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Structural Study on the Free Lipid A Isolated from Lipopolysaccharide of *Porphyromonas gingivalis*

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The chemical structure of lipid A isolated from *Porphyromonas gingivalis* lipopolysaccharide was elucidated by compositional analysis, mass spectrometry, and nuclear magnetic resonance spectroscopy. The hydrophilic backbone of free lipid A was found to consisted of β(1,6)-linked D-glucosamine disaccharide 1-phosphate. (*R*)-3-Hydroxy-15-methylhexadecanoic acid and (*R*)-3-hydroxyhexadecanoic acid are attached at positions 2 and 3 of the reducing terminal residue, respectively, and positions 2′ and 3′ of the nonreducing terminal unit are acylated with (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid and (*R*)-3-hydroxy-13-methyltetradecanoic acid, respectively. The hydroxyl group at position 4′ is partially replaced by another phosphate group, and the hydroxyl groups at positions 4 and 6′ are unsubstituted. Considerable heterogeneity in the fatty acid chain length and the degree of acylation and phosphorylation was detected by liquid secondary ion-mass spectrometry (LSI-MS). A significant pseudomolecular ion of lipid A at *m*/*z* 1,769.6 $[M-H]^-$ corresponding to a diphosphorylated GlcN backbone bearing five acyl groups described above was detected in the negative mode of LSI-MS. Predominant ions, however, were observed at *m*/*z* 1,434.9 $[M-H]^-$ and *m*/*z* 1,449.0 $[M-H]^-$, each representing monophosphoryl lipid A lacking (*R*)-3-hydroxyhexadecanoic and (*R*)-3-hydroxy-13-methyltetradecanoic acids, respectively. The presence of mono- and diphosphorylated lipid A species was also confirmed by LSI-MS of de-*O*-acylated lipid A (*m*/*z* 955.3 and 1,035.2, respectively).

The lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, an oral anaerobic gram-negative rod, exhibits bone resorption activity and induces various inflammatory cytokines in human gingival fibroblast cultures (14, 19, 30, 38, 42). It is therefore thought to be one of the most important virulence factors, along with proteases, collagenase, and adhesins, that cause host tissue destruction in adult periodontal disease (3, 7, 39, 40). *P. gingivalis* LPS displays the overall endotoxic activities of enterobacterial LPS, but the potency of the former is far more moderate than that of the latter (16, 17, 28, 30). Furthermore, *P. gingivalis* LPS contains a significant amount of protein and stimulates the splenocytes of endotoxin-nonresponsive C3H/HeJ mice to undergo mitosis (9, 21, 22). These interesting biological properties may be explained by the unusual structure of the lipid moiety of the LPS lipid A, which contains relatively longer chains of 3-hydroxy fatty acids (15 to 17 carbon atoms) (5, 9, 20, 22) than enterobacterial LPS, which consists mainly of 3-hydroxytetradecanoic acid (34, 36).

To clarify the reason for these novel actions of *P. gingivalis* LPS and to obtain direct evidence that it is implicated in these events, it is necessary to use well-defined material, preferably lipid A, the endotoxic center of LPS. However, lipid A of *P. gingivalis* has not been isolated by conventional methods in free form as *Bacteroides* LPS has (8, 43, 44). We therefore isolated the *P. gingivalis* lipid A and elucidated its chemical structure by compositional analyses, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

Microbes. *P. gingivalis* SU 63, isolated from a periodontal pocket, was grown anaerobically under the conditions reported previously (24). The cells were heated (121°C, 15 min), harvested by centrifugation (7,000 × *g*, 20 min), and washed successively with distilled water and acetone.

Preparation of LPS. LPS was extracted from acetone-dried cells with hot phenol-water (45); digested with RNase A (Sigma Chemical Co., St. Louis, Mo.), DNase I (Sigma), and proteinase K (Sigma) (37); and then purified by repeated ultracentrifugation (105,000 × *g*, 12 h, 15°C). The LPS was washed successively with phenol-chloroform-petroleum ether (2:5:8 [vol/vol/vol]) (10) and acetone and then lyophilized. The purified LPS (yield, 4.2% from dried cells) contained 39.2% total carbohydrate, 20.1% total lipid, 19.7% amino sugar, 2.5% total phosphate, and 2.2% protein.

Isolation of lipid A. Crude lipid A (434 mg) was recovered from hydrolysates (1% acetic acid, 100°C, 1.5 h) of LPS (1.25 g) according to the methods described previously (32, 33). It was purified by passage through a Dowex 50 (H^+) column (1 by 5 cm) with chloroform-methanol (3:1 [vol/vol]) as the eluent and gel permeation chromatography with a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column (2.5 by 100 cm) with the same solvent as the eluent (flow rate, 24 ml/h). Synthetic lipid A analog 506 (*Escherichia coli* type) was purchased from Daiichi Pure Chemicals (Tokyo, Japan).

Chemical degradation and modification of lipid A. De-*O*-acylation and dephosphorylation of lipid A were performed by the method described previously (12). To prepare phosphoryl-methylated lipid A, free lipid A (10 mg) was dissolved in chloroform-methanol (3:1 [vol/vol]) and passed through a Dowex 50 (H^+) column (0.6 by 4 cm). The sample was immediately methylated with diazomethane for 1 min in an ice bath and dried under a stream of nitrogen.

The lipid A backbone was prepared from the de-*O*-acylated LPS according to the method of Hase and Rietschel (15). Briefly, acid hydrolysis (0.1 M HCl, 100°C, 30 min) of LPS was followed by reduction ($NaBH_4$, room temperature, 16 h), hydrazinolysis (anhydrous hydrazine, 100°C, 40 h), and N acetylation (acetic acid anhydride-NaOH).

Analytical methods. Total amounts of phosphate, lipid, and carbohydrate were determined by methods described elsewhere (25, 27). The amounts of protein and ethanolamine were determined with a L-8500 amino acid analyzer (Hitachi) after hydrolysis (110°C, 24 h) in 6 M HCl containing 1% phenol. Amounts of neutral and amino sugars were estimated by gas-liquid chromatography (GLC) and GLC-MS by using temperature program B according to the procedure described previously (26) (0.01 M HCl, 100°C, 15 min for 4-amino-4-deoxy-L-arabinose). The amino sugar content was determined by the Morgan-Elson reaction (41, 46).

Total fatty acids were released by hydrolysis in 4 M HCl (100°C, 4 h) followed by heating in 1 M NaOH (100°C, 2 h) (11). Ester-bound fatty acids were selectively liberated by 0.25 M CH_3ONa treatment (37°C, 15 h) (35). Amide-bound acyloxyacyl residues were analyzed according to the method of Wollenweber et al. (47). All fatty acids were determined by GLC and GLC-MS (program A or C) as the methyl esters. The absolute configuration of (*R*)-3-hydroxy fatty acids was determined by ^1H NMR spectroscopy with the shift reagent Tris-[3-(heptaflu-

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TABLE 1. Chemical composition of LPS and lipid A from *P. gingivalis*

Constituent	Amt (nmol/mg) in:	
	LPS	Lipid A[a]
GlcN	862.6	838.9 (2.0)
Phosphate	255.1	499.2 (1.2)
Ethanolamine	433.3	64.1 (0.2)
$iC_{15:0}$	75.2	54.8 (0.1)
$aC_{15:0}$[b]	25.8	18.5 (0.1)
$C_{16:0}$	143.2	317.4 (0.8)
$iC_{17:0}$	36.1	73.9 (0.2)
3-OH-$iC_{15:0}$	62.2	235.7 (0.6)
3-OH-$C_{16:0}$	74.9	282.2 (0.7)
3-OH-$iC_{17:0}$	222.6	841.6 (2.0)
Neutral sugars	2,088.9	ND[c]

[a] Results in parentheses are expressed as moles per 2 mol of GlcN.
[b] $aC_{15:0}$, 12-methyltetradecanoic acid.
[c] ND, not detected.

oropropylhydroxymethylene)-(+)-camphorato] europium(III) derivative (2). Authentic (*R*)- and (*S*)-3-hydroxyhexadecanoic acid methyl esters were used as the reference compounds.

Methylation proceeded according to the method of Hakomori (13). The methylated material was purified by using a SEP-PAK C_{18} cartridge (Waters, Milford, Mass.) and analyzed by GLC and GLC-MS (program D). Acetolysis, reduction ($NaBDH_4$), and acetylation were performed as described previously (29).

GLC conditions. GLC was performed with a GC-14A instrument (Shimadzu, Kyoto, Japan) equipped with a chemically bonded DB-5 fused silica capillary column (DB5MS, 30 m by 0.32 mm; J&W Scientific, Folsom, Calif.) with nitrogen as the carrier gas. The temperature settings for programs A to D were 120, 140, 150, and 250°C, respectively, each for 3 min, increasing to 250°C at 3°C/min for programs A and B and to 300°C at 5°C/min for programs C and D.

MS. GLC-MS was performed with a DX-300 instrument (JEOL, Tokyo, Japan) equipped with the same column described above, with helium as the carrier gas. The electron impact-MS (EI-MS) was recorded at 70 eV, and ammonia was used as the reactant gas for chemical ionization-MS (CI-MS).

Liquid secondary ion-MS (LSI-MS) of intact and chemically modified lipid A was performed with a ZAB-2SEQ instrument (VG Analytical, Manchester, England) operated at 8 keV in the negative and positive modes. The cesium gun was operated at 30 keV, and current-controlled scans were acquired at a rate of 30 s per decade. The matrix was 3-nitrobenzyl alcohol.

Fast atom bombardment-tandem MS (FAB-MS/MS) was carried out with an SX/SX-102A instrument (JEOL) operated at 10 keV in the negative-ion mode. The xenon gun was operated at 6 keV, and current-controlled scans were acquired at a rate of 30 s per decade. Helium was used as the collision gas.

NMR spectroscopy. ^1H NMR analysis to determine the absolute configuration of 3-hydroxy fatty acids (methyl esters) was performed with a VXR-400S (Varian, Palo Alto, Calif.) in $CDCl_3$ at 30°C. Tetramethylsilane (0.00 ppm) was used as an internal standard. The ^{31}P NMR spectrum of de-*O*-acylated lipid A (4 mg) was recorded at 161.9 MHz in D_2O containing 50 mM EDTA (pH 11.0 with triethylamine) at 30°C. Chemical shifts were determined relative to 80% phosphoric acid (0.00 ppm) as an external standard. The ^{31}P NMR spectrum of phosphoryl-methylated lipid A was measured with an JEOL A-600 instrument in methyl $(CD_3)_2SO/C_6D_6$ (1:9 [vol/vol]) with triphenyl phosphate (−18.00 ppm) as an external standard.

Field gradient $^1H,^1H$-homonuclear correlation spectroscopy (HH-COSY) (6, 31) and a $^{31}P,^1H$-heteronuclear multiple-quantum coherence (PH-HMQC) experiment (1) of phosphoryl-methylated lipid A were performed with an JEOL A-600 instrument by using methyl $(CD_3)_2SO/C_6D_6$ (1:9 [vol/vol]) as the solvent. In HH-COSY, the 2K × 1K datum points were processed with square sine bell functions for resolution enhancement in the f_2 and f_1 dimensions, respectively, and were zero-filled to give 2K × 2K real datum points. In the PH-HMQC experiment, the 1K × 0.5K datum points were processed with the square sine bell function for the f_2 dimension and with the Brackman Harriss function for the f_1 dimension and zero-filled to give 1K × 1K real datum points. All measurements were done by using JEOL software.

RESULTS

Chemical composition of lipid A. Chemical analysis revealed that *P. gingivalis* lipid A consists of the components listed in Table 1. A small amount of protein (1.9%) was also present in the lipid A preparation. The 4-amino-4-deoxy-L-arabinose present in the form of a polar head group in the lipid A's of *Salmonella* spp., some species of *Pseudomonas*, and *Proteus* spp. (36) was not detected.

TABLE 2. Ester-bound fatty acid analysis of *P. gingivalis* lipid A

Fatty acid	Amt (nmol/mg)	
	Without carboxymethylation	With carboxymethylation
$iC_{15:0}$	40.4	42.9
$aC_{15:0}$	14.3	14.7
$C_{16:0}$	225.8	262.4
$iC_{17:0}$	50.6	59.9
3-OH-$iC_{15:0}$[a]	189.6	214.3
3-OH-$C_{16:0}$[a]	170.6	187.2
3-OH-$iC_{17:0}$[a]	62.4	70.6

[a] The amounts of these fatty acids include each 3-*O*-methoxy fatty acid derived from the nucleophilic addition of methoxide to methyl Δ^2-fatty acids caused by β-elimination during the reaction.

The absolute configuration of (*R*)-3-hydroxy fatty acids was determined by ^1H NMR studies of the methyl esters. The carboxymethyl proton, normally resonating at 3.71 ppm in $CDCl_3$ shifts in a dose-dependent manner to a lower field in the presence of the shift reagent Europium(III) complex, and the proton shift induced by this reagent is expressed more efficiently by the *S* configuration than by the *R* configuration. In the ^1H NMR analysis of the fatty acid fraction obtained from *P. gingivalis* lipid A, carboxymethyl protons of 3- and non-hydroxy fatty acids were detected at 3.79 and 3.67 ppm, respectively. The signal shift of 3-hydroxy fatty acids to a lower field in response to the reagent was the same as that of authentic (*R*)-3-hydroxyhexadecanoic acid methyl ester, indicating that the 3-hydroxy fatty acids present in the *P. gingivalis* lipid A have the *R* configuration.

Analysis of lipid A backbone. The chemical structure of the lipid A backbone was determined by methylation analysis. GLC analysis of the product yielded a major peak identified as 6-*O*-[2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-β-D-glucopyranosyl]-2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-glucitol derived from GlcN disaccharide. The CI-mass spectrum had the predicted molecular mass of the derivative whose ion was recognized at *m/z* 553 $[M+H]^+$. EI-MS also revealed the ion at *m/z* 552 $[M]^+$ and fragment ions at *m/z* 276 and 260, representing cleavage of the glycosidic linkage of the disaccharide. A fragment ion at *m/z* 218 corresponding to the cleavage between the C-4–C-5 bond, which is characteristic of the derivative of the (1,6)-linked GlcN disaccharide backbone (4), was also detected. Other characteristic ions were observed at *m/z* 228 (*m/z* 260 minus methanol), *m/z* 130 (cleavage between C-2–C-3), *m/z* 174 and 378 (C-3–C-4).

The derivative was further acetolyzed, reduced ($NaBD_4$), acetylated, and then analyzed by GLC and GLC-MS, yielding 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-[1-D]-glucitol; 6-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-glucitol; and 1,6-di-*O*-acetyl-3,4,5-tri-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-glucitol (data not shown). This last derivative was an artificial product produced by acetylation (18). These results indicate that the backbone of *P. gingivalis* lipid A consists of a (1,6)-linked GlcN disaccharide.

Fatty acid linkages. Ester-bound fatty acids were investigated by treating intact lipid A with CH_3ONa. As shown in Table 2, the methyl esters of hexadecanoic acid ($C_{16:0}$), (*R*)-3-hydroxy-13-methyltetradecanoic acid [(*R*)-3-OH-$iC_{15:0}$], and (*R*)-3-hydroxyhexadecanoic acid [(*R*)-3-OH-$C_{16:0}$] were detected in the methanolysates as ester-bound fatty acids by

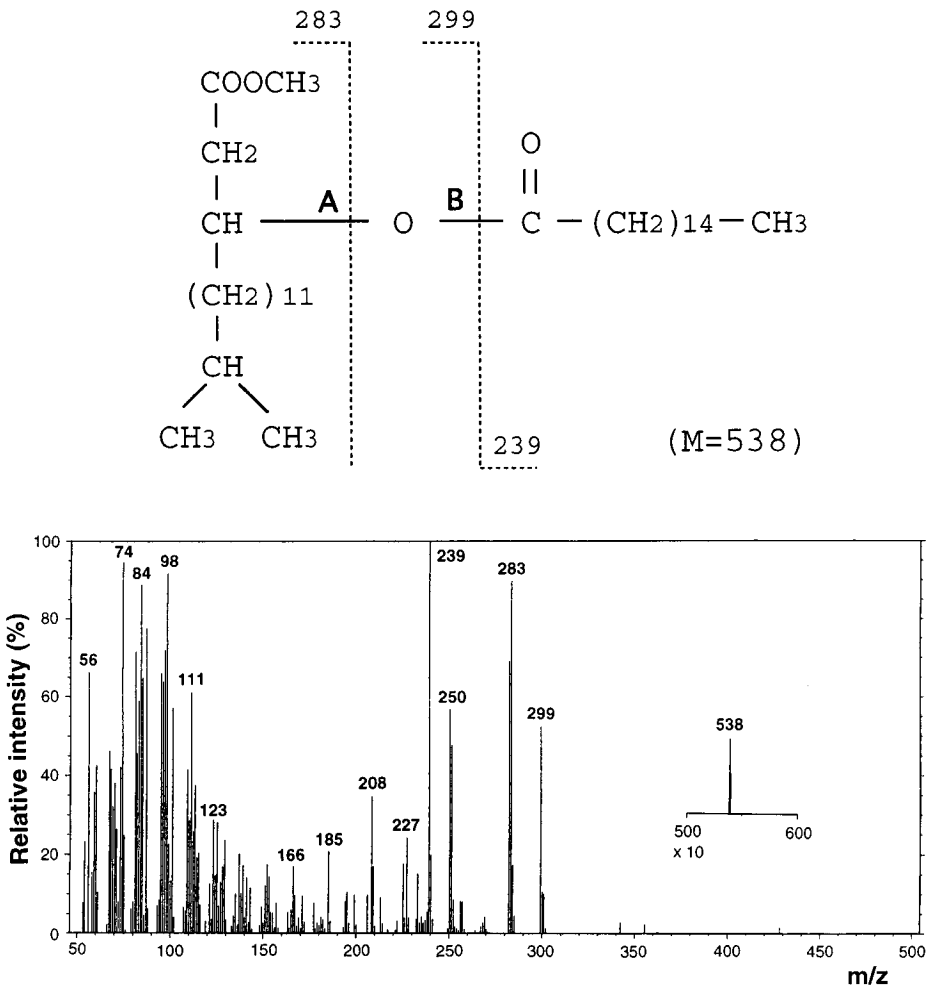

FIG. 1. EI-mass spectrum and fragmentation pattern of (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid methyl ester found in *P. gingivalis* lipid A. M, molecular weight.

direct GLC analysis. No fatty acids were found by GLC analysis after carboxyl methylation (Table 2), showing that the hydroxyl residue of the ester-bound 3-hydroxy fatty acids linked directly to lipid A backbone was not esterified by a second acyl group. On the other hand, (*R*)-3-hydroxy-15-methylhexadecanoic acid [(*R*)-3-OH-$iC_{17:0}$] (2.0 mol/2 mol of GlcN) was released from de-*O*-acylated lipid A as the sole amide-linked fatty acid.

In the analysis to determine amide-bound 3-acyloxyacyl residues (47), an acyloxyacyl group was detected on GLC and was identified as (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid [(*R*)-3-*O*-($C_{16:0}$)-$iC_{17:0}$] methyl ester by EI-MS (Fig. 1), in which characteristic fragment ions based on the cleavage of the linkage sites A and B were detected in addition to the molecular ion. The molecular mass was also confirmed as 538 Da (*m/z* 556 $[M+NH_4]^+$) by CI-MS (data not shown).

The presence of the acyloxyacyl fatty acid also indicated that the remaining ester-linked fatty acids, (*R*)-3-OH-$C_{16:0}$ and (*R*)-3-OH-$iC_{15:0}$, are bound directly to the lipid A backbone.

LSI-MS and FAB-MS/MS of lipid A. The distribution of fatty acids in the lipid A backbone was analyzed by MS. The negative-ion mode of LSI-MS of intact lipid A revealed considerable heterogeneity on the basis of both the fatty acid chain length and the degrees of phosphorylation and acylation, as shown in Fig. 2 and Table 3. Predominant ions were observed at *m/z* 1,434.9 $[M-H]^-$ and *m/z* 1,449.0 $[M-H]^-$, representing monophosphoryl lipid A bearing four fatty acid residues. The significant ion at *m/z* 1,769.6 $[M-H]^-$ was recognized as the ion with highest mass, corresponding to diphosphoryl GlcN disaccharide replaced by the five acyl groups indicated in Table 3, and its monophosphorylated species was also detected at *m/z* 1,689.3 $[M-H]^-$. A tetraacyl compound lacking both phosphates and monophosphoryl-triacyl lipid A molecules were detected at *m/z* 1,369.2 $[M-H]^-$ and 1,194.1 $[M-H]^-$, respectively. In addition, diphosphoryl compounds which lacked (*R*)-3-OH-$C_{16:0}$ or (*R*)-3-OH-$iC_{15:0}$ were determined (*m/z* 1,514.7 or 1,529.3, respectively). Fragment ions at both *m/z* 780.6 and 766.5 (intensity ratio, 3:2) are presumed to be the reducing-terminal GlcN unit (GlcN I) on the basis of the results of standard LSI-MS with synthetic lipid A analog 506 (*E. coli* type), in which the fragment ion (*m/z* 710.5) corresponding to GlcN I is easily assigned, while the fragment ion of the non-reducing-terminal GlcN unit (GlcN II) could not be assigned in the spectrum under the conditions that we employed (data not shown).

In the negative-ion mode of LSI-MS with de-*O*-acylated lipid A, the quasimolecular ions at *m/z* 955.3 $[M-H]^-$ and *m/z* 1,035.2 $[M-H]^-$ were detected at an intensity ratio of 7.7:1.0, as

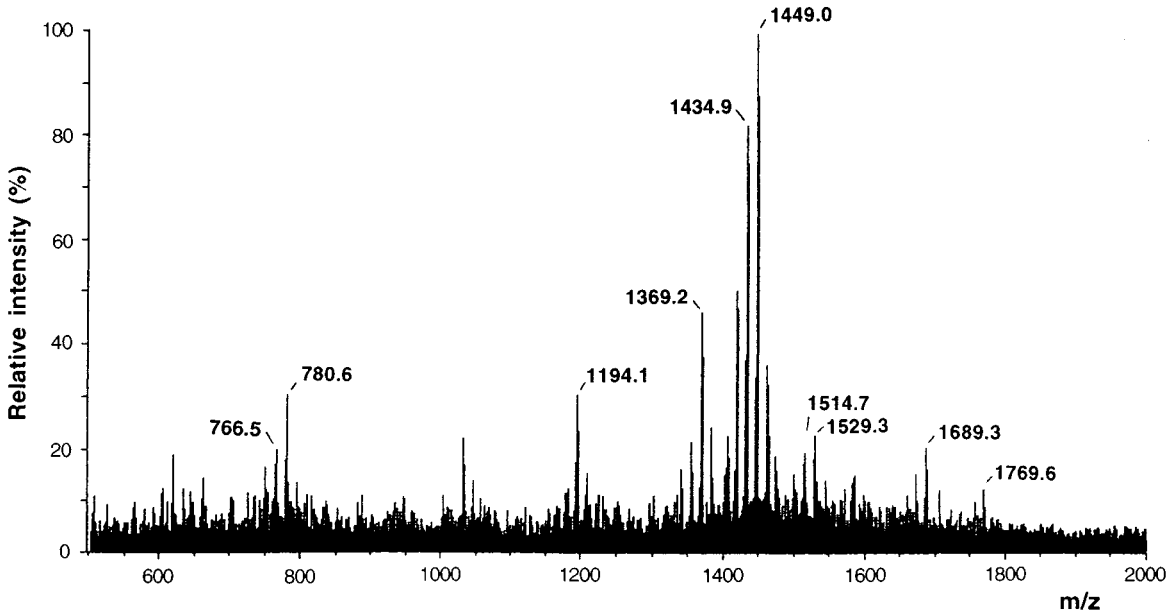

FIG. 2. LSI-mass spectrum of *P. gingivalis* native lipid A in negative-ion mode. The matrix was 3-nitrobenzyl alcohol.

shown in Fig. 3a. The predominant peak (*m/z* 955.3) was identified as monophosphoryl GlcN disaccharide bearing 2 molecules of amide-bound (*R*)-3-OH-$iC_{17:0}$, and the small peak (*m/z* 1,035.2) was assigned to the diphosphorylated form.

The mass spectrum of dephosphorylated lipid A in the positive-ion mode of LSI-MS is shown in Fig. 3b. The peak at *m/z* 1,633.1 $[M+Na]^+$ represents a molecule consisting of GlcN, (*R*)-3-OH-$iC_{15:0}$, $C_{16:0}$, (*R*)-3-OH-$C_{16:0}$, and (*R*)-3-OH-$iC_{17:0}$ in a molar ratio of 2:1:1:1:2, and the ion groups around the peak are also related to a pentaacyl lipid A disaccharide species on the basis of the heterogeneity of the fatty acid chain lengths. Pseudomolecular ions at *m/z* 1,378.9 $[M+Na]^+$ and *m/z* 1,393.1 $[M+Na]^+$ corresponded to the tetraacyl species, which lack (*R*)-3-OH-$C_{16:0}$ and (*R*)-3-OH-$iC_{15:0}$, respectively, in the lipid A molecule (*m/z* 1,769.6), and the peak at *m/z* 1,137.6 $[M+Na]^+$ was the triacyl component representing the absence of both fatty acids. Since the β(1,6)-linkage of the lipid A backbone is partially cleaved during dephosphorylation (23), the peaks at *m/z* 708.4 and 723.6 were detected as $[M+Na]^+$ values of GlcN II and GlcN I, respectively, which did not appear in LSI-MS of intact lipid A. These results indicate that the predominant molecule (*m/z* 1,449.0 $[M-H]^-$) in *P. gingivalis* lipid A consists of GlcN II with 1 molecule each of GlcN and amide-linked (*R*)-3-*O*-($C_{16:0}$)-$iC_{17:0}$ and of GlcN I with 1 mol each of GlcN, phosphate, amide-bound (*R*)-3-OH-$iC_{17:0}$, and ester-bound (*R*)-3-OH-$C_{16:0}$. These findings were also confirmed by the characteristic fragmentation pattern derived from the cleavage processes A to E, detected in negative-ion mode of FAB-MS/MS of the parent ion (*m/z* 1,449.0 $[M-H]^-$), as shown in Fig. 4.

Position of free hydroxyl and phosphate groups. NMR analysis of phosphoryl-methylated lipid A was performed to determine the location of free hydroxyl and phosphate groups. The ^1H NMR data assigned by the field gradient HH-COSY experiment are summarized in Table 4. An anomeric proton in the αGlcN I unit was detected at 6.04 ppm (*dd* [doublet doublet] $J_{H\text{-}1,H\text{-}2}$ of 3.02 Hz). The resonance of H-1 had shifted to a 0.5-ppm-lower field than in the nonsubstituted compound (5.46 ppm) (31), indicating that position 1 of GlcN I was replaced by a phosphate group ($J_{H\text{-}1,P}$ of 5.77 Hz). Hydroxyl

TABLE 3. Compositional summary of *P. gingivalis* lipid A species detected as pseudomolecular ions $[M-H]^-$ in negative-ion mode of LSI-MS

m/z[a]	Mol wt[b]	Peak intensity (%)[c]	No. of residues					
			GlcN	Phosphate	$C_{16:0}$	3-OH-$iC_{15:0}$	3-OH-$C_{16:0}$	3-OH-$iC_{17:0}$
1,769.6	1,770.4	12.4	2	2	1	1	1	2
1,689.3	1,690.4	20.6	2	1	1	1	1	2
1,529.3	1,530.0	22.8	2	2	1	0	1	2
1,514.7	1,516.0	19.5	2	2	1	1	0	2
1,449.0	1,450.0	100.0	2	1	1	0	1	2
1,434.9	1,436.0	82.2	2	1	1	1	0	2
1,369.2	1,370.0	46.4	2	0	1	0	1	2
1,194.1	1,195.6	30.5	2	1	1	0	0	2
780.6	782.0	30.4	1	1	0	0	1	1
766.5	768.0	20.0	1	1	0	1	0	1

[a] m/z of each pseudomolecular ion $[M-H]^-$ detected by LSI-MS.
[b] Molecular weight was calculated as the average weight.
[c] The values shown are the relative intensities, taking that of the ion at *m/z* 1,449.0 as 100%.

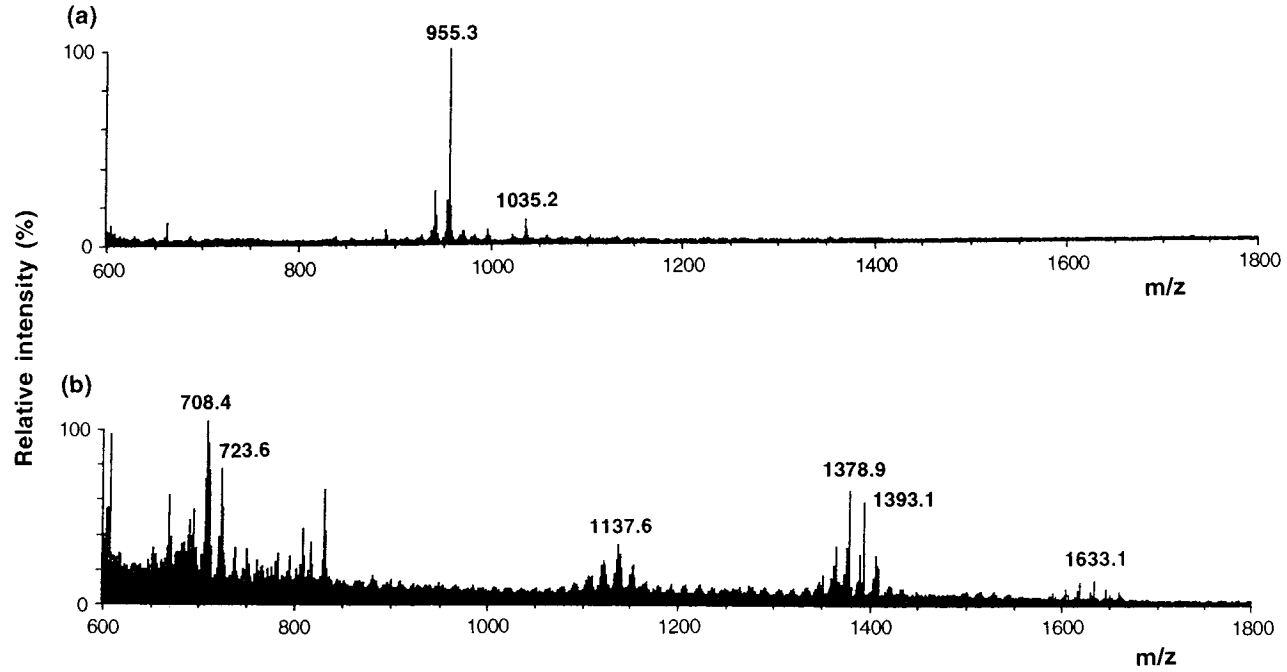

FIG. 3. LSI-mass spectra of *P. gingivalis* de-*O*-acylated lipid A in negative-ion mode (a) and *P. gingivalis* dephosphorylated lipid A in positive-ion mode (b). The matrix was 3-nitrobenzyl alcohol.

proton cross-coupling to the H-4 signal of GlcN I was observed at 5.80 ppm, proving that position 4 of GlcN I is not substituted. Two nonreducing-terminal GlcN units (GlcN II) were assigned. The resonance of H-1′ of GlcN II had shifted to a 0.39- to 0.43-ppm-higher field, indicating a glycosidic linkage. The $J_{H\text{-}1',H\text{-}2'}$ constant (8.52 Hz) proved to be in the β configuration. Two different of H-4′ signals were observed, at 3.96 and 4.35 ppm. The former was assigned to the H-4′ proton of

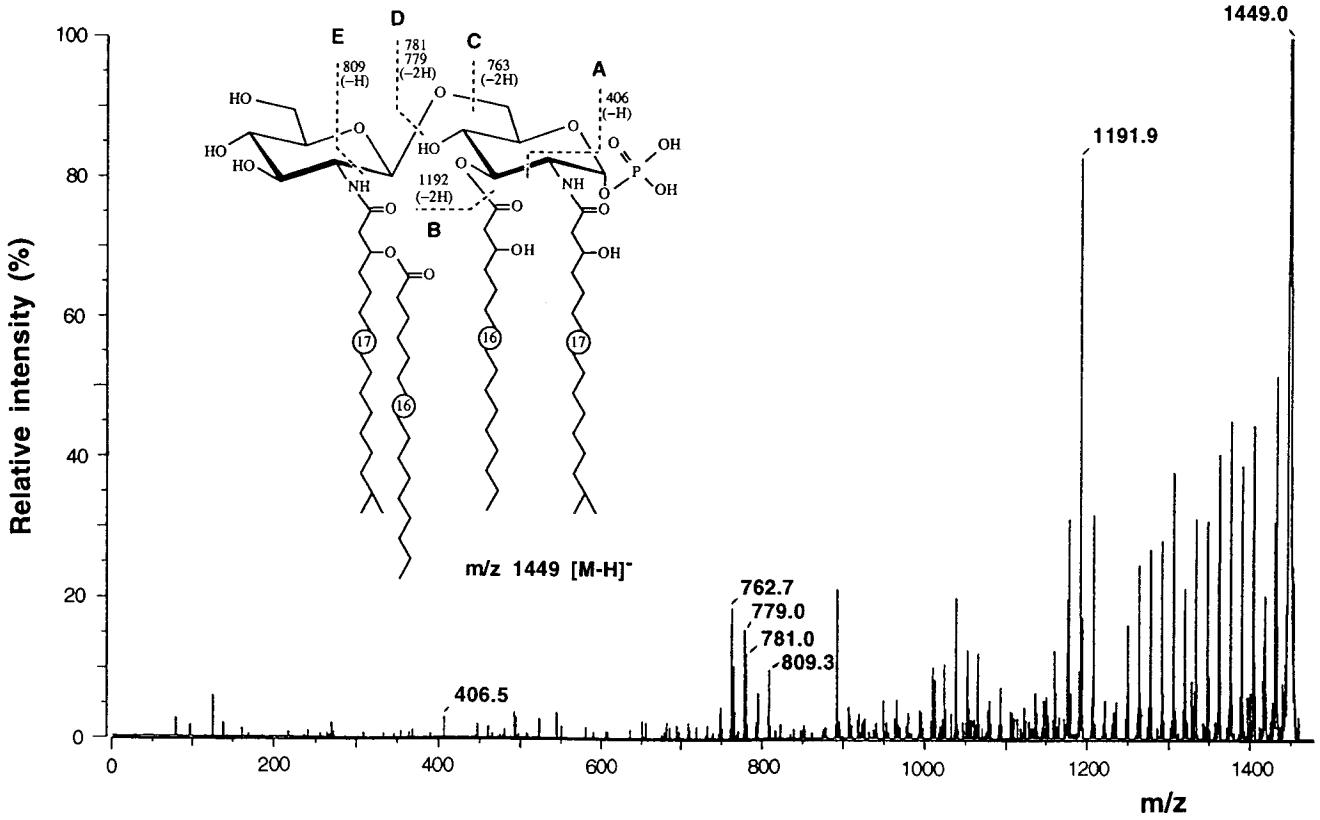

FIG. 4. FAB-MS/MS of *P. gingivalis* native lipid A in negative-ion mode. The matrix was 3-nitrobenzyl alcohol.

TABLE 4. Chemical shift and coupling constant for *P. gingivalis* phosphomethylated lipid A by FG-HH-COSY

Proton[a]	Value for chemical shift and coupling constant[b]			
	GlcN I	GlcN II[c]		Fatty acid
		Without phosphate	With phosphate	
H-1	6.04 ($J_{1,2}$ = 3.02)			
H-2	4.71			
H-3	5.67			
H-4	3.95			
H-5	4.45			
H-6a	4.06			
H-6b	4.30			
2-NH	8.17 ($J_{NH,2}$ = 8.25)			
4-OH	5.80			
H-1′		5.07 ($J_{1',2'}$ = 8.52)	5.03 ($J_{1',2'}$ = 8.53)	
H-2′		4.35	4.00	
H-3′		5.51	5.49	
H-4′		3.96	4.35	
H-5′		3.70	3.65	
H-6′a		4.20	4.20	
H-6′b		4.25	4.25	
2′-NH		8.16 ($J_{NH',2'}$ = 7.97)	8.11 ($J_{NH',2'}$ = 7.69)	
4′-OH		5.64		
6′-OH		4.85	4.85	
$H_{\alpha 1}$				2.40
$H_{\alpha 2}$				2.54
$H_{\alpha 3}$				2.76
$H_{\alpha 4}$				2.86
$H_{\beta 1}$				4.26
$H_{\beta 2}$				4.22
$H_{\beta 3}$				4.25
$H_{\beta 4}$				5.69
$H_{\beta 1\text{-OH}}$				4.54
$H_{\beta 2\text{-OH}}$				4.95
$H_{\beta 3\text{-OH}}$				4.52

[a] 4-OH and $H_{\beta\text{-OH}}$ are the hydroxyl protons at the 4-carbon of the reducing-terminal GlcN residue (GlcN I) and the β-carbon of the unsubstituted hydroxy fatty acids, respectively; 4′- and 6′-OH are the hydroxyl protons at the 4′- and 6′-carbons of the nonreducing-terminal GlcN residue (GlcN II), respectively. H_α and H_β are the protons at the α- and β-carbons of the hydroxy fatty acids, respectively. Other peaks originating from the heterogeneity were also detected (data not shown).

[b] Chemical shift is expressed in parts per million; coupling constant (J) is expressed in hertz.

[c] Two types of GlcN II were assigned with regard to the absence (no substitution) or presence of a phosphate group at position 4′.

GlcN II, which is unsubstituted at position 4′, because the signal was directly J-coupled with a free-hydroxyl proton (5.64 ppm). The latter was identified as the H-4′ of GlcN II, which has a phosphate residue at position 4′. Hydroxyl proton cross-coupling to H-6′ of GlcN II was determined at 4.85 ppm, proving that position 6′ of GlcN II is also unsubstituted. The ^{1}H NMR study also showed that there is an acyloxyacyl residue in *P. gingivalis* lipid A and that position 3 of the other 3-hydroxy fatty acids is not esterified.

The location of phosphate groups on lipid A was investigated by ^{31}P NMR spectroscopy. Two signals were observed for de-*O*-acylated lipid A, a predominant signal resonating at 2.26 ppm and a small signal at 4.50 ppm (Fig. 5a). The signal at 2.26 ppm was assigned to a glycosidic phosphate at the C-1 position, and the latter was identified as an ester-linked phosphate. Two phosphate signals were also detected in the ^{31}P NMR spectrum of phosphoryl-methylated lipid A, at −0.63 and 0.24 ppm (f_1 dimension of Fig. 5b). The PH-HMQC experiment (Fig. 5b) showed that the phosphate detected at −0.63 ppm is attached at position 1 of GlcN I and that the other is attached at position 4′ of GlcN II. Signals A and D were identified as the cross-peaks between phosphate and H-1 (GlcN I) and phosphate and H-4′ (GlcN II). Signals E and F were assigned to the cross-peaks between phosphate and the phosphoryl-methyl protons. Signals B and C suggested that ethanolamine is attached to a phosphate group at position 1 of GlcN I.

DISCUSSION

In the present study we isolated and chemically characterized lipid A from *P. gingivalis*. Its proposed structure is shown in Fig. 6. The backbone of the predominant molecule in the lipid A complex consists of a β(1,6)-linked GlcN disaccharide 1-phosphate, in which amino groups at positions 2 and 2′ are acylated with (*R*)-3-OH-iC$_{17:0}$ and (*R*)-3-O-(C$_{16:0}$)-iC$_{17:0}$, respectively. Positions 3 and 3′ are esterified with (*R*)-3-OH-C$_{16:0}$ and (*R*)-3-OH-iC$_{15:0}$, respectively, which may be reversible. However, the predominant structures lacked one or the other of these ester-linked acyl residues. Positions 4 and 6′

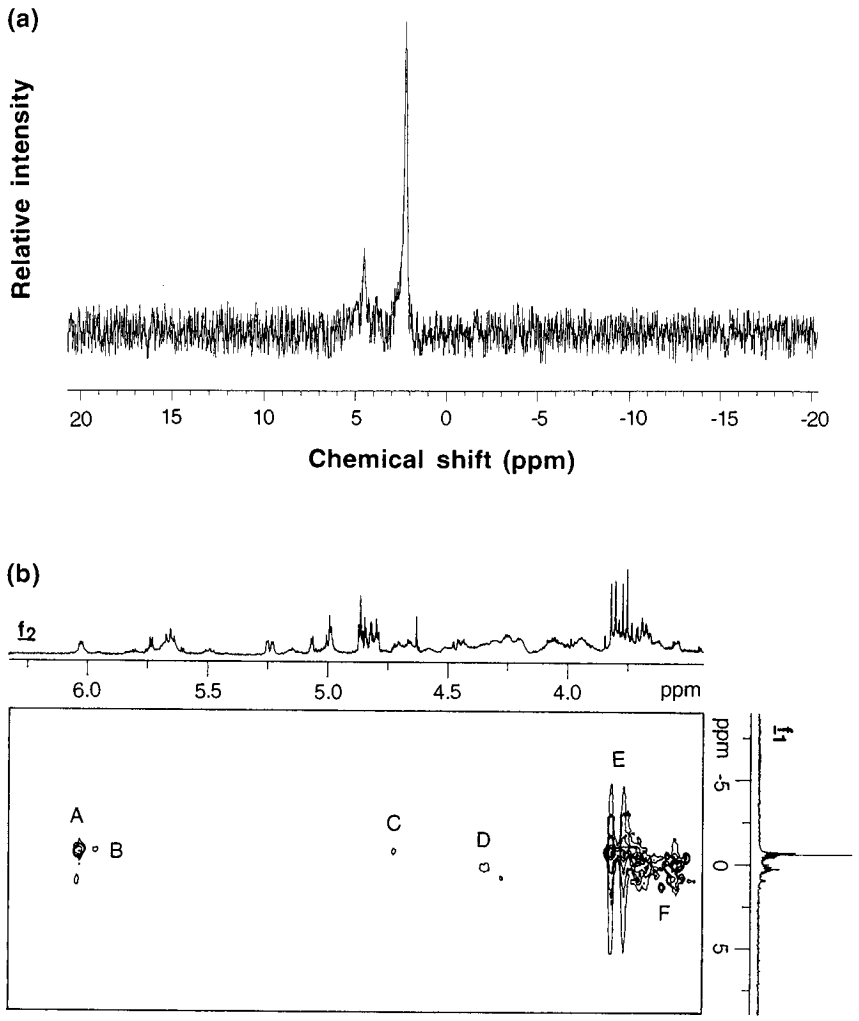

FIG. 5. ^{31}P NMR spectrum of *P. gingivalis* de-*O*-acylated lipid A (a) and PH-HMQC NMR spectrum of *P. gingivalis* phosphoryl-methylated lipid A (b). The samples were dissolved in D_2O containing 50 mM EDTA (pH 11.0) (a) and methyl $(CD_3)_2SO/C_6D_6$ (1:9 [vol/vol]) (b). The f_2 and f_1 dimensions of the PH-HMQC spectrum represent ^{1}H and ^{31}P NMR spectra, respectively.

were unsubstituted, because two hydroxyl protons at these positions which J-coupled directly with H-4 and H-6′, respectively, were assigned in the HH-COSY spectrum. Chemical and physicochemical experiments revealed the presence of the lipid A species as a minor component, whose position 4′ is replaced by phosphate residues. Moreover, the phosphate group linked α glycosidically to position 1 may be further esterified by a small amount of ethanolamine in the form of a polar head group, as in *S. minnesota* lipid A (36).

In the genus *Bacteroides*, the lipid A of *Bacteroides fragilis* was previously isolated and structurally defined (44). It has structural features similar to those of the *P. gingivalis* lipid A determined here in terms of fatty acid composition and backbone structure. However, these two lipid A's differ with regard to phosphate substitution. As do the lipid A's of *Rhodomicrobium vannielii* (18) and *Bacteroides intermedius* LPSs (20), *B. fragilis* lipid A (44) lacks the ester-linked phosphate group at position 4′ of the lipid A backbone, the constituent common to enterobacterial lipid A's (34). Using mass spectra of both native and de-*O*-acylated lipid A complexes and NMR analyses, we demonstrated in this study that *P. gingivalis* synthesizes diphosphorylated lipid A species, although a minor component of lipid A complexes.

The relationship between the chemical structure of lipid A and its endotoxic activity has been examined by many investigators using both natural and chemically synthesized lipid A analogs. In principle, these investigators reached a tentative conclusion, namely, that both of the phosphates, at positions 1 and 4′, considerably influence its activity. Fatty acids play an especially important role in endotoxic activity. Their number, binding sites, and type appear to be critical determinants of the potency of endotoxic activity. In fact, some of the non- or low-toxic lipid A preparations found thus far have characteristic fatty acids with the usual diglucosamine backbone and phosphates. In view of this, it is possible that all of the unusual structural features of *P. gingivalis* lipid A shown in this study are associated with low endotoxicity and a unique biological property, namely, induction of mitosis by *P. gingivalis* LPS in spleen cells of endotoxin-nonresponsive mice. The isolation of lipid A from *P. gingivalis* LPS and its structural determination encourage further studies on the relationship between structure and biological activity at the native lipid A level. Moreover, biological data obtained by certain investigators using LPSs should be confirmed by using free form lipid A, including its action in C3H/HeJ mice. An investigation of the overall synthesis of lipid A is now in progress in our laboratory, in

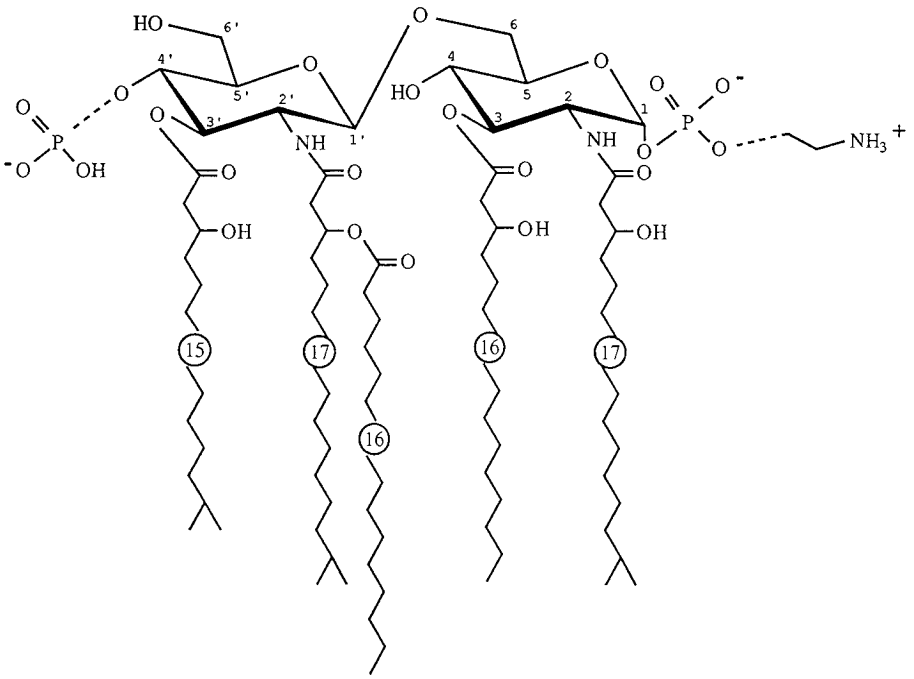

FIG. 6. Proposed structure of *P. gingivalis* lipid A. A dotted bond means minor substitution. Positions 3 and 3′ are esterified with (*R*)-3-OH-$C_{16:0}$ and (*R*)-3-OH-$iC_{15:0}$, respectively, which may be reversible. Considerable heterogeneity of the fatty acid chain length and the degree of acylation (especially at positions of 3 and 3′ [Table 3]) was found in lipid A.

order to clarify the participation of the protein residue in lipid A (1.9%) in stimulation of C3H/HeJ mice.

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