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Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of *Yersinia pestis*

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Abstract—Following a report of variations in the lipopolysaccharide (LPS) structure of *Yersinia pestis* at mammalian (37 °C) and flea (25 °C) temperatures, a number of changes to the LPS structure were observed when the bacterium was cultivated at a temperature of winter-hibernating rodents (6 °C). In addition to one of the known *Y. pestis* LPS types, LPS of a new type was isolated from *Y. pestis* KM218 grown at 6 °C. The core of the latter differs in: (i) replacement of terminal galactose with terminal D-*glycero*-D-*manno*-heptose; (ii) phosphorylation of terminal oct-2-ulosonic acid with phosphoethanolamine; (iii) a lower content of GlcNAc, and; (iv) the absence of glycine; lipid A differs in the lack of any 4-amino-4-deoxyarabinose and presumably partial (di)oxygenation of a fatty acid(s). The data obtained suggest that cold temperature switches on an alternative mechanism of control of the synthesis of *Y. pestis* LPS.

Keywords: *Yersinia pestis*; Lipopolysaccharide; Structure modification; Lipid A; Core oligosaccharide

1. Introduction

The natural environmental temperatures for *Y. pestis*, the cause of bubonic and pneumonic plague, may vary from 0 to 42 °C. Significant variations in the lipopolysaccharide (LPS) structure were observed when the bacteria are cultivated at 25–28 or 37 °C, including alternation of terminal core monosaccharides [D-*glycero*-D-*manno*-heptose (DDHep) vs D-galactose; 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) vs D-*glycero*-D-*talo*-octulosonic acid (Ko)][1] and change in the content of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and the degree of acylation in lipid A.[1–3] These variations were accompanied by alteration in the LPS bioactivity suggesting a role for overcoming the defense systems of both warm-blooded mammals (host) and cold-blooded insects (vector). We now studied the LPS structure in a *Y. pestis* strain grown at 6 °C (LPS-6) to mimic the conditions in animals during winter hibernation, and compared it with those of the LPS from the same strain cultivated at 25 and 37 °C (LPS-25 and LPS-37, respectively).

2. Results and discussion

LPS-6 was isolated by phenol/chloroform/light petroleum extraction from *Y. pestis* KM218 grown at 6 °C

Abbreviations: Ara4N, 4-amino-4-deoxyarabinose; DDHep, D-*glycero*-D-*manno*-heptose; LDHep, L-*glycero*-D-*manno*-heptose; ESI FTICR, electrospray ionization Fourier transform ion-cyclotron resonance; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid; LA, lipid A; LPS, lipopolysaccharide; EtN*P*, phosphoethanolamine; 12:0, 16:1, 3HO14:0, lauroyl, palmitoleoyl, 3-hydroxymyristoyl groups.

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in a casein hydrolysate medium. In SDS-PAGE, the electrophoretic mobility of LPS-6 was practically the same as that for the previously described LPS-25[1] (data not shown).

The electrospray ionization Fourier transform ion-cyclotron resonance (ESI FTICR) mass spectrum of LPS-6 (not shown) indicated a mixture of two LPS types called LPS-6A and LPS-6B. The former was identified as one of the Ko-containing LPS-25 types with known structure[1] shown in Figure 1. Indeed, as in LPS-25, lipid A in LPS-6A bears four to six acyl groups and phosphate groups are substituted with 4-amino-4-deoxyarabinose (Ara4N) nearly stoichiometrically. The core of LPS-6A contains only Gal and predominantly Ko as terminal core monosaccharides, which is in agreement with our previous finding that DDHep and Kdo are preferentially incorporated into the core at 37 °C, whereas Gal and Ko are incorporated at lower temperatures.[1]

In contrast, LPS-6B was significantly different from the known *Y. pestis* LPS types.[1] For the structural elucidation, LPS-6 was purified using the Bligh & Dyer procedure,[4] and LPS-6B was recovered from the chloroform/methanol/water extract. As from the ESI FTICR MS data, the LPS-6B preparation was

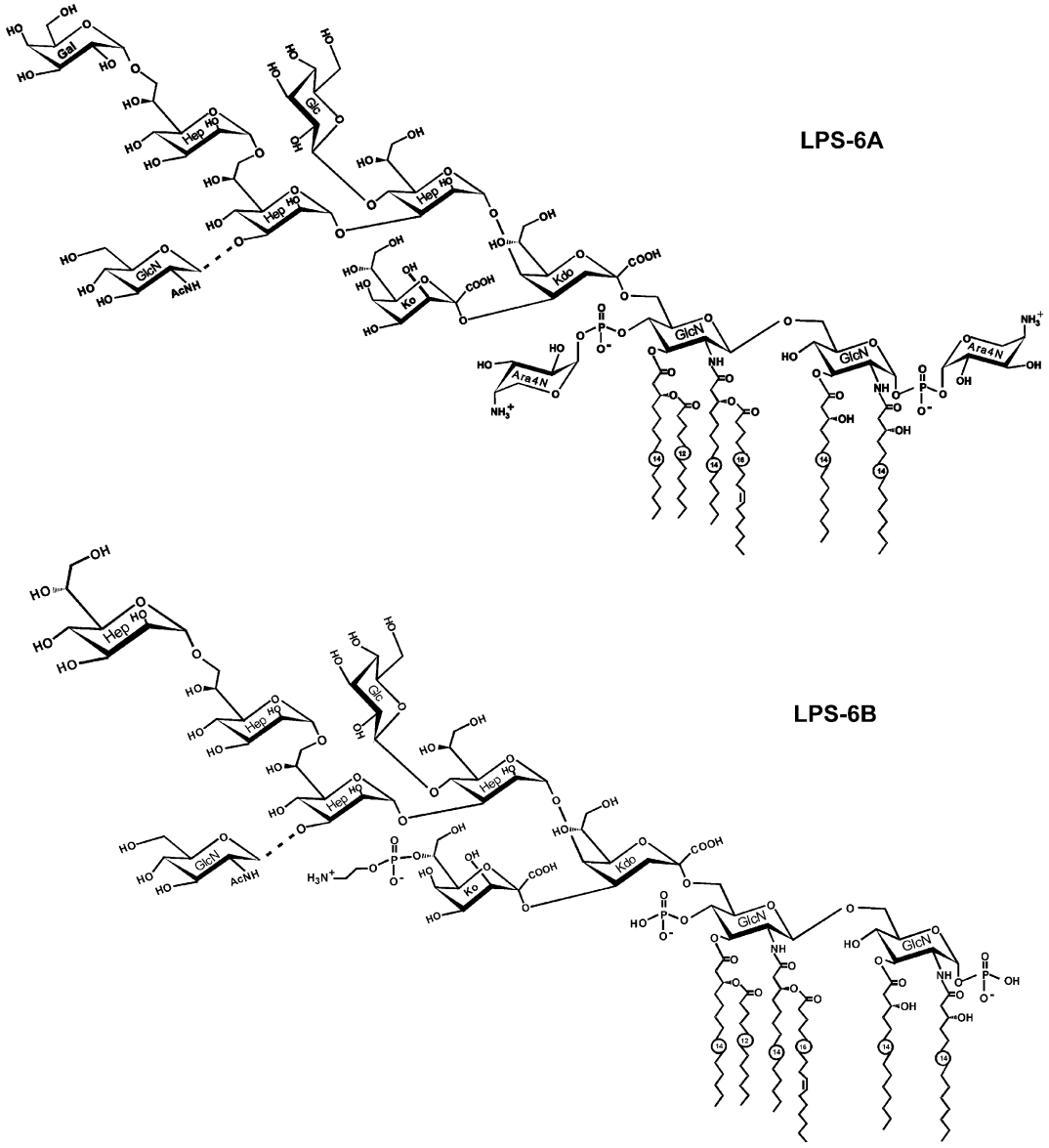

Figure 1. Structures of hexaacyl LPS-6A (LPS-$6A_{hexa}$)[1] and hexaacyl LPS-6B (LPS-$6B_{hexa}$) (this work) from *Y. pestis* KM218 cultivated at 6 °C. Pentaacyl LPS-6A lacks 16:1 and tetraacyl LPS-6A lacks both 16:1 and 12:0; tetraacyl LPS-6B lacks 16:1 and one of the 3-HO14:0 groups. In a minority of molecules of both LPS-6A and LPS-6B, terminal Ko is replaced with terminal Kdo. The dotted line indicates a non-stoichiometric substitution with GlcNAc (~50% in LPS-6A or ~15% in LPS-6B). Some molecules of LPS-6A contain glycine at an unknown position in the core. In lipid A of LPS-6B, an O-linked fatty acid(s) is partially (di)oxygenated.

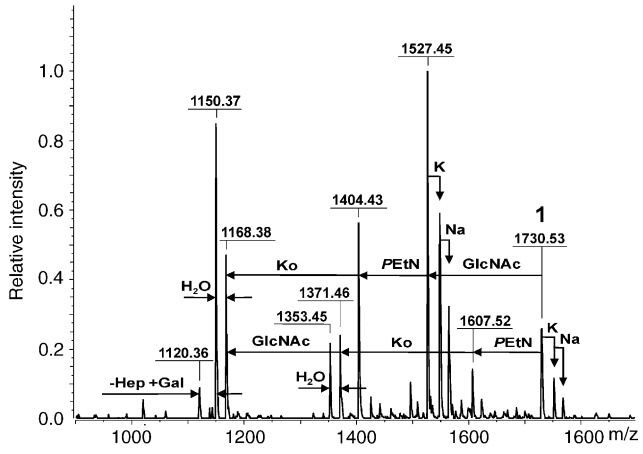

Figure 2. Negative ion ESI FTICR mass spectrum of the core oligosaccharides isolated from LPS-6B. For the structure of compound **1** see Figure 3.

essentially free from LPS-6A. LPS-6B was degraded under mild acid conditions to cleave the linkage between the core and lipid A moieties.

As opposed to the LPS-6A core, ions consistent with the presence of DDHep-containing compounds were abundant in the ESI FTICR mass spectrum of the core oligosaccharide mixture (Fig. 2), whereas ions consistent with Gal-containing oligosaccharides possessed only minor intensities. The spectrum showed structural heterogeneity owing to the presence or absence of GlcNAc ($\Delta m/z$ 203), Ko ($\Delta m/z$ 236), and/or phosphoethanolamine (EtN*P*, $\Delta m/z$ 123). The total content of GlcNAc in the core of LPS-6B was estimated as ~15% (compare ~50% in LPS-6A and ~90% in LPS-25 and LPS-37[1]). In addition, Ko-containing molecules formed K- and Na-salts ($\Delta m/z$ 38 and 22, respectively), and Ko-lacking molecules occurred in both normal and anhydro forms ($\Delta m/z$ 18).

The Ko-containing and Ko-lacking oligosaccharides were separated by HiTrap Q anion-exchange chromatography, the former reduced with borohydride, and the structure of the major *P*EtN-containing compound (**1**) was determined by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy as described.[5,6]

Comparison of the NMR spectra of **1** and the corresponding oligosaccharide isolated earlier from another Ko-containing LPS-25 type[1] showed that both have the identical carbohydrate backbone, the only distinction with **1** is the presence of EtN*P*. After assignment of the spectra (the key NMR chemical shifts are shown in Table 1), the location of EtN*P* at position 7 of terminal Ko was established by a 2D ^{1}H, ^{31}P HMQC experiment, which showed correlations of phosphorus at δ 0.9 with protons of ethanolamine at δ 4.19 (CH_2O) and δ 3.30 (CH_2N) as well as with H-7 of Ko at δ 4.52 (Fig. 3). This conclusion was confirmed by downfield displacements, due to a phosphate deshielding effect, of both H-7 and C-7 signals of EtN*P*–Ko to δ_H 4.52 and

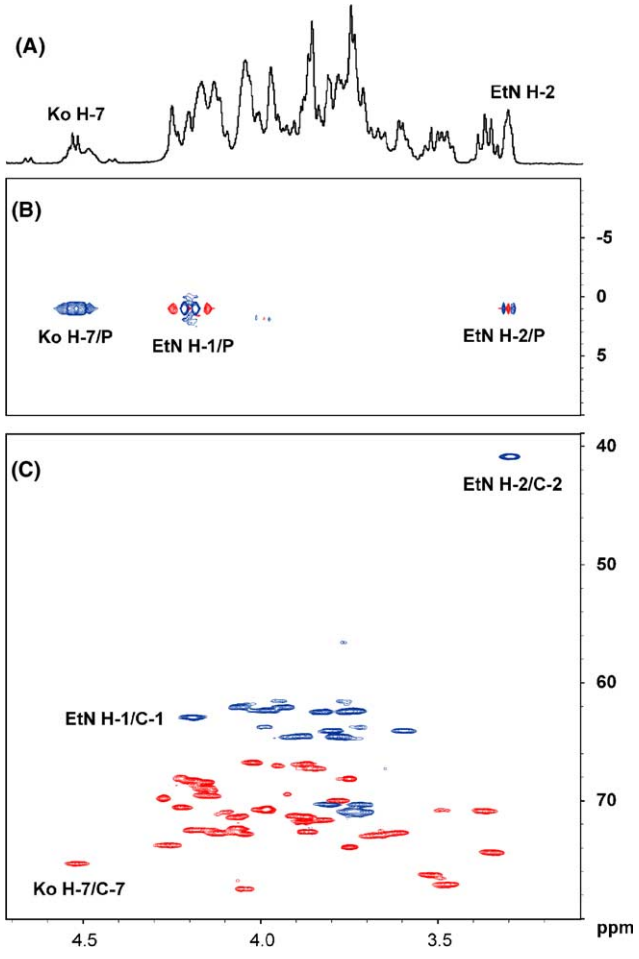

Figure 3. The corresponding parts of ^{1}H NMR (A), 2D ^{1}H,^{31}P HMQC (B), and 2D ^{1}H,^{13}C gHSQC (C) spectra of the Ko-containing oligosaccharides isolated from LPS-6B.

Table 1. NMR chemical shifts of EtN*P*–Ko in oligosaccharide **1** (δ, ppm)

Residue	Nucleus	Atom								
		1	2	3	4	5	6	7	8a	8b
Ko	^{1}H			4.05	4.02	4.15	3.89	4.52	3.96	4.06
	^{13}C			72.8	66.9	68.6	71.7	75.4	62.0	
EtN*P*	^{1}H	4.19	3.30							
	^{13}C	63.0	40.9							
	^{31}P	0.9								

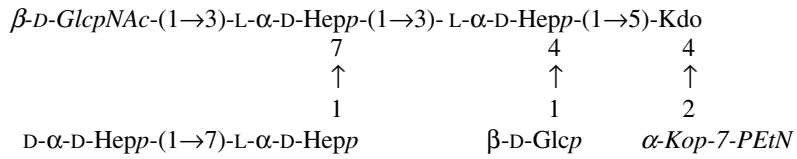

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Figure 4. Structure of the core oligosaccharides isolated from LPS-6B. Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid, D-α-D-Hep and L-α-D-Hep, D-*glycero*- and L-*glycero*-D-*manno* heptose, *P*EtN, phosphoethanolamine. Non-stoichiometric constituents are shown in italics. In a minority of molecules, terminal D-α-D-Hep is replaced with terminal β-D-Gal.

δ_C 75.4 from δ_H 4.11 and δ_C 72.2 in non-phosphorylated Ko (data of the 2D $^1H,^{13}C$ HSQC spectrum). The general structure of **1** shown in Figure 4 was confirmed by determination by ESI FTICR MS of the molecular masses of the LPS-6B-derived core oligosaccharides (Fig. 2), which differed from the calculated molecular masses by <0.03 Da.

The positive ion ESI FTICR mass spectrum of lipid A released by mild acid degradation of LPS-6B (Fig. 5) showed the major ion consistent with bisphosphoryl hexaacyl lipid A (LA-6B$_{hexa}$, *m*/*z* 1925.41) containing four 3-hydroxymyristoyl groups (3-HO14:0) and one group each of lauroyl (12:0) and palmitoleoyl (16:1) groups.[1,7] Less intense was an ion for tetraacyl lipid A (LA-6B$_{tetra}$, *m*/*z* 1517.00) that lacks 12:0 and one of the 3-HO14:0 groups. There were also ions of minor intensity from pentaacyl and triacyl lipid A, which differed from LA-6B$_{hexa}$ and LA-6B$_{tetra}$ in the lack of 12:0 (Δ*m*/*z* −182) and 16:1 (Δ*m*/*z* −236), respectively, as well as monophosphoryl lipid A species resulting from the loss of one of the phosphate group (Δ*m*/*z* −80) during mild acid degradation of LPS-6B. The assignment of the ions was in agreement with fatty acid analysis data of LPS-6B (Table 2). In addition, the spectrum contained ions consistent with the presence of tetraacyl, pentaacyl, and hexaacyl lipid A species having a molecular mass higher by 32 Da than the corresponding counterparts with the defined composition (Fig. 5). These ions could reflect (di)oxygenation of an O-linked fatty acid(s); however, no major hydroxylated fatty acid other than 3-HO14:0 was detected in fatty acid analysis of LPS-6B. Therefore, the major ions at *m*/*z* 1957.35 and 1549.00 must remain unassigned.

Table 2. GLC analysis of O-linked fatty acid composition of LPS-6B

Fatty acid	Retention time (min)	Relative detector response
12:0	8.05	1.00
3-HO12:0 + 14:0	11.99	0.14
$\Delta^2$14:1[a]	12.66	0.42
16:1	15.16	0.60
3-HO14:1	15.33	0.08
16:0	15.55	0.22
3-HO14:0	15.78	1.75
18:1	17.18	Trace
18:0	17.40	Trace

Given are data of the trimethylsilylated methyl esters of fatty acids released by alkaline hydrolysis of the LPS.
[a] From 3-HO14:0 by β-elimination of 12:0 or 16:1.

With the core and lipid A structures characterized, the ESI FTICR mass spectrum of the whole LPS-6B (Fig. 6) could be assigned. An ion at *m*/*z* 3536.76 evidently belonged to hexaacyl LPS-6B with the core terminated

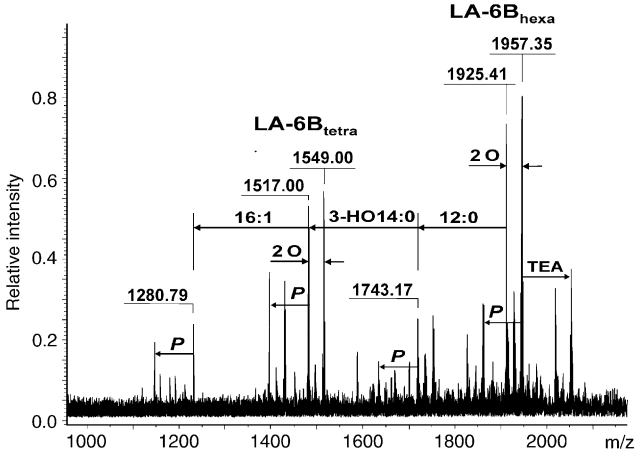

Figure 5. Positive ion ESI FTICR mass spectrum of lipid A isolated from LPS-6B. LA-6B$_{hexa}$ and LA-6B$_{tetra}$ stand for hexaacyl and tetraacyl lipid A. Given are mass numbers of monoadducts with triethylamine (TEA), which was added to improve the signal-to-noise ratio.

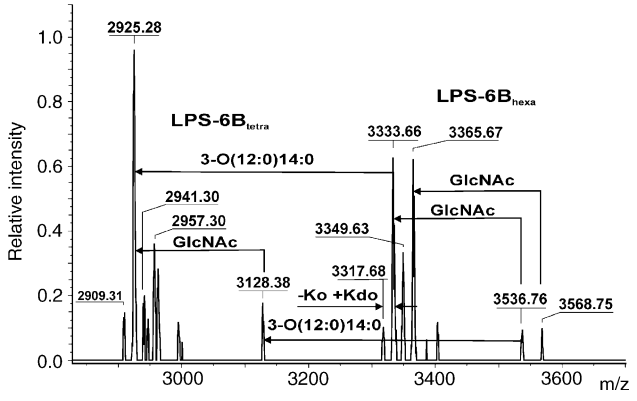

Figure 6. Negative ion ESI FTICR mass spectrum of the purified whole LPS-6B from *Yersinia pestis* KM218 cultivated at 6 °C. LPS-6B$_{hexa}$ and LPS-6B$_{tetra}$ stand for hexaacyl and tetraacyl LPS species. For the structure of LPS-6B$_{hexa}$, see Figure 1B.

with one residue each of DDHep and EtN*P*–Ko as shown in Figure 1. Other compounds differ in the absence of GlcNAc ($\Delta m/z$ −203) and replacement of EtN*P*–Ko with EtN*P*–Kdo ($\Delta m/z$ −16) in the core as well as in the absence of 12:0 and one of the 3-HO14:0 groups from lipid A ($\Delta m/z$ −408). Neither ions for pentaacyl or triacyl lipid A species, nor those for phosphate- or EtN*P*-lacking molecules were observed in the mass spectrum of the whole LPS-6B, and, hence, in the isolated core and lipid A they were artifacts caused by mild acid degradation of the LPS. The absence of ions consistent with the presence of Gal-containing compounds (expected $\Delta m/z$ −30), which were detected as minor species in the isolated core (Fig. 2), could be accounted for by a too low content in intact LPS-6B. Again, there were ions of higher m/z values from dioxygenated compounds ($\Delta m/z$ 32), whose structures could not be determined.

Overall, cold temperature induced significant structural modifications in the LPS structure of *Y. pestis*. While LPS-6A possesses the same structure as the already determined structure of one of the LPS-25 types, LPS-6B is a new *Y. pestis* LPS type. The LPS-6B structure is distinguished by phosphorylation with phosphoethanolamine of terminal Ko or Kdo, whereas no core phosphorylation had been hitherto observed in any *Y. pestis* LPS. Another peculiar feature of LPS-6B is the complete lack of Ara4N in lipid A, which is a major component in other LPS types from strain KM218, including both LPS-25 and LPS-37, as well as in all other *Y. pestis* strains studied by us[1] and others[2,3] earlier. Remarkably, as opposed to LPS-6B, a high degree of substitution of lipid A phosphate groups with Ara4N was also observed in LPS-6A. Furthermore, LPS-6B is distinguished by the absence of glycine, a lower content of GlcNAc and replacement of Gal with DDHep in the core, oxygenation in lipid A and different fatty acid components of the tetraacyl species. These data suggest different mechanisms of control of the synthesis of LPS-6A (LPS-25, LPS-37) and LPS-6B. The latter switches on at cold temperatures, and one can speculate that the production of LPS-6B may be beneficial for the asymptomatic persistence of the bacterium in the host during winter hibernation. Comparative studies of bioactivity of LPS-6, LPS-25, and LPS-37 and structure–activity correlation of the *Y. pestis* LPS will be reported elsewhere.

3. Experimental

3.1. Growth of bacteria, isolation of LPS, and SDS-PAGE

Y. pestis strain KM218, a plasmidless derivative of the Russian vaccine strain EV line NIIEG, was grown at 6 °C in liquid aerated media containing fish-flour hydrolysate and yeast autolysate as described.[1] The lipopolysaccharide (LPS-6) was extracted from dried cells with phenol/$CHCl_3$/light petroleum[8] and purified by enzymatic digestion of nucleic acids and proteins followed by repeated ultracentrifugation (105,000*g*, 4 h). LPS-6B was extracted from the LPS-6 preparation by the Bligh & Dyer procedure as described.[4] SDS-glycine polyacrylamide gel electrophoresis was performed and gels were silver stained as described.[9]

3.2. Mild acid degradation of LPS

LPS-6 was degraded with aq 2% AcOH at 100 °C for 4 h, the water-insoluble crude lipid A precipitate (LA-6) was separated by centrifugation (13,000*g*, 15 min), washed with water, suspended in water, lyophilized, and the solid preparations were purified away from phospholipid contaminations by treatment with 1:1 $CHCl_3$–MeOH.

The water-soluble supernatant was fractionated by GPC on a column (70 × 2.6 cm) of Sephadex G50 (S) (Amersham Biosciences, Sweden) using pyridinium acetate buffer (4 mL pyridine and 10 mL AcOH in 1 L water) as eluant and monitoring with a differential refractometer (Knauer, Germany), followed by anion-exchange chromatography on a HiTrap Q column (5 mL; Amersham Biosciences, Sweden) using water to elute neutral contaminants (10 min) and then a 0 → 1 M gradient of NaCl in water over 50 min to give two acidic fractions, which were desalted by GPC on Sephadex G-15. For NMR spectroscopic studies, the fraction eluted last was reduced with $NaBH_4$ in water (20 °C, 2 h).

3.3. Fatty acid analysis

For O-linked fatty acid analysis, LPS-6 was saponified with 1 M NaOH (85 °C, 20 min), and then neutralized with 1 M HCl. Fatty acids were extracted with $CHCl_3$, methylated with diazomethane, trimethylsilylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, and analyzed by GLC–MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5 ms column (Hewlett-Packard) using a temperature gradient of 150 °C (3 min) → 320 °C at 5 °C min^{-1}.

3.4. NMR spectroscopy

Prior to measurements, samples were exchanged twice with D_2O. NMR spectra were recorded on a Varian Inova 500 spectrometer in D_2O solns at 25 °C with acetone as an internal standard (δ_H 2.225, δ_C 31.5 ppm). Standard pulse sequences were used in 2D NMR experiments, including COSY, TOCSY (mixing time 120 ms), NOESY (400 ms), ^1H,^{13}C gHSQC, ^1H,^{13}C HMQC-TOCSY (100 ms), HMBC (100 ms), and ^1H,^{31}P HMQC

(optimized for $J_{P,H}$ 11 Hz). Spectra were assigned using the computer program Pronto.[10]

3.5. Mass spectrometry

High-resolution ESI FTICR MS was performed on an ApexII-instrument (Bruker Daltonics, Billerica, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. For negative ion MS, samples ($\sim$10 ng μL^{-1}) were dissolved in 50:50:0.001 2-propanol–water–Et_3N (TEA) and sprayed at a flow rate of 2 $\mu L\ min^{-1}$. When the whole LPS and lipid A were studied, TEA was added stepwise not to exceed pH 9 to avoid cleavage of O-linked fatty acids. For positive ion MS, a sample solution in 50:50:0.001 2-propanol-1 mM $AcONH_4$-TEA was adjusted to pH 4.5 with conc AcOH. Capillary entrance voltage was set to 3.8 kV and dry gas temperature to 150 °C. The spectra were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses.

Acknowledgments

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