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Structural study of a highly *O*-acetylated core of *Legionella pneumophila* serogroup 1 lipopolysaccharide

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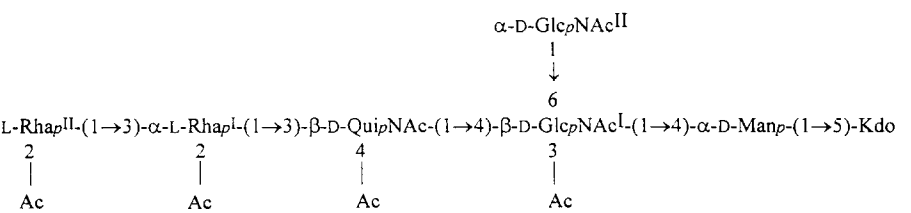
Abstract

A core oligosaccharide was obtained after mild acid degradation of *Legionella pneumophila* serogroup 1 lipopolysaccharide (LPS). On the basis of chemical, GLC-MS, ^{1}H, and ^{13}C NMR spectroscopic data, it was found that the oligosaccharide obtained is a highly *O*-acetylated heptasaccharide having the following structure:



where Kdo is 3-deoxy-D-*manno*-octulosonic acid and QuiNAc is 2-acetamido-2,6-dideoxyglucose. In the LPS, the O-specific polysaccharide chain is linked to position 3 of the terminal rhamnosyl group and is cleaved during degradation of the LPS. The degradation also induced partial migration and partial removal of the *O*-acetyl group from the terminal rhamnosyl group which, together with the occurrence of the reducing Kdo residue in multiple forms, contributes to the

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heterogeneity of the isolated core oligosaccharide. No such highly *O*-acetylated core oligosaccharide has been reported so far for LPS of Gram-negative bacteria. © 1996 Elsevier Science Ltd.

Keywords: Lipopolysaccharide, degradation; Core oligosaccharide, structure; *O*-Acetyl groups; *Legionella pneumophila*

1. Introduction

Legionella pneumophila is a facultative intracellular human pathogen causing severe respiratory infection in humans [1]. Data on the lipopolysaccharide (LPS) of this microorganism, which may play an important role in the infection, are scanty (see ref. [2] and refs. cited therein). Fatty acid [3] and sugar composition [4] have been reported for the LPS characteristic for the *L. pneumophila* serogroup 1, which is most frequently isolated from both environmental and clinical specimens [5,6]. Recently, the structure of the O-specific polysaccharide chain of this LPS has been elucidated as a homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid (legionaminic acid) [2], the configuration of which was revised later in favour of the L-*glycero*-D-*galacto* isomer [7,8].

Here we describe a structural study of the core region of *L. pneumophila* serogroup 1 LPS. Preliminary results have been reported elsewhere [9].

2. Results

The LPS was isolated as described previously [2]. In SDS-PAGE (Fig. 1), the LPS revealed several intense zones of ladder-like bands of S-type molecules containing a polysaccharide chain attached to the core–lipid A region. A characteristic narrow distance between the individual bands reflects a small size of the monosaccharide repeating unit in the polysaccharide chain. No faster moving bands which might belong to R-type molecules lacking the polysaccharide chain were detected, thus suggesting the content of the LPS molecules with an unsubstituted core to be below the SDS-PAGE sensitivity.

Degradation of the LPS with sodium acetate buffer (pH 4.4) resulted in cleavage of the lipid moiety. GPC of a water-soluble portion on Sephadex G-50 yielded five fractions (Fig. 2). GLC analysis of acetylated methyl glycosides showed that each of fractions 1–4 contained mannose, rhamnose, GlcN, 2-amino-2,6-dideoxyglucose (QuiN), and 3-deoxy-D-*manno*-octulosonic acid (Kdo) in similar ratios. The same sugars and, in addition, 2,3-diamino-2,3-dideoxyglucose, which is a constituent of lipid A backbone [9], have been found in the LPS of *L. pneumophila* [4]. None of fractions 1–4 contains phosphate and, hence, like the O-polysaccharide chain [2], the core of the LPS is not phosphorylated.

The ^{1}H NMR spectra of fractions 1–3 showed the presence of O-polysaccharide chains of a legionaminic acid homopolymer having different length [2], while no legionaminic acid was found in fraction 4, which appeared to be an unsubstituted core oligosaccharide. The amount of fraction 4 varied depending on the duration of LPS

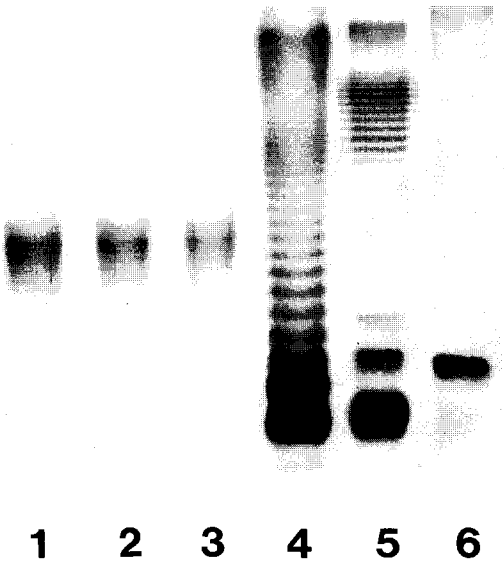


Fig. 1. SDS-PAGE (14%) of *L. pneumophila* serogroup 1 LPS (lanes 1–3 with 1.0, 0.75, and 0.5 μg LPS applied, respectively). S-type LPS of *Pseudomonas aeruginosa* immunotype 2 (lane 4) and *Escherichia coli* O111 (lane 5) and SR-type LPS of *Salmonella enterica* sv. Typhimurium mutant SH777 (lane 6) were applied for comparison.

hydrolysis from a negligible amount within 1 h to 1% of the LPS weight within 4 h. Accordingly, each of fractions 1–3 afforded fraction 4 on further hydrolysis under the same conditions. These and SDS-PAGE data suggested that fraction 4 was derived by the cleavage of the polysaccharide chain from the core rather than corresponding to the occurrence of the unsubstituted core in the native LPS.

In the low molecular weight fraction 5, mainly Kdo was detected, together with trace amounts of Man, GlcN, and phosphate.

The ^{13}C NMR spectrum of fraction 4 contained a number of signals for *O*-acetyl groups at δ 21.2–21.9 (CH_3) indicating that it was a mixture of partially *O*-acetylated related oligosaccharides (OS). This was treated with aq ammonia and the resulting *O*-deacetylated core (OS_{NH_4OH}) was reduced with sodium borohydride to give a reduced *O*-deacetylated core (OS_{NaBH_4}). Both OS_{NH_4OH} and OS_{NaBH_4} had the same amino and neutral sugar composition as OS and, hence, as expected, the reducing end of the core was occupied by a Kdo residue. The Rha:Man:QuiN:GlcN ratios determined for OS_{NH_4OH} by GLC of alditol acetates were 2.1:1.1:1:1.4, and the QuiN:GlcN ratio determined using an amino acid analyser was 1:2.

The ^1H NMR spectrum of OS_{NH_4OH} contained, *inter alia*, signals for three *N*-acetyl groups at δ 2.05 (s, 3 H) and 2.08 (2 s, 6 H) and three CH_3 groups of 6-deoxy sugars at δ 1.25, 1.30, and 1.34 (all d, each 3 H, $J_{5,6}$ 6.0–6.5 Hz, H-6). Three of the H-1 signals present in the spectrum belonged to sugars with the *gluco* configuration (QuiNAc and two GlcNAc residues), one of which was α-linked (δ 4.90, d, $J_{1,2}$ 4 Hz) and two others were β-linked (δ 4.58 and 4.62–4.63, both d, $J_{1,2}$ 8.8 Hz) (Fig. 3). The splitting of the

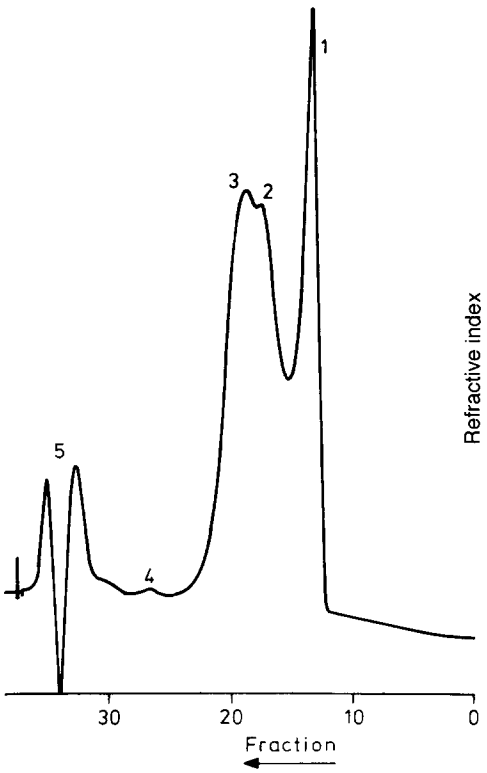


Fig. 2. Profile of elution from Sephadex G-50 of carbohydrate portion of *L. pneumophila* serogroup 1 LPS after degradation with sodium acetate buffer (pH 4.4). Fractions 1–3 correspond to the core substituted with longer and shorter chain polysaccharides, fraction 4 corresponds to the unsubstituted core (OS), and fraction 5 contains monosaccharides, lower oligosaccharides, and phosphate.

H-1 signal at δ 4.62–4.63 was most likely due to a structural heterogeneity (see below). Two more H-1 signals at δ 4.85 and 5.02 (both d, $J_{1,2}$ 1.5–2 Hz) and a number (> 6) of less intense signals at δ 4.99–5.21 (all d, $J_{1,2}$ 2–2.2 Hz) belonged to sugars with the *manno* configuration (Man and two Rha residues). Such splitting of the H-1 signal of one of the sugars may be accounted for by its attachment to Kdo, which is known to occur at the reducing end in multiple, mainly anhydro forms as artefacts after mild acid hydrolysis [8,10,11]. For the same reason, signals for the CH_2 group of Kdo were not clearly seen in the spectrum.

The ^{13}C NMR spectrum of OS_{NH_4OH} contained, *inter alia*, signals for three *N*-acetyl groups [CH_3 at δ 23.2 and 23.4 (2 C); CO at δ 175.0, 175.5, and 175.7], three CH_3 groups (C-6) of 6-deoxy sugars at δ 17.6, 17.8, and 17.9, and three carbons bearing nitrogen at δ 54.9, 56.3, and 56.6. There were also C-1 signals of five sugar residues at δ 99.0, 101.8, 102.5 (2 C), and 103.6, whereas that for the sixth sugar (Man attached to Kdo) was split to a number of components not clearly recognisable in the spectrum (Fig. 3).

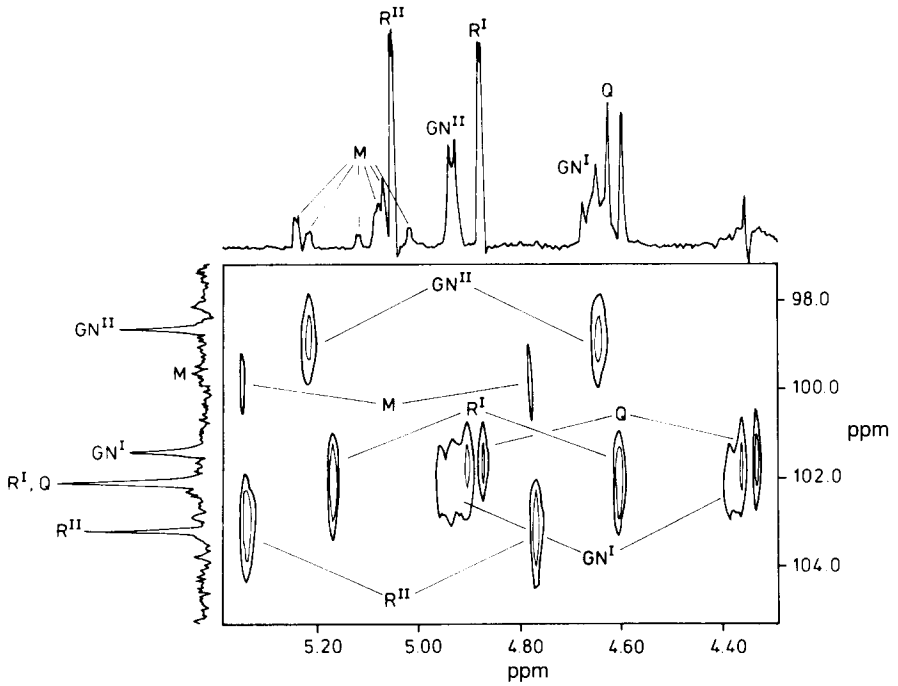


Fig. 3. Part of a 300-MHz 2D H-detected $^{1}H,^{13}C$ HMQC spectrum of *O*-deacetylated core oligosaccharide (OS_{NH_4OH}) derived from *L. pneumophila* serogroup 1 LPS. The corresponding parts of 1D ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Of multiple Man H-1 cross-peaks, only one, at 5.04/100.1, is present on the slice shown. Designations: GN, GlcNAc; M, Man; R, Rha; Q, QuiNAc.

These data suggested that OS_{NH_4OH} is a heptasaccharide containing one residue each of mannose, QuiNAc, and Kdo, and two residues each of rhamnose and GlcNAc. GLC of acetylated glycosides with optically active secondary alcohols [12,13] showed that mannose and both amino sugars are D and rhamnose is L.

Together with the monosaccharide methyl glycosides, mild methanolysis of OS_{NH_4OH} afforded methyl glycosides of three disaccharides, which were analysed by GLC-MS as peracetylated derivatives, using published data [14]. Two of them had molecular weights 649 and 648 a.m.u. (CI-MS data) and exhibited in EI-MS the same ion with *m/z* 330 corresponding to the nonreducing end sugar and different ions with *m/z* 303 and 302 for the reducing end sugar. Thus, they were assigned as per-*O*-acetylated HexNAc → Hex → OMe and HexNAc → HexNAc → OMe, respectively. The third disaccharide derivative was per-*O*-acetylated Hex → Kdo → OMe present in three isomeric forms, each of which had molecular weight 722 a.m.u. and exhibited ions with *m/z* 331 and 375 for the nonreducing and reducing end sugars, respectively. Taking into account the given sugar composition, these disaccharide glycosides could be identified as GlcNAc → GlcNAc → OMe, GlcNAc → Man → OMe, and Man → Kdo → OMe, and, thus, the following sequence could be deduced: GlcNAc → GlcNAc → Man → Kdo.

Methylation analysis of OS_{NaBH_4} resulted in identification of 2,3,4-tri-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylmannose, and 2-amino-2,6-dideoxy-2,4-di-*N*,*O*-methylglucose, which were derived by hydrolysis with 2 M trifluoroacetic acid and identified by GLC-MS as alditol acetates in the ratios 1:0.9:0.5:0.7. In addition, methyl 5-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctonate was identified in trace amounts. Similar analysis of carboxyl-reduced OS_{NaBH_4} resulted in the same partially methylated derivatives and 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyloctitol in the ratios 1:0.8:0.4:0.8:0.3, together with small amounts of partially methylated disaccharide derivatives corresponding to Man → Kdo and Rha → Rha fragments. No GlcN derivative was detected upon this hydrolytic procedure. When hydrolysis conditions were changed to acetolysis according to Stellner et al. [15], almost equal amounts of 2,3,6-tri-*O*-methylmannose, 2-amino-2,6-dideoxy-2,4-di-*N*,*O*-methylglucose, 2-amino-2-deoxy-2,3,4,6-tetra-*N*,*O*-methylglucose, and 2-amino-2-deoxy-2,3-di-*N*,*O*-methylglucose, with rhamnose derivatives being practically absent from the mixture.

Therefore, OS_{NH_4OH} is branched with Rha and GlcNAc at the terminal nonreducing ends; the second GlcNAc residue is at the branching point and substituted at positions 4 and 6, the second Rha residue and QuiNAc are 3-substituted, Man is 4-substituted, and Kdo 5-substituted. These data and the identification of a number of disaccharides obtained in mild methanolysis and methylation analysis suggested the following sugar sequences in OS_{NH_4OH}:

GlcNAc
↓
Rha→Rha→QuiNAc→GlcNAc→Man→Kdo

In the ^1H NMR spectrum of OS_{NH_4OH}, all signals for QuiNAc, both Rha residues (Rha^I and Rha^{II}), and both GlcNAc residues ($GlcNAc^I$ and $GlcNAc^{II}$) were assigned using 2D COSY and COSY with one- and two-step relayed coherence transfer (Table 1). The positions of multiple signals for H-1,2,3,4 of Man were found as well.

With the ^1H NMR spectrum assigned, the signals for the anomeric carbons in the ^{13}C NMR spectrum of OS_{NH_4OH} were assigned using a 2D H-detected $^1H,^{13}C$ HMQC experiment (Fig. 3). Relatively large $^1J_{H\text{-}1,C\text{-}1}$ coupling constant values, 169–171 Hz, determined from the nondecoupled HMQC spectrum for $GlcNAc^{II}$, Man, Rha^I, and Rha^{II} (correlation peaks at δ_H/δ_C 4.90/99.1, 5.04/100.1, 4.85/102.5, and 5.02/103.6, respectively) showed these sugar residues to be α-linked, whereas relatively small $^1J_{C\text{-}1,H\text{-}1}$ values of 165 Hz for QuiNAc and $GlcNAc^I$ (δ_H/δ_C 4.58/101.8 and 4.62/102.5, respectively) indicated their β linkage [16]. Carbons C-6 of $GlcNAc^I$ and $GlcNAc^{II}$ resonated at δ 67.6–67.7 and 61.8, respectively; hence, the former is 6-substituted [17] and thus located at the branching point, whereas the latter is the terminal sugar residue in the side chain.

Attachment of $GlcNAc^{II}$ at position 6 of $GlcNAc^I$ was demonstrated by the appearance of NOE signals on H-6 protons of the latter at δ 3.70 and 3.83 as a result of selective preirradiation of H-1 of the former at δ 4.90. The linkage and sequence patterns of the 6-deoxy sugar region of OS_{NH_4OH} were confirmed by similar selective

1D NOE experiments, which revealed the interresidue NOE contacts Rha^{II} H-1,Rha^{I} H-3 at δ 5.02/3.78 and Rha^{I} H-1,QuiNAc H-3 at δ 4.85/3.61. No unambiguous conclusion could be made from experiments with preirradiation of QuiNAc H-1 and GlcNAc^{I} H-1 because of signal coincidences.

The data obtained suggested that OS_{NH_4OH} has the structure **1**.

α-D-Glc*p*NAc^{II}
1
↓
6
α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Qui*p*NAc-(1→4)-β-D-Glc*p*NAc^{I}-(1→4)-α-D-Man*p*-(1→5)-Kdo

1 (OS_{NH_4OH})

The substitution pattern of the *O*-acetyl groups was revealed by detailed NMR analysis of the initial fraction 4 oligosaccharide (OS). The ^{1}H NMR spectrum of OS was assigned using 2D COSY and relayed COSY. The following protons were found to shift downfield because of a strong deshielding effect of *O*-acetyl groups, as compared with their position in the spectrum of OS_{NH_4OH}: Rha^{I} H-2 (δ 4.97 and 4.99 versus 3.87), QuiNAc H-4 (δ 4.71 versus 3.32), and GlcNAc^{I} H-3 (δ 5.17–5.18 versus 3.74). Thus, three of the *O*-acetyl groups are located at position 2 of Rha^{I}, position 4 of QuiNAc, and position 3 of GlcNAc^{I}. The positions of the signals for Man and GlcNAc^{II} were almost the same in both spectra, and, hence, these residues are not *O*-acetylated. As for Rha^{II}, this was present in three *O*-acetylated forms with the signals for H-2 (major), H-3, and H-4 shifted downfield (δ 5.15, 4.90, and 4.84 versus 4.06, 3.83, and 3.42, respectively) and a non-*O*-acetylated form in the ratios 1:0.5:0.3:0.2. The presence of an *O*-acetyl group at position 2 in the major form of Rha^{II} and its absence from the three other forms seem to account for the splitting of the neighbouring Rha^{I} H-1 signal (δ 4.97 and 4.99).

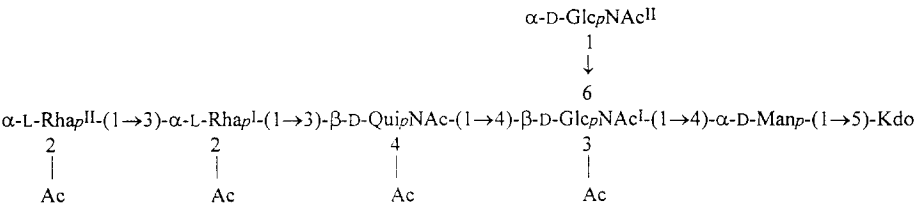
Table 1
360-MHz ^{1}H NMR chemical shifts for OS_{NH_4OH} (δ in ppm related to internal acetone, δ_H 2.225) [a]

	H-1	H-2	H-3	H-4	H-5	H-6
α-L-Rha *p*^{II}-(1 →	5.02	4.06	3.83	3.42	3.77	1.30
→ 3)-α-L-Rha *p*^{I}-(1 →	4.85	3.87	3.78	3.54	4.01	1.25
→ 3)-β-D-Qui *p*NAc-(1 →	4.58	3.85	3.61	3.32	3.54	1.34
α-D-Glc *p*NAc^{II}-(1 →	4.90	3.94	3.86	3.50	3.73	3.81, 3.92
→ 4)-β-D-Glc *p*NAc^{I}-(1 →	4.62–4.63	3.76	3.74	3.62	3.76	3.70, 3.83
6 ↑						
→ 4)-α-D-Man *p*-(1 →	4.99–5.21	4.02–4.13	3.97–4.02	3.75–3.80		

[a] Chemical shifts for the *N*-acetyl groups: δ 2.05 (3 H) and 2.08 (6 H).

Since position 3 of Rha^{II} is the site of attachment of the polysaccharide chain (see below), it is suggested that in the LPS Rha^{II} is *O*-acetylated at position 2 and three other, minor forms are artefacts resulting from partial migration (O-2 → O-3 → O-4) as well as partial removal of the *O*-acetyl group during mild acid degradation of the LPS. The presence in OS of four *O*-acetyl groups was further corroborated by the ratios of the integral intensities, 4:3:3, of the signals for CH_3 of the *O*-acetyl groups, *N*-acetyl groups, and 6-deoxy sugars, respectively, in the ^{13}C NMR spectrum of OS.

Therefore, the major form of OS, which, it is suggested, corresponds to the intact core of *L. pneumophila* LPS, has the structure **2**.



2 (OS)

Of three polysaccharide-containing fractions obtained by mild acid degradation of the LPS, fraction 3 was eluted from Sephadex G-50 last (Fig. 2) and, thus, had the shortest polysaccharide chain. As judged by the ratios of intensities of the signals for legionaminic acid and core constituents in the ^1H NMR spectrum, it contained on average ca. 10 residues of legionaminic acid.

Fraction 3 was methylated and hydrolysed with 2 M trifluoroacetic acid as described above for fraction 4. GLC-MS of the alditol acetates derived revealed the same partially methylated sugars as in methylation analysis of fraction 4, except for 2,3,4-tri-*O*-methylrhamnose. This showed that Rha^{II}, which is terminal in the unsubstituted core, serves as the attachment site of the polysaccharide chain in the LPS.

This conclusion was confirmed by a significant displacement of the signal for Rha^{II} H-3 from δ 3.83 in the ^1H NMR spectrum of OS_{NH_4OH} to δ 4.32 in the spectrum of fraction 3, while positions of the signals for the other core sugars changed insignificantly.

Smith degradation of the *O*-deacetylated fraction 3, including mild acid hydrolysis at pH 4.4, resulted in a polysaccharide product isolated by GPC on Sephadex G-50. Sugar analysis and ^1H NMR data showed that this product consisted of the undegraded, but *O*-deacetylated, polysaccharide chain and a modified core oligosaccharide containing Rha^{II}, Rha^{I}, QuiNAc, $GlcNAc^{I}$, and erythritol (Ery-ol) derived from oxidised Man. The H-1 signals for the core sugar constituents were at δ 5.00, 4.85 (both d, $J_{1,2}$ 2 Hz), 4.69, and 4.61 (both d, $J_{1,2}$ 8 Hz), respectively.

Therefore, the core-derived region of the Smith-degraded product has the structure **3**,

which further confirmed the structure **1** of OS_{NH_4OH} and the site of the attachment of the polysaccharide chain to the core.

→3)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Qui*p*NAc-(1→4)-β-D-Glc*p*NAc^{I}-(1→2)-Ery-ol

3

3. Discussion

A peculiar feature of *L. pneumophila* LPS is the presence of only those molecular species that contain an O-polysaccharide chain (S-type LPS), and no species with an unsubstituted core (R-type LPS) or with a core containing one O-antigen oligosaccharide unit attached (SR-type LPS), which are typical of most other S-type LPS-containing bacteria studied.

One might expect that, in accordance with this feature, mild acid degradation of the LPS at pH 4.4, used to cleave the linkage between the core and lipid A, will release no free core oligosaccharide. However, this was not the case, due to the cleavage of the linkage between the O-polysaccharide chain and the core during the degradation of the LPS.

The O-specific polysaccharide itself, which is an α-(2 → 4)-linked homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (legionaminic acid) [2,7,8], is stable under these and even more drastic conditions (e.g., 0.1 M HCl at 100 °C [2]). The fact that the linkage between the polysaccharide chain and the core is much more labile than the ketosidic linkage of the legionaminic acid within the homopolymer may be due to the fact that the configuration of the anomeric centre of the legionaminic acid residue attached to the core is different from that within the polysaccharide chain. In fact, the ketosidic linkage of the L-*glycero*-L-*manno* isomer of legionaminic acid (pseudaminic acid) is known to be much more stable when the carboxyl group is axial (as in the legionaminic acid homopolymer), as compared with the anomer having the equatorial carboxyl group [18,19]. The ^{1}H NMR spectrum of fraction 3, however, provided no evidence for the characteristic H-3ax and H-3eq protons of a β-ketosidic linked legionaminic acid residue attached to the terminal Rha^{II} in the core oligosaccharide. This fact was probably due to the excess of about 10 α-linked legionaminc acid residues in this oligosaccharide fraction 3 (Fig. 2) [2].

The core oligosaccharide obtained on mild acid hydrolysis of *L. pneumophila* LPS was heterogeneous owing to the occurrence of the Kdo residue at the reducing end and the rhamnosyl group at the nonreducing end in multiple forms. The former phenomenon is well known and is associated mainly with formation of anhydro derivatives of Kdo [10,11]. The latter appeared to come from partial migration and partial removal of the *O*-acetyl group present originally at position 2 of Rha^{II}, which occurred during the cleavage of the polysaccharide chain.

In spite of such heterogeneity, the structure of the core was elucidated using sugar and methylation analysis, and ^{1}H and ^{13}C NMR spectroscopy before and after *O*-deacetylation of the free core, and was confirmed by study of the *O*-deacetylated core substituted by a short polysaccharide chain. This also allowed determination of the site of the attachment of the polysaccharide chain to the core as position 3 of the terminal nonreducing rhamnosyl group.

The core of *L. pneumophila* serogroup 1 LPS lacks L-*glycero*-D-*manno*-heptose, which is the sugar linked to Kdo in most bacterial LPSs. Instead, the homomorphic D-mannose is attached to Kdo at position 5 as occurs also in LPS of a few other bacteria [20,21]. Phosphate is also absent, but there are present at least four *O*-acetyl groups concentrated in the outer region of the core remote from lipid A. Together with the *N*-acetyl groups of the amino sugars (QuiNAc and GlcNAc) and the methyl groups of the 6-deoxy sugars (Rha and QuiNAc), the *O*-acetyl groups endow the outer region of the core with characteristic hydrophobicity, also found for the polysaccharide chain attached [2].

The negatively charged inner region of the core contains at least one Kdo residue and is not excluded from containing more of them than are found in many other bacterial LPS studied [20,21]. The full structure of the Kdo region of *L. pneumophila* LPS, including the configuration of the glycosidic linkage of Kdo, remains to be determined.

4. Experimental

Miscellaneous methods.—GPC was performed on a column (45 × 2.4 cm) of Sephadex G-50 (S) (Pharmacia) using a pyridinium acetate buffer (pH 4.5) and monitored with a Knauer differential refractometer. GLC was performed with a Varian Model 3700 chromatograph equipped with a capillary column of SPB-5 using a temperature gradient 150 → 320 °C at 5°/min. GLC-MS in both CI (with ammonia) and EI modes was carried out with a Hewlett–Packard Model 5989 instrument equipped with a capillary column of HP-1 under the same chromatographic conditions as in GLC. SDS-PAGE was carried out in 14% polyacrylamide gel using a Mini-PROTEAN II System (Bio-Rad), and LPS bands were visualised by a modified silver staining technique [22].

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AM-360 spectrometer for solutions in D_2O at 60 °C with acetone (δ_H 2.225, δ_C 31.45) as internal standard. Standard Bruker software was used in 1D NOE and 2D COSY experiments. A 2D H-detected $^{1}H,^{13}C$ HMQC experiment was performed with a Bruker AM-300 spectrometer equipped with a BSV-3 generator as described [23].

Bacterial strain, isolation and degradation of LPS.—Strain Philadelphia 1 of *L. pneumophila* ATCC 33152 (serogroup 1) was grown on buffered charcoal yeast agar plates as described [3]. The LPS was isolated from dry cells by a modified phenol–chloroform–petroleum ether procedure with the yield 4.5% (w/w) as described previously [2,9].

The LPS (420 mg) was degraded with 0.1 M NaOAc–AcOH buffer (pH 4.4, 50 mL) at 100 °C for 4 h, a precipitate (118 mg) was removed by centrifugation, and the

supernatant was freeze-dried and fractionated by GPC on Sephadex G-50 to give fractions 1–4 (70, 108, 32, and 5 mg, respectively) as well as fraction 5 containing low molecular weight LPS-derived compounds contaminated by salt (see Fig. 2).

Sugar analysis.—Monosaccharides were analysed by GLC after methanolysis with 2 M HCl/MeOH (120 °C, 16 h) and acetylation with Ac_2O in pyridine (70 °C, 0.5 h) or after hydrolysis with 2 M CF_3CO_2H (121 °C, 2 h) followed by borohydride reduction and acetylation. Amino sugars were analysed conventionally using an LKB Alpha plus 4151 amino acid analyser after hydrolysis with 4 M HCl (100 °C, 16 h). Disaccharides were analysed by GLC after methanolysis with 0.5 M HCl/MeOH (80 °C, 6 h) and acetylation.

O-Deacetylation.—Fraction 4 (OS, 10 mg) was treated with aq 12% ammonia (1 mL, 37 °C, 16 h) to give OS_{NH_4OH} (8 mg). Similarly, fraction 3 (30 mg) gave an *O*-deacetylated product (27 mg).

Methylation analysis.—*O*-Deacetylated and borohydride-reduced fraction 4 (OS_{NaBH_4}, 2 mg) was methylated with MeI in Me_2SO in the presence of solid NaOH [24]. Partially methylated neutral sugars were derived by hydrolysis with 2 M CF_3CO_2H (100 °C, 2 h), reduced with $NaBD_4$, acetylated, and analysed by GLC-MS using published data [25]. For Kdo analysis, carboxyl-reduction with $NaBH_4$ was performed prior to hydrolysis. For amino sugar analysis, hydrolysis (acetolysis) (0.25 M H_2SO_4 in glacial AcOH, 80 °C, 16 h) was applied and followed by hydrolysis, borohydride reduction, and acetylation [15].

Smith degradation.—*O*-Deacetylated fraction 3 (15 mg) was oxidised with 0.1 M $NaIO_4$ (1 mL, 20 °C, 48 h, in dark), the excess of $NaIO_4$ was reduced by several drops of ethylene glycol, the product was reduced with $NaBH_4$ (15 mg), desalted by GPC on Sephadex G-50, and hydrolysed with 0.1 M NaOAc–AcOH buffer (pH 4.4, 2 mL) at 100 °C for 2 h, and the Smith-degraded product (9 mg) was isolated by GPC on Sephadex G-50.

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