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Structural Analysis of the Deep Rough Lipopolysaccharide from Gram Negative Bacterium *Alteromonas macleodii* ATCC 27126^T: The First Finding of β-Kdo in the Inner Core of Lipopolysaccharides

Valeria Liparoti,[a] Antonio Molinaro,*[a] Luisa Sturiale,[b] Domenico Garozzo,[b] Evgeny L. Nazarenko,[c] Raisa P. Gorshkova,[c] Elena P. Ivanova,[d] Luidmila S. Shevcenko,[c] Rosa Lanzetta,[a] and Michelangelo Parrilli[a]

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Alteromonas macleodii ATCC 27126^T is a Gram-negative marine bacterium isolated from a sea water sample collected from around the Hawaiian Islands. The structure of the lipooligosaccharide derived from its outer membrane has been fully determined using either alkaline or acid hydrolysis. Alkaline treatment, aimed at recovering the complete carbohydrate backbone, was carried out by mild hydrazinolysis (de-*O*-acylation) followed by de-*N*-acylation using hot KOH and furnished a single core glycoform. Mild acid hydrolysis was employed to obtain the lipid A moiety which was selectively de-*O*-acylated and analysed to determine its primary structure. The structural elucidation of both fractions was carried out by chemical analyses, 2D NMR spectroscopy and MALDI-TOF mass spectrometry and revealed a novel lipooligosaccharide with an unusual structure.

Introduction

Many microorganisms isolated from aquatic environments are heterotrophic bacteria that can be divided into two subgroups depending on their capacity to ferment carbohydrates. Within the nonfermentative group is the genus *Alteromonas* belonging to the family *Alteromonadaceae*. The genus *Alteromonas* was established by Baumann et al.[1] for marine Gram-negative bacteria with a single polar flagellum and an oxidative metabolism. Later, on the basis of phylogenetic comparisons, the taxonomic structure of the genus *Alteromonas* was revised. This revision led to the partition of the genus *Alteromonas* into two genera, *Pseudoalteromonas* and *Alteromonas*,[2] which so far include one validly described species, *A. macleodii*, and two subspecies, the taxonomic status of which has not yet been validated. Whilst this type strain of the species has been isolated from surface sea water, few other strains have been isolated from extreme environments, for example, deep-sea hydrothermal vents where bacteria survive harsh conditions: high pressures, high temperature gradient and high concentrations of toxic elements.

Like the majority of Gram-negative bacteria, *A. macleodii* possesses lipopolysaccharides (LPSs) in the outer leaflet of the external cellular membrane. LPSs are crucial amphiphilic constituents of the outer membrane of the Gram-negative bacterial cell wall.[3] Depending on the size of the saccharide portion, there exist two different types of lipopolysaccharides: smooth (S-LPSs) and rough (R-LPSs) forms. S-LPSs are typically composed of three structurally and biogenetically different regions:[4] a glycolipid moiety (lipid A), a saccharide portion composed of up to 15 monosaccharides (core region)[4,5] and a polysaccharide (O-specific chain). R-LPSs do not possess a polysaccharide part and have been found in wild-type strains and in mutant strains that harbour mutations in the genes encoding the enzymes for the biosynthesis and/or the transfer of the O-specific polysaccharide. The only structural element that is present in all LPSs is the 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo) in an α configuration, a residue that links the core oligosaccharide to the lipid A.

Lipopolysaccharides from marine bacteria, living in extreme conditions, frequently show peculiar chemical features as a consequence of their adaptation to their environment. In this work we performed the complete structural elucidation of the R-LPS of the type strain of *A. macleodii* ATCC 27126^T.

[a] Dipartimento di Chimica Organica e Biochimica, Università degli studi di Napoli "Federico II",
Via Cinthia, 80126 Napoli, Italy
Fax: +39-081-674393
E-mail: molinaro@unina.it
[b] Istituto di Chimica e Tecnologia dei Polimeri, ICTP – CNR, Viale R. Margherita 6, I-95123 Catania, Italy
[c] Pacific Institute of Bioorganic Chemistry, Far-East Branch of the Russian Academy of Sciences,
690022 Vladivostok 22, Russian Federation
[d] Swinburne University of Technology,
P. O. Box 218, Hawthorn, Vic 3122, Australia

Results and Discussion

Compositional Analysis

The dried cells of *A. macleodii* were extracted by the phenol/water method.[6] The lipooligosaccharide fraction was found exclusively in the water phase and was purified by gel-permeation chromatography. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) showed, after silver nitrate gel staining, a typical migration pattern to the bottom of the gel, in accordance with the low molecular weight of the R-LPS form. The monosaccharide compositional analysis of R-LPS showed the presence of L-*glycero*-D-*manno*-heptose (L,D-Hep), 2-amino-2-deoxy-D-glucose (D-GlcN) and Kdo. Methylation analysis of the dephosphorylated R-LPS fraction showed the presence of 6-substituted GlcN, 7-substituted Hep, 5-substituted Kdo and terminal Kdo. Fatty acid analysis revealed the presence of (*R*)-3-hydroxydodecanoic acid [C12:0 (3-OH)], (*R*)-3-hydroxyundecanoic acid [C11:0 (3-OH)] both in amide and ester linkages, and dodecanoic acid (C12:0) exclusively in an ester linkage, as the major components. C10:0 (3-OH) and C13:0 (3-OH) were also found in minor amounts.

Primary Structure Determination of the Oligosaccharide Obtained by Alkaline Degradation

Hydrazinolysis and hot KOH treatment of the lipooligosaccharide fraction produced an oligosaccharide which was subjected to full 2D NMR analysis in order to establish its primary structure. The ^{1}H NMR spectrum (Figure 1) showed three anomeric signals, corresponding to three different spin systems, denoted as **A**, **B** and **C**, according to their decreasing chemical shift values. Anomeric configurations were assigned on the basis of $^1J_{C\text{-}1,1\text{-}H}$ values measured in a coupled HSQC experiment, whereas the relative configurations were established on the basis of the chemical shifts and the $^3J_{H,H}$ values obtained from the DQF-COSY spectrum. All monosaccharide residues were found to be present as pyranose rings on the basis of either the ^{13}C chemical shift values or to the occurrence of a long-range correlation between C-1/1-H and 5-H/C-5 in the ^{1}H,^{13}C HMBC spectrum (for Kdo residues between C-2 and 6-H). Complete assignment of the ^{1}H and ^{13}C resonances was achieved (Table 1) by carrying out DQ-COSY, TOCSY, ROESY, HSQC and HMBC experiments, whereas ^{31}P NMR spectroscopy allowed phosphate substitutions to be detected. The signal from the 1-H atom of spin system **A** was present as a double doublet with $^3J_{1\text{-}H,2\text{-}H}$ = 3.1 Hz and $^3J_{1\text{-}H,P}$ = 7.6 Hz. *O*-Phosphorylation at the anomeric position was confirmed by the presence of a cross peak at 5.70/0.47 ppm in the ^{1}H,^{31}P HSQC spectrum. On the basis of the $^3J_{H,H}$ values, characteristic of an α-*gluco* configuration, and the resonance of C-2 at δ = 54.2 ppm, correlating with a nitrogen-bearing carbon atom, it was possible to identify **A** as a 2-deoxy-2-amino residue, the α-GlcN (GlcN I) of the lipid A backbone. The anomeric signal of the **B** spin system was present as a singlet with $^3J_{1\text{-}H,2\text{-}H} < 2$ Hz. From the 2-H atom of the residue it was possible to identify all the other resonances of the ring protons in the TOCSY spectrum up to the 7-H proton signals, leading to the identification of this spin system as an α-heptose ($J_{1\text{-}H,C\text{-}1}$ = 174 Hz). The **C** residue is present in a β-*gluco* configuration according to the $^3J_{1\text{-}H,2\text{-}H}$ and $^1J_{1\text{-}H,C\text{-}1}$ values and in fact, in the ROESY experiment, intra-residue NOE connectivity from 1-H to 3-H and to 5-H was observed. Thus the **C** residue was recognised as the GlcN II of the lipid A backbone since in the HSQC spectrum 2-H was correlated with a nitrogen-bearing carbon signal at δ = 55.6 ppm. The characteristic diastereotopic methylene signals of two Kdo residues were present in the high-field region of the ^{1}H NMR spectrum at 1.84 (3-H$_{ax}$) and 2.44 (3-H$_{eq}$) ppm (residue **D**) and 2.02 (3-H$_{ax}$) and 2.22 (3-H$_{eq}$) ppm (residue **E**). On the basis of the chemical shifts of the 3-H proton signals,[7,8] it was possible to assign a β configuration to Kdo **D** and an α configuration to Kdo **E**. This latter spin system was also characterised by a visible downfield shift of the 4-H signal, typically due to phosphorylation at the O-4 position (see below). The ^{13}C chemical shifts could be assigned by carrying out an HSQC experiment using the interpreted ^{1}H NMR spectrum. Three anomeric signals, numerous ring carbon signals and two nitrogen-bearing carbon signals (lipid A skeleton) were present. In addition, at high fields the two methylene carbon signals of the Kdo units were found. By comparison with the ^{13}C chemical shifts of nonsubstituted residues,[9] low-field-shifted signals indicated substitutions at the O-6 residues of **A** and **C**, O-7 of **B** and O-5 of **E**.

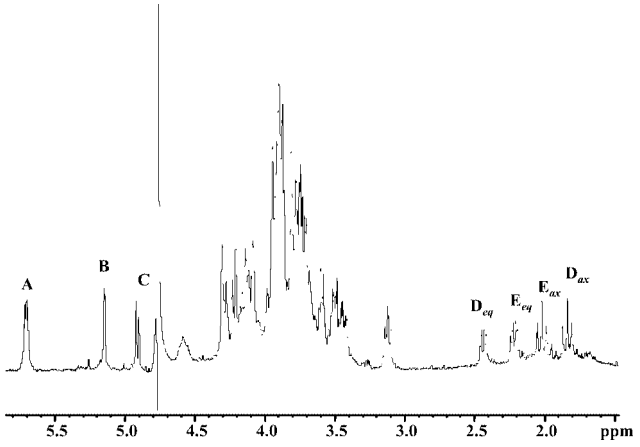

Figure 1. ^{1}H NMR spectrum of *A. macleodii* oligosaccharide obtained after strong alkaline treatment of the LPS. The anomeric signals of the spin systems are as designated in Table 1.

The sequence of the monosaccharide residues was obtained from the NOE data of the ROESY spectrum (not shown). A typical lipid A carbohydrate backbone was revealed by an inter-residual NOE contact between 1-H of **C** and 6a,b-H of **A**. The Kdo unit **E** is substituted by heptose **B** as indicated by the NOE effect found between 1-H of **B** and 5-H and 7-H of **E** and, in addition, between 5-H of **B** and 3-H$_{ax}$ of **E**. The above NOE contacts found in the ROESY spectrum between the **B** and **E** residues are only

Table 1. ^{1}H, ^{13}C (bold) and ^{31}P (italic) NMR chemical shifts (δ) of the *Alteromonas macleodii* oligosaccharide obtained by alkaline degradation.[a]

	Chemical shift, δ ($^{1}H/^{13}C/^{13}P$) [ppm]							
Unit	1	2	3	4	5	6	7	8
A	5.704	3.432	3.908	3.488	4.143	3.874/4.289		
6-α-GlcN	**91.4**	**54.2**	**69.7**	**69.9**	**72.7**	**69.3**		
	0.47							
B	5.147	4.086	3.888	3.736	4.222	4.218	3.592/3.974	
7-α-Hep	**100.4**	**70.2**	**70.7**	**66.9**	**73.3**	**70.2**	**64.7**	
C	4.914	3.121	3.889	3.884	3.772	3.513/3.729		
6-β-GlcN	**99.2**	**55.6**	**74.4**	**71.8**	**74.0**	**62.4**		
				1.26				
D			2.44/1.84	3.763	3.949	3.596	3.934	3.766/3.884
t-β-Kdo			**34.5**	**67.7**	**65.6**	**73.5**	**69.2**	**64.2**
E			2.22/2.02	3.894	4.308	3.814	3.812	3.693/3.932
5-α-Kdo			**34.2**	**70.1**	**72.4**	**71.9**	**69.4**	**63.6**
				1.27				

[a] NMR experiments were carried out at 25 °C and calibrated with respect to internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm). 85% Phosphoric acid was used as external reference (δ = 0.00 ppm) for ^{31}P NMR spectroscopy.

possible when both residues have identical absolute configurations.[10] GLC analysis showed the presence of the L-*glycero*-D-*manno*-heptose residue **B**; thus, Kdo **E** possesses a D configuration and, therefore, the characteristic sequence of the inner core of LPSs composed of L,D-heptose-(1→5)-α-D-Kdo was confirmed. The terminal β-Kdo (unit **D**) is attached to O-7 of the heptose, as verified by HMBC correlation between C-2 of this residue and 7-H of **B** and confirmed by the downfield shift of O-7 of **B** which experienced a mild glycosylation effect, consistent with substitution by a ketose residue. The substitution pattern and the sequence of the residues are in accord with the methylation data.

The ^{31}P NMR spectrum showed the presence of three monophosphate monoester signals (Table 1). The site of substitution was deduced from the $^{1}H,^{31}P$-HSQC spectrum, which showed correlations of ^{31}P signals with 1-H of **A** (GlcN I), 4-H of **C** (GlcN II) and 4-H of **E** (α-Kdo). Thus, the primary structure of the fully deacylated oligosaccharide backbone of the lipooligosaccharide from *A. macleodii* was identified as follows, with the GlcN disaccharide being substituted by a core trisaccharide with two Kdo residues:

D B E C A

β-Kdo-(2→7)-α-**Hep**-(1→5)-α-**Kdo4*P***-(2→6)-β-**GlcN4*P***-(1→6)-α-**GlcN1*P***

A MALDI-TOF mass spectrum (not shown) of the oligosaccharide confirmed the above structural hypothesis. In fact, the spectrum showed an ion peak at *m/z* = 1212.1 built up of two HexN, one Hep, two Kdo and three phosphate groups.

The Non-Carbohydrate Substituents of the Lipid A from *A. macleodii*: Fatty Acid and Polar Head Substitution

With all the above information on the saccharide portion, it was possible to study the MALDI-TOF mass spectrum (Figure 2a) of the intact lipooligosaccharide. It could be divided into two subspectra (Figure 2b,c). At high molecular masses (Figure 2b), in the range 1800–2400 Da, four series of ion peaks were present. The peak at *m/z* = 2186.0 (average mass) is consistent with the molecular ion $[M - H]^-$, corresponding to the expected core trisaccharide fragment (Kdo_2Hep) linked to a pentaacylated lipid A carrying four C12:O (3-OH) residues as primary fatty acids and one C12:O as a secondary fatty acid, whereas the ion at *m/z* = 1987.9 was assigned to the tetraacylated species ($\Delta m/z$ = 198) and the ion at *m/z* = 1790.4 to the triacylated species. Note that each species has an ion fragmentation pattern in which all peaks differ by 14 Da, thus denoting a high heterogeneity of the fatty acids. The fourth group of peaks correlates with the presence of a linked 2-aminoethylphosphate (PEtN, $\Delta m/z$ = 123). At lower molecular masses (Figure 2c) it was possible to detect fragments arising from bond cleavage between Kdo and lipid A, originating in an in-source β elimination[11] of the Kdo-containing oligosaccharide ions. In particular, the ion at *m/z* = 1276.3 was identified as a tetraacylated lipid A carrying one C12:O (3-OH) residue in an ester linkage and two C12:O (3-OH) in amide linkages as primary fatty acids and one C12:O residue as a secondary fatty acid. The ion at *m/z* = 1474.6 is consistent with a pentaacylated lipid A bearing an additional primary C12:O (3-OH) residue. All the other peaks, differing by 14 Da, arise from the high heterogeneity of the primary fatty acids, which is also reflected in the heterogeneity of the molecular species. Further analysis of the fragment ions allowed us to establish that the PEtN residue is linked to the core oligosaccharide. In fact, in addition to the ion at 711.2 Da, attributable to the core oligosaccharide (Figure 2a), another peak was present at 834.6 Da consistent with the same skeleton and bearing an additional PEtN residue.

Phosphate substitution of the core oligosaccharide moiety was definitely inferred by ^{31}P NMR spectroscopy carried out in denaturing conditions on the intact lipooligosaccharide: a typical pyrophosphate signal at 9.5 ppm was visible in the ^{31}P NMR spectrum. In this case, pyrophosphate existence is only possible if PEtN is linked to the phosphate substituting the Kdo unit at O-4.

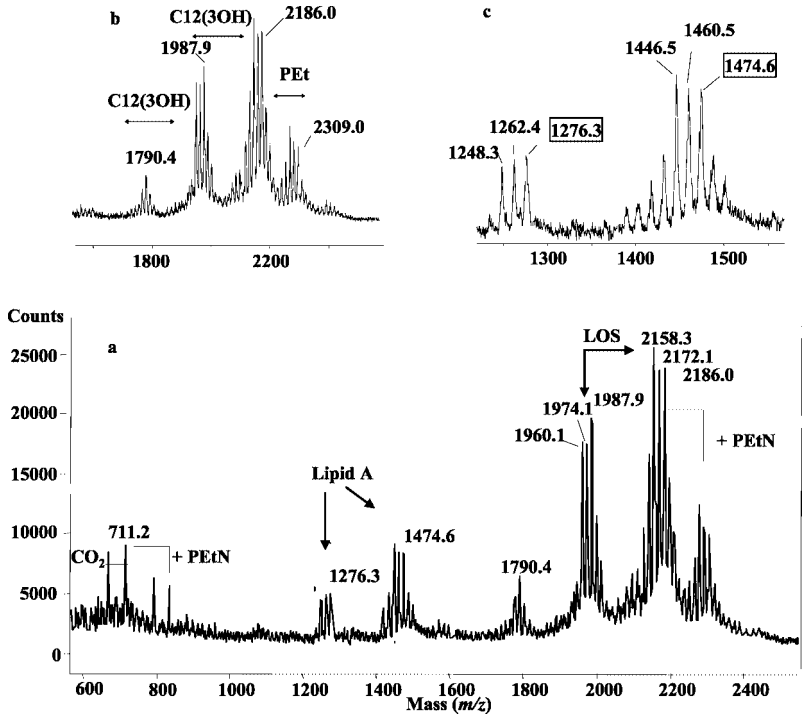

Figure 2. a) Negative-ion MALDI-TOF mass spectrum of the native lipooligosaccharide fraction in which the fragments from lipid A and from the core are visible, b) expansion of the molecular ions mass range and c) expansion of the lipid A mass range.

At this stage, most of the structural elucidation of the LPS from *A. macleodii* was complete and only the secondary fatty acid location was still to be assigned. Thus, lipid A was obtained from the lipooligosaccharide fraction by mild acetic acid hydrolysis, exploiting the labile ketoside linkage of Kdo with β-GlcN, and was further selectively de-*O*-acylated with 12% NH_4OH and then analysed by MALDI-TOF mass spectrometry. This soft chemical approach leaves the amide-bound acyloxyacyl moieties unaltered, if present,[12] and allows the location of secondary fatty acids to be determined. The spectrum (Figure 3a), recorded with a negative polarity, contained a main ion peak centred at *m*/*z* = 1077.8, attributable to the molecular ion $[M - H]^-$ related to a triacylated bis-phosphorylated lipid A species with two amide-linked C12:O (3-OH) residues, one of which bears an additional secondary C12:O residue. The other ion peaks were separated by 14 Da owing to the presence of fatty acids of different lengths, while the ion at *m*/*z* = 997.8 was assigned to the same lipid A backbone lacking the phosphate residue, likely lost during the above acid hydrolysis. This analysis has allowed us to establish that the secondary fatty acid, always present in the lipid A, is exclusively linked through an acyloxyacyl amide moiety. In order to define the location of this acyloxyacyl moiety on the disaccharide backbone, the positive-ion mode MALDI-TOF spectrum was analysed. The spectrum contained several pseudomolecular ions $[M + Na]^+$, clearly in agreement with the expected molecular structure of the lipid A. In the lower mass range (Figure 3b), the peak at *m*/*z* = 622.6 was assigned to the oxonium ion originating from the cleavage of the glycoside linkage of the nonreducing GlcN residue in lipid A. This last structure, in particular, was identified as the diacylated GlcN II, carrying a C12:O (3-OH) and a C12:O residue, and testified to the presence of the secondary fatty acid exclusively linked to the primary amide bound fatty acid in GlcN II. Oxonium fragments, related to the presence of fatty acids of different lengths (±14 Da), were also visible.

Thus, the complete structure (Figure 4) of the lipooligosaccharide from *A. macleodii* strain ATCC 27126^T has been established.

The genus *Alteromonas* includes Gram-negative heterotrophic bacteria associated mainly with severe marine habitats. In particular, *Alteromonas* shows competitive advantages in nutrient acquisition and colonisation for its algicidal activities against phytoplankton.[13] It is clear that the outer membrane is involved in this process. Thus we have studied and completely characterised the major component, the short-chain LPS produced from *Alteromonas macleodii* strain ATCC 27126^T. From a chemical point of view, this is the first time that an *Alteromonas* LPS has been isolated and structurally analysed. *Alteromonas macleodii* produces a native deep-rough lipooligosaccharide with a trisaccharide inner-core region composed of a phosphorylated Kdo residue substituted by a Hep residue and unusually ends with a terminal β-Kdo. A Kdo residue with a β-anomeric configuration is rather rare in polysaccharides in general, is sometimes found in polysaccharide capsules[14] and is very rare in LPSs. In the O chain of the LPS from *Serratia marcescens*, β-Kdo is present as a single residue at the end of the chain functioning as a monosaccharide cap in the regulation of the length of the molecule.[15] It is unclear whether

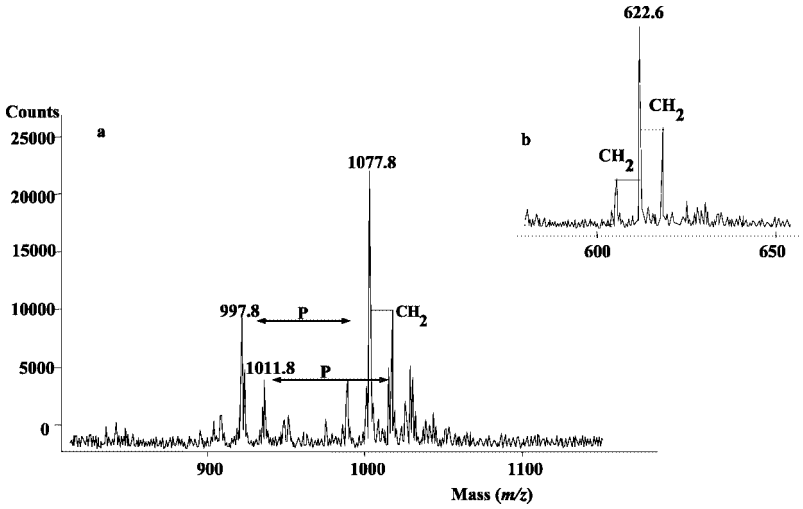

Figure 3. a) Negative-ion MALDI-TOF mass spectrum of the ammonium hydroxide treated lipid A fraction of *Alteromonas macleodii* and b) positive-ion MALDI-TOF mass spectrum of the ammonium hydroxide treated lipid A (oxonium ion mass range).

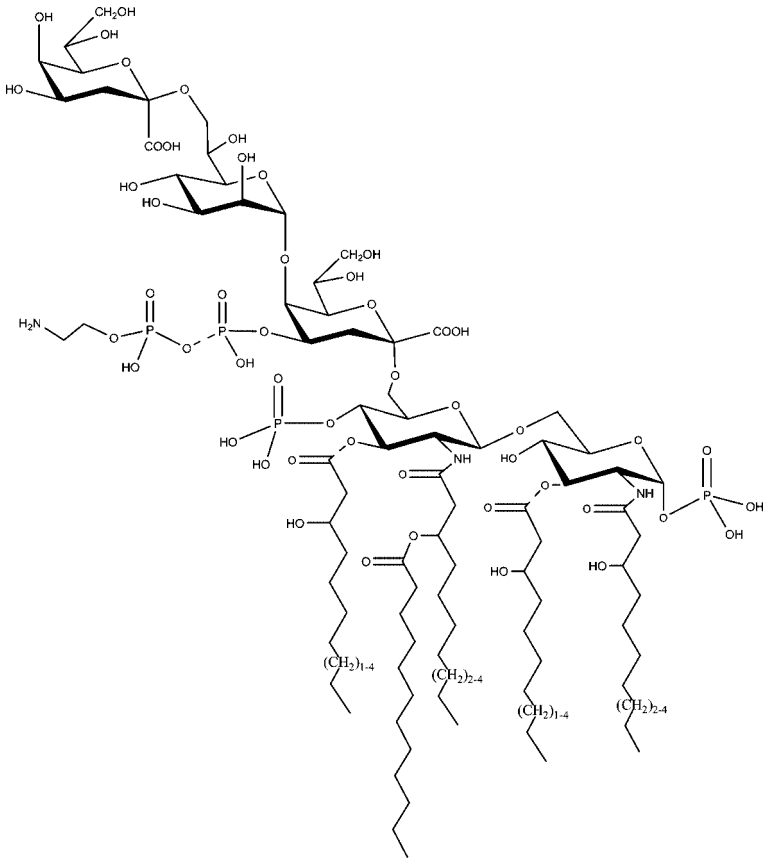

Figure 4. The complete structure of the lipooligosaccharide of *A. macleodii*. Dotted bonds indicate non-stoichiometric substitutions.

this residue is attached by a multifunctional Kdo transferase or whether two different enzymes are involved in the transfer of the two Kdo residues. Surely this second residue of Kdo and the phosphate residues contribute equally to enhance the density of the negative charge on this small lipooligosaccharide. It is generally believed that these negatively charged groups allow ionic bridges to be established between LPS molecules through electrostatic interactions with bivalent cations (Ca^{2+}, Mg^{2+}), contributing to the rigidity and stability of the Gram-negative cell wall.[16,17] Thus, the high number of negative charges together with a very short saccharide chain could be important for supporting the integrity of the outer membrane exposed to a extreme environment.

Experimental Section

Bacteria and Bacterial LPSs: The type strain of *Alteromonas macleodii* ATCC 27126^T was cultivated on a liquid medium containing glucose (1 g L^{-1}), pepton (5 g L^{-1}), yeast extract (2.5 g L^{-1}), K_2HPO_4 (0.2 g L^{-1}), $MgSO_4$ (0.05 g L^{-1}), sea water (750 mL) and distilled water (250 mL). Cells were collected by centrifugation, washed with water and next dried with acetone (three times) to yield around 12 g of dried cells from 20 L of the cultural fluid. Dried cells were extracted three times with a mixture of aq. 90% phenol/water (1:1 v/v) as described previously.[18] The water phase was purified on a Sephadex S-500 column (120 × 1.5 cm; Pharmacia). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) was performed. The gel was stained with silver nitrate for detection of LPSs.[19]

Isolation of Oligosaccharide and Lipid A: An aliquot of LPS (20 mg) was dissolved in anhydrous hydrazine (1 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (20 mL) and allowed to precipitate. The precipitate was then centrifuged (3000 *g*, 30 min), washed twice with ice-cold acetone, dried and then dissolved in water and lyophilised (15 mg, 80% of LPS). A portion of this material (10 mg) was subsequently de-*N*-acylated with 4 M KOH (120 °C, 16 h) as described before.[20] After desalting on a Sephadex G-10 column (50 × 1.5 cm; Pharmacia), the resulting oligosaccharide fraction represented the carbohydrate backbone of the lipooligosaccharide (5 mg).

Free lipid A was obtained by hydrolysis of the lipooligosaccharide (10 mg) with 1% acetic acid (100 °C, 2 h. Mild de-*O*-acylation of lipid A was performed by treatment (200 μg) with 32% ammonium hydroxide (200 μL, 20 °C, 16 h).[12]

General and Analytical Methods: Monosaccharide analysis was carried out by GLC analysis of their *O*-methyl glycoside derivatives, whereas the absolute configuration of the glucosamine and heptose residues was assigned by GLC analysis of their 2-(+)-*O*-octyl glycol side derivatives.

Methylation analysis was carried out on a dephosphorylated sample obtained with 48% HF (4 °C, 48 h). For methylation analysis of the Kdo region, the lipooligosaccharide was carboxy-methylated with methanolic HCl (0.1 M, 5 min) and consecutively with diazomethane in order to improve its solubility in DMSO. Methylation was carried out as described previously.[21] The lipooligosaccharide was hydrolysed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxymethylated as before, carboxy-reduced with $NaBD_4$ (4 °C, 18 h) and acetylated and analysed by GLC–MS. Methylation of the complete core region was carried out as described previously,[22,23] and the sample was hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxy-reduced, acetylated and analysed by GLC–MS.

GLC and GLC–MS were all carried out on a Hewlett–Packard 5890 instrument and a SPB-5 capillary column (0.25 mm × 30 m, Supelco). For sugar methylation analysis and *O*-methyl glycoside derivatives the temperature program was as follows: 150 °C for 2 min, then 2 °C min^{-1} to 200 °C for 0 min, then 10 °C min^{-1} to 260 °C for 11 min, then 8 °C min^{-1} to 300 °C for 20 min. For fatty acid analysis the temperature program was 80 °C for 2 min, then 8 °C min^{-1} to 300 °C for 15 min.

NMR Spectroscopy: For structural assignment of the oligosaccharides, 1D and 2D ^{1}H NMR spectra were recorded for a solution of 2 mg of product in 0.6 mL of D_2O. Experiments were carried out at 25 °C with a Varian Inova 500 spectrometer and ^{31}P NMR spectra were recorded with a Bruker DRX-400 spectrometer. Spectra were calibrated with respect to internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm). 85% Phosphoric acid was used as external reference (δ = 0.00 ppm) for ^{31}P NMR spectroscopy. For ^{31}P NMR spectroscopy of the lipooligosaccharide, the 1D spectrum was recorded in a 700 μL solution of 1% deuteriated SDS with 5 μL of 32% NH_4OH (pD 9.5, uncorrected value).

ROESY experiments were performed with data sets of 512 × 1024 points and 32 scans were acquired. A mixing time of 200 ms was employed. The double quantum-filtered phase-sensitive COSY experiment was performed with a 0.258 acquisition time with data sets of 4096 × 1024 points and 64 scans were acquired. The TOCSY experiment was performed with spinblock time of 120 ms and data sets of 512 × 1024 points; 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution-enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D DQF-COSY experiments.[24,25] The HSQC and HMBC experiments were measured using data sets of 2048 × 256 points and 64 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.[26] The ^{1}H,^{13}C, HMBC spectrum was optimised for a 6 Hz coupling constant and the ^{1}H,^{31}P HSQC spectrum for an 8 Hz coupling constant. In all the heteronuclear experiments the data matrix was extended to 2048 × 1024 points by forward linear prediction extrapolation.[27,28]

All the NMR analyses were carried out following literature procedures[29] and spectra were assigned with the help of the computer program Pronto,[30] which allows the simultaneous display of different two-dimensional spectra and individual labelling of cross peaks.

MALDI-TOF Analysis: MALDI-TOF mass spectra were recorded with negative and positive polarity in linear mode on a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated by 24 kV. The mass spectra reported are the result of 256 laser shots. Resolution was about 1500.

The lipid A sample was dissolved in $CHCl_3/CH_3OH$ (50:50, v/v) at a concentration of about 25 pmol μL^{-1}. The matrix solution was prepared by dissolving 2,4,6-trihydroxyacetophenone (THAP) in CH_3OH/0.1% trifluoroacetic acid/CH_3CN (7:2:1, v/v) at a concentration of 75 mg mL^{-1}. A sample/matrix solution mixture (1:1, v/v) was deposited (1 μL) onto a stainless-steel gold-plated 100-sample MALDI probe tip and left to dry at room temperature.

The R-LPS sample required a more laborious preparation than the recently reported case.[31] Briefly, a small amount of the intact R-LPS was first suspended in a mixture of methanol/water (1:1) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and allowed to dissolve by a brief ultrasonication. A few microlitres of the mixture obtained were then desalted on a small piece of Parafilm™ with some grains of cation-exchange beads (Dowex 50WX8-200, Sigma–Aldrich) previously converted into the ammonium form; a sample of this solution (0.3 μL) was finally deposited, together with the same volume of 20 mM dibasic ammonium citrate, as a thin layer of an homogeneous matrix film obtained from a solution whose components were 2,4,6-trihydroxyacetophenone (200 mg mL^{-1}) in methanol and nitrocellulose (Trans-blot membrane, BioRad; 15 mg mL^{-1}) in acetone/propan-2-ol (1:1 v/v) mixed in a 4:1 v/v ratio.

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