{13}C signals) it followed that it is a pentofuranose, and comparison of the ^{13}C chemical shifts with published values [18] showed that it had α-xylo-configuration. Anomeric proton of the residue E gave no HMBC correlations but many NOE correlations, which could not be reliably explained. Thus its position was not identified. Also in the region of resonances of methyl groups of 6-deoxy-sugars and similar compounds the H/C-6 signals of the other monosaccharides were present, not included in the analyzed structure.

Aurantose, identified in LGLA-Hy, was a novel branched tetradeoxydecose (Fig. 5). Aurantose had labile glycoside bond and could be selectively cleaved from the polymer by 2% AcOH (100°, 4 h). It was isolated in pure form, $[\alpha]_D$ + 5.2° (c 1.5, water) from the hydrolysate of the LGLA and analyzed by NMR (Table 2). Its carbon skeleton had a branching at quarternary C-4. In free state it existed in two dominant pyranose forms, with pyranose ring including O-5 in $^{4}C_1$ conformation. Observation of a NOE correlation between β-anomeric proton ($J_{1,2}$ 8.1 Hz) and H-3ax and H-5 proved simultaneously position of cyclization via O-5 (alternatively ring could be formed through O-7), β-anomeric configuration and $^{4}C_1$ ring conformation. β-Anomer was dominating in the free monosaccharide. Intramolecular NOEs were observed between H-5 and H-8, and between H-6 and H-7 (Fig. 6). Inspection of the structural model showed that NOE H-5–H-8 is only possible in case of (4*S*)-isomer (assuming D-configuration based on C-5, which was confirmed in another experiment, see below). For the determination of the configuration at C-7 molecular modeling was used. Minimum energy conformation of the dihedral angles at the C-4–C-7 and C-7–C-8 bonds was found using grid search at 30° steps. The resulting optimal structures of the C-7 epimers had significantly different H-6–H-7 distances; observed strong NOE between these protons could be possible in the case of (7*R*)-isomer. There was no data that could be used for the determination of the configuration at C-9. Full structure of the aurantose may be determined after its chemical synthesis. In LGLA, aurantose was α-linked according to the $J_{1,2}$ of 4 Hz and position of C-3 and C-5 signals close to that in the α-anomer of the free sugar (Table 2). Aurantose completely disappears after strong acid hydrolysis used for monosaccharide analysis, but its alditol acetate was detected on GC-MS analysis of the products of partial hydrolysis with 0.5 M TFA or acetic acid.

Partial hydrolysis of LGLA with 0.5 M TFA (100°, 1 h) gave large number of products, which were separated by gel- and reverse phase chromatography after $NaBH_4$ reduction. The polymeric product gave very bad NMR spectra and was not analyzed. Two pure oligosaccharides were obtained, OS1 and OS2 (Fig. 5). NMR data for OS 1 are given in Table 1; they agree well with the data for LGLA-Hy.

LGLA was further partially cleaved by anhydrous HF treatment at −15°C (ice-salt bath). The main product of this reaction was OS3 (Fig. 5), isolated by gel chromatography.

Table 1 NMR data for LGLA-Hy and oligosaccharides (data for aurantose are shown in Table 2)

Unit, compound	1	2	3	4	5 (5a)	6 (5b)	7 (6b)	7b
α-Rha A, LGLA	5.03	4.04	3.81	3.47	3.93	1.28		
	102.4	*70.7*	*70.8*	*72.7*	*69.7*	*17.3*		
β-Rha C, LGLA	4.79	4.26	3.64	3.47	3.45	1.33		
	101.2	*67.7*	*77.2*	*70.9*	*72.6*	*17.5*		
β-Xyl D, LGLA	4.42	3.41	3.57	3.73	3.30	4.19		
	104.6	*74.1*	*75.3*	*78.8*	*64.7*			
α-Xyl*f* E, LGLA	5.44	4.16	4.32	4.25	3.70	3.78		
	101.9	*77.2*	*76.0*	*79.0*	*61.2*			
α-Fuc F, LGLA	5.18	4.00	3.91	3.98	4.26	1.30		
	94.2	*68.5*	*75.0*	*80.5*	*68.3*	*15.7*		
α-Rha A, **1**	4.97	4.08	3.82	3.52	3.95	1.34		
	102.9	*71.4*	*71.1*	*72.8*	*70.2*	*17.6*		
β-Rha C, **1**	4.83	4.11	3.64	3.40	3.44	1.36		
	101.9	*71.5*	*73.5*	*72.9*	*72.9*	*17.7*		
β-Xyl D, **1**	4.60	3.46	3.65	3.77	3.41	4.24		
	105.5	*74.3*	*75.1*	*79.0*	*65.2*			
Fuc-ol F, **1**	3.70	4.06	3.98	3.79	4.13	1.35		
	63.5	*72.1*	*79.7*	*84.6*	*68.2*	*19.7*		
DD-β-glcHep T, **3**	4.44	3.25	3.44	3.48	3.50	4.02	3.65	3.75
	104.4	*73.8*	*76.6*	*71.1*	*77.8*	*72.3*	*62.5*	
β-GlcNAc S, **3**	4.59	3.82	3.76	3.48	3.45	3.75	3.90	
	100.5	*55.6*	*82.8*	*71.1*	*76.6*	*61.7*		
α-Man Z, **3**	5.18	4.07	3.98	3.72	3.82	3.62	3.80	
	92.1	*78.0*	*68.9*	*78.0*	*72.3*	*62.2*		
β-DD-Hep Q, **3**	4.64	4.00	3.60	3.65	3.45	4.03	3.70	3.78
	101.4	*71.5*	*74.1*	*68.1*	*78.4*	*72.3*	*62.5*	
β-Glc* T, LGLA-ox	4.57	3.47	3.65	3.59	3.49	3.73	3.90	
	103.5	*73.4*	*86.9*	*69.4*	*76.3*	*61.3*		
β-GlcNAc S, LGLA-ox	4.95	3.85	3.84	3.56	3.46	3.78	3.93	
	101.4	*55.7*	*83.5*	*69.7*	*76.2*	*61.8*		
β-Man Z, LGLA-ox	4.88	4.40	3.82	3.74	3.54	3.67	3.90	
	102.3	*76.5*	*71.6*	*77.9*	*76.3*	*62.1*		
β-Man* Q, LGLA-ox	4.71	4.23	3.70	3.71	3.46	3.76	3.93	
	101.0	*68.1*	*78.8*	*65.9*	*77.2*	*61.4*		
α-Fuc F, LGLA-ox	5.07	3.83	3.94	3.83	4.30	1.21		
	96.6	*69.0*	*70.5*	*72.9*	*67.9*	*16.1*		
α-Par* B, OS4	4.93	3.84	1.79/2.14		3.91	1.20		
	95.5	*67.9*	*35.4*	*70.5*	*69.8*	*17.3*		
β-Rha C, OS4	4.79	4.23	3.67	3.52	3.46	1.35		
	100.4	*68.9*	*78.7*	*71.4*	*73.3*	*18.0*		
Gro*, OS4	3.71	3.90	3.64/3.73					
	62.6	*81.3*	*61.6*					

LGLA-Hy spectra were obtained at 60° with 5% fully deuterated SDS, LGLA-ox in D_2O at 55°C, oligosaccharide spectra at 25° in D_2O. ^{1}H shifts are in normal font, ^{13}C in italic. Asterisk marked sugars are deuterated at C-6 (Man, Glc), C-4 (Par) or C-1 (Gro)

Analysis of OS3 was complicated by the presence of the reducing end mannose in three forms: α-fluoride, free OH-1, and 1,6-anhydro-β-pyranoside. Fluoride was converted into reducing monosaccharide by heating of NMR sample for 4 h at 110°. This process was monitored by NMR and a long reaction time was indeed necessary to hydrolyze all fluoride. Besides that, DDHep Q was partly missing (NMR data for glycosyl fluoride, 1,6-anhydro derivative and for the oligosaccharides without residue Q are not shown). The structure of the OS3 was determined by NMR (Table 1), monosaccharide and methylation analyses, which were all in agreement with the presented formula.

Longer oligosaccharides, containing components found in the OS3, were also present among HF cleavage products, but no pure compound could be isolated.

In order to obtain further structural information, several attempts were made to deacylate the GlcNAc residue for subsequent deamination. Treatment of LGLA with 4 M

Table 2 NMR data for free aurantose and for the α-aurantose residue B in the LGLA

Compound		1	2	3ax	3eq	4	5	6	7	8a	8b	9	10
Free α (minor)	^{1}H	5.15	4.01	1.70	1.94		4.12	1.09	3.75	1.48	1.51	4.01	1.23
	$J_{n,n+1}$	3.6	$J_{2,3eq}$ 4.9	$J_{2,3ax}$ 12.6	$J_{3ax,3eq}$ 12.9		6.5		10.3			5.9	
	^{13}C	92.6	65.7	31.0		77.2	67.6	13.9	72.5	41.0		65.7	24.3
Free β (major)	^{1}H	4.56	3.67	1.51	2.18		3.84	1.14	3.75	1.48	1.51	4.01	1.22
	$J_{n,n+1}$	8.1	$J_{2,3eq}$ 5.2	$J_{2,3ax}$ 12.8	$J_{3ax,3eq}$ 13.4		6.5		10.3			5.9	
	^{13}C	99.5	69.0	36.3		77.4	75.8	14.2	72.5	41.0		65.7	24.3
LGLA	^{1}H	4.98	4.04	1.79	1.93		4.24	1.06	3.71	1.40	1.50	3.97	1.20
	^{13}C	94.6	65.3	31.0		76.4	67.2	13.0	71.9	40.3		64.9	23.5

KOH at 120°C and even 150°C for 24 h, as well as treatment with anhydrous hydrazine at 120°C for a week resulted in recovery of slightly depolymerized starting compound with *N*-acetyl group fully retained.

Periodate oxidation of the LGLA with subsequent $NaBD_4$ reduction and 2% AcOH hydrolysis gave two main products, a polysaccharide and an oligosaccharide 4. Both compouds were of high purity and their study gave information about the structure of the main chain of the polymer and absolute configuration of the aurantose. NMR analysis of the oxidized polymer (Table 1, Fig. 7) led to the structure presented in Fig. 5. DDHep Q and DDglcHep T were converted to 6-deutero-Man and 6-deutero-Glc due to oxidation-reduction, and showed respectively inverted H/C-6 signals in HSQC-DEPT spectrum. Polymeric chain consisted of the repeating oligosaccharide 3, linked through O-3 of the β-Glc T (former β-DDglcHep). It contained additionally α-Fuc residue, linked to the side-chain Man (former DDHep) Q. This indicated the attachment position of the side-chain pentasaccharide, which was identified by the NMR analysis of the whole LGLA-Hy. The structure of the oxidized polymer was confirmed by methylation analysis, which was in full agreement with the proposed structure.

Analysis of the OS4 showed that it contained 4-C-deutero-paratose (3,6-dideoxy-D-*ribo*-hexopyranose) at the non-reducing end. It was derived from the aurantose by the periodate oxidation of 4,7-diol and subsequent reduction. Reduction should also give C-4-epimeric compound, which probably was formed in minor amount, had less stable glycoside bond and was lost during AcOH hydrolysis. Glycerol at the reducing end was obviously formed from the oxidized Xyl*p*. Because of the absence of H-4 identification of the configuration of Par was based on coupling constants $J_{1,2}$, $J_{2,3ax}$, and ^{13}C NMR shifts. Calculation of the ^{13}C NMR shifts [19] for the α-Par-(1-3)-β-L-Rha unit showed that Par should have D-configuration: for the combination of D-Par with L-Rha calculated values of Par C-1 chemical shift is 95.5 ppm (experimental 96.7 ppm), C-2 and C-3 of Rha 68.3 and 79.5 ppm (experimental 68.9 and 78.7, respectively); calculated signal positions for the combination of L-Par with L-Rha are far from experimental: C-1 of Par 101.6, C-2 of Rha 71.4, C-3 of Rha 82.6 ppm. Identity of D-paratose was confirmed by GLC-MS of alditol acetate and of acetylated (*R*)-2-octyl glycosides. Thus aurantose had D-configuration.

Taken together these results show that LGLA had a structure with a main chain, consisting of polymerized oligosaccharide 3, and branches built up from the B-C-D-(A-)-F oligosaccharide (Fig. 5). Methylation analysis of the LGLA (Fig. 8) was in agreement with this structure but showed additional information. A peak of the terminal fucopyranose was present. This sugar was not identified by any of the above experiments, although one can see some H-5:H-6 correlation in COSY spectrum of LGLA which could belong to Fuc residue. Of the heptose peaks only that of 3-substituted DDHep was identified. There were no standards available to determine the configuration of alditols of disubstituted heptose derivatives. But since 3- and 2,3-substituted heptose peaks were smaller than 3,4-disubstituted one, it is reasonable to suggest that DDHep was partially substituted at position 3 and partially at 2,3. Then β-DDglcHep was 3,4-disubstituted in the polymer. Additional substituents on the heptose residues were α-Xyl*f* and Fuc*p*, whose respective positions remained unknown.

TLR activation and Limulus gel-clot assay for biological activity of LGLA

LGLA demonstrated significantly less endotoxic activity in a LAL gel-clot assay [three endotoxin units (EU)/mg] than that observed for *E. coli* O113 control LPS (1×10^{7} EU/mg). An IL-8 promoter fused to a Firefly luciferase reporter in either HeLa cells transfected with TLR2, or RAW cells, was not activated by LGLA (data not shown). A whole blood activation assay with ELISA to assess TLR-mediated release of TNF-α following stimulation of numerous variant TLRs did not detect any appreciable release of TNF-α. following exposure of the whole blood cells to LGLA (Fig. 9).

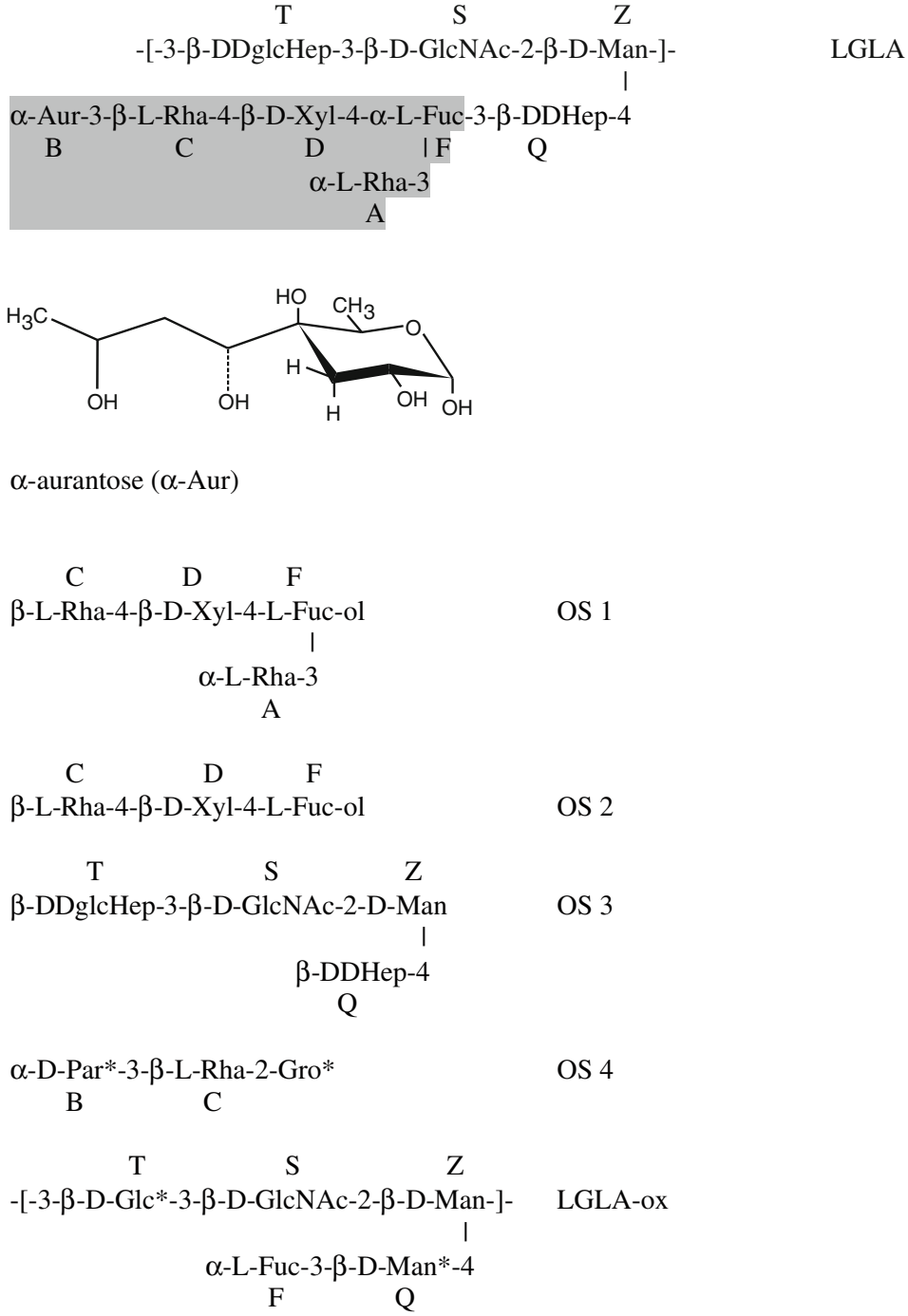

Fig. 5 Partial structure of the LGLA and the structure of the isolated oligosaccharides. *Grey shadow* on LGLA structure indicates the fragment that was inferred from NMR analysis of LGLA-Hy. * indicates deuterium labeled components of the $NaIO_4$ oxidation products

Conclusion

Although *S. aurantia* is considered a Gram negative bacterium, its major glycolipid LGLA possesses neither the LPS structural components of core or lipid A nor the biological properties associated with LPS, such as the ability to stimulate gelation of Limulus amoebocyte lysate, or to act as a TLR agonist. LGLA contained some fatty acids and migrated in SDS-PAGE, which indicates that it is a glycolipid, because polysaccharides without lipid component do not enter the gel. However, the nature of the lipid part remained unknown. Lipid part was lost after periodate oxidation and mild hydrolysis, which indicate the presence of some oxidizable components between the periodate-stable polymeric part and the lipid. Repeating subunits of LGLA contain 11 monosaccharides including the newly

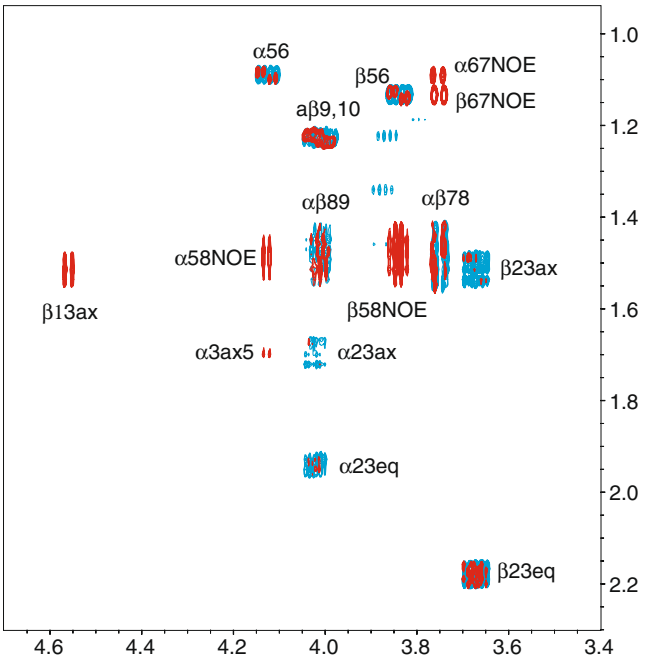

Fig. 6 Fragment of the overlapped COSY (*blue*) and ROESY (*red*) spectra of the free aurantose

identified tetradeoxydecose, aurantose. Analysis of the LGLA did not produced the complete structure of the repeating unit; the exact location of two of the 11 monosaccharides remained unknown. Probably this structure could be completely resolved using genetic modifications, which will remove some components and facilitate NMR analysis. As a whole molecule, LGLA is too densely packed, which leads to signal broadening due to fast relaxation, which prevents detailed analysis of the intact structure by NMR.

Large and extensively branched LGLA differs strikingly from most other known spirochete glycolipids, which

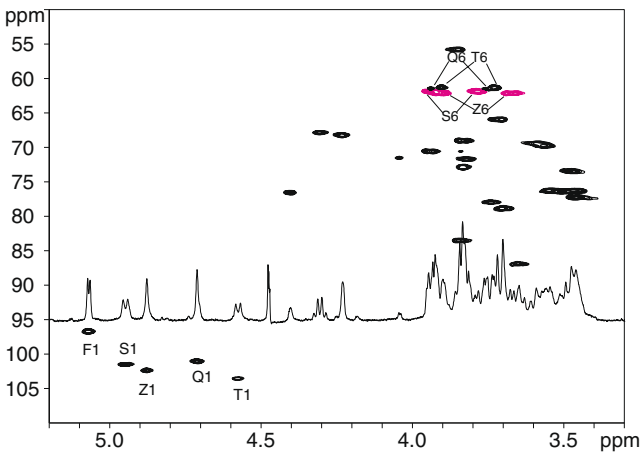

Fig. 7 Fragment of the HSQC spectrum of LGLA-ox polysaccharide (D_2O, 55°C). *Black signals* belong to CH-groups, *red ones* to CH_2-groups. Deuterated H/C-6 of the residues Q and T appear as CH

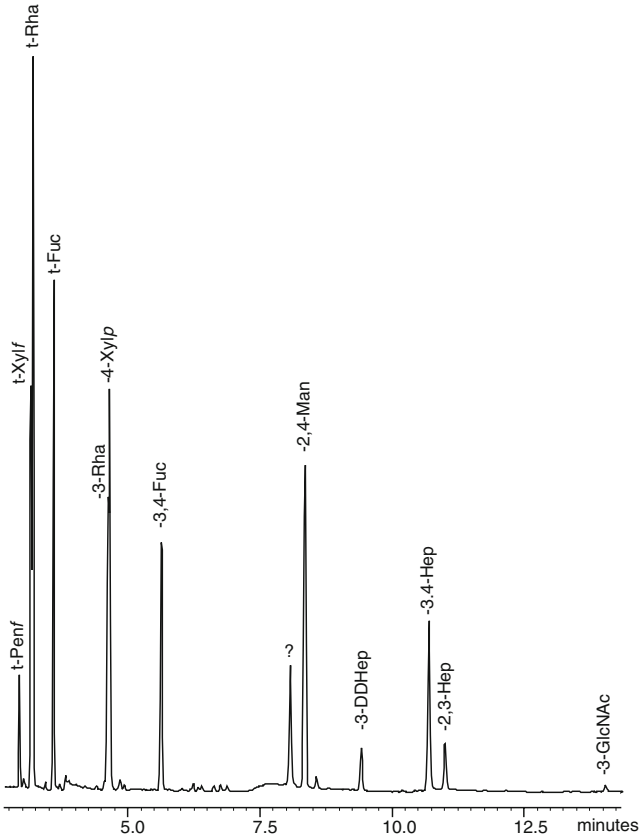

Fig. 8 Chromatogram of the GC-MS analysis of the methylated LGLA

contain only a few monosaccharides [7, 8, 14]. The only other non-LPS large carbohydrate structure reported in spirochetes was a polysaccharide isolated from a glycoconjugate of the oral treponeme, *Treponema medium* [20], which also showed no endotoxin-like biological properties. The glycoconjugate of *T. medium* binds CD14 and LPS binding protein, blocking host cell activation by other periodontopathic bacteria, rather than stimulating the release of IL-8 by host cells [21]. A similar action by LGLA may account for the lack of TNF-α release in the whole blood assay, although the ability of the *S. aurantia* glycolipid to block the activation of host cells by other bacterial pathogens has not been examined.

Genetic modification has not yet been reported for *S. aurantia*. Several genes with similarity to those involved in the biosynthesis of paratose were identified in *S. aurantia* (C.J. Paul, unpublished, Genbank accession number: DQ832182) and could indicate the location of the LGLA biosynthesis genes however the complete genome sequence for *S. aurantia* is not yet available. With the adaptable Leptospiraceae producing LPS and the more host-restricted pathogenic members of the Spirochaetaceae family producing smaller glycolipids of only a few monosaccharides, comparison of the complete genome sequences of the

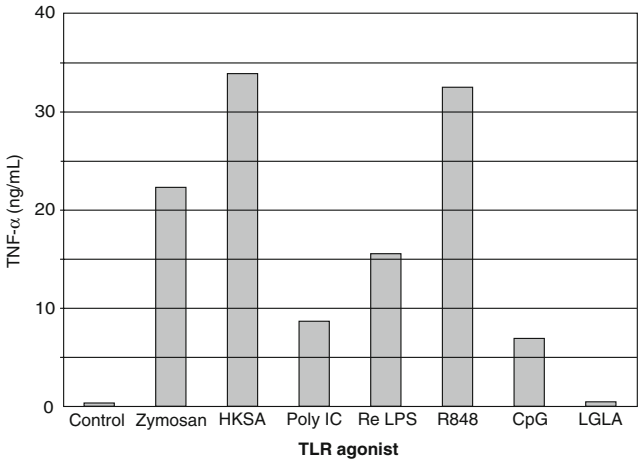

Fig. 9 TLR activation test. LGLA (5 μg/mL) did not cause the release of TNF-α when exposed to whole blood cells in contrast to detection of TNF-α released in the presence of known TLR agonists (TLR2: zymosan (5×10^9 particles/mL) and heat-killed *Staphylococcus aureaus* (2.5×10^6 particles/mL); TLR3: Poly IC (50 μg/mL); TLR4: Re595 LPS (20 ng/mL); TLR7: R848 (1 μg/mL); TLR9; CpG Oligo (2 μM). *Error bars* represent the standard deviation of cellular activation assays performed in triplicate

available spirochete genomes with a complete genome sequence of *S. aurantia* could provide insights into the evolution of the diverse membrane-associated carbohydrate structures in these bacteria.

The LGLA structure is completely different from the previously characterized *S. aurantia* glycolipid, LGLB [14]. LGLA does not contain any charged components and has long chains with repeating units, whereas LGLB has no repetitive elements and is densely charged due to the presence of uronic acids and free amino groups.

Experimental section

Bacterial strains and culture conditions S. aurantia strain M1 (E.P. Greenberg, Ohio State University, Columbus, OH) was propagated in spirochete growth media (SGM) containing 0.4% maltose (wt/vol; Sigma-Aldrich), 0.2% tryptone and 0.2% yeast extract (Difco) at pH 7.5. Cells were grown at 30°C with gentle aeration (30 rpm; orbital shaker, Forma Scientific) for 24 or 48 h. Cell stocks were maintained in SGM media in liquid nitrogen. *E. coli* DH5α were propagated in selective LB with ampicillin when required, with all stocks maintained in DMSO at −70°C.

Isolation of LGL from S. aurantia Bacteria were harvested from a total of 55 L of SGM and extracted following the method of Darveau and Hancock [15]. The final product was extracted once with cold 95% ethanol and twice with chloroform:methanol (2:1; vol/vol) to remove phospholipids and carotenoids, dissolved in dH20 and digested with pronase (25 μg/mL, 18 h at 37°C). A final extraction by chloroform:methanol (2:1), was followed by dialysis against water using Slide-a-lyzer® 10K cassettes (Pierce Chemical Company), and lyophilization.

Denaturing gel electrophoresis (SDS-PAGE) SDS-PAGE 8% or 15% resolving gel were used with 4.5% stacking gel was conducted as described elsewhere [22]. Low molecular weight LPS and LGL were resolved by Tricine SDS-PAGE with a 15% resolving gel 10% spacer gel and 4.5% stacking gel [23]. LPS from *Salmonella typhimurium* was purchased from Sigma Aldrich. Products in acrylamide gels were visualized by silver staining [24].

Immuno- and lectin blotting SDS-PAGE gels were transferred following standard protocols [22]. Membranes were incubated overnight in a 1:1,000 dilution of chicken LGLA polyclonal antibody (RCH Antibodies, Kingston, ON, Canada) in 3% skim milk in 0.1% Tween 20 phosphate buffered saline (PBS-T), washed three times in PBS-T, incubated with horse radish peroxidase (HRP)-conjugated donkey anti-chicken antibody (Sigma) diluted in 3% skim milk in PBS-T to 1:20,000 for 1 h, washed and developed (see below). Membranes for Western blots using the MAb 5c-177 [25], were boiled for 2.5 h in 10% (vol/vol) acetic acid, following transfer, to remove polysaccharides, blocked with 5% skim milk in PBS-T and incubated overnight with MAb 5c-177 hybridoma culture supernatant. Following PBS washes, the membrane was incubated with a 1:20,000 dilution of anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 h. For lectin blotting, SDS-PAGE and transfer were as described above with blocking in 0.1% Tween 20 Tris-buffered saline (TBS-T; 50 MM Tris, pH 7.5, 0.15 M NaCl). Membranes were incubated with 5 μg/mL biotinylated *Maackia amurensis* lectin II (MALII, Vector Laboratories) for 1 h, washed three times in TBS-T with detection by HRP-conjugated streptavidin (Vector Laboratories). All Western blots and lectin blots were developed by colorimetric detection of HRP by 1-Step™ TMB-Blotting (Pierce Chemical Company).

Assays for biological activity of LGLA Limulus amebocyte lysate (LAL) assay for LGLA was conducted by Associates of Cape Cod, Inc. (MA, U.S.A.). To measure activation of any TLR receptor, the production of TNF-α by whole blood cells, in response to a panel of TLR agonists, was measured as described previously [14, 26]. Agonists examined were: *S. aurantia* LGLA (5 μg/mL); Zymosan (5×109 particles/mL, Molecular Probes); heat-killed *Staphylococcus aureus* (HKSA) (2.5×106 particles/mL; Molecular Probes); Poly IC (50 μg/mL, Sigma Genosys); Re595

LPS (20 ng/mL; R848 (1 μg/mL, Invitrogen); and, CpG Oligo (2 μM; Sigma Genosys)

Electron microscopy Freeze substitution was performed as described previously, with some modifications [27, 28]. The pellet from a 100 mL overnight culture of *S. aurantia* was resuspended in 750 μL SGM media injected into a thin piece of copper tubing. Sections of tubing were then frozen in a Leica EMpact high pressure freezer (Leica Microsystems, Wetzlar, Germany) at −196°C. Tubing sections were removed and cut end to end, while remaining chilled in liquid nitrogen, then immersed substitution medium [2% (wt/vol) osmium tetroxide (OsO_4; BCN Chemicals Inc., Beaconsfield, Quebec, Canada) and 2% (wt/vol) uranyl acetate in anhydrous acetone]. Samples were incubated for 72 h at −80°C for substitution, then allowed to reach room temperature, washed six times for 15 min each in anhydrous acetone, and infiltrated overnight at room temperature in acetone-Epon 812 (Can EM, Guelph, Ontario, Canada; 1:1) or acetone-Spurr's resin (Marivac Ltd., Halifax, Nova Scotia, Canada; 1:1). Samples were embedded in either fresh Epon 812 or Spurr's resin and polymerized at 60°C for 36 or 24 h, respectively, then thin-sectioned on a Reichert Jung Ultracut ultramicrotome, and mounted on Formvar carbon-coated copper grids. Sections were poststained with 2% (wt/vol) aqueous uranyl acetate and lead citrate. Electron microscopy was performed with a Philips EM300 operating at 60 kV under standard conditions with a liquid nitrogen cold trap in place.

Column chromatography Crude LGL (1.5 g) was dissolved in sample buffer (20 mM Tris–HCl, pH 8; 50 mM EDTA; 10% SDS) and fractionated on a 5.5×40 cm column of Sephacryl S-300 HR (Sigma-Aldrich) at room temperature (column buffer: 10 mM Tris–HCl, pH 8; 10 mM EDTA; 0.2M NaCl; 0.3% SDS). Fractions of ~2.1 mL were collected at an average flow rate of 1.5 mL/min. The character of the eluted material was assessed by determining total carbohydrate and hexosamine [29, 30] content and by Tricine–SDS PAGE (see above). Fractions containing molecules with similar molecular weight, and carbohydrate and hexosamine content, were pooled, precipitated with cold 0.375M $MgCl_2$ in 95% ethanol, suspended in distilled water and subjected to a second chromatography to ensure homogeneity of the final distinct fractions. Material was then re-precipitated, suspended in distilled water, dialyzed, lyophilised and weighed.

Structural characterization of glycolipid Kdo and fatty acid analysis LGLA was assessed for the presence of Kdo [31]. Identification of fatty acids was done as previously described [14].

Monosaccharide analysis LGLA (0.5 mg) was hydrolyzed (0.2 mL of 3 M TFA, 120°C, 2 h), followed by evaporation 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 GLC on a HP1 capillary column (30 m×0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient from 170°C to 260°C at 4°C/min and by GC-MS on Varian Saturn 2000 ion-trap instrument on the same column. For the identification of paratose in the OS4 monosaccharides were analysed as acetylated *O*-methyl glycoside derivatives as reported [32]. The absolute configuration was determined by analysis of the acetylated chiral 2-octylglycosides using synthetic D-paratose as a standard [33].

NMR spectroscopy NMR spectra were recorded at 25°C, 55°C or 60°C in D_2O on Varian UNITY INOVA 600 instrument, using acetone as a reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, NOESY (mixing time of 200 ms), TOCSY (spinlock time of 120 ms), HSQC, and gHMBC (long-range transfer delay of 100 ms) were used.

Gel chromatography Gel chromatography 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.

Preparation of aurantose LGLA (25 mg) was hydrolyzed by 2% AcOH (2 mL, 3 h, 100°C), precipitate removed by centrifugation at 12000 rpm, soluble products separated by gel chromatography on a Sephadex G50 column. Monosaccharide fraction was separated by paper chromatography on Whatman 4 paper in pyridine–butanol–water (3:2:1). Paper was cut in 6 strips, substances eluted by water and analyzed by NMR.

O-deacylation of LGLA LGLA (50 mg) was dissolved in anhydrous hydrazine, kept 1 h at 50°C, cooled, poured in cold acetone (50 mL), precipitate collected by centrifugation, washed with acetone, dissolved in water and lyophilized. The product was separated by anion-exchange chromatography on a Hitrap Q column (Pharmacia) in water for 20 min, then in linear gradient of 0 to 1 M NaCl over 60 min. with UV detection at 220 nm. Fractions were desalted by gel chromatography on Sephadex G-15 column.

Partial acid hydrolysis LGLA (50 mg) was hydrolyzed with 0.5 M TFA (100°, 1 h), freeze-dried, separated on Sephadex G-15 to give three oligosaccharide and a monosaccharide fractions. Oligosaccharides were further separated on C18 reverse phase column (Phenomenex Aqua, 9×250 mm) in water to give large number of fractions analyzed by NMR. Major oligosaccharides were studied in detail to identify reducing end monosaccharides, then reduced and repurified to obtain cleaner compounds; among them oligosaccharides 1 and 2; several disaccharides were also obtained.

HF treatment LGLA (60 mg) was treated by anhydrous HF (10 mL) at −15°C (ice-salt bath) for 30 min. Reaction was poured onto wide plastic Petri dish for rapid evaporation, dissolved in water and residual HF neutralized by $CaCO_3$. The products were separated by gel chromatography on Sephadex G-50 column and analyzed by NMR.

Periodate oxidation LGLA (40 mg) was treated with 4 mL of 0.02 M $NaIO_4$ for 24 h in the dark, excess of periodate was destroyed by ethylene glycol (0.2 mL), the polymer was desalted by gel chromatography on Sephadex G-15 column, reduced with excess of $NaBD_4$, desalted, and hydrolysed with 2% AcOH (100°C, 3 h). LGLA-ox and OS4 were isolated by gel chromatography on Sephadex G-15 column.

Methylation analysis $NaBH_4$ reduced oligosaccharide 3 (0.5 mg) was dissolved in anhydrous DMSO (1 mL), dry powdered NaOH (~50 mg) was added, mixture stirred for 1 h, MeI (0.3 mL) was added, stirred for 30 min, excess MeI removed by air blow, remainder diluted with water (5 mL), extracted with $CHCl_3$ (5 mL). Organic layer was washed 3 times with water, dried, methylated product converted into alditol acetates as described for monosaccharide analysis using $NaBD_4$ and analyzed by GC-MS.

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