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The Outer Membrane of the Marine Gram-Negative Bacterium *Alteromonas addita* is Composed of a Very Short-Chain Lipopolysaccharide with a High Negative Charge Density

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The complete structure of the lipopolysaccharide isolated from the Gram-negative marine bacterium *Alteromonas addita*, type strain KMM 3600^T = R10SW13^T, has been elucidated by means of a combined chemical approach and state-of-the-art NMR and MS analyses. Isolation and characterisation of the lipid A moiety and the core oligosaccharide were pursued separately after either acid or alkaline treatment of the lipopolysaccharide. The structure detected was identified as a novel, highly negatively charged, deep-rough lipopolysaccharide in which a trisaccharide subunit is connected to a typical lipid A glucosamine backbone. Within the core oligosaccharide, a phosphodiester bridge connects a glucose unit to a heptose residue.

Introduction

Alteromonas addita KMM 3600^T is the type strain of a new species recently added to the genus *Alteromonas*. This Gram-negative bacterium was isolated from a sea water sample collected in the Pacific Ocean region of Chazma Bay (Sea of Japan) during a study on free-living microbial colonies in radionuclide contaminated environments.[1] Although genetically related to the other defined *Alteromonas* species, *A. addita* can be distinguished by a combination of phenotypic, genotypic and phylogenetic characteristics, for example the range of salinity and temperature growth, the presence of haemolytic activity and the ability to hydrolyse agar. In fact, this microorganism is neutrophilic, halophilic and psycrotolerant, exhibiting an optimal growth temperature between 4 and 37 °C. The interaction of *A. addita* KMM 3600^T with the unfriendly habitat in which it was first isolated is mediated by the molecules harboured in the outermost limit of the bacterial cell, namely the outer membrane (OM), surrounding the peptidoglycan cell wall. The external surface of Gram-negative OMs is principally made of lipopolysaccharides (LPSs), characteristic and vital molecules of this class of bacteria. LPSs typically possess an architecture in which three structurally and biogenetically distinct regions can be defined:[2] a glycolipid portion, the lipid A, that anchors the molecule to the cell membrane, composed of a variously acylated bisphosphorylated glucosamine backbone; the oligosaccharide region of the core, usually showing molecular motifs conserved among species belonging to the same genus that contains archetypal monosaccharide residues, namely, L-*glycero*-D-*manno*-heptose (L,D-Hep) and 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo); the highly variable polysaccharide region, the O-specific chain (or O-chain). The occurrence within the molecule structure of all three portions defines the "smooth" type LPSs (S-LPS), but Gram-negative bacteria can express LPSs lacking the polysaccharide portion, the "rough" type LPSs (R-LPS) or lipooligosaccharides (LOSs) composed of the lipid A and the covalently linked core oligosaccharide.[3,4] In this paper, the structure of the LOS fraction isolated from *Alteromonas addita* KMM 3600^T is described. This interesting glycolipid showed some similarity with the one isolated from *A. macleodii* ATCC 27126^T, the only other *Alteromonas* LPS described so far.[5] Complete structural elucidation was achieved by chemical "dissection" of the glycolipid and rigorous structural elucidation of the intact molecule by using state-of-the-art methodologies such as 2D NMR and MALDI-TOF MS spectrometry.

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Results and Discussion

LPS Isolation and Chemical Analyses

Cultivated cells of *Alteromonas addita* KMM 3600^T were washed with aqueous 1% phenol to remove contaminants and subsequently subjected to LPS extraction following the phenol/chloroform/light petroleum (PCP)[6] and hot phenol/water[7] procedures described previously. The extracted fractions, further purified by enzymatic digestion, were checked for LPS content by SDS-PAGE.[8] A LOS, migrating to the bottom of the run, was detected both in the PCP extract and in the water phase of the phenol/water extraction, whereas no S-LPS was detected in any fraction. Monosaccharide and fatty acid analyses revealed the two fractions have the same chemical composition and the same ratios of constituents so that the following analyses were carried out on both fractions.

Chemical analyses for monosaccharide determination were performed on the intact LOS sample and revealed the presence of 2-deoxy-2-amino-D-glucose (D-GlcN), L-*glycero*-D-*manno*-heptose (L,D-Hep), D-glucose (D-Glc) and 3-deoxy-*manno*-oct-2-ulopyranosonic acid (Kdo) in the ratio 2:1:1:1. Methylation analysis showed the presence of 6-substituted-GlcN, 5-substituted-Kdo, terminal-Hep and terminal-Glc, all pyranose rings. Fatty acids were analysed by GC–MS analysis of their methyl ester derivatives and were identified as ester-linked (*R*)-3-hydroxydecanoic acid [10:0(3-OH)], (*R*)-3-hydroxydodecanoic acid [12:0(3-OH)], (*R*)-3-hydroxytridecanoic acid [13:0(3-OH)], dodecanoic acid [12:0] and tetradecanoic acid [14:0] and amide-linked (*R*)-3-hydroxytetradecanoic acid [14:0(3-OH)]. A minor amount of [13:0(3-OH)] was also found to be present as an amide substituent.

Characterization of the Lipid A

In order to isolate the lipid A moiety from the LOS of *Alteromonas addita* KMM 3600^T, an aliquot of the sample was hydrolysed in mildly acidic conditions with acetate buffer, exploiting the acid lability of the Kdo linkage to the lipid A. The lipid A fraction was then collected as a precipitate by centrifugation and analysed by mass spectrometry. The negative-ion MALDI-TOF spectrum (Figure 1) showed two distinct ion clusters (**1**,**2**). The more abundant species (**1**) was represented by the ion peak at *m*/*z* = 1501.9, which is comprised of two glucosamines, two phosphate groups (P) and two 14:0(3-OH), one 12:0(3-OH), one 10:0(3-OH) and one 12:0 residues. This penta-acylated species is the most abundant form of the lipid A family isolated from *A. addita* KMM 3600^T. Ion peaks differing by 14 and 28 Da were detected, explained by diverse fatty acid chain lengths and identified on the basis of a previous chemical analysis. A minor amount of monophosphoryl penta-acyl lipid A was also present, as demonstrated by peaks differing from related species in the main cluster by 80 Da, a phosphate group. The proposed molecular formulae are collected in Table 1.

Species **2** was identified as the tetra-acyl lipid A with the main peak at *m*/*z* = 1303.8, lacking the 12:0(3-OH) residue of the peak at *m*/*z* = 1501.9. Monophosphoryl tetra-acyl lipid A was revealed by the presence of ion peaks differing by 80 Da, as reported in Table 1. Since mass differences of 80 Da were not detected in the native LOS mass spectra (see below), the monophosphorylated species are likely generated by the cleavage of one phosphate group during the acid hydrolysis performed in the preparation of the lipid A samples. In order to locate the fatty acids on the disaccharide backbone, an aliquot of lipid A was treated with

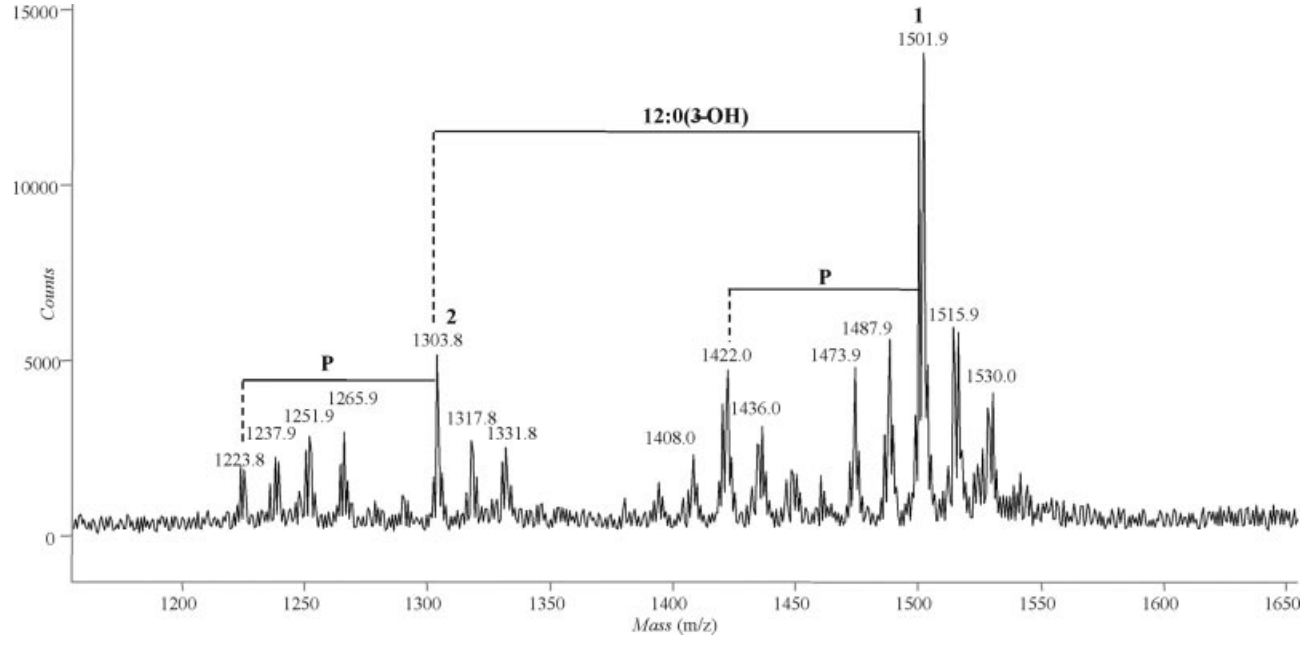


Figure 1. Negative-ion MALDI-TOF mass spectrum of the intact lipid A fraction from *Alteromonas addita* KMM 3600^T. Numbers refer to the relevant ion peak clusters described in Table 1 and in the text.

Table 1. Proposed acyl and phosphate content of the molecular species composing the lipid A fraction from *Alteromonas addita* KMM 3600^T. Species lacking a phosphate group are artefacts produced by acid treatment.

Observed ion (*m*/*z*)	Species	Fatty acids and phosphate substitution
1501.9	**1**	$GlcN_2$-P_2-[14:0(3-OH)]$_2$-12:0(3-OH)-10:0(3-OH)-12:0
1515.9	**1**	$GlcN_2$-P_2-[14:0(3-OH)]$_2$-13:0(3-OH)-10:0(3-OH)-12:0
1530.0	**1**	$GlcN_2$-P_2-[14:0(3-OH)]$_2$-12:0(3-OH)-10:0(3-OH)-14:0
1487.9	**1**	$GlcN_2$-P_2-14:0(3-OH)-13:0(3-OH)-12:0(3-OH)-10:0(3-OH)-12:0
1473.9	**1**	$GlcN_2$-P_2-[13:0(3-OH)]$_2$-12:0(3-OH)-10:0(3-OH)-12:0
1422.0	**1**	$GlcN_2$-P-[14:0(3-OH)]$_2$-12:0(3-OH)-10:0(3-OH)-12:0
1436.0	**1**	$GlcN_2$-P-[14:0(3-OH)]$_2$-13:0(3-OH)-10:0(3-OH)-12:0
1408.0	**1**	$GlcN_2$-P-14:0(3-OH)-13:0(3-OH)-12:0(3-OH)-10:0(3-OH)-12:0
1303.8	**2**	$GlcN_2$-P_2-[14:0(3-OH)]$_2$-10:0(3-OH)-12:0
1317.8	**2**	$GlcN_2$-P_2-14:0(3-OH)-13:0(3-OH)-12:0(3-OH)-12:0
1331.8	**2**	$GlcN_2$-P_2-[14:0(3-OH)]$_2$-10:0(3-OH)-14:0
1223.8	**2**	$GlcN_2$-P-[14:0(3-OH)]$_2$-10:0(3-OH)-12:0
1237.9	**2**	$GlcN_2$-P-14:0(3-OH)-13:0(3-OH)-12:0(3-OH)-12:0
1251.9	**2**	$GlcN_2$-P-[14:0(3-OH)]$_2$-10:0(3-OH)-14:0
1265.9	**2**	$GlcN_2$-P-14:0(3-OH)-13:0(3-OH)-12:0(3-OH)-14:0

NH_4OH, treatment that selectively cleaves ester-linked acyloxyacyl moieties whilst not affecting the acyloxyacylamide substituents.[9] The negative-ion MALDI-TOF spectrum (Figure 2) showed a single main peak at *m*/*z* = 1134.0, originating from species containing two GlcN, two phosphate groups, two 14:0(3-OH) residues as amide substituents and one secondary 12:0 residue. The minor peak at *m*/*z* = 1120.0 indicates a species containing 13:0(3-OH) instead of a 14:0(3-OH) residue, as expected on the basis of the chemical analysis of the *N*-linked fatty acids. The peak at *m*/*z* = 1054.0 originates from the triacyl monophosphoryl lipid A, while the peak at *m*/*z* = 951.7 indicates a species lacking the 12:0 residue. In order to clarify the position of the secondary 12:0 residue on the GlcN disaccharide, a positive-ion MALDI-TOF spectrum was recorded (not shown). Beside the pseudomolecular ion of the above described species, it was possible to observe, at lower molecular masses, the oxonium ion originating from an "in source" disaccharide cleavage corresponding to the non-reducing GlcN of the lipid A bearing a phosphate group, one 14:0(3-OH) residue and the 12:0 substituent at *m*/*z* = 650.8, allowing us to locate the 12:0 residue on the acylamide group on GlcN II. In order to assign the ester-linked primary fatty acids, a positive-ion MALDI-TOF spectrum of the intact lipid A sample was also recorded (Figure 3) that indicated the presence of a 12:0(3-OH) residue as a primary substituent at *O*-

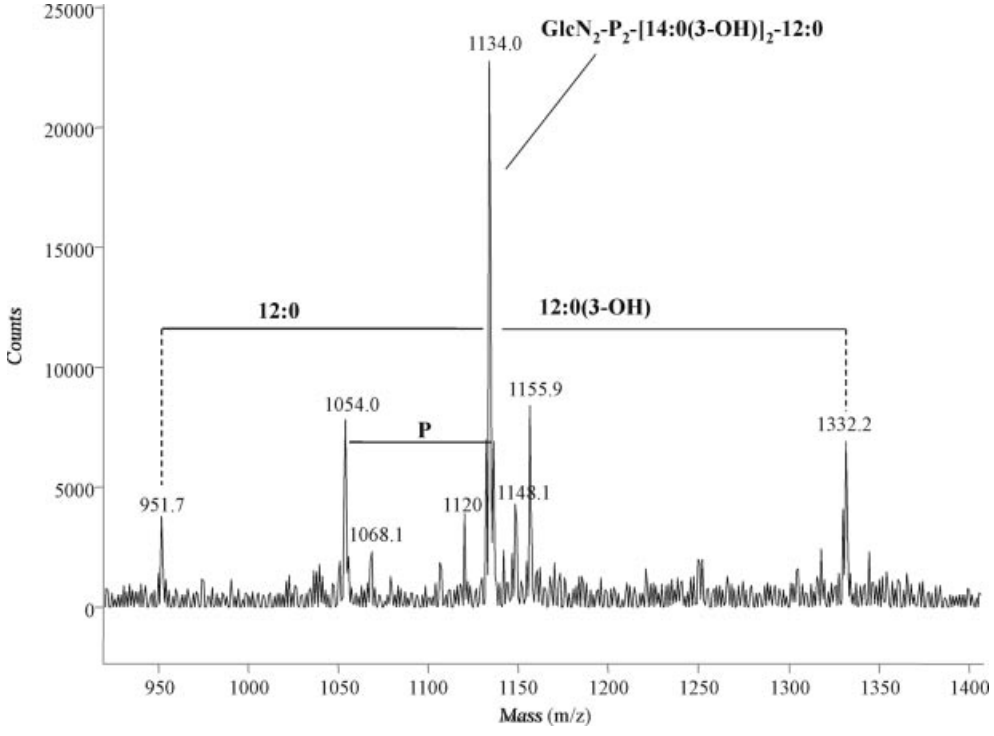


Figure 2. Negative-ion MALDI-TOF mass spectrum of the ammonium treated lipid A fraction from *Alteromonas addita* KMM 3600^T. The peak at *m*/*z* = 1332.2 is indicative of a minor amount of incompletely reacted lipid A.

3 of the non-reducing GlcN unit. Still, the molecular mass of the intact lipid A points to one more primary 10:0(3-OH) residue that has to be placed at *O*-3 of the reducing GlcN residue. The anomeric configurations of the glucosamine disaccharide backbone and the position of the glycosidic linkage and phosphate groups have been inferred by analyses of the deacylated oligosaccharide portion, as described below. On the basis of these observations, we were able to identify the main lipid A species from *Alteromonas addita* KMM 3600^T, as depicted in Figure 4.

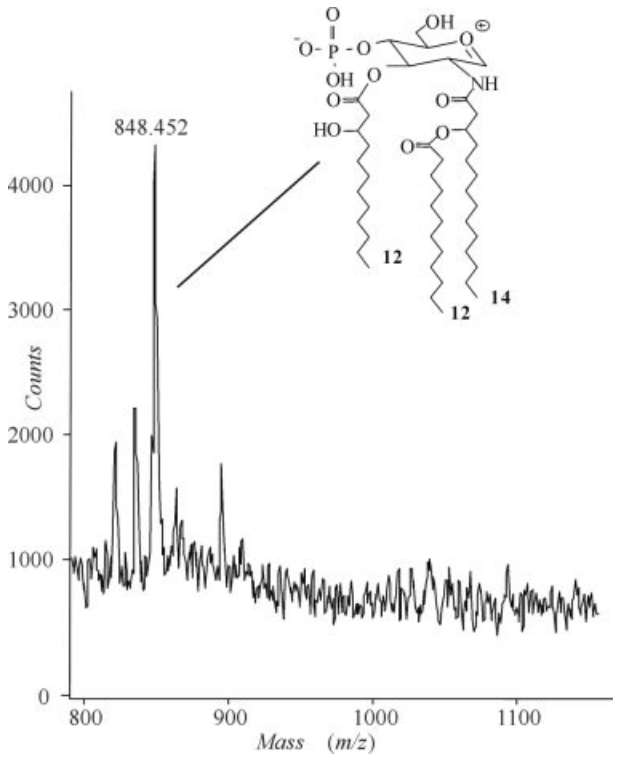


Figure 3. Section of the positive-ion MALDI-TOF mass spectrum of the intact lipid A fraction from *Alteromonas addita* KMM 3600^T. The structure sketched shows fatty acid substitution.

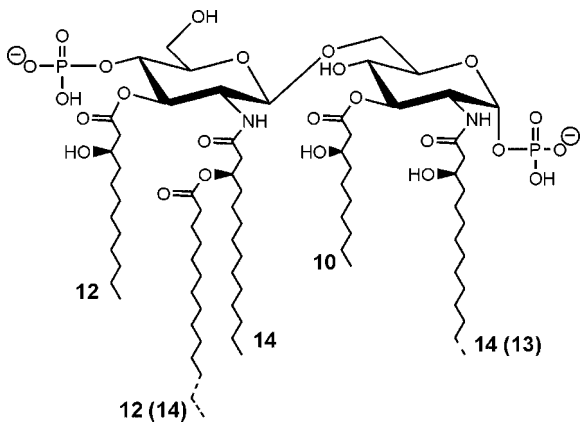


Figure 4. Complete structure of the penta-acyl lipid A species from *Alteromonas addita* KMM 3600^T. Dotted lines indicate variable acyl chain length.

Characterization of the Core Oligosaccharide

In order to isolate and characterise the oligosaccharide fraction, alkaline treatment with hydrazine followed by strong hydrolysis with KOH was realised to *O*,*N*-deacylate the LOS. The ^{1}H NMR spectrum of the resulting oligosaccharide fraction (Figure 5) was quite simple in its appearance. Three signals could be easily recognised within the anomeric region of the spectrum (**A**–**C**, in order of decreasing chemical shift) and allowed the identification of three distinct spin systems. Moreover, at high fields, signals typical of the diastereotopic methylene group of a Kdo residue (**D**) could be identified at 2.092 and 2.200 ppm. The full assignment of the proton and carbon resonances for the identified monosaccharides was realised on the basis of a complete set of 2D NMR experiments. In particular, from the TOCSY and DQF-COSY spectra, the full proton resonance pattern was established for each spin system. Subsequently, the carbon and phosphate chemical shifts were derived from the ^{1}H,^{13}C-HSQC and ^{1}H,^{31}P-HSQC spectra (not shown), respectively. All of these data are collected in Table 2. The anomeric signal at 5.660 ppm (1-H**A**) appeared, in the ^{1}H NMR spectrum, as a double doublet, with $^3J_{1H,2H}$ = 3.0 Hz and $^3J_{1H,P}$ = 7.9 Hz, diagnostic of an α-configured residue phosphorylated at *O*-1. Phosphorylation was also confirmed by the observation in the ^{1}H,^{31}P-HSQC spectrum of a cross peak with a phosphate signal at δ = 1.46 ppm. The $^3J_{H,H}$ values for the other ring position indicated that this residue adopted a *gluco* configuration and the chemical shift for C-2 (δ = 55.4 ppm) allowed the identification of residue **A** as the α-GlcN belonging to the lipid A disaccharide backbone. The glycosylation at *O*-6 was proven by observation of a down-field displacement typical of a C-6 resonance (δ = 70.2 ppm), in agreement with the methylation data. Anomeric signal 1-H**B** appeared as a singlet as a result of the small $^3J_{1H,2H}$ value (<2 Hz). This residue was identified, on the basis of the typical chemical shift values, as the expected L,D-Hep residue with an α configuration. Moreover, the down-field displacement of 3-H (4.362 ppm) and the phosphate group at δ =

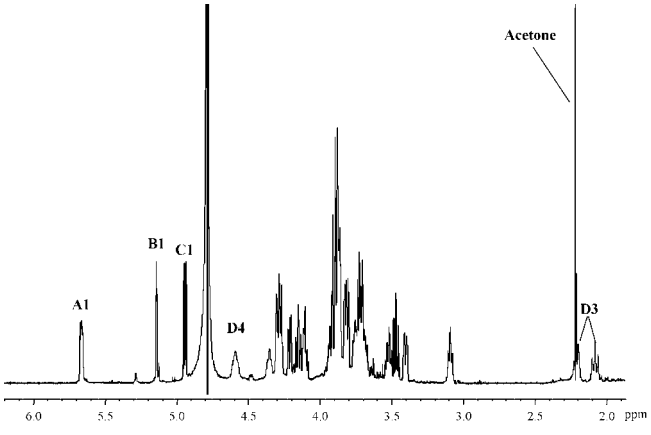


Figure 5. ^{1}H NMR spectrum of the *Alteromonas addita* oligosaccharide obtained after strong alkaline treatment of LOS. Capital letters refer to relevant signals of the spin systems described in Table 2.

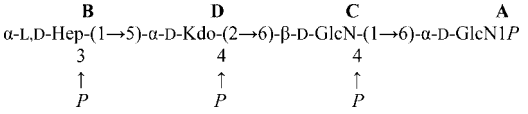
Table 2. ^{1}H, ^{13}C and ^{31}P NMR chemical shifts of the oligosaccharide obtained by alkaline degradation of the LOS derived from *Alteromonas addita* KMM 3600^{T}.[a]

Residue		1(3_{ax})	2(3_{eq})	3(4)	4(5)	5(6)	6(7)	7(8)
A	^{1}H	5.660	3.404	3.899	3.488	4.160	3.860/4.270	
6-α-GlcN1*P*	^{13}C	91.69	55.4	69.7	71.0	73.7	70.2	
	^{31}P	1.46						
B	^{1}H	5.147	4.309	4.362	3.902	4.218	4.117	3.733/3.818
t-α-Hep3*P*	^{13}C	101.5	70.3	76.8	67.1	74.4	73.5	63.3
	^{31}P			1.33				
C	^{1}H	4.951	3.094	3.877	3.869	3.762	3.523/3.729	
6-β-GlcN4*P*	^{13}C	100.23	56.5	73.2	75.3	75.2	63.7	
	^{31}P				1.85			
D	^{1}H	2.092	2.200	4.595	4.283	3.829	3.915	3.693/3.927
5-α-Kdo4*P*	^{13}C	35.1		71.5	74.4	72.8	70.7	64.7
	^{31}P			2.03				

[a] Atom numbers in parentheses refer to the Kdo residue. 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.

1.33 ppm in the ^{1}H,^{31}P-HSQC spectrum pointed to phosphorylation at *O*-3. Residue **C** was identified as the non-reducing β-GlcN belonging to the lipid A backbone on the basis of anomeric and ring proton and carbon chemical shifts and the high $^{3}J_{H,H}$ coupling constants. The intra-residual dipolar correlations typical of a residue with a β-*gluco* configuration, namely 1-H/3-H, 1-H/5-H, were observed in the 2D ROESY spectrum. Also in this case, phosphorylation was detected at *O*-4 (δ_P = 1.85 ppm), in accordance with the typical LipA backbone architecture. Finally, the resonances arising from the Kdo residue **D** were fully assigned starting with the high-field signals of the methylene group at C-3. On the basis of the chemical shift values found for 3_{ax}-H (2.092 ppm) and 3_{eq}-H (2.200 ppm), it was possible to establish the α configuration of this residue.[10,11] The signal for 4-H was down-field shifted and appeared in the ^{1}H NMR spectrum as a broad signal owing to couplings with the 3_{ax}-H, 3_{eq}-H, 5-H protons and a phosphate group. Also, phosphorylation at *O*-4 was confirmed by a correlation with a phosphate signal at δ = 2.03 ppm in the ^{1}H,^{31}P-HSQC spectrum. No signal arising from the D-Glc residue was detected, as expected on the basis of chemical analyses.

The monosaccharide sequence within this oligosaccharide was established on the basis of the NOE contacts detected in the 2D ROESY spectrum. The typical lipid A glucosamine backbone was proven by inter-residual NOE correlation between 1-H**C** and 6-H**A**. The linkage between the heptose residue **B** and O-5 of the Kdo residue **D** was confirmed by the dipolar correlation between 1-H**B** and 5-H**D** and 7-H**D** and, in addition, between 5-H**B** and 3_{ax}-H**D**. These NOE contacts are also diagnostic of the D configuration of the Kdo residue as they are only possible if the two monosaccharide rings possess the same absolute configuration.[12] On the basis of GC–MS analyses, the absolute configuration of the heptose has already been stated, namely, L-*glycero*-D-*manno*-heptose, thus, Kdo **D** must possess a D configuration. Therefore, the structure identified so far for the oligosaccharide derived from *A. addita* KMM 3600^{T} is as given in Scheme 1.



Scheme 1. Structure of the oligosaccharide isolated from *A. addita* KMM 3600^{T}.

This tetrasaccharide structure was further confirmed by the MALDI-TOF spectrum (not shown) in which a peak is visible at *m*/*z* = 1072.0 representing the molecular mass of the oligosaccharide. In a small quantity, a peak at Δ*m*/*z* = 80 due to the absence of a phosphate group was present resulting from the strong alkaline treatment. Neither NMR nor MS data showed signals arising from the missing glucose residue, previously identified by means of chemical analyses.

In order to find and locate this monosaccharide, an aliquot of intact LOS was analysed by MALDI-TOF MS. The negative-ion spectrum (Figure 6) presented three main series of peaks all characterised by the expected heterogeneity of the lipid A, in terms of the type and number of fatty acids contained. The most intense ion peak, around *m*/*z* = 2236.0, was attributed to the intact, penta-acyl LOS species, while the ions around *m*/*z* = 2065.5 were generated by minor amounts of tetra-acyl LOS, in accordance with the lipid A analyses. Moreover, it was possible to detect peaks related to the lipid A at *m*/*z* = 1502.1 that arise from the described "in source" fragmentation of the Kdo residue and the glucosamine backbone.[13,14] The molecular mass of the intact LOS inferred by the peaks corresponded to the species LipA-Kdo-Hep-Hex-P_2. On the basis of this observation, it was possible to deduce that the Glc unit present in the intact molecule was quantitatively lost on alkaline treatment and was thus connected to the molecule through a base-labile linkage. In order to clarify the nature of such a linkage, mild *O*-deacylation with hydrazine was performed (de-*O*-LOS). The negative-ion MALDI-TOF spectrum (Figure 7) showed an ion peak for the *O*-deacylated LOS at *m*/*z* = 1686.6 that matches the species $GlcN_2$-Kdo-Hep-Hex-P_4-[14:0(3-OH)]$_2$. It was also possible to detect a

peak at *m*/*z* = 952.1 originating from the *O*-deacylated lipid A, namely, $GlcN_2$-P_2-[14:0(3-OH)]$_2$, testifying that the Glc residue belonged to the core and not to the lipid A region. In order to locate it, a full NMR analysis was realised on this sample as well. To overcome the poor solubility of the partially acylated sample and avoid the formation of micelle aggregates, all these experiments were executed under denaturing conditions using a solution of 1 mg/mL perdeuteriated SDS in D_2O. In this way, a full series of 2D NMR spectra, namely, TOCSY, DQF-COSY, ROESY, ^1H,^{13}C-HSQC and ^1H,^{31}P-HSQC, could be recorded. The ^1H NMR spectrum of de-*O*-LOS (Figure 8) showed, within the anomeric region, four signals. The high-field signal of the methylene group of the Kdo residue partially merged with the signals of the fatty acid residues. Nevertheless, all chemical shifts and $^3J_{H,H}$ coupling constants could clearly be distinguished from the 2D NMR spectra. On the basis of the same consideration made for the fully delipidated oligosac-

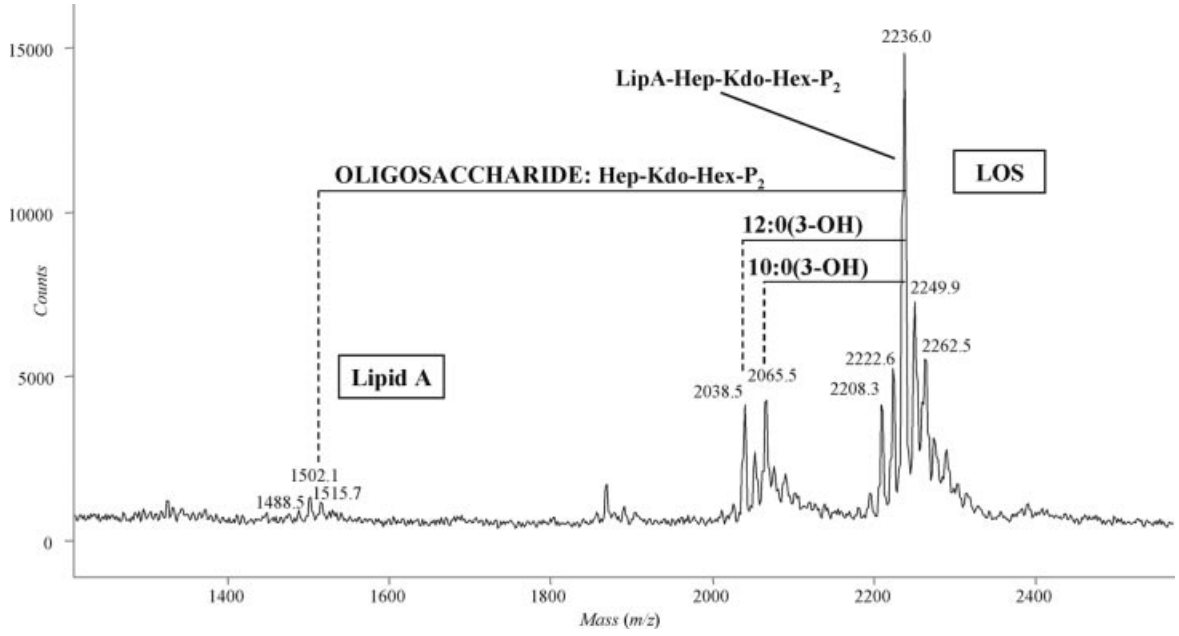


Figure 6. Negative-ion MALDI-TOF mass spectrum of the native lipooligosaccharide fraction obtained from *Alteromonas addita* KMM 3600^T. The visible peaks originate from the lipid A portion following cleavage of the linkage with the core oligosaccharide.

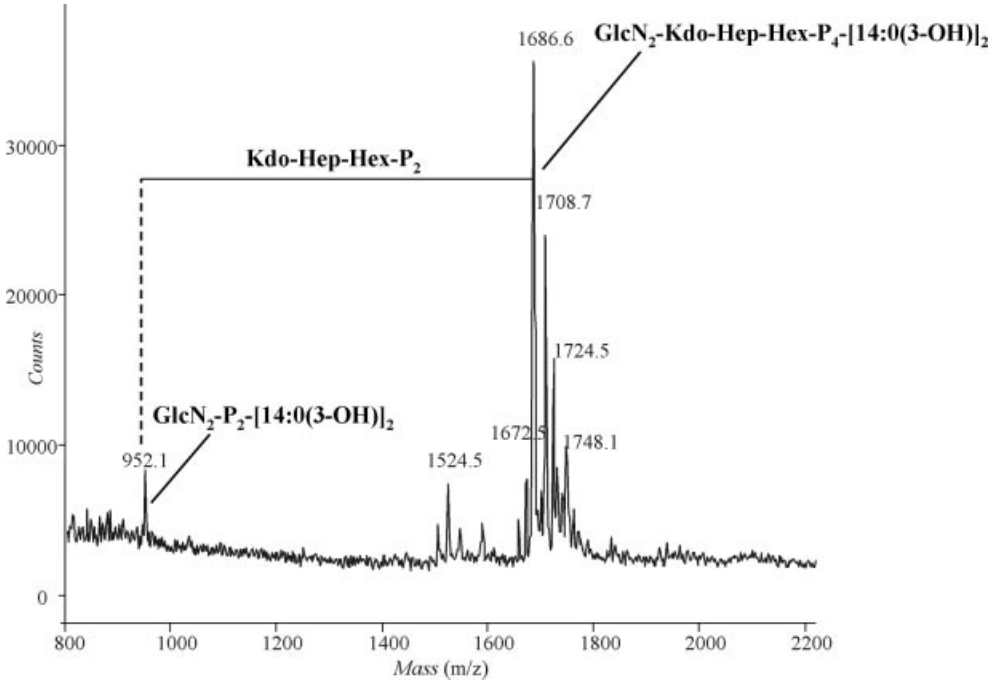


Figure 7. Negative-ion MALDI-TOF mass spectrum of the hydrazine-treated lipooligosaccharide fraction obtained from *Alteromonas addita* KMM 3600^T.

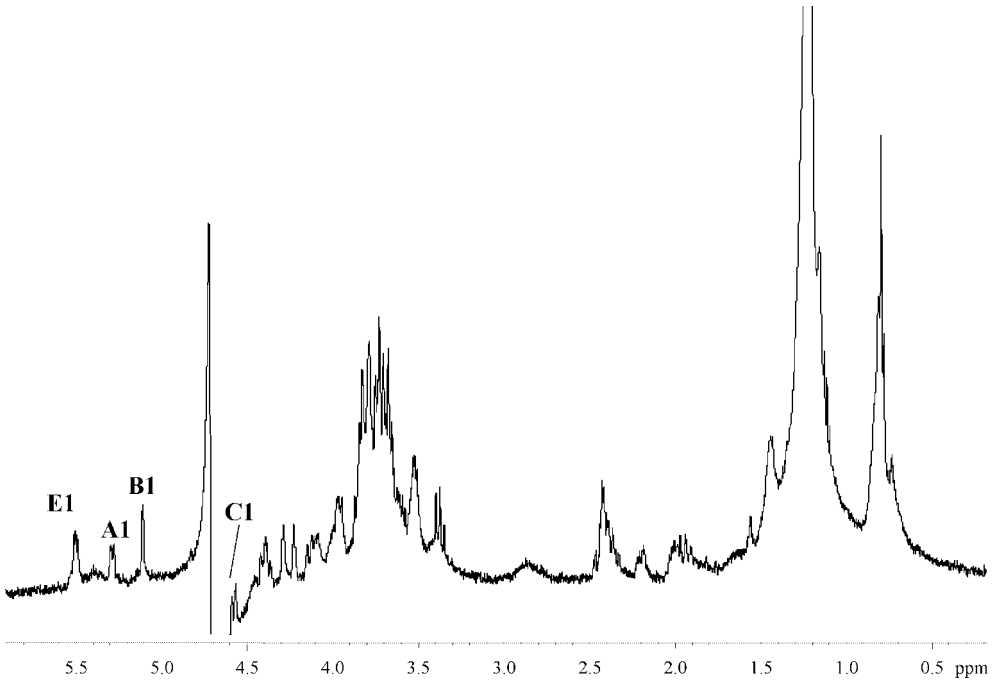


Figure 8. ^{1}H NMR spectrum recorded under denaturing conditions of the hydrazine-treated lipooligosaccharide fraction obtained from *Alteromonas addita* KMM 3600^{T}. The capital letters refer to the identified spin systems described in Table 3.

Table 3. ^{1}H, ^{13}C, and ^{31}P NMR chemical shifts of the *O*-deacylated LOS obtained from *Alteromonas addita* KMM 3600^{T} by mild alkaline degradation.[a]

Residue		1(3_{ax})	2(3_{eq})	3(4)	4(5)	5(6)	6(7)	7(8)
A	^{1}H	5.297	3.841	3.710	3.515	3.956	4.141/3.825	
6-α-GlcN1*P*	^{13}C	92.4	54.1	71.4	69.7	71.1	68.2	
	^{31}P	3.18						
B	^{1}H	*5.111*	4.292	4.401	3.759	4.095	3.641	3.764/3.648
t-α-Hep3*P*	^{13}C	99.9	69.2	76.1	66.4	71.4	73.2	61.9
	^{31}P			–1.18				
C	^{1}H	4.576	3.755	3.636	3.718	3.758	3.692/3.533	
6-β-GlcN4*P*	^{13}C	100.4	54.9	74.4	74.7	73.0	62.9	
	^{31}P				3.94			
D	^{1}H	1.930	2.200	4.476	4.231	3.842	3.793	3.645
5-α-Kdo4*P*	^{13}C	34.5		68.6	72.6	71.9	70.4	63.8
	^{31}P			4.74				
E	^{1}H	5.511	3.522	3.677	3.383	3.826	3.660/3.772	
t-α-Glc1*P*	^{13}C	95.7	71.4	73.2	69.5	73.2	61.8	
	^{31}P	–1.18						

[a] Atoms in parentheses refer to the Kdo residue. NMR experiments were carried out in 1 mg/mL D_2O solution of perdeuterated SDS at 25 °C with 5 μL NH_4OH 32%.

charide, it was possible to assign the proton and carbon resonances of the identified spin systems (Table 3). The residues **A**, **B**, **C** and **D** were respectively identified as the corresponding spin systems in the previously analysed oligosaccharide. The anomeric signal at δ = 5.511 ppm (1-H**E**) appeared as a double doublet with $^{3}J_{1H,2H}$ = 3.0 Hz, diagnostic of an α-configured residue, and $^{3}J_{1H,P}$ = 8.0 Hz. On the basis of the chemical shifts values, the residue was recognised as the expected α-Glc and the *O*-phosphorylation at the anomeric position suggested the occurrence of a phosphodiester bridge connecting this residue to the oligosaccharide. This hypothesis was confirmed by the observation in the ^{1}H,^{31}P-HSQC spectrum (Figure 9) of a cross peak with a phosphate group at δ = –1.18 ppm, in turn correlating with the proton at δ = 4.401 ppm, namely 3-H of the Hep residue **B**. Thus, this phosphodiester linkage was cleaved by the strong alkaline treatment, leaving the phosphate group attached to the Hep residue in the previously described oligosaccharide. The complete structure of the LOS obtained from *Alteromonas addita* KMM 3600^{T} is depicted in Figure 10.

The occurrence of a phosphodiester bridge connecting two monosaccharide residues is an uncommon feature of the core region of lipopolysaccharides that has so far been found mainly in marine Gram-negative bacterial LPSs. It has, in fact, been detected in the oligosaccharides of the LPSs of *Shewanella oneidensis*[15] and *Arenibacter certesii*,[16] both isolated from marine habitats.

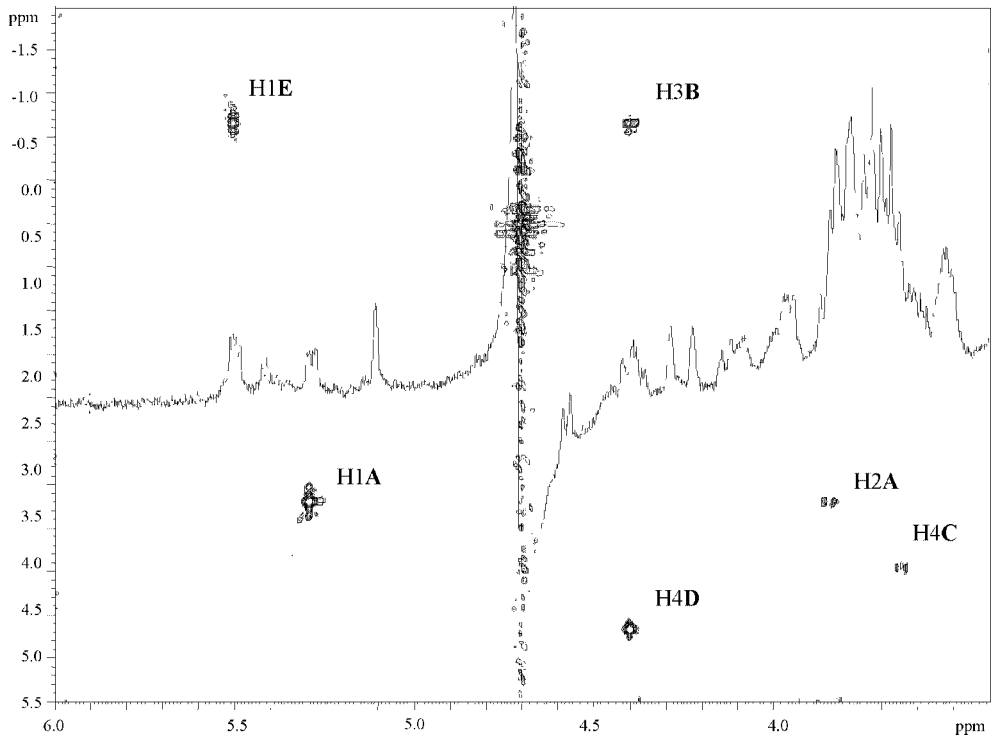


Figure 9. Zoom of the $^{1}H,^{31}P$-HSQC spectrum and of the ^{1}H NMR spectrum of the *O*-deacylated LOS fraction obtained from *Alteromonas addita* KMM 3600^{T}. The spectrum shows cross peaks relevant to the localisation of the phosphate groups on the residues described in Table 3. In addition, the long-range correlation of the anomeric phosphate group of residue **A** with H2 is also visible.

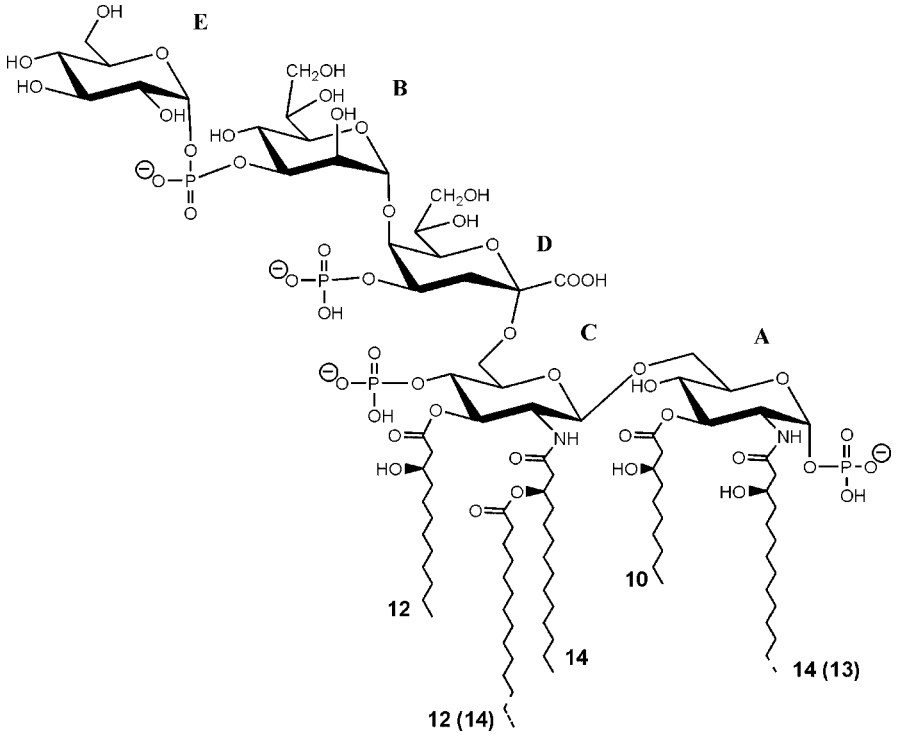


Figure 10. The complete structure of the LOS obtained from *Alteromonas addita* KMM 3600^{T}.

Bacteria belonging to different *Alteromonas* species have always been isolated in harsh marine environments. It appears that the structure and physiology of the outer cell membrane is strictly involved in the adaptation to such habitats. As found in *A. macleodii* ATCC 27126^{T}[5] LPS,[5] the structure is characterised by a very short carbohydrate chain and a high negative charge density, inferred by the presence of four phosphate groups and a Kdo residue. It

has actually been ascertained that the presence of several negatively charged groups is one of the factors that most effects the permeability and resistance of the bacterial membrane towards external factors, making it possible for the lipopolysaccharide molecule to build ionic bridges through the complexation of metallic bivalent cations.[17] Thus, the short-chain, highly negatively charged lipooligosaccharide of *Alteromonas addita* KMM 3600^T is one of the chemical factors that allows the survival of the bacterium in unfriendly marine habitats, by counteracting the negative effects of pressure and salinity on the membrane's correct physiology.

This is the second structural elucidation of the LPS obtained from *Alteromonas*. The genus *Alteromonas*, which was established in 1972 by Baumann et al.,[18] was subsequently revised in 1995 and divided into two, *Alteromonas* and the new genus *Pseudoalteromonas*, up to now containing about 30 different species.[19] Interestingly, it should be noted that all *Pseudoalteromonas* possess S-LPSs,[20] all having acid sugar residues frequently present as nonulosonic acid residues. Conversely, these characteristics are not present in the LPS obtained from *Alteromonas* which produces only a very small lipooligosaccharide. Therefore, this complex glycolipid also represents a distinctive molecular hallmark of the *Alteromonas* genus.

Experimental Section

Cell Culture and LPS Extraction: The type strain of *Alteromonas Addita* KMM 3600^T was cultivated on a liquid medium containing glucose (1 g/L), pepton (5 g/L), yeast extract (2.5 g/L), K_2HPO_4 (0.2 g/L), $MgSO_4$ (0.05 g/L), sea water (750 mL) and distilled water (250 mL). Cells were collected by centrifugation, washed with water and then dried with acetone (three times) to obtain around 12 g of dried cells from 20 L of the cultured fluid. LOS was isolated from the dried cells (2.0 g) after washing with aqueous 1% phenol to remove exocellular polysaccharides followed by conventional aqueous 90% phenol/chloroform/light petroleum (50 mL, 2:5:8 v/v/v) extraction.[6] After removal of the organic solvents under vacuum, the LOS fraction was precipitated from phenol with water, washed first with aqueous 80% phenol and then three times with cold acetone, each time centrifuged and lyophilised (22 mg, 1.1% of the dry mass). Cells were then extracted three times with aqueous 90% phenol/water (1:1 v/v) as described previously.[7] The phenol and water phases were digested with DNase, RNase and Proteinase K, dialysed and freeze-dried. Silver-stained sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE 12%) was performed to detect the LPSs (54 mg).[8]

General and Analytical Methods: Monosaccharide analysis was realised by GC–MS analysis of their *O*-methyl glycoside derivatives. The absolute configurations of Glc, GlcN and Hep residues were assigned by GLC analysis of their 2-(+)-*O*-octyl glycoside derivatives and comparison with genuine standards as described previously.[21]

Methylation analysis was carried out on the dephosphorylated sample after treatment with 48% HF (4 °C, 48 h). For methylation analysis of Kdo, LOS was carboxy-methylated with methanolic HCl (0.1 M, 5 min) and then with diazomethane in order to improve its solubility in DMSO. Methylation was carried out as described by the Ciucanu and Kerek procedure.[22] The lipooligosaccharide was then hydrolysed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated as before, carboxy-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analysed by GC-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 GC–MS.

The total fatty acid content was determined after strong hydrolysis of lipid A,[24] first with 4 M HCl (100 °C, 4 h) and subsequently with 5 M NaOH (100 °C, 30 min). Fatty acids were then extracted with chloroform, methylated with diazomethane and analysed by GC–MS. Ester bound fatty acids were analysed after selective alkaline hydrolysis with 0.5 M NaOH/MeOH (1:1 v/v, 85 °C, 2 h). After acidification and extraction with chloroform, fatty acids were methylated with diazomethane and analysed by GC–MS. The absolute configurations of fatty acids were determined as described previously.[25]

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

Isolation of the Lipid A and the Core Oligosaccharide: To isolate the lipid A fraction, an aliquot (10 mg) of LOS was hydrolysed with 100 mM acetate buffer (1 mL, pH 4.7, 100 °C, 3 h). After centrifugation (3000*g*, 60 min, 4 °C) the lipid A was collected as a precipitate, washed twice with water and lyophilised (5 mg).

To selectively *O*-deacylate the sample, a second aliquot of LOS 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 (12 mg). A portion of this material (6 mg) was subsequently de-*N*-acylated with 4 M KOH (120 °C, 16 h) as described previously.[26] After desalting on a Sephadex G-10 column (50 × 1.5 cm; Pharmacia), the resulting oligosaccharide fraction represented the carbohydrate backbone of the lipooligosaccharide (3 mg).

NMR Spectroscopy: For structural assignment of the oligosaccharide, 1D and 2D ^1H NMR spectra of a solution of 3 mg of product in 0.6 mL of D_2O were recorded. Experiments were carried out at 25 °C with a Bruker DRX-600 spectrometer equipped with a cryogenic probe. Spectra were calibrated relative to internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm). ^{31}P NMR spectra were recorded with a Bruker DRX-400 spectrometer and 85% phosphoric acid was used as the external reference (δ = 0.00 ppm). The NMR spectra of the *O*-deacylated LOS were recorded on a 600 μL solution of 1 mg/mL deuteriated SDS with 32% NH_4OH (5 μL, pD 9.5, uncorrected value).

ROESY experiments were carried out 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 an acquisition time of 0.258 s with data sets of 4096 × 1024 points; 64 scans were acquired. The TOCSY experiment was performed with a spin-lock time of 120 ms, data sets of 512 × 1024 points and 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.[27,28] The HSQC experiments were performed 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.[29]

MALDI-TOF Analysis: Negative- and positive-ion MALDI-TOF mass spectra were recorded 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. A resolution of about 1500 was used.

The lipid A sample was dissolved in $CHCl_3/CH_3OH$ (50:50, v/v) at a concentration of about 25 pmol/μL. 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. 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 LOS and *O*-deacylated LOS were prepared as recently reported.[14] Briefly, a small amount of LOS was first suspended in a mixture of methanol/water (1:1) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and allowed to dissolve after a brief ultrasonication. A few microlitres of the obtained mixture 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; 0.3 μL of this sample solution was finally deposited, together with the same volume of 20 mM dibasic ammonium citrate, in a thin layer of an homogeneous matrix film obtained from a solution of 2,4,6-trihydroxyacetophenone (THAP) (200 mg/mL in methanol) and nitrocellulose (Trans-blot membrane, BioRad, 15 mg/mL) in acetone/propan-2-ol (1:1 v/v), mixed in a 4:1 v/v ratio.

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