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Structural study on a lipopolysaccharide from *Coxiella burnetii* strain Nine Mile in avirulent phase II

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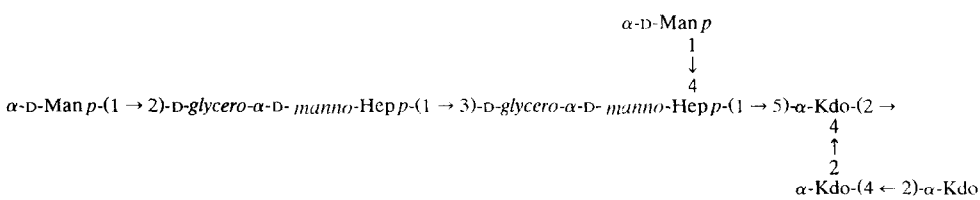
Abstract

A lipopolysaccharide isolated from *Coxiella burnetii* strain Nine Mile in avirulent phase II contains in the lipid A proximal region D-mannose, D-*glycero*-D-*manno*-heptose, and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in the molar ratio 2:2:3. The primary structure **1** of the heptasaccharide was determined by glycose analysis, methylation analysis, ESI-MS, and FABMS.



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Keywords: Lipopolysaccharide; *Coxiella burnetii*; Strain Nine Mile

1. Introduction

Coxiella burnetii, the etiological agent of Q fever, is found worldwide and is responsible for an acute and potentially severe disease characterized by pneumonitis,

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hepatitis, and a significant incidence of neurologic complications [1]. Following infection, *C. burnetii* has been observed to persist in animal tissues for long periods [2,3]. Persistent infections in humans may lead to chronic disease in the form of endocarditis, which affects between 4 and 11% of those who contract acute Q fever [4]. The best studied therapeutic agents in the treatment of Q fever are the tetracyclines [4,5]. However, these drugs are suppressive rather than curative in chronic disease and consequently, *C. burnetii*-induced endocarditis is often fatal [4]. Q fever is a zoonosis which affects feral and domestic large and small animals. The microorganism is found in excreta and products of conception and contaminates both carcasses and milk of infected animals [6]. It is extremely resistant to harsh environmental conditions due to spore formation, it readily becomes airborne, and it is highly infectious for humans [7].

C. burnetii is unique among Rickettsiae in that it undergoes a virulent (phase I) to avirulent (phase II) transition upon serial laboratory passages in eggs or tissue culture [8]. This phase variation resembles in many aspects the well known S → R variation found with many Gram-negative bacteria and is accompanied by dramatic changes in both composition and structure of the lipopolysaccharide (LPS). Phase I organisms express a smooth (S) LPS I with *O*-polysaccharide chain, whereas phase II organisms express a rough (R) LPS II [9–11]. Like that in other Gram-negative bacteria, the LPS is localized in the outer membrane of the *C. burnetii* cell. Its composition and structure determine to a great extent interactions between the microorganism and host including the pathogenicity and immunogenicity of the agent [12].

The structure of the carbohydrate region of the LPS II was investigated [9,13] in the past but the results presented were ambiguous. In the papers cited, it has been assumed that the LPS II contains a "Kdo-like substance" in the lipid A proximal region that is not identical to enteric Kdo. Further, the sequence of other constituent sugars, namely of D-mannose and D-*glycero*-D-*manno*-heptose (D,D-heptose) could only be anticipated from the analytical methods applied. In fact, to our knowledge, no complete experimental data have ever been provided in this respect.

In our previous paper [14], the presence of Kdo in the lipid A proximal region of the LPS II was indicated. Further studies, including methylation analysis, have demonstrated [15] that the LPS II contains three Kdo residues with a structural arrangement similar to that found in enterobacterial LPSs. The present work was undertaken with the aim to bring additional evidence for the presence of Kdo in the LPS II and to establish the overall carbohydrate composition and structure of the LPS II.

2. Experimental

Gel electrophoresis.—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [16] elsewhere.

TLC.—TLC was performed on precoated Silica Gel 60 plates (Merck, Darmstadt, Germany) with 3:2 (v/v) 2-methylpropanoic acid–1 M ammonium hydroxide. Spots were revealed by charring.

Colorimetric methods.—Protein content was estimated according to Hartree [17]. Total hexosamine content was determined by the Morgan–Elson reaction as modified by Swann and Balazs [18]. For direct hexosamine estimation, the same method was applied

but the hydrolysis step was omitted. Phosphate was determined according to Lowry et al. [19], and differential estimation of the Kdo residues was performed by a modified [20] thiobarbituric acid (TBA) assay.

GC-MS.—A Hewlett–Packard Model 5890 A gas chromatograph coupled with a Hewlett–Packard Model 5971 A mass-selective detector and a Finnigan MAT SSQ 710 mass spectrometer both with helium as the carrier gas were used. Mass spectra were recorded at an electron energy of 70 eV and an ion-source temperature of 150 °C. Acetylated and methylated glycose derivatives were analyzed using SP-2330 (30 m × 0.25 mm; Supelco, Bellefonte, USA) and SE-54 (25 m × 0.25 mm; Weeke, Muehlheim, Germany) fused silica capillary columns. The column temperature programs were 80 (2 min) to 235 °C at 30 °C/min with a 38 min hold and 160 (3 min) to 305 °C at 3 °C/min for the former and the latter column, respectively. Trimethylsilyl methyl glycosides were separated in a DB-1 column (60 m × 0.25 mm; Fison, Loughborough, UK) at 180 °C for 2 min, then increasing to 300 °C at 2 °C/min. GC was performed with a Hewlett–Packard Model 5890 A chromatograph, using the same columns and temperature programs as just described and with flame ionization detection.

Electrospray ionization mass spectrometry.—ESI-MS was performed with an API III biomolecular mass analyzer (PE-Sciex, Thornhill, Canada) interfaced to a Macintosh IIfx data station. The mass spectrometer was operated in the positive ion mode with an ion spray voltage of 5000 V and orifice potential of 35 V. Solution (1 μg/μL) of oligosaccharide fraction A (see below) in aq 50% acetonitrile containing 0.1% formic acid and 2 mM ammonium formate was introduced into the ESI source at 2 μL/min using a syringe infusion pump (Harvard Apparatus, S. Natick, MA, USA). The mass range was scanned from m/z 200 to 1800. Ten scans were collected and averaged.

FABMS.—A VG ZAB-SE mass spectrometer (VG Analyticai, Manchester, UK) operating at low resolution (1:1000) with an accelerating voltage of 8 kV was used. Prior to the analysis in the positive ion mode, the oligosaccharide fraction A was acetylated [21] using trifluoroacetic anhydride and acetic acid, dissolved in MeOH (10 μg/μL), and 1 μL of this solution was mixed on the probe tip with thioglycerol (2 μL). Scans were taken over the mass range m/z 300–2600.

Cultivation and purification of C.burnetii *phase II cells.—C. burnetii* strain Nine Mile, serologically in phase II (yolk sac passage 165 in our laboratory), was propagated in chicken embryo yolk sacs. After cultivation, the *C. burnetii* cells were purified by a modified procedure of Schramek et al. [22]. Briefly, yolk sacs with the rickettsial cells were suspended in aq 1 M NaCl containing 0.2% formaldehyde to 20% concentration and the suspension was allowed to stand for 5 days at 5 °C. The mixture was then centrifuged at 21,500 *g* for 40 min and the sediment was treated with 0.5% trypsin (EC 3.4.21.4, 1:250, from bovine pancreas, Serva, Heidelberg, Germany) in phosphate-buffered saline (PBS) at 37 °C for 30 min. After dilution with 0.15 M NaCl and centrifugation, the sediment was digested with trypsin as in the preceding step and the cells were purified further by extraction with ether. The aqueous layer was centrifuged at 15,000 *g* for 30 min and the cells were subjected to final treatment with 0.25% trypsin in PBS at 37 °C for 30 min. The suspension was diluted with 0.15 M NaCl and extracted again with ether. Purified *C. burnetii* phase II cells were centrifuged from aqueous phase and stored preferentially at 5 °C in 0.15 M NaCl containing 0.2% formaldehyde.

Isolation of the LPS II.—The cells (1 g) were extracted with 2:1 (v/v) $CHCl_3$–MeOH at 20 °C overnight. The extraction was repeated with fresh solvent mixture for 2 h. The cell suspension was centrifuged at 3000 *g* for 10 min and the sediment was washed with acetone and ether. The dried cells were suspended in preheated distilled water (50 mL, 68 °C) and extracted with an equal volume of aq 90% phenol as described [23]. The aqueous phase was collected after centrifugation. The phenol phase was re-extracted with water twice and the combined aqueous phases were evaporated to a low volume and dialyzed against distilled water for 4 days. The crude LPS II (38 mg) was solubilized in 50 mM Tris–HCl buffer (4 mL, pH 7.5) and treated simultaneously with RNase (EC 3.1.27.5) and DNase I (EC 3.1.21.1), both from bovine pancreas (Boehringer Mannheim, Germany) for 4 h at 37 °C, and then with proteinase K (EC 3.4.21.14, from Tritirachium album, Sigma, St. Louis, MO, USA) for 6 h at 37 °C. After dialysis against distilled water and lyophilization, the LPS II was purified by ultracentrifugation at 120,000 *g* for 4 h. The final yield was 32.3 mg of the LPS II corresponding to 3.2% of the *C. burnetii* phase II cells.

Identification of glycose constituents.—*(a) As alditol acetates.* The LPS II (300 μg) in 2 M CF_3CO_2H (300 μL, TFA) was kept in a sealed tube for 2 h at 100 °C. The mixture was brought to dryness, and water was evaporated from the residue until it became neutral. Sodium borodeuteride in 1 M ammonium hydroxide solution (2 mg/mL, 0.5 mL) was then added, followed, 15 h later, by dilute AcOH (final pH 3–4). Solvents were removed and a 9:1 (v/v) of mixture of MeOH and AcOH was added (3 × 0.5 mL) to, and evaporated from, the residue, which was then dissolved in Ac_2O (0.1 mL) and pyridine (0.1 mL) and sealed. After 1 h at 100 °C, solvents were removed and the residue was dissolved in $CHCl_3$ (500 μL). Water (500 μL) was added and the mixture was vortexed and then centrifuged for 4 min. The organic layer was evaporated to dryness and the final residue was dissolved in acetone and injected directly onto a GC column.

(b) As trimethylsilyl methyl glycosides. The LPS II (200 μg) in 2 M HCl in MeOH (200 μL) was kept in a sealed tube for 2 h at 60 °C. The mixture was brought to dryness and MeOH (3 × 0.5 mL) was added to remove the residual HCl. Tri-Sil reagent (200 μL, Pierce, Rockford, IL, USA) was then added and the mixture was heated in a sealed tube for 20 min at 80 °C. The residue remaining after removal of the reagent was mixed with hexane (1 mL), vortexed, and centrifuged for 3 min. The solution in hexane was evaporated to a small volume and injected to a GC column.

Nitrous acid deamination.—Fatty acids were released from LPS II by acid and alkaline hydrolyses [24]. After extraction of fatty acids with 1:1 (v/v) $CHCl_3$–EtOAc, the dried residue (1 mg) was dissolved in water (0.3 mL), treated with aq 33% AcOH (0.3 mL) and aq 5% $NaNO_2$ (0.3 mL), and kept for 3 h at 20 °C. The mixture was deionized and the carbohydrate residue was reduced with $NaBH_4$ and acetylated prior to GC-MS analysis.

Methylation analysis.—The LPS II (3 mg) was methylated and purified as described previously [15]. The methylated LPS II (3 mg) was carboxyl-reduced with lithium triethylborodeuteride (500 μL, Super-Deuteride, Aldrich-Chemie, Germany) for 90 min at 20 °C. After addition of AcOH (to compose the excess of the reagent) and dialysis, the recovered material (2.5 mg) was partially hydrolyzed with 50 mM TFA (1 mL) at

100 °C for 20 min, and then carbonyl-reduced with $NaBD_4$ (10 mg) in water (1 mL) for 16 h at 20 °C. The mixture was divided into two parts. One (500 μg) was acetylated with 1:1 (v/v) Ac_2O–pyridine for 1 h at 100 °C and the other (1.8 mg) was re-methylated [25], purified on Sep-Pak C_{18} cartridge [26], hydrolyzed with 2 M TFA (100 °C, 2 h), reduced with $NaBH_4$, and acetylated. Both samples were analyzed by GC-MS.

Mild acid hydrolysis of the LPS II and fractionation of the hydrolyzate on a Sephadex G-15 column.—The LPS II (17 mg) was hydrolyzed in aq 1% AcOH (3.5 mL) for 2 h at 100 °C. The precipitated lipid A was removed from the suspension by centrifugation and the supernatant was lyophilized. The lyophilized material (8 mg) was dissolved in water (0.3 mL) and loaded onto a column (1.7 × 20 cm) of Sephadex G-15. The column was eluted with water at a flow rate of 0.1 mL/min and the effluent was monitored with an RI detector (Knauer, Model 2050/70, Berlin, Germany). The oligosaccharide fraction A (4 mg) eluted first from the column was used for further analytical work as described.

Chromium trioxide oxidation of the oligosaccharide fraction A.—Fraction A (2 mg) was suspended in a 1:1, (v/v) mixture of Ac_2O–pyridine (0.9 mL) and heated in a sealed tube for 3 h at 100 °C. Powdered chromium trioxide (150 mg) was added to a solution of the acetylated product in AcOH (3 mL), and the mixture was sonicated at 45 °C. Aliquots were withdrawn at 0, 1, and 2 h, and each was immediately diluted with water. The solution was extracted four times with $CHCl_3$, and the extracts were combined, dried (Na_2SO_4), and evaporated to dryness. The product was deacetylated with 0.1 M NaOMe for 2 h, deionized, and hydrolyzed with 2 M TFA for 1 h, and the alditol acetates were analyzed by GC-MS. After 2 h of oxidation, mannose (82%) and D,D-heptose (75%) could be found in amounts comparable to their original content in the fraction A.

Enzymatic hydrolysis of the oligosaccharide fraction A.—The fraction A (500 μg) in 10 mM sodium acetate buffer (500 μL, pH 4.5) was treated with α-D-mannosidase (EC 3.2.1.24, from jack beans, Sigma, St. Louis, MO, USA) for 24 h at 20 °C. Mannose was released as detected by GC-MS. Similar treatment with β-D-mannosidase (EC 3.2.1.25, from snail acetone powder, Sigma, St. Louis, MO, USA) at pH 4.0 did not result in a release of mannose.

3. Results and discussion

The LPS II of *C. burnetii* was isolated from the purified cells by a conventional hot phenol–water procedure. The LPS II was found in the aqueous layer. After dialysis and lyophilization, the preparation contained 15% protein. The presence of nucleic acid was also detected by UV spectrometry. A treatment of the crude LPS II with RNase, DNase, and proteinase K, gave after centrifugation, a pure LPS II in a yield of 3.2%. When the method of Wu et al. [27] was employed for the isolation of the LPS II, no substantial increase in the yield was observed. Likewise, the phenol–chloroform–petroleum ether procedure [28] did not give a higher yield of the LPS II from the *C. burnetii* phase II cells.

The LPS II gave a single band characteristic for the R-LPSs on SDS-PAGE

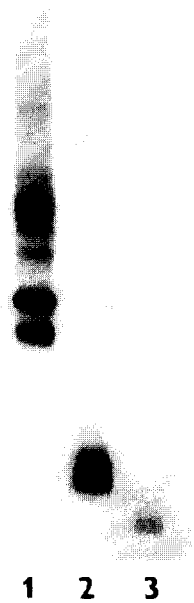

Fig. 1. SDS-PAGE silver stain of the LPS I, LPS II, and *S. minnesota* Re 595 LPS (lanes 1, 2, and 3). The amounts of LPS used per lane 1–3 were 24, 3, and 12 μg, respectively.

electrophoresis (Fig. 1). Its mobility was lower than that of the LPS from *Salmonella minnesota* Re 595 (Sigma, St. Louis, MO, USA). Substantially different banding patterns of the LPS II and the LPS I clearly indicate deep changes in both composition and structure of the LPS I which take place during the serial laboratory passages of the microorganism in embryonated chicken eggs and its transition from virulent phase I to avirulent phase II. When SDS-PAGE electrophoresis was performed in the presence of 4 M urea in the gel, the LPS II gave again a single band whereas *S. minnesota* Re 595 LPS exhibited two distinct bands (not shown). In addition, on TLC, the LPS II gave one strong and one very faint spot (R_f 0.40 and 0.45, respectively) whereas *S. minnesota* Re 595 LPS gave four spots (R_f 0.49, 0.52, 0.54, and 0.56) of approximately the same intensity. Thus, a high degree of homogeneity in the LPS II could be inferred.

When the LPS II was hydrolyzed with TFA, and the hydrolyzate was treated with borodeuteride and then acetylated, GC-MS revealed the presence of a deuterated, per-*O*-acetylated hexitol (mannitol by retention time) and a heptitol (a single peak that had the retention time of per-*O*-acetyl-D-*glycero*-D-*manno*-heptitol) in the molar ratio of ~ 1:1. This result was consistent with that published [9] earlier. Methanolysis of the LPS II and subsequent trimethylsilylation of the mixture gave on GC-MS analysis the corresponding derivatives of mannose, D,D-heptose, and Kdo in a molar ratio of ~ 2:2:3.3. The composition of fatty acids in lipid A was examined [29] exhaustively in the past and, thus, it was not a feature of the present study. Glucosamine was identified

Table 1
Colorimetric analyses of the LPS II from *C. burnetii* strain Nine Mile

Compound	Amount present	
	(nmol/mg)	(μg/mg)
Glucosamine [a]	239	43
Glucosamine [b]	32	6
Phosphate	289	28
Kdo	354	84
Kdo [c]	220	52
Kdo [d]	105	25

[a] Estimated as hexosamine by the Morgan–Elson reaction.
[b] Estimated as hexosamine without prior hydrolysis.
[c] Estimated by the TBA assay after hydrolysis in 0.1 M sodium acetate buffer (pH 4.4; 100 °C, 1 h).
[d] Estimated by the TBA assay without prior hydrolysis.

as 2,5-anhydromannitol by GC-MS after acid and alkaline hydrolyses of the LPS II, and nitrous acid deamination followed by reduction and acetylation. Colorimetric assays (Table 1) gave the molar ratio of glucosamine, phosphate, and Kdo of 2:2.4:3, respectively. For glucosamine, the quantitative determination accomplished by the Morgan–Elson reaction gave the value of 239 nmol/mg of the LPS II. The direct estimation, without the hydrolysis step, gave 32 nmol/mg, indicating that some C-1 of the glucosamine disaccharide were not phosphorylated. The quantitative determination of Kdo by the TBA assay (Table 1) yielded 354 nmol/mg of the LPS II. When the TBA assay was performed without hydrolysis prior to periodate oxidation, 105 nmol/mg was found, indicating that approximately two thirds of the total Kdo was substituted at position C-4 or C-5 or both. When the LPS II was hydrolyzed in 0.1 M sodium acetate buffer (pH 4.4) about two thirds (220 nmol/mg) of the total Kdo was released, thus indicating that one Kdo residue is probably heptosyl-substituted. The results reported bring additional evidence in support of our previous finding [15] on the presence of three variously linked Kdo residues in the LPS II. The lipid A proximal Kdo has been shown to be substituted at C-4 by a Kdo-(2 → 4)-Kdo disaccharide, and this structural arrangement is similar to that found [30] in enterobacterial LPSs.

In ref. [15] the methylation-linkage analysis of the Kdo region of the LPS II was of primary interest. In our hands, however, the widely applied [31] sodium borohydride/deuteride reduction of the Kdo methyl ester did not exceed ≈ 70%. Therefore, we decided to repeat the methylation-linkage analysis of the LPS II with the aim to increase the reduction of Kdo methyl ester by the use of a more suitable reducing agent and to determine the mode of linkage of other constituent sugars, namely of D-mannose and D,D-heptose. Thus, the LPS II was permethylated, carboxyl-reduced with lithium triethylborodeuteride [32] in tetrahydrofuran, and hydrolyzed under mild conditions. The hydrolyzate was carbonyl-reduced with $NaBD_4$ and divided into two portions. One portion was acetylated and directly analyzed by GC-MS. Isomeric [33] 1,2,6-tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl(1,1,2-3^{2}H)octitols and 1,2,4,6-tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyl(1,1,2-3^{2}H)octitols giving characteristic fragment ions [15] represented ≈ 93% of the analyzed sample in the molar ratio of ~ 1:1. The other portion

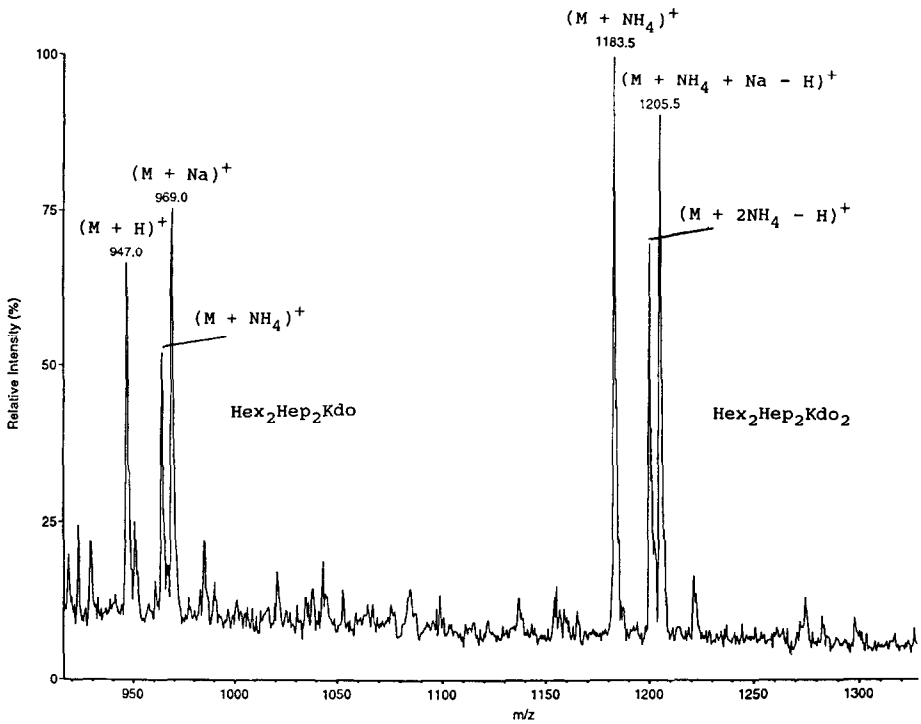

Fig. 2. ESI-MS spectrum of the oligosaccharide fraction A from the LPS II of *C. burnetii*.

was re-methylated, hydrolyzed, carbonyl-reduced with $NaBH_4$, and acetylated. GC-MS revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol, 1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methylheptitol, and 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyl(1,1,2-3^2H)octitol isomers [15,33] in the ratio of ~ 2:1:1:1, respectively. From all these results, the presence of two terminal mannoses, 2- and 3,4-linked heptoses, terminal, 4- and 4,5-linked Kdo residues could be established in the LPS II.

For ESI-MS and FABMS investigations of the sequence of sugar units in the LPS II, the lipid A was removed by mild acid hydrolysis and the carbohydrate portion was separated on Sephadex G-15. The oligosaccharide fraction A eluted first from the column was analyzed by ESI-MS. Singly charged ions $(M + NH_4)^+$, $(M + 2NH_4 - H)^+$, and $(M + NH_4 + Na - H)^+$ having molecular masses 1184, 1201, and 1206, respectively, could be seen in the positive ion mode (Fig. 2). This corresponds to a hexamer $Hex_2Hep_2Kdo_2$ that arose by cleavage of one Kdo residue, most probably the terminal one, during the mild acid hydrolysis of the LPS II. Further, ions $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ with molecular masses 947, 964, and 969 were also detected (Fig. 2) and corresponded to a pentamer Hex_2Hep_2Kdo. However, attempts to obtain data on the sequence of sugar residues by MS/MS were unsuccessful. The fraction A was then analyzed by FABMS. When the sample was peracetylated with trifluoroacetic anhydride–acetic acid mixture and analyzed in the positive ion mode, the main fragments at

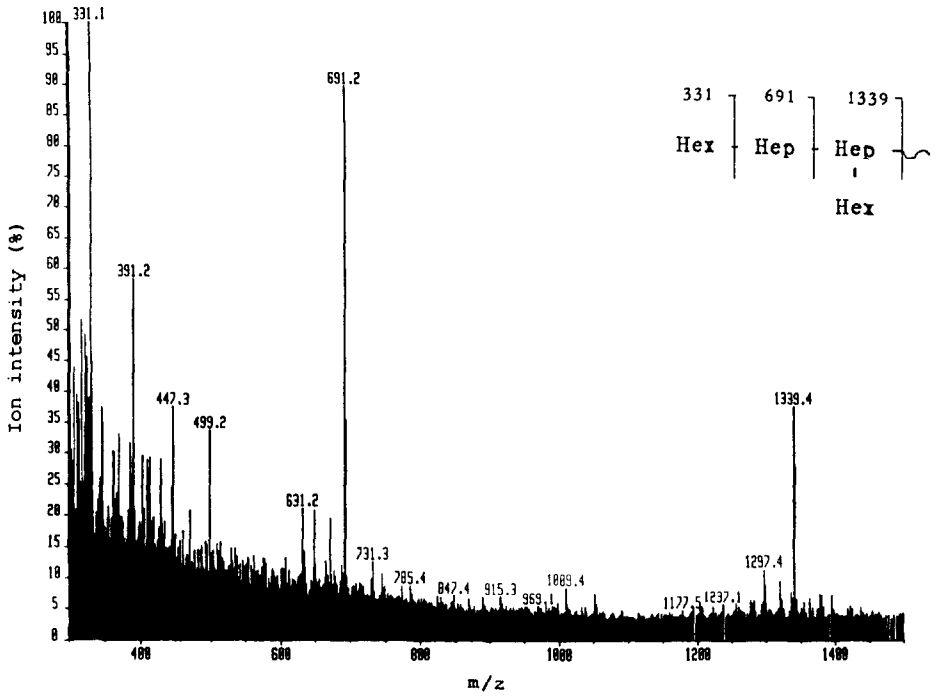

Fig. 3. FABMS spectrum of the peracetylated tetrasaccharide from the LPS II of *C. burnetii*.

m/z 1339, 691, and 331 were clearly seen (Fig. 3). Thus, the sequence of hexose and heptose residues in the LPS II was unambiguously established as shown in Fig. 3.

Chromium trioxide oxidation [34] of the peracetylated fraction A revealed only a minor decomposition of mannose and D,D-heptose (18 and 25%, respectively), suggesting that both sugars are α-linked. Enzymatic hydrolysis of the fraction A with α- and β-D-mannosidases released mannose only with the former enzyme. The mannose residues are located in terminal positions and should equally be accessible to both the enzymes. Thus, the enzymic treatment indicates that the two mannoses are α-D. The α-anomeric configuration for the Kdo residues in the LPS II is suggested on biosynthetic grounds [35] and on vast structural and immunological evidence in the literature, e.g., in ref. [30].

From the results reported, structure **1** of the heptasaccharide proximal to lipid A, present in the LPS II may be proposed.

α-D-Man*p*
1
↓
4
α-D-Man*p*-(1 → 2)-D-*glycero*-α-D-*manno*-Hep*p*-(1 → 3)-D-*glycero*-α-D-*manno*-Hep*p*-(1 → 5)-α-Kdo-(2 →
4
↑
2
α-Kdo-(4 ← 2)-α-Kdo

1

The only major ambiguity is the correct linkage assignment for the 3,4-linked D,D-heptose. It is well known [30] that the two heptoses proximal to lipid A and located in the main core chain are α-(1 → 3)-linked in most LPSs investigated, and therefore, this structural arrangement is anticipated also in structure **1**. Nevertheless, a structural arrangement cannot be excluded in which C-3 of this D,D-heptose is a branch point and C-4 is involved in the backbone linkage. Further studies towards elucidation of this problem are in progress.

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