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Note

Identification of an α-D-Man*p*-(1 → 8)-Kdo disaccharide in the inner core region and the structure of the complete core region of the *Legionella pneumophila* serogroup 1 lipopolysaccharide

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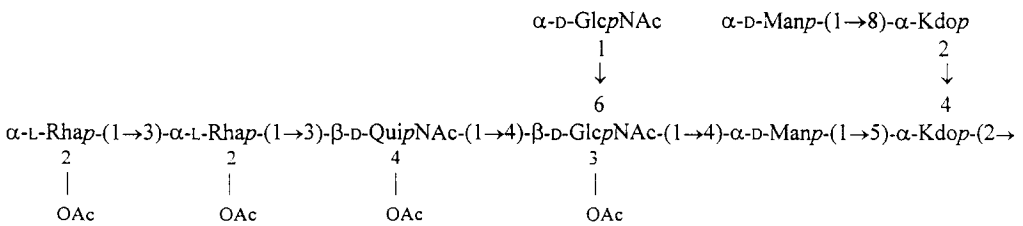
Abstract

A disaccharide α-D-mannopyranosyl-(1 → 8)-3-deoxy-D-*manno*-octulosonic acid [α-D-Man*p*-(1 → 8)-Kdo] was released by mild acid degradation of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1) lipopolysaccharide (LPS) and identified using NMR spectroscopy and GLC–MS of derived products. These data, together with methylation analysis of the native LPS and previously reported data [Y.A. Knirel, H. Moll, and U. Zähringer, *Carbohydr. Res.*, 293 (1996) 223–234], allowed elucidation of the complete core region of the LPS as having the following nonasaccharide structure:



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A facultative intracellular human pathogen *Legionella pneumophila* is the causative factor of legionellosis, a severe respiratory disease in susceptible individuals [1]. Outer membrane lipopolysaccharide (LPS, endotoxin, O-antigen) plays an important role in infections due to enterobacteria [2], and it is likely that LPS contributes to the virulence of *Legionella* as well. LPS is also a serogroup-specific antigen used for serological classification of bacterial strains, including *L. pneumophila* [3,4].

In our chemical and immunochemical studies of *Legionella* LPS, we have determined the structure of the O-specific polysaccharide chain of LPS of *L. pneumophila* serogroup 1 (strain Philadelphia 1) as an α-2,4-linked homopolymer of a legionaminic acid derivative, 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid [5–7]. In addition to the O-chain polysaccharide with variable number of legionaminic acid residues, mild acid hydrolysis of LPS resulted in heptasaccharide **1** with multiple *O*-acetyl groups, which was devoid of legionaminic acid and, thus, represented a partial structure of the core oligosaccharide [8] (see structure below, where QuiNAc is 2-acetamido-2,6-dideoxyglucose and Kdo is 3-deoxy-D-*manno*-octulosonic acid). The O-chain polysaccharide was found to be attached to the terminal rhamnose residue at position 3 [8].

Besides that, mild acid degradation released from LPS two low-molecular-mass products: a 5,7-diacetamido-8-*O*-acetyl-3,5,7,9-tetradeoxynonulosonic acid [9] and a Man → Kdo disaccharide. In this paper we report on the identification of this disaccharide and suggest the structure of the complete core region of *L. pneumophila* serogroup 1 LPS.

LPS was isolated as described [2], degraded with sodium acetate buffer (pH 4.4) and, after deionization of the carbohydrate portion, a low-molecular-mass fraction was isolated by GPC on Sephadex G-50. ^{1}H and ^{13}C NMR spectroscopic studies showed that this was a mixture of a 5,7-diacetamido-8-*O*-acetyl-3,5,7,9-tetradeoxynonulosonic acid [9] and a Man-(1 → 8)-Kdo disaccharide with Kdo present in multiple forms, in accordance with the published data [10], the predominant form being α-pyranose (see disaccharide **2** below).

Sugar analysis revealed mannose as alditol acetate and Kdo as a mixture of aldonic acid methyl ester and lactone acetates. GLC–MS analysis of fully methylated derivatives indicated the presence of a methylated Man → Kdo disaccharide which was characterized by a quasimolecular ion $[M + NH_4]^+$ at *m/z* 544 in CI MS and typical fragmentation in EI MS with formation of ions at *m/z* 467 ($[M - COOMe]^+$), 219 (A-series fragment for Man), 291 and 351 (D-series fragments for Kdo) [11]. Methylation of the carbonyl and carboxyl reduced disaccharide resulted in a mixture of two permethylated mannosyl → 3-deoxyoctitol stereoisomers which displayed a peak of $[M + NH_4]^+$ at *m/z* 546 in CI MS and peaks of fragment ions at *m/z* 219 (for Man), 293 and 353 (for 3-deoxyoctitol) in EI MS. After methanolysis and acetylation, isomers of 8-*O*-acetyl-3-deoxy-1,2,4,5,6,7-penta-*O*-methyloctitol were identified and, hence, the Kdo residue in the disaccharide is 8-substituted.

Disaccharide **2** was purified from the mixture using anion-exchange HPLC at high pH. The ^{1}H NMR spectrum showed that a number of minor disaccharides with different forms of Kdo were still present, most likely, due to mutarotation. The spectrum of disaccharide **2** was assigned using a COSY experiment and spin systems for Man*p* and Kdo*p* were identified based on the coupling constant values (Ta-

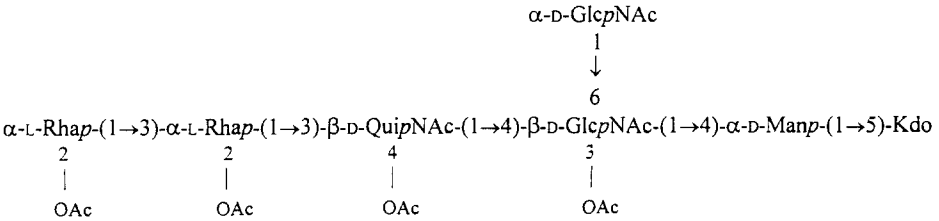

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Table 1
^{1}H NMR data for disaccharide **2** (δ, ppm; J, Hz)

Sugar residue	Proton							
	H-1	H-2	H-3eq H-3ax	H-4	H-5	H-6a H-6b	H-7	H-8a H-8b
α-D-Man$p$(1 →	4.86	3.97	3.84	3.64	3.70	3.84 3.71		
	$J_{1,2}$ 1.8	$J_{2,3}$ 3.5	$J_{3,4}$ 9.6	$J_{4,5}$ 10	$J_{5,6a}$ 2.7	$J_{5,6b}$ 5 $J_{6a,6b}$ 11		
→ 8)-α-Kdo$p$			1.93 2.05	4.09	4.05	3.93	4.03	3.82 3.65
			$J_{3eq,4}$ 5 $J_{3eq,3ax}$ 12.7	$J_{3ax,4}$ 12	$J_{4,5}$ 3.0	$J_{5,6}$ 0.9	$J_{6,7}$ 9.5 $J_{7,8a}$ 4.5	$J_{7,8b}$ 2.5 $J_{8a,8b}$ 11

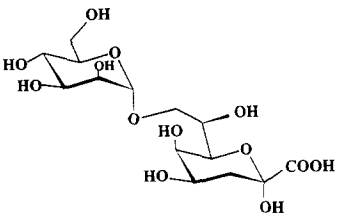
ble 1). The chemical shifts for Man in **2** were close to those in α-D-mannopyranose [12] and different from the chemical shifts in the corresponding β anomer, and, hence, Man is α-linked (e.g., cf. the chemical shifts for H-3 and H-5 of Man at δ 3.84 and 3.70 in **2**, at δ 3.86 and 3.82 in α-mannopyranose, but at δ 3.66 and 3.38 in β-mannopyranose [12], respectively). Similar comparison of the chemical shift and coupling constant values allowed the conclusion that Kdo in **2** is in the α-pyranose form (e.g., cf. the chemical shifts for H-3ax and H-3eq of Kdo at δ 1.93 and 2.05 in **2**, and at δ 1.88 and 1.99 in 3-deoxy-α-D-*manno*-octulopyranosonic acid, respectively, but at δ 2.08, 2.30, 2.38, and 2.60 in the two furanose forms [10]).

Based on the ^{1}H NMR spectrum, the ^{13}C NMR spectrum of disaccharide **2** was assigned using an ^{1}H,^{13}C HMQC experiment (Table 2). Comparison of the ^{13}C chemical shifts with published data [10,12,13] confirmed the α-pyranose form of both Man and Kdo, and the terminal non-reducing position of the former. Attachment of Man to Kdo at position 8 followed from characteristic displacements of the signals for C-7 and C-8 of Kdo from δ 70.32 and 63.97 in the unsubstituted monosaccharide [10] to δ 68.7 and 69.2 in **2**, respectively, while chemical shifts for the other carbon signals were significantly the same.

Table 2
^{13}C NMR data for disaccharide **2** (δ, ppm)

Sugar residue	Carbon							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Disaccharide **2**								
α-D-Man$p$-(1 →	100.5	71.1	71.7	68.0	73.6	62.1		
→ 8)-α-Kdo$p$	174.8	96.8	34.5	67.2	66.8	72.1	68.4	68.9

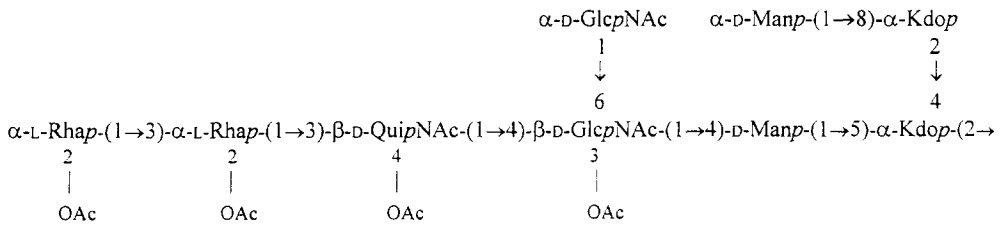
These data indicated that disaccharide **2** is α-D-mannopyranosyl-(1 → 8)-3-deoxy-α-D-*manno*-octulopyranosonic acid [α-D-Man$p$-(1 → 8)-α-Kdo].



2

In addition to partially methylated Rha, Man, QuiNAc, and GlcNAc derived from the outer core region (see structure **1**), methylation analysis of LPS revealed terminal Man, 8-substituted Kdo, 4,5-disubstituted Kdo, and no terminal Kdo. Mild methanolysis of the methylated LPS afforded a fully methylated Man → Kdo disaccharide identical by GLC–MS data to that obtained from disaccharide **2** (see above).

Therefore, taking together the data obtained in this work and the structure **1** elucidated previously [8], it could be concluded that the core region of the *L. pneumophila* serogroup 1 LPS has the structure **3**. The reducing Kdo residue of the core is attached to position 6′ of the 2,3-diamino-2,3-dideoxy-D-glucose disaccharide lipid A backbone [14]. The configurations of the two Kdo residues were not confirmed and are shown in the formula as α as it was established in all other studied bacterial LPSs containing the Kdo-(2 → 4)-Kdo disaccharide [15–18] (see structure below).



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Although substitution of the lateral Kdo residues in LPS is known with a number of various sugars and phosphate substituents, including substitution at position 8 [15], the occurrence of a Man*p*-(1 → 8)-Kdo disaccharide has not been reported hitherto. Together with an isomeric disaccharide, Man*p*-(1 → 5)-Kdo, it constitutes the inner, hydrophilic region of the LPS core. In contrast, the outer region of the core is enriched with 6-deoxy sugars and *N*- and *O*-acetylated sugars and, together with a highly *N*- and *O*-acylated O-chain [5], confers to a high hydrophobicity of LPS and of the bacterial surface as a whole. While the presence of a hydrophilic, negatively charged inner region in LPS is important to maintain the integrity of the outer membrane, the hydrophobic outer region supports the outspread of *L. pneumophila* (e.g., its concentration in aerosols) and the adherence to alveolar macrophages at the early step of pulmonary infection and, thus, may be considered as a factor of virulence of *L. pneumophila* [14].

1. Experimental

Growth of bacterium and isolation of LPS.—Bacterial strain Philadelphia 1 of *L. pneumophila* (ATCC 33152, serogroup 1) was grown on buffered charcoal yeast agar [19]. LPS was isolated from dried cells by a modified phenol–chloroform–petroleum ether procedure [20] using diethyl ether instead of water as solvent for precipitating LPS [5].

Isolation of disaccharide **2**.—LPS was degraded with 0.1 M NaOAc–HOAc buffer (pH 4.4) at 100 °C for 6 h. After centrifugation, deionization with an IR-120 (H^+) cation-exchange resin, and lyophilization, the carbohydrate portion was fractionated by GPC on Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer (pH 4.5) monitored with a Knauer differential refractometer. The fraction eluted last was treated with aq 12% NH_4OH (50 °C, 2 h) and fractionated by anion-exchange HPLC on a preparative column of CarboPac PA-1 at 4 mL/min in a gradient of NaOAc (0 → 0.1 M) in 0.1 M NaOH for 50 min with monitoring using a pulse amperometric detector (Dionex, USA). Deionization of the main fraction with a Dowex 50W-X8 (H^+) cation-exchange resin afforded disaccharide **2**.

Sugar analysis.—Hydrolysis was performed with 2 M CF_3COOH (4 h, 100 °C), the products were reduced with $NaBH_4$, esterified with 0.5 M HCl–MeOH (5 min at room temperature followed by evaporation thrice with the same solution), acetylated with Ac_2O in pyridine, and analyzed by GLC–MS on a Hewlett–Packard 5989A instrument equipped with an HP-5 column using a temperature gradient of 150 °C (3 min) → 320 °C at 5 °C/min. The D-configuration of mannose was confirmed by GLC of acetylated (*R*)-2-octyl glycosides [21].

Methylation analysis.—Methylation was performed with MeI in Me_2SO in the presence of solid NaOH [22] and followed by GLC–MS analysis without cleavage of the disaccharide. Alternatively, the disaccharide was reduced with $NaBH_4$, esterified with 0.5 M HCl–MeOH at room temperature, reduced again with $NaBH_4$, methylated [22], methanolyzed with 2 M HCl–MeOH (4 h, 85 °C), and acetylated. LPS was methylated [22] and methanolyzed with 2 M HCl–MeOH (45 min, 85 °C) or hydrolyzed with 2 M CF_3COOH (2 h, 100 °C), reduced with $NaBH_4$, esterified with 0.5 M HCl–MeOH, reduced again with $NaBH_4$, and acetylated.

NMR spectroscopy.—One-dimensional ^1H NMR and two-dimensional spectra were recorded with a Bruker DRX-600 spectrometer and ^{13}C NMR spectra with a Bruker AMX-360 spectrometer for solutions in D_2O at 305 K using internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) or acetone (δ_C 31.45) as references. Two-dimensional NMR experiments were performed using standard Bruker software.

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