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

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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 ^{1}H, ^{13}C, and ^{31}P NMR techniques on a de-*O*-acylated Lipid A sample. The Lipid A has a β-(1,6)-glucosamine disaccharide structure which is substituted by phosphomonoesters 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 ^{1}H 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 β-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 uncapped (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 diphosphodiglucosamine 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[3] techniques and fast atom bombardment

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[3] Abbreviations used: NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry; KDO, 3-deoxy-D-manno-

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(FAB) mass spectrometry (MS). Other chemical methods were used to further confirm the structure of the Lipid A. The identity and position of the ester bound fatty acids were established by FAB-MS on the intact Lipid A.

MATERIALS AND METHODS

Isolation and purification of LPS. *P. aeruginosa* strain K799 wildtype [International Antigen Typing Scheme and Habs Serotype O-5] 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 Sephacryl S-200 column (2.6 × 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 3-deoxy-D-manno-2-octulosonic acid (KDO)-containing fractions (i.e., rough LPS) were pooled and dialyzed against distilled water for 2 days. SDS-polyacrylamide 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 12,000*g* for 30 min. The precipitate was dissolved in $CHCl_3$/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 $CHCl_3$/MeOH (4/1) and separated on a Sephadex LH20 column (1.4 × 17 cm) as described (18). The eluants were monitored by TLC on silica gel 60 plates. The plates were developed with $CHCl_3$:MeOH:H_2O:NH_3 (50:25:4:2) and visualized with iodine. A fast moving fraction **1** (R_f = 0.90) and a slow moving fraction **2** (R_f = 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 (20). Fatty acids were analyzed by gas liquid chromatography (GLC) as methyl esters after methanolysis 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 derived alditol acetates. Samples were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h and coevaporated with water two to three times to remove residual acid, followed by reduction with sodium borohydride ($NaBH_4$ 10 mg/ml in 0.1 M NH_4OH) and derivatization with acetic anhydride in pyridine. The alditol acetates were extracted into $CHCl_3$. Methylation was performed according to the Hakomori method (21), using KH instead of NaH to generate the dimsyl 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 30 min.

Liberation of Lipid A with time. Native LPS (20 mg) was dissolved in 10 mM NaOAc (1 ml) and heated at 100°C, and aliquots (100 μl) were removed at 15-min intervals until 2 h. The aliquots were lyophilized and washed three times with acetone. Then the samples were extracted three times with $CHCl_3$/MeOH 2/1. The acetone extracts, chloroform extracts, and remaining residues were analyzed for the presence of fatty acid as their methyl esters.

Chromatographic techniques. Gas–liquid chromatography was performed on a Hewlett–Packard HP5890 instrument fitted with a flame ionization detector. A DB1 capillary column (30 m) programmed at 90°C for 4 min, 8°C/min 220°C was used for analysis of the fatty acyl methyl esters. Alditol acetates and partially methylated alditol acetates were analyzed on a SE54 column (30 m) 180°C for 1 min, 5°C/min 220°C, 10°C/min 250°C and 150° for 1 min, 5°C/min 220°C, respectively.

Instrumental methods. Proton NMR spectra were recorded at 600.14 MHz on a Bruker AMX-600 spectrometer. Spectra were obtained using a spectral width of 6.1 KHz, a 16-K data block, and a 90° pulse angle. Broad-band, proton-decoupled ^{13}C NMR spectra were obtained at 150.92 MHz using the same instrument at a spectral width of 30 KHz, a 32-K data block, and a 90° pulse employing WALTZ decoupling (22). Chemical shifts were referenced to acetone (δ_H, 2.225 ppm; δ_C, 31.07 ppm). All spectra were recorded at 37°C. Lipid A compounds **1** and **2** were dissolved in $CDCl_3$/methanol-*d*4 (4/1, v/v) at concentrations of 60 mg/ml. Spectral measurements were made on the de-*O*-acylated compound **1** in D_2O solution (5 mg/ml, pD-5). Two dimensional homonuclear COSY and NOESY experiments were carried out as previously described (23) and the data were processed to give magnitude or phase sensitive spectra, respectively.

Heteronuclear $^{13}C/^1H$ chemical shift correlations were measured in the ^1H-detected mode *via* multiple quantum coherence ($^1H\{^{13}C\}$ HMQC) with a Bruker 5-mm inverse broad-band probe using reverse electronics. The HMQC experiments employed the pulse sequence described by Bax *et al.* (24), and ^{13}C decoupling during ^1H acquisition was achieved using the GARP-1 composite pulse sequence (25). A data matrix ($t_1 \times t_2$) of 256 × 1024 complex points was employed over the full ^{13}C and ^1H spectral regions and 128 scans were acquired for each t_1 value. A squared sine-bell window function shifted over 60° was applied in both dimensions and the data are presented in the absorption mode in f_1 (^{13}C) and the absolute value mode in f_2 (^1H).

Phosphorous-31 spectra were recorded at 202.45 MHz on a Bruker AMX 500 spectrometer by employing a spectral width of 10 kHz, a 16-K data set, and a 90° pulse. Chemical shifts are referenced to that of an external sample of aqueous 85% phosphoric acid (δ_p, 0.0 ppm). Two dimensional $^{31}P/^1H$ chemical shift correlations were made by ^1H-detected multiple quantum coherence ($^1H\{^{31}P\}$HMQC) for a 64 × 1024 data matrix, 64 scans per t_1 value, and delays of 40, 60, or 80 ms.

FAB-MS analyses were carried out using a JEOL AX505H double-focusing mass spectrometer operating at an accelerating voltage of 3 kV and a mass resolution of 1500. *m*-Nitrobenzyl alcohol or glycerol–thioglycerol (1:3) were used as the supporting matrix for positive ion FAB-MS, while triethanolamine (TEA) was employed for negative FAB analysis. The matrix was applied to the stainless steel probe tip and was mixed with a solution (1 μl) of the sample in methylene chloride or water. A Xe atom beam of 6 kV was used to sputter and ionize the sample and spectra were calibrated with Ultramark 1621.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was done with a Kratos MS80RFA mass spectrometer fitted with a Kratos Carlo Erba Series 4160 gas chromatograph.

RESULTS

Compounds **1** and **2** (isolated by purification of Lipid A on a Sephadex LH 20 column) were analyzed for the presence of fatty acids, sugars, KDO, and phosphate (Table I). KDO was found in negligible amounts in **1**. A lower percentage of GlcN was obtained due to incomplete removal of phosphate. Compound **2** did not contain reasonable percentages of Lipid A components. NMR spectral data indicated that **2** may be a mixture of free fatty

2-octulosonic acid; LPS, lipopolysaccharide; GLC, gas–liquid chromatography; TFA, trifluroacetic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; COSY, correlation spectroscopy; NOESY, 2D nuclear Overhauser enhancement spectroscopy; GlcN, glucosamine; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; TEA, triethanolamine;

TABLE I

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

Lipid A compound	KDO (%)	Phosphate (as P) (%)	Fatty acids (total %)	Mole ratio					Sugars GlcN (%)
				$C_{10\beta}$	C_{12}	$C_{12\alpha}$	$C_{12\beta}$	C_{16}	
1	0.1	2.0	27.0	0.8	0.2	2.2	2.0	0.44	8.2
2	0.0	0.0	0.3	0.8	0.2	1.5	2.0	0.4	0.0
Native LPS	3.4	6.6	5.0	0.8	0.3	2.0	2.0	3.7[a]	2.0

Note. $C_{10\beta}$, 3-hydroxydecanoic acid; C_{12}, dodecanoic acid; $C_{12\alpha}$, 2-hydroxydodecanoic acid; $C_{12\beta}$, 3-hydroxydodecanoic acid; C_{16}, hexadecanoic acid.

[a] The amount of C_{16} fatty acid found in the native LPS, as well as in other prepartions, 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.

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 ($NaBH_4$) and acetylated to produce partially methylated alditol acetates. Three amino sugar derivatives were detected by GLC–MS analysis: 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-1,5-di-*O*-acetyl glucitol (10%), 3,4-di-*O*-methyl-2-*N*-acetamido-1,5,6-tri-O-acetyl glucitol (40%), and 3,6-di-*O*-methyl-2-*N*-methylacetamido-1,4,5 tri-*O*-acetyl glucitol (50%). The small amount of tri-*O*-methylated product resulted from the removal of the phosphate monoester from position 4′ of the terminal nonreducing glucosamine of the diglucosamine backbone, while the 3,6-di-*O*-methyl derivative was obtained from the same sugar with the phosphate intact. The reducing glucosamine contained a glycosidically linked phosphate moiety. Thus the presence of the phosphate may have been the cause for lack of methylation of the amino group at position 2 of this sugar, resulting in the *N*-acetamido-3,4-di-*O*-methyl derivative only. Since position 6 was not methylated, it follows that this was the position of linkage of the terminal sugar to the glucosamine-1-phosphate residue.

The ^1H NMR spectrum of compound **1** showed an intense envelope of resonances between 1.2 and 1.3 ppm and an apparent singlet at 0.8 ppm arising from the fatty acyl protons. The protons attached to the sugar backbone could not be detected. Correspondingly, only resonances due to fatty acid side chains were readily discernable in the ^{13}C NMR spectrum. Two poorly resolved signals arising from the two phosphate groups were observed in the ^{31}P NMR spectrum.

When compound **1** was de-O-acylated with anhydrous hydrazine, the NMR spectra (D_2O) showed vast improvement. In the ^{31}P NMR spectrum of this sample, resonances were clearly observed at −1.57 and 1.18 ppm which were indicative of two phosphomonoesters. The proton and carbon-13 resonances associated with the two 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 $^1H/^{13}C$ chemical shift correlation map are shown in Figs. 1, 2, and 3. The ^1H and ^{13}C chemical shift assignments are recorded in Table II.

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

The high-field anomeric proton resonance at 4.68 ppm showed a $J_{1,2}$ value of 7.8 Hz which was characteristic of a β-linked glucosamine residue ($GlcN_{II}$). In accordance with the assignment, this proton resonance corresponded to the C-1′ resonance at 101.3 ppm in the $^1H/^{13}C$ correlation map (Fig. 2B). 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 $GlcN_I$, which unequivocally established the β-(1,6)-linkage to the α-glucosamine residue. The H-6

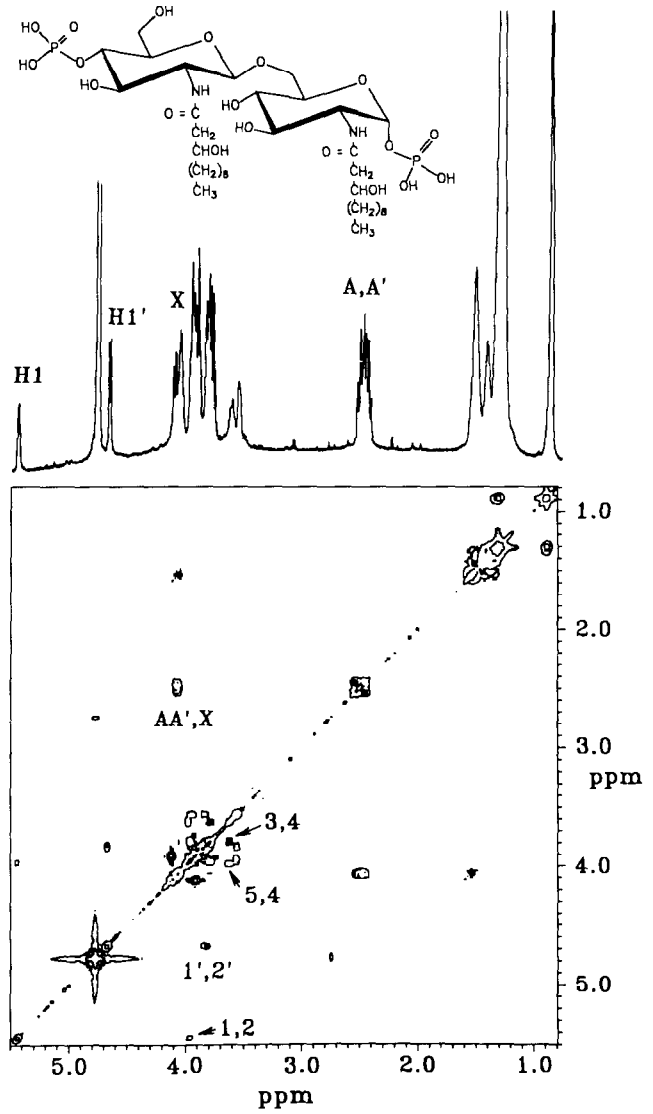


FIG. 1. Contour plot of the 2D COSY spectrum of the de-*O*-acylated sample of the lipid A (compound **3**). The spectrum was measured over the full spectral region (3500 Hz) in deuterium oxide using a data matrix of 256 × 1024 points. Cross-peaks relating H-1 and H-2 of the two glucosamines as well as H-3, H-4, and H-5 of the α-glucosamine residue ($GlcN_I$) 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 $GlcN_I$ 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 $GlcN_{II}$ occurred in a region of the ^{13}C 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 $GlcN_{II}$ (73.4 ppm) could be attributed to substitution by phosphate at that position. Indeed, a connectivity between the ^{31}P resonance at 1.18 ppm confirmed the positioning of the phosphomonoester at C-4′ of $GlcN_{II}$.

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 A_1-type fragment ions (27) at *m/z* 799 ($M-H_3PO_4$) and 440 (Fig. 4). Correspondingly, the negative ion FAB-MS showed abundant ions at *m/z* 917 (MNa–H), 895 (M–H),

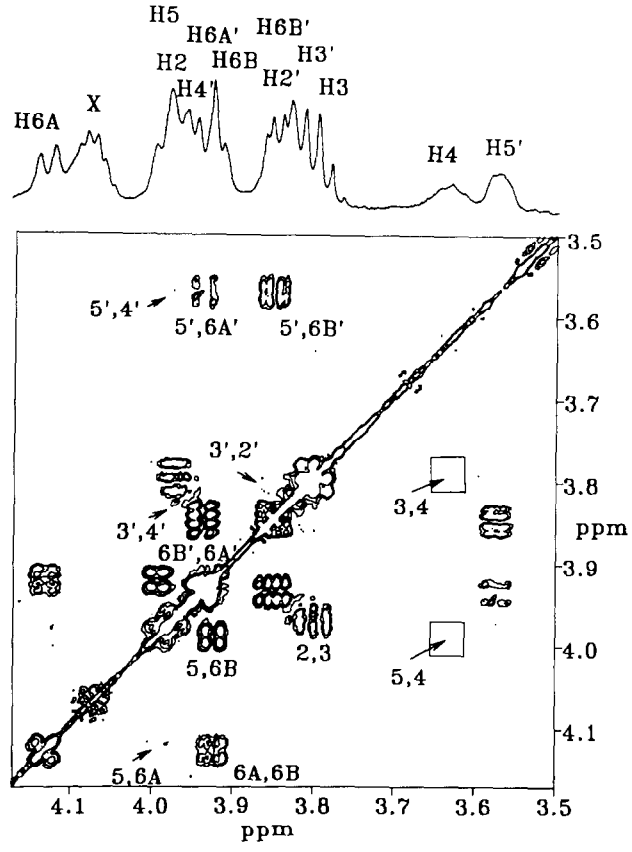


FIG. 2. Contour plot of the ring proton region (4.2–3.5 ppm) of the COSY spectrum of the de-*O*-acylated lipid A (compound **3**) measured using a narrow sweep width (1800 Hz) and 512 × 2048 data points. Assignments of cross-peaks corresponding to $GlcN_I$ (H-1–H-6B) and $GlcN_{II}$ (H-1′–H-6B′) are shown below and above the diagonal, respectively. Cross peaks relating H-3, H-4, and H-5 of $GlcN_I$ were very weak and are not visible at the indicated contour level; see Fig. 1 for assignments.

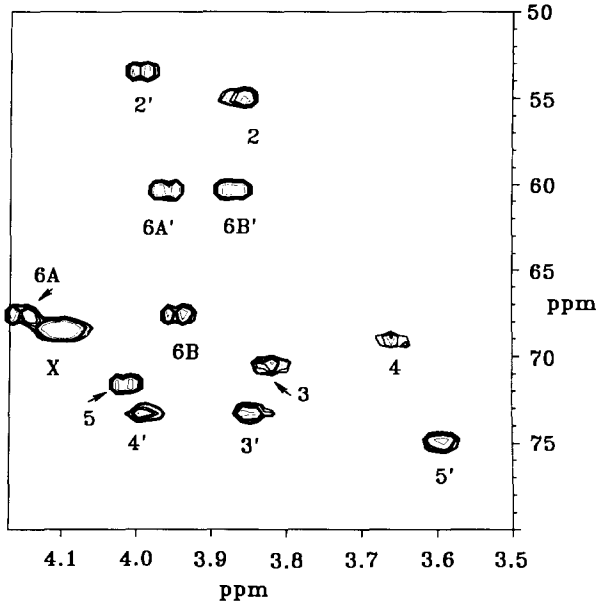


FIG. 3. Heteronuclear ^{1}H–^{13}C 2D chemical shift correlation map ($^{1}H(^{13}C)$HMQC) of the ring proton region (4.2–3.5 ppm) of the de-*O*-acylated lipid A (compound **3**). The contour indicated by X is due to the methine resonances of the 3-hydroxy dodecanyl groups.

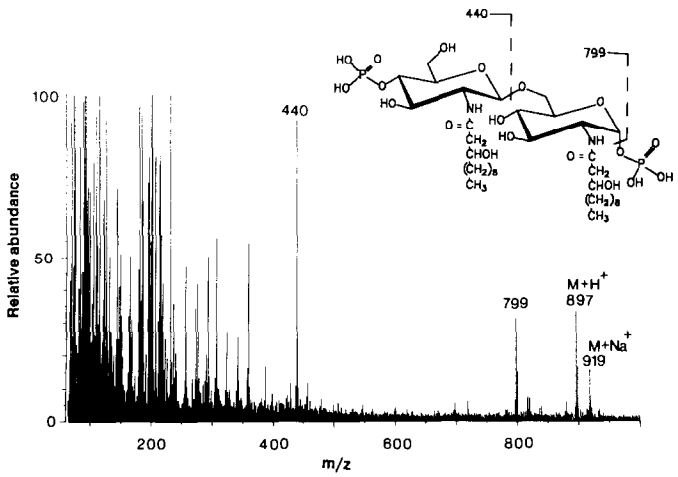


FIG. 4. Positive ion FAB mass spectrum of the de-*O*-acylated lipid A (compound **3**). Fragment ions arising from A_1-type cleavage are indicated.

and 815 ($M-H_2PO_3$) (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-hydroxdodecanoic 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 β-cleavage (27) in which the charge is retained at the reducing end of the molecule (i.e., $GlcN_I$). 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 β-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 $GlcN_{II}$. Correspondingly, the occurrence of the fragment at *m/z* 825 can be assigned to the β-cleavage product from the hexaacyl species (Table III).

TABLE II

^{1}H and ^{13}C NMR Data for *P. aeruginosa* de-*O*-acylated Lipid A (3)

Position:	1	2	3	4	5	6A	6B
^{1}H ($GlcN_I$)	5.45 ($J_{1,2} \sim 3$ Hz) ($J_{H,P} \sim 5$ Hz)	3.98	3.80	3.63	3.99	4.13	3.92
^{13}C ($GlcN_I$)	93.5	53.3	70.5	69.0	71.8	67.8	
^{1}H ($GlcN_{II}$)	4.68 ($J_{1,2}$ 7.8 Hz)	3.83	3.81	3.95	3.57	3.93	3.85
^{13}C ($GlcN_{II}$)	101.3	55.0	73.4	73.4	75.0	60.5	

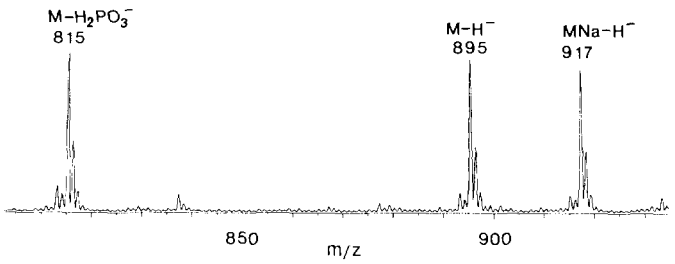


FIG. 5. Molecular ion region of the negative ion FAB mass spectrum of the de-*O*-acylated lipid A (compound **3**).

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 $GlcN_I$ 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* 1145 (**D**), 1113 (**E**), 979 (**A**), and 947 (**B**). Molecular ions due to diphosphorylated 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 diphosphorylated 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 diglucosaminyldiphosphate. 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 $CHCl_3$/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-hydroxydecanoic: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-hydroxydecanoic 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-hydroxydecanoic 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 hexaacyl 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

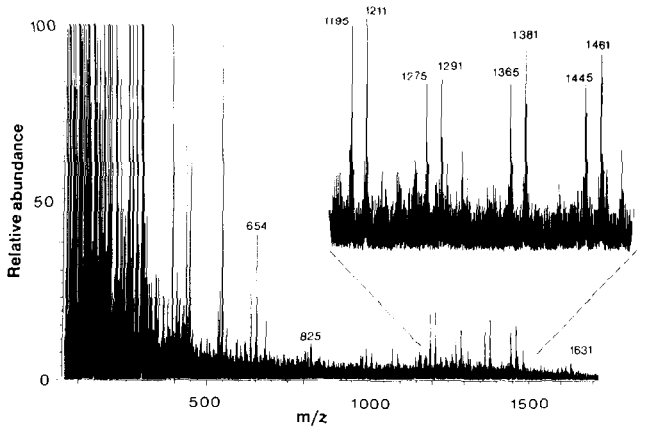
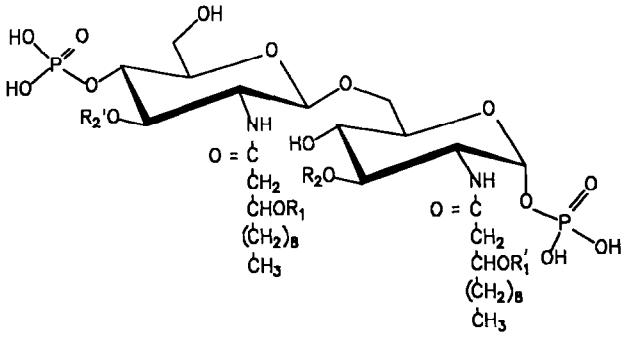


FIG. 6. Negative ion mass spectrum of the intact lipid A (compound **1**). Inset shows expansion of the molecular ion region from the penta- and tetraacyl species; see Table III for assignments.

TABLE III

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



Lipid A species	R'_1	R_1	R'_2	R_2	M_r	Negative FAB M-1	Negative FAB M-P[a]	Negative FAB M-$GlcN_{II}$[b]
Hexaacyl	2OHC12	2OHC12	3OHC10	3OHC10	1632	1631	1551	825
	2OHC12	C12	3OHC10	3OHC10	1616	1615	1535	
Pentaacyl	2OHC12	2OHC12	3OHC10	H	1462	1461	1381	654
	2OHC12	C12	3OHC10	H	1446	1445	1365	
Tetraacyl	2OHC12	2OHC12	H	H	1292	1291	1211	654
	2OHC12	C12	H	H	1276	1275	1195	

[a] $M-H_2PO_3$.

[b] β-Cleavage by loss of $GlcN_{II}$ (27).

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.

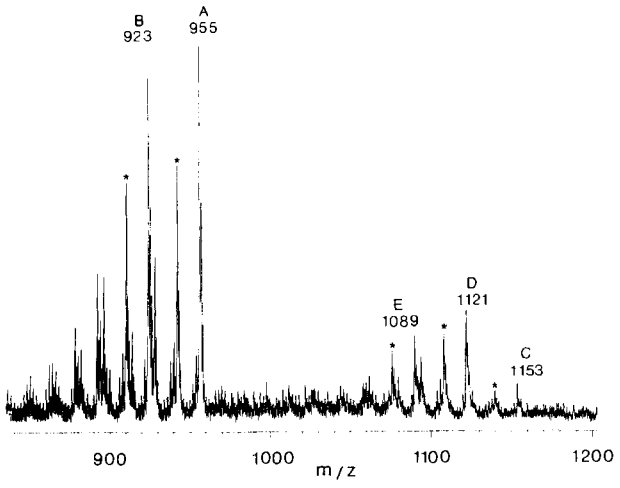


FIG. 7. Molecular ion region of the negative ion FAB mass spectrum of permethylated compound 1. The molecular species ($M-H^-$) are labeled (see Table IV for assignments). Peaks marked by an asterisk differ from the closest labeled peak by 14 mass units (i.e., B-14, A-14, E-14, D-14, and C-14) and are probably due to undermethylation.

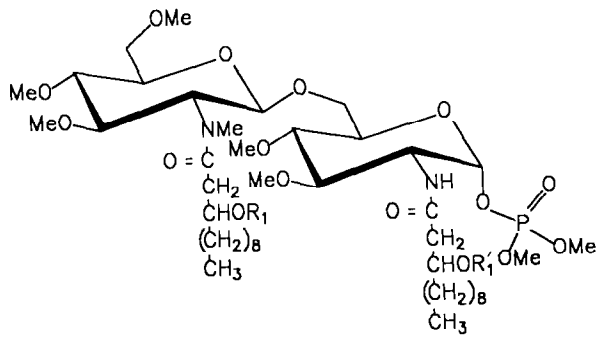
Lipid A species substituted by hexadecanoic acid were not detected in the FAB-MS and the position of substitution therefore remains uncertain. The presence of ester bound hexadecanoic acid in intact *P. aeruginosa* Lipid A has been previously inferred from plasma desorption MS data (28). The varying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

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

TABLE IV

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



Species	R_1/R'_1	Loss of MeOH[a]	M_r	Negative FAB M-1	Negative FAB M-$GlcN_{II}$[b]	Positive FAB (M + Na)
A	Me/Me	—	956	955	526	979
B	Me/Me	−32	924	923	526/494	947
C	Me/OMeC12		1154	1153		—
D	Me/OMeC12	−32	1122	1121		1145
E	Me/OMeC12	−64	1090	1089		1113

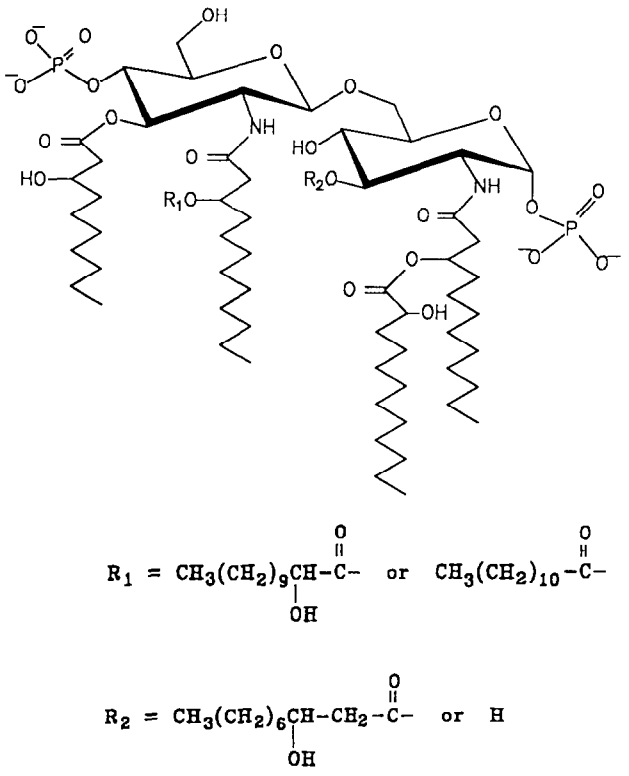
[a] Resulting from elimination due to the strongly basic Hakomori methylation conditions.
[b] β-cleavage product.

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-hydroxydecanoic acid, and dodecanoic acids which were only ester bound. A Lipid A species **3** consisting only of the amide liked fatty acids was obtained by hydrazinolysis of **1**. Positive and negative fast atom bombardment mass spectrometry of this species **3** 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–^{13}C 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 ^{13}C 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 **1** 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 $GlcN_{II}$ 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



R_1 = $CH_3(CH_2)_9CH(OH)-C(=O)-$ or $CH_3(CH_2)_{10}-C(=O)-$

R_2 = $CH_3(CH_2)_6CH(OH)-CH_2-C(=O)-$ or H

FIG. 8. Proposed structure of *P. aeruginosa* lipid A.

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.

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