Characterization of Lipid A from Pseudomonas aeruginosa O-Antigenic B Band Lipopolysaccharide by 1D and 2D NMR and Mass Spectral Analysis

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The Lipid A from the lipopolysaccharide of Pseudomonas aeruginosa was examined by high-field nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). The backbone structure and the position of phosphate substituents were unambiguously established by one- and two-dimensional 1H, 13C, and 31P NMR techniques on a de-O-acylated Lipid A sample. The Lipid A has a α-(1,6)-glucosamine disaccharide structure which is substituted by phosphonoesters through glycosidic bonds at C-1 and at C-4'. The configuration of the glycosidically linked phosphate at position C-1 was identified as α which is the same as that of Enterobacterial Lipid A. Chemical analysis revealed that the Lipid A contained 2-hydroxydodecanoic, 3-hydroxydodecanoic, dodecanoic, 3-hydroxydecanoic, and hexadecanoic acids in the approximate molar ratios 2:2:2:0:0:2:0:8:0:4. From 1H NMR and fast atom bombardment (FAB) mass spectrometry on the de-O-acylated Lipid A, it was established that both glucosamine residues were N-acylated by 3-hydroxydodecanoic acid. The identity and positions of the ester bound fatty acids in the intact Lipid A were investigated by negative ion FAB-MS. In addition to the hexaacyl and pentaacyl Lipid A species, a tetraacyl species was identified. Heterogeneity due to hydroxylated and nonhydroxylated dodecanoic acid esters could be uniquely localized to the nonreducing α-glucosamine residue from the fragmentation pattern observed in the negative ion FAB-MS. The complete structure of the Lipid A from P. aeruginosa will be useful in understanding the determinants responsible for the endotoxin activity of this molecule.

Pseudomonas aeruginosa is a gram-negative opportunistically pathogenic bacterium. The lipopolysaccharide isolated from P. aeruginosa has been shown to contain a heterogeneous mixture of molecules of different polysaccharide lengths (1, 2). Lipopolysaccharide consists of the Lipid A and core oligosaccharide, either unrapped (90% of LPS molecules) or capped by different lengths of O-antigen polysaccharide. The isolated Lipid A has endotoxic activity for mammalian cells like those Lipid A's of Enterobacteriaceae (3).

Lipid A of gram-negative Enterobacteriaceae consists of a β-(1,6)-linked diglucosamine disaccharide backbone (4, 5) to which phosphates are attached at positions 1 and 4' and saturated, hydroxy fatty acids are ester and amide linked at positions 2, 3, 2', and 3'. The Lipid A backbone of Pseudomonas is similar to that of the Enterobacteriaceae only in the diphosphoglucosamine moiety (6, 7). The fatty acids attached to this backbone differ considerably. There is no hydroxytetradecanoic acid in Pseudomonas, but 2- and 3-hydroxydodecanoic acids are found which are ester and amide linked, respectively.

Pseudomonas Lipid A has been studied by various groups in order to characterize the determinants involved in endotoxic activity. The structures of the Lipid A analyzed by chemical methods have been reported (8-14). However, to date there has been no precise assignment of the locations of dodecanoic and 2-hydroxydodecanoic fatty acids or of the configuration of the phosphate at position 1. In this paper, the structure of the Lipid A backbone devoid of the ester linked fatty acids is unequivocally determined by one- and two-dimensional high-field NMR techniques and fast atom bombardment

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1 This investigation has been supported by the Canadian Bacterial Diseases Network.

2 To whom correspondence should be addressed.

3 Abbreviations used: NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry; KDO, 3-deoxy-D-manno-
**MATERIALS AND METHODS**

**Isolation and purification of LPS.** *P. aeruginosa* strain K799 wildtype (International Antigen Typing Scheme and Habs Serotype O-51) was grown in proteose peptone No. 2 nutrient medium in a 100-l fermenter. Bacteria were harvested by centrifugation and lyophilized to yield a dry pellet. LPS was isolated by the Darveau-Hancock method (15). In order to separate smooth, O-antigen-containing and O-antigen-lacking LPS, bulk LPS was solubilized in a deoxycholate buffer containing 0.2 M NaCl, 10 mM Tris at pH 8.0, 0.02% sodium azide, 5 mM EDTA, and 0.25% sodium deoxycholate and fractionated on a Sepharcl S-200 column (2.6 X 100 cm) using the same column buffer at a flow rate of 26 ml/h (16). The eluants were monitored by the thiobarbituric assay and the later-eluting 2-deoxy-D-manno-2-octulosonic acid (KDO)-containing fractions (i.e., rough LPS) were pooled and dialyzed against distilled water for 2 days. SDS-polycrylamide gel electrophoresis (17) confirmed that the LPS isolated was rough LPS. The KDO and phosphate contents were 3.7 and 4.2% (as P), respectively.

**Preparation of Lipid A.** Purified rough LPS (556 mg) was heated in 10 mM NaOAc, pH 3.6 (35 ml), at 100°C for 2 h. Lipid A was isolated by floatation after freezing and thawing four times and centrifugation at 12,000g for 30 min. The precipitate was dissolved in CHCl₃/MeOH (2/1) and washed with water. Lipid A was obtained in 31% yield (wt/wt).

**Purification of Lipid A.** Lipid A (165 mg) was dissolved in CHCl₃/MeOH (4/1) and separated on a Sephadex LH20 column (1.4 X 17 cm) as described (18). The eluants were monitored by TLC on silica gel 60 plates. The plates were developed with CHCl₃/MeOH/H₂O/NH₃ (50:25:4:2) and visualized with iodine. A fast moving fraction 1 (Rₜ = 0.90) and a slow moving fraction 2 (Rₜ = 0.50) were both observed as single spots as the major components.

**Chemical methods.** Phosphate was determined according to Ames and Dubin (19). KDO was determined by the thiobarbituric acid method (70). Fatty acids were analyzed by gas liquid chromatography (GLC) as methyl esters after methanalysis with 2 M methanolic HCl at 100°C for 18 h, with pentadecanoic acid as the internal standard. Sugars were analyzed by GLC as the derivatized alditol acetates. Samples were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h and evaporated with water two to three times to remove residual acid, followed by reduction with sodium borohydride (NaBH₄, 10 mg/ml in 0.1 M NH₄OH) and derivatization with acetic anhydride in pyridine. The alditol acetates were extracted into CHCl₃. Methylation was performed according to the Hocomori method (21), using KH instead of NaH to generate the dimethyl anion. Methylated sugars were analyzed by GLC-MS after hydrolysis with 4 M TFA at 100°C for 2 h, after subsequent conversion to partially methylated alditol acetate derivatives. Removal of ester linked fatty acids was enabled by treatment with anhydrous hydrazine at 37°C for 4 h, in order to separate smooth, O-antigen-containing and O-antigen-lacking LPS, bulk LPS was solubilized in a deoxycholate buffer containing 0.2 M NaCl, 10 mM Tris at pH 8.0, 0.02% sodium azide, 5 mM EDTA, and 0.25% sodium deoxycholate and fractionated on a Sepharcl S-200 column (2.6 X 100 cm) using the same column buffer at a flow rate of 26 ml/h (16). The eluants were monitored by the thiobarbituric assay and the later-eluting 2-deoxy-D-manno-2-octulosonic acid (KDO)-containing fractions (i.e., rough LPS) were pooled and dialyzed against distilled water for 2 days. SDS-polycrylamide gel electrophoresis (17) confirmed that the LPS isolated was rough LPS. The KDO and phosphate contents were 3.7 and 4.2% (as P), respectively.

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**T labels**

**2-octulosonic acid.** LPS, lipopolysaccharide; GLC, gas-liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; COSY, correlation spectroscopy; NOESY, 2D nuclear Overhauser enhancement spectroscopy; GlcN, glucosamine; HMQCl, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; TEA, triethanolamine;
acids and further analysis of 2 was not attempted. The fatty acid profile of compound 1 was the same as that obtained for intact LPS.

Methylation of 1 was performed by the Hakomori procedure. The strongly basic conditions employed in this method resulted in the removal of the ester linked fatty acids. The partially methylated sugars obtained following hydrolysis of the methylated product were reduced (NaBH₄) and acetylated to produce partially methylated alditol acetates. Three amino sugar derivatives were dehydrolysis of the methylated product were reduced following the isolation of the diglucosamine backbone, while the 3,6-di-O-methyl derivative was obtained from the same sugar. The reducing glucosamine moieties of the disaccharide unit were completely assigned by homo- and heteronuclear chemical shift correlation techniques. Contour plots of the COSY spectra and the ¹H/¹³C chemical shift correlation map are shown in Figs. 1, 2, and 3. The ¹H and ¹³C chemical shift assignments are recorded in Table II.

The low field anomeric proton resonance at 5.46 ppm showed a ¹H/¹³C correlation to the carbon resonance at 93.5 ppm (Fig. 3). The chemical shift values and the magnitude of the vicinal proton coupling constant (J₁₂ = 3 Hz) were indicative of a β-linked glucosamine. From the COSY spectrum, it was evident that the ¹H resonance showed additional heteronuclear coupling which could be attributed to phosphorous-31 (J₁₂ = 8 Hz), thereby indicating this glucosamine residue (GlcN) to be α-glycosidically linked to phosphate. In agreement with this conclusion, a strong connectivity was observed between the ³¹P signal at -1.57 ppm and the downfield anomeric ¹H resonance (5.45 ppm) in the 2D ³¹P/¹H chemical shift correlation experiment.

The high-field anomeric proton resonance at 4.68 ppm showed a J₁₂ value of 7.8 Hz which was characteristic of a β-linked glucosamine residue (GlcN). In accordance with the assignment, this proton resonance corresponded to the C-3' resonance at 101.3 ppm in the ¹H/¹³C correlation map (Fig. 2D). In the narrow-sweep-width COSY (Fig. 2), cross-peaks relating H-3/H-4 and H-4/H-5 of the α-linked glucosamine (I) were very weak. However, under the low resolution conditions of the full-scale COSY (Fig. 1), these cross-peaks, as well as the corresponding cross-peaks from the β-linked glucosamine (II), were clearly visible. An unambiguous assignment of H-3' and H-5' was obtained from the occurrence of intraresidue nuclear Overhauser effects (NOE's) to the β-anomeric proton resonance. In the NOESY experiment, trans-glycosidic NOE's also related the β-anomeric proton (4.67 ppm) to H-6A (4.13 ppm), H-6B (3.92 ppm), and H-5 (3.99 ppm) of GlcN, which unequivocally established the β-(1,6)-linkage to the α-glucosamine residue. The H-6

<table>
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<tr>
<th>Lipid A compound</th>
<th>KDO (%)</th>
<th>Phosphate (as P) (%)</th>
<th>Fatty acids (total %)</th>
<th>Mole ratio C₁₀₆</th>
<th>C₁₂</th>
<th>C₁₂ᵣ</th>
<th>C₁₂ᵣ</th>
<th>C₁₆</th>
<th>Sugars GlcN (%)</th>
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<td>2.0</td>
<td>2.0</td>
<td>3.7</td>
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Note. C₁₀₆, 3-hydroxydecanoic acid; C₁₂, dodecanoic acid; C₁₂ᵣ, 2-hydroxydodecanoic acid; C₁₂ᵣ, 3-hydroxydodecanoic acid; C₁₆, hexadecanoic acid.

*The amount of C₁₆ fatty acid found in the native LPS, as well as in other preparations, was variable (0.2-3.7 mol/Lipid A) depending on the batch of LPS isolated. This may be present as a contaminant from outer membrane products.

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TABLE I

Chemical Composition of 1 and 2 Isolated from Lipid A of Pseudomonas aeruginosa Wild Type K799

<table>
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<tr>
<th>Mole ratio</th>
<th>Sugars GlcN (%)</th>
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<td>C₁₀₆</td>
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<td>0.8</td>
<td>0.3</td>
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</table>

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For the detailed chemical composition of lipids and fatty acids, please refer to Table I.
H1, c; -2.0; B. EJ D AA', X. -3.0; -mm /

FIG. 1. Contour plot of the 2D COSY spectrum of the de-0-acylated sample of the lipid A (compound 3). The spectrum was measured over the full spectral region (3500 Hz) in deuterium oxide using a data matrix of 256 X 1024 points. Cross-peaks relating H-1 and H-2 of the two glucosamines as well as H-3, H-4, and H-5 of the α-glucosamine residue (GlcN₂) are indicated. Resonances corresponding to the AA'X spin systems from the N-linked 3-hydroxy dodecanyl groups are also shown.

proton pair of the GlcN₁ was correlated to the low-field methylene carbon resonance at 67.8 ppm (Fig. 3) which confirmed substitution at the C-6 position (26). In contrast, the C-6' resonance of GlcN₁ occurred in a region of the ¹³C NMR spectrum (60.5 ppm) characteristic of unsubstituted hydroxy methylene groups (26).

In unsubstituted β-glucosamines, C-4 usually resonates at ca. 70 ppm. The lower field chemical shift value for the C-4' resonance from GlcN₁ (73.4 ppm) could be attributed to substitution by phosphate at that position. Indeed, a connectivity between the ³¹P resonance at 1.18 ppm confirmed the positioning of the phosphomonoester at C-4' of GlcN₁.

The amide linked fatty acids were determined to be 3-hydroxy acids from the full scale COSY experiment (Fig. 1); two overlapping AA'X spin systems were identified which could be attributed to the α-methylene protons (AA' part, 2.5 ppm) and the proton attached to the hydroxylated carbon (X part ca. 4.1 ppm) clearly indicating β-hydroxy acids. Signals at 0.9 ppm could be attributed to the methyl protons of the fatty acyl chains, while the large envelope of resonances centered at 1.3 ppm were due to the distal methylene protons of those chains.

The MS data on the hydrazinolysis sample permitted the nature of the fatty N-acyl groups to be identified. The positive ion FAB-MS (glycerol/thioglycerol, 1/3) showed abundant molecular ions at m/z 897 (M + H) and 919 (M + Na). Sequential cleavage of the glycosidic bonds gave A₁-type fragment ions (27) at m/z 799 (M-H₂PO₄) and 440 (Fig. 4). Correspondingly, the negative ion FAB-MS showed abundant ions at m/z 917 (M-Na-H), 895 (M-H), 873 (M-H₂O), 595 (M-HNa).
FIG. 3. Heteronuclear $^1$H-$^1$C 2D chemical shift correlation map ($^1$H($^1$C)HMQC) of the ring proton region (4.2–3.5 ppm) of the de-$O$-acylated lipid A (compound 3). The contour indicated by X is due to the methine resonances of the 3-hydroxy dodocanoyl groups.

and 815 (M–H,PO$_4$) (Fig. 5). The FAB-MS results are consistent with $N$-acylation of each GlcN by 3-hydroxydodecanoic acid and this is in agreement with the structures recently proposed by Bhatt et al. (14) and Kulshin et al. (12).

The location of the ester bound fatty acids were deduced from the fragmentation observed in the FAB mass spectrum of the intact Lipid A. The negative FAB-MS of the intact Lipid A compound 1 is shown in Fig. 6. Major pairs of molecular ions could be attributed to hexaacyl (m/z 1615, 1631), pentaacyl (m/z 1461, 1445) and tetraacyl species (m/z 1291, 1275) which arise from sequential loss of C-10 hydroxy fatty acids. Fragment ions arising from the loss of one phosphate group also occurred in this region. The difference in 16 mass units between each pair of ions could be attributed to the presence of either a hydroxylated or non hydroxylated dodecanoic acid residue (see Table III).

A similar doubling pattern has been recently observed by laser desorption (12), and by plasma desorption (28) MS of $P$. aeruginosa Lipid A preparations, the indicated heterogeneity is thought (12, 14) to arise from esterification of the amide bound 3-hydroxydodecanoic acid residues by either 2-hydroxydodecanoic acid or dodecanoic acid. In the negative FAB-MS, a significant fragment ion was observed at m/z 654 (Fig. 6). The most likely mode of fragmentation at the glycosidic linkage occurs by a mechanism involving $\beta$-cleavage (27) in which the charge is retained at the reducing end of the molecule (i.e., GlcN$_1$). Fragmentation by this mechanism is predicted from a consideration of the Lipid A structural model proposed by Bhatt et al. (14). Thus the fragment ion at m/z 654 can be attributed to $\beta$-cleavage of the pentaacyl and tetraacyl species. The occurrence of this fragment, together with the lack of significant ion 16 mass units lower at m/z 638, would indicate that the nonhydroxylated dodecanoic acid esters reside solely on GlcN$_1$. Correspondingly, the occurrence of the fragment at m/z 825 can be assigned to the $\beta$-cleavage product from the hexaacyl species (Table III).

### TABLE II

<table>
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<tr>
<th>Position</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6A</th>
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<tr>
<td>$^1$H (GlcN$_1$)</td>
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<td>53.3</td>
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Permethylated compound 1 (the composition of which was analyzed as partially methylated alditol acetates and discussed earlier) was subjected to negative FAB-MS (Fig. 7). Two groups of molecular ions were observed which could be attributed to the presence of molecular species showing the absence of methylation at the amide nitrogen of GlcN1 and the phosphate group at the 4'-position (Table IV). An abundant molecular ion at m/z 955 corresponds to the permethylated de-O-acylated monophosphorylated species, A. An ion of similar intensity at m/z 923 (B) probably arises from loss of methanol from one of the amide linked 3-methoxydodecanoic acids of A due to the strongly basic conditions employed for Hakomori methylation. In the higher mass group, the weak ion observed at m/z 1153 (C) can be attributed to further substitution of A by an ester linked methoxydodecanoic acid, while the ions at m/z 1121 (D) and 1089 (E) arise from sequential elimination of methanol from C during the methylation procedure. Fragments due to β-cleavage were observed at m/z 494 and 526 (Table IV). In the positive ion FAB mass spectrum, pseudomolecular ions (M + Na) were observed for each of the four major species at m/z 1146 (D), 1118 (E), 979 (A), and 847 (B). Molecular ions due to diphasphorylated species were not observable in either the negative or the positive FAB-MS, although their presence was inferred from the methylation analysis results. The absence of identifiable molecular ions due to diphasphorylated species may be due to poor relative FAB ionization efficiencies or to rapid fragmentation. The most complete structure of the Lipid A from P. aeruginosa was established as a hexaacyl diglucosaminylidiphosphate. Whether the pentaacyl and tetraacyl moieties were native or breakdown products in the isolation procedure was examined next. An experiment using the same reagent employed to isolate Lipid A (10 mM NaOAc, pH 3.6) was performed. A sample of LPS was dissolved in the NaOAc reagent and aliquots were removed at specific time intervals. The reaction was allowed to run 2 h at 100 °C to duplicate the conditions used to isolate Lipid A. Each aliquot was then lyophilized, extracted with acetone to remove released fatty acids, and extracted with CHCl₃/MeOH 2/1 to remove liberated Lipid A. The acetone extracts, chloroform extracts, and remaining residues were analyzed for the presence of fatty acids (as fatty acid methyl esters). It was evident that no fatty acids were released under these conditions. The proportion of Lipid A liberated increased with time and reached a constant value after 1 hr 45 min. The ratios of the different fatty acids were 1.1:0.6:2.0:2.0 for 3-hydroxydodecanoic:dodecanoic:2-hydroxydodecanoic:3-hydroxydodecanoic acids.

The FAB-MS of the Lipid A (compound 1) showed molecules containing different amounts of fatty acids. A small percentage (Fig. 6) contained a hexaacyl species consisting of 2 mol each of 2-hydroxydodecanoic, 3-hydroxydodecanoic, and 3-hydroxydodecanoic acids per Lipid A molecule (m/z 1631), and an approximately equal percentage of a hexaacyl species having a 2-hydroxydodecanoic acid replaced by dodecanoic acid (m/z 1615). The pentaacyl species corresponding to the removal of 3-hydroxydodecanoic acid from the hexacyl species (m/z 1461, 1445) and that due to additional removal of phosphate (m/z 1381, 1365) were observed in significant and almost equal amounts. The amount of each of these species containing hydroxylated and nonhydroxylated fatty acid (m/z 1461 and 1445, and m/z 1381 and 1365) were nearly equal. The tetraacyl species formed by removal of both 3-hydroxy dodecanoic acids (m/z 1291, 1275) and that due to loss of phosphate (m/z 1211, 1195) were also quite abundant. In addition, minor amounts of triacyl species corresponding to loss of a 2-hydroxydodecanoic acid from the tetraacyl species (m/z 1093, 1077) and triacyl species with additional loss of phosphate (m/z 1013, 997) were observed. The small percentages of the hexacyl species observed in the FAB-MS (~4%) is to a large extent due to poor ionization efficiencies related to matrix solubility (i.e., in TEA); when glycerol/thioglycerol was used as the matrix, none of the hexaacyl species were observed. The ratios of fatty acids as methyl esters obtained by analysis of Lipid A indicate that the ratio of pentaacyl species is probably about three to four times that of the hexaacyl species.
### TABLE III

Negative FAB-MS Data and Proposed Structure of *P. aeruginosa* Lipid A

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<th><strong>M-P</strong>&lt;sup&gt;+&lt;/sup&gt;</th>
<th><strong>M-GlcN&lt;sub&gt;H&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</strong></th>
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<td>1446</td>
<td>1445</td>
<td>1365</td>
</tr>
<tr>
<td>Tetraacyl</td>
<td>20HC&lt;sub&gt;12&lt;/sub&gt;</td>
<td>20HC&lt;sub&gt;12&lt;/sub&gt;</td>
<td>H</td>
<td>M</td>
<td>1292</td>
<td>1291</td>
<td>1211</td>
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<td></td>
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<td>C12</td>
<td>H</td>
<td>M</td>
<td>1276</td>
<td>1275</td>
<td>1195</td>
</tr>
</tbody>
</table>

* M-H<sub>3</sub>P<sub>3</sub>.

* β-Cleavage by loss of GlcN<sub>H</sub> (27).

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species, and that the substitution by nonhydroxylated dodecanoic acid is about 30%. Due to the high volatility of both the 3-hydroxydecanoic and the dodecanoic acids, it could be possible that the ratios of these two fatty acids should be higher than what was obtained. Since the FAB-MS shows that the different species are obtained as breakdown products, the ratio of pentaacyl to hexaacyl species in native LPS may be lower than three to four times. The percentage of substitution by nonhydroxylated dodecanoic acid is about 40-45% from the FAB-MS, which indicates that the dodecanoic acid ratio is probably greater than that obtained by fatty acid analysis.

Lipid A species substituted by hexadecanoic acid were not detected in the FAB-MS and the position of substitution therefore remains uncertain. The presence of ester bound hexadecanoic acid in intact *P. aeruginosa* Lipid A has been previously inferred from plasma desorption MS data (28). The varying amounts of hexadecanoic acid (2-8%) observed in different preparations of LPS indicate that it may not be a part of the Lipid A.

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**DISCUSSION**

The complete structure of the Lipid A from *P. aeruginosa* has now been established (Fig. 8). It has a backbone comprising two glucosamine units which are β,1-6 linked. There are two phosphates, one attached to the 4' position of the terminal glucosamine and the second in the α anomeric configuration at carbon 1 of the reducing sugar. The anomeric configuration of the phosphoester had until now been unknown. This basic structure is identical to those of Escherichia coli and Salmonella Lipid A backbones (7). The similarities end there since the nature of
the fatty acids attached to the backbone differ considerably in P. aeruginosa. The fatty acids identified were 3-hydroxydodecanoic acid, which was only found amide bound, and 2-hydroxydodecanoic acid, 3-hydroxydodecanoic acid, and dodecanoic acid which were only ester bound. A Lipid A species consisting only of the amide linked fatty acids was obtained by hydrazinolysis of 1. Positive and negative fast atom bombardment mass spectrometry of this species confirmed the presence of two hydroxydodecanoic acids, two phosphate esters bound to two glucosamine moieties, each sugar carrying one fatty acid, and one phosphate. The positions of the phosphates were assigned by a series of NMR spectroscopy experiments. Two dimensional proton COSY and NOESY experiments were performed to assign the proton chemical shifts, and a heteronuclear $^1H-^1C$ correlation experiment was used for assigning the carbon chemical shifts. The position of attachment of the phosphate esters was deduced from the $^{31}P-^1H$ heteronuclear correlation experiment. The fatty acid was shown to contain a 3-hydroxy methylene group. This is the first example involving complete $^1H$ and $^13C$ assignment of a sugar backbone which contains both phosphate groups.

The Lipid A isolated by treatment with 10 mM NaOAc, pH 3.6, for 2 h at 100°C was purified on Sephadex LH20 to yield a fraction showing a single spot on TLC. The FAB-MS of this compound showed molecular ions due to molecules containing variable amounts of fatty acids attached to the glucosamine disaccharide. From the values obtained for the fatty acids of the intact LPS, it is evident that the major species is the pentaacyl Lipid A. It was confirmed that no fatty acids were being removed during the isolation and purification of Lipid A. From the ratios of the fatty acids in the LPS and the relative abundance of molecular ions in the FAB-MS, the proportion of the hexaacyl species could not be determined exactly, but is probably greater than 25%. This is not in agreement with the observation made by Kulshin et al. (12), which was based entirely on the ratios of fatty acids.

It is known that the Lipid A showed heterogeneity with respect to hydroxylated and nonhydroxylated dodecanoic acid, and documented that the nonhydroxylated dodecanoic acid could be ester bound to either of the amide linked 3-hydroxy dodecanoic acids (11, 12). In this paper it was shown that the FAB-MS of the intact Lipid A was consistent with both the pentaacyl and the hexaacyl species showing heterogeneity only in GlcNII where the 2-hydroxydodecanoic acid ester bound to the N-linked 3-hydroxydodecanoic acid is replaced by dodecanoic acid. The extent of replacement of the hydroxylated fatty acid by the nonhydroxylated fatty acid was estimated to be about 40%.

The native LPS was found to contain a high percentage of phosphate. It is known that the core region contains most of these phosphates as ortho- and pyrophosphates in the mono- and diester forms. The Lipid A species that was generated only showed two phosphomonoesters. It is possible that any pyrophosphates attached to Lipid A may

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**TABLE IV**

FAB-MS Data of Permethylated 1 and Assignment of Molecular Fragments

<table>
<thead>
<tr>
<th>Species</th>
<th>RI/R</th>
<th>Loss of MeOH</th>
<th>$M_r$</th>
<th>$M-1$</th>
<th>$M-GlcNII$</th>
<th>Positive FAB (M + Na)</th>
<th>Negative FAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Me/Me</td>
<td>—</td>
<td>956</td>
<td>955</td>
<td>526</td>
<td>979</td>
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<tr>
<td>B</td>
<td>Me/Me</td>
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<td>924</td>
<td>923</td>
<td>526/494</td>
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<td>C</td>
<td>Me/OMeCl2</td>
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<td>1154</td>
<td>1153</td>
<td>—</td>
<td>—</td>
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<td>Me/OMeCl2</td>
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<td>1122</td>
<td>1121</td>
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<tr>
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<td>1090</td>
<td>1089</td>
<td>—</td>
<td>1113</td>
<td></td>
</tr>
</tbody>
</table>

*a* Resulting from elimination due to the strongly basic Hakomori methylation conditions.

*b* $\beta$-cleavage product.
have hydrolyzed under the acidic conditions employed to yield the monophosphate containing species. We are currently attempting to locate the positions of attachment of the pyrophosphate groups in the native LPS.

REFERENCES