A Methylated Phosphate Group and Four Amide-linked Acyl Chains in *Leptospira interrogans* Lipid A

THE MEMBRANE ANCHOR OF AN UNUSUAL LIPOPOLYSACCHARIDE THAT ACTIVATES TLR2*

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*Leptospira interrogans* differs from other spirochetes in that it contains homologs of all the *Escherichia coli* *lpx* genes required for the biosynthesis of the lipid A anchor of lipopolysaccharide (LPS). LPS from *L. interrogans* cells is unusual in that it activates TLR2 rather than TLR4. The structure of *L. interrogans* lipid A has now been determined by a combination of matrix-assisted laser desorption ionization time-of-flight mass spectrometry, NMR spectroscopy, and biochemical studies. Lipid A was released from LPS of *L. interrogans* serovar Pomona by 100 °C hydrolysis at pH 4.5 in the presence of SDS. Following purification by anion exchange and thin layer chromatography, the major component was shown to have a molecular weight of 1727. Mild hydrolysis with dilute NaOH reduced this to 1338, consistent with the presence of four *N*-linked and two *O*-linked acyl chains. The lipid A molecules of both the virulent and nonvirulent forms of *L. interrogans* serovar Icterohemorrhagiae (strain Verdun) were identical to those of *L. interrogans* Pomona by the above criteria. Given the selectivity of *L. interrogans* LpxA for 3-hydroxylaurate, we propose that *L. interrogans* lipid A is acylated with *R*-3-hydroxylaurate at positions 3 and 3′ and with *R*-3-hydroxypalmitate at positions 2 and 2′. The hydroxyacyl chain composition was validated by gas chromatography and mass spectrometry of fatty acid methyl esters. Intact hexa-acylated lipid A of *L. interrogans* Pomona was also analyzed by NMR, confirming the presence a β-1′,6-linked disaccharide of 2,3-diamino-2,3-dideoxy-D-glucopyranose units. Two secondary unsaturated acyl chains are attached to the distal residue. The 1-position of the disaccharide is derivatized with an axial phosphate moiety, but the 4′-OH is unsubstituted. 1H and 31P NMR analyses revealed that the 1-phosphate group is methylated. Purified *L. interrogans* lipid A is inactive against human THP-1 cells but does stimulate tumor necrosis factor production by mouse RAW264.7 cells.

Nearly all of the diverse eubacteria that are enclosed by two membranes synthesize lipid A as the hydrophobic anchor of their outer membrane lipopolysaccharide (LPS)1 (1, 2). Several spirochetes of clinical importance, such as *Treponema pallidum*, *Treponema denticola*, and *Borrelia burgdorferi*, possess an outer membrane but do not make LPS (3–5). Accordingly, they lack the *lpx* genes (6, 7), which are required for lipid A assembly in *Escherichia coli* and other Gram-negative organisms (2). The absence of lipid A in the outer membranes of *T. pallidum*, *T. denticola*, and *B. burgdorferi* may be compensated for by alternative lipids (8), lipoproteins (9), or other complex glycoconjugates (4). Whatever the explanation, spirochetes lacking LPS are not easily cultivated outside of their hosts (10, 11).

Disease-causing serovars of *Leptospira interrogans* spp. are members of a distinct spirochete group (12, 13) that can survive or proliferate either in a mammal or in the environment, typically in fresh water contaminated by the urine of infected animals (14). *L. interrogans* causes a hemorrhagic fever in humans known as Weil’s disease, which may be fatal in untreated cases because of liver, kidney, or pulmonary damage (14, 15). Early biochemical, serologic, and genetic studies showed that LPS is present in *Leptospira*, but its covalent structure has not been characterized (12, 16–20). However, numerous studies have shown that leptospiral LPS possesses much lower endotoxic activity than typical Gram-negative LPS (21). Werts et al. (22) found that highly purified *L. interrogans* LPS is unusual because it activates TLR2 rather than TLR4. The latter is the classical signaling receptor of the innate

1 The abbreviations used are: LPS, lipopolysaccharide; Kdo, 2-keto-3-deoxy-d-manno-octulosonic acid; UDP-GlcNAc3N, UDP 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose; GlcN3N, 2,3-diamino-2,3-dideoxy-D-glucopyranose; COSTY, correlation spectroscopy; HMQC, heteronuclear multiple-quantum coherence; NOE, nuclear Overhauser effect; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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immune system that detects the lipid A moiety of most other Gram-negative LPSs (23–26).

The recently completed sequencing of the L. interrogans serovar Lai genome strongly supports the idea that these spirochetes synthesize lipid A and LPS, because the genome encodes a complete set of Lpx orthologs and LPS-related glycosyl transferases (15). Likewise, the earlier studies of Adler and coworkers demonstrated the existence of typical O-antigen gene clusters in various strains of L. interrogans, indicating that some form of LPS must be present (12, 27).

Given the unusual bioactivity of L. interrogans LPS toward TLR2 (22) and the lack of structural studies, we now report methods for the purification and characterization of L. interrogans lipid A. A combination of mass spectrometry, NMR spectroscopy, bioinformatics, and enzymology was used to show that L. interrogans makes lipid A molecules in which the usual glucosamine units are replaced with the analog 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcN3N). As anticipated from glucosamine units are replaced with the analog 2,3-diamino-

that UDP 2-acetamido-3-amino-2,3-dideoxy-

contains significant full-length orthologs of the enzymes GnnA/H9251

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Further purification of the L. interrogans lipid A by preparative TLC was carried out as described by Que et al. (42, 48) for Rhizobium etli lipid A. Briefly, the lipid A was redissolved in 2 ml of CHCl3, MeOH (4:1, v/v), and a 0.2–0.5-mg sample was applied in 10-μl spots along a line at the origin of four 20 × 20-cm Silica Gel 60 analytical TLC plates (Whatman DE-52), equilibrated in the acetate form in CHCl3/MeOH/H2O (2:3:1, v/v/v) and loaded onto the column. Most of the putative L. interrogans lipid A eluted with CHCl3, MeOH, 30 mM NH4 Ac (2:3, v/v), suggesting that it is not strongly anionic (42, 45, 46). Fractions from the 30 mM NH4 Ac wash were pooled and, following removal of the solvents (42), stored at −20 °C.

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O-Decylation of L. interrogans Lipid A by Mild Alkaline Hydrolysis—Complete hydrolysis of all ester-linked fatty acids was achieved by resuspending ~0.1 mg of pure lipid A in a 1-ml glass vial equipped with a Teflon-lined cap in 120 μl of CHCl3, MeOH, 0.6 M NaOH (2:3, v/v) and allowing the hydrolysis to proceed at room temperature for 30–60 min. The final mild alkaline hydrolysis mixture was converted into an acidic two-phase Bligh-Dyer system by the addition of 220 μl of CHCl3, 220 μl of MeOH, and 206 μl of 0.1 M HCl. The lipid A was dissolved with a stream of N2, and the sample was stored at −20 °C. The deacylated lipid A was further purified using a 0.25-ml DEAE-cellulose column to remove residual silica chips. The purified preparations were stored dry at −20 °C.

Materials—Glass-backed 0.25-mm silica gel 60 TLC plates were obtained from Merck. Chloroform, ammonium acetate, and sodium acetate were from EM Sciences, whereas pyridine, methanol, and formic acid were from Pierce. Deuterated solvents (CD3OD, CDCl3 containing 0.1% tetramethylsilane, and D2O) and 5-mm NMR tubes were from Sigma-Aldrich. Triton X-100 and the bicinchoninic assay kit were from Pierce. Fatty acid analysis by gas chromatography/mass spectrometry (GC/MS) was performed using a Varian 3400 Varian-MAT system equipped with a 30-m, 0.25-mm, 1% phenylmethylsilicone column (J&W Scientific, Folsom, CA) and a 30-7MS Capillary column (1/8 inch i.d., 0.25 micrometer thickness, 0.25 mm i.d., 20 m, fused-silica capillary column, J&W Scientific, Folsom, CA). The deacylated lipid A was further purified using a 0.25-ml DEAE-cellulose column, equilibrated, and eluted as above. The O-deacylated lipid A was eluted with CHCl3, MeOH, 30 mM NH4 Ac (2:3, v/v), recovered by acid-cationic two-phase Bligh-Dyer partitioning, and was stored dry at −20 °C.

Purification of LPS—The method described by Westphal and Jann (38) was used to extract LPS from lyophilized cells of L. interrogans serovar Pomona (5 g dry weight) (19). LPS from L. interrogans serovar Icterohaemorrhagiae (strain Verdon) was obtained from the Centre de Reference des Leptospires (Institut Pasteur, Paris, France). Both the virulent and the avirulent variants of the Verdun strain were cultured as described previously (22). The bacteria were grown in a special medium with added pyruvate at 30 °C. The pellet was resuspended in endotoxin-free phosphate-buffered saline to an optical density of 1 at 570 nm. The sample mixtures were allowed to react at room temperature for 30 min. After incubation for only 5 min at room temperature, the solution was converted into an acidic two-phase Bligh-Dyer mixture by the addition of 220 μl of CHCl3, 220 μl of MeOH, and 206 μl of 0.1 M HCl. The lipid A was dissolved with a stream of N2, and the sample was stored at −20 °C. MALDI-TOF Mass Spectrometry of L. interrogans Lipid A—Spectra were acquired in the negative ion and the positive ion linear modes using a Kratos Analytical (Manchester, UK) MALDI-TOF mass spectrometer, equipped with a 337-nm nitrogen laser, a 20-kV extraction voltage, and time-delayed extraction (42). Each spectrum was the average of 50 shots. The lipid A samples were prepared for MALDI-TOF analysis by deposing 0.3 μl of the sample dissolved in chloroform, methanol (4:1, v/v), followed by 0.3 μl of the matrix, which was a mixture of saturated 6-aza-2-thiophenemine in 50% acetonitrile and 10% tribasic ammonium citrate (9.1, v/v). The mixture was allowed to dry at room temperature. The acylated lipid A from E. coli (Sigma) set at m/z 1797 was used as an external standard for calibration.

Fatty Acid Analysis by Gas Chromatography/Mass Spectrometry—Typically, ~1 mg of purified L. interrogans lipid A was dissolved in 200 μl of toluene and 400 μl of fresh 1% sulfuric acid in methanol. The sample was 4 h at 100 °C. After which 1 ml of hexane was added. The mixture was mixed for 50 s, followed by centrifugation for ~10 min at room temperature. The upper phase containing the methyl esters was removed and dried down under a stream of nitrogen. Fatty acid methyl ester analyses were analyzed at the University of Minnesota Mass Spectrometry Facility Department of Chemistry using a Finnigan MAT MAT Mass spectrometer coupled to a Hewlett-Packard Series I model 5890 gas chromatograph.

NMR Analysis—NMR spectroscopy was carried out at the Duke University NMR Spectroscopy Center (48, 49). The L. interrogans lipid A was dissolved in 0.6 ml of CDCl3, CD3OD, D2O (2:3:1,v/v/v) in a 5-mm
NMR tube. Proton and carbon chemical shifts are reported relative to internal tetramethylsilane at 0.00 ppm.

NMR spectra were recorded on Varian Unity 500 or 600 NMR spectrometers, each equipped with a Sun Ultra 10 computer and a 5-mm Varian probe. Two-dimensional NMR experiments (COSY, NOE spectroscopy, total correlation spectroscopy, and HMQC) were performed at 600 MHz (48, 50). Directly detected $^1$H-decoupled $^{31}$P NMR spectra were recorded at 202.37 MHz with a spectral window of 12143.3 Hz digitized into 25,280 data points (digital resolution of 1 Hz/point or $0.005 \text{ ppm/point}$), a $60^\circ$ pulse flip angle (8 $\mu$s), and a 1.6-s repeat time. $^{31}$P chemical shifts were referenced to 85% H$_3$PO$_4$ at 0.000 ppm. Inverse decoupled difference spectra were recorded as $^1$H-detected $^{31}$P-decoupled heteronuclear NMR experiments (49, 50).

RESULTS

Weak Binding of L. interrogans Lipid A to DEAE-cellulose—The lipid A recovered from L. interrogans LPS after hydrolysis at 100 °C in 12.5 mM sodium acetate, pH 4.5, migrated as a discrete band during TLC (Fig. 1). Given its elution from DEAE-cellulose with CHCl$_3$, MeOH, 30 mM aqueous ammonium acetate (2:3:1, v/v/v), this substance is predicted to have a net negative charge of about -1 (42, 45). Typical lipid A 1,4-bisphosphate species from E. coli elute with CHCl$_3$, MeOH, 240 mM ammonium acetate (2:3:1, v/v/v) (46, 50), because they can carry up to four negative charges. The weak binding of the L. interrogans material to DEAE-cellulose resembles what is seen with R. etli lipid A in which both phosphate groups are missing and replaced with carboxylate-containing sugars (42).

Identical Lipid A Molecules in L. interrogans Serovars Pomona and Icterohaemorrhagiae—Negative ion mode MALDI-TOF mass spectrometry of the lipid A isolated from L. interrogans serovar Pomona (Fig. 2A) revealed a major peak at m/z 1726.4, likely representing [M - H]$^-$ of the major molecular species. Smaller peaks differing by 28 atomic mass units presumably reflect fatty acyl chain length heterogeneity. The lipid A of both the virulent and the avirulent forms L. interrogans strain Verdun (22) yielded virtually identical spectra (Fig. 2, B and C). Because much greater quantities of LPS were available from the Pomona serovar (12, 19), all of the NMR studies were performed with Pomona lipid A.

One-dimensional NMR Spectroscopy L. interrogans Lipid A—The $^{31}$P NMR spectrum of L. interrogans lipid A at 202 MHz revealed a single resonance at 0.676 ppm (Fig. 3), demonstrating that this material contains only one phosphate group. The chemical shift of the phosphorus signal is in the range of what is expected for a monophosphomonoester unit (0.5 to 2 ppm) under these conditions (50). However, a monophosphodiester group cannot be excluded based solely upon the chemical shift position. The presence of only one phosphate group is consistent with the low affinity of L. interrogans lipid A for DEAE-cellulose.

The one-dimensional 600 MHz $^1$H NMR spectrum of L. interrogans lipid A in CDCl$_3$, CD$_3$OD, D$_2$O (2:3:1, v/v/v) revealed well resolved peaks in the sugar (3.5–5.5 ppm) and acyl chain (0.9–2.8 ppm) regions (Fig. 4, upper and left margins), similar to E. coli or R. etli lipid A (48–50). However, the L. interrogans spectrum contains an unusually prominent doublet at 3.61 ppm, which integrates to three protons and shows an apparent
coupling constant of 10.9 Hz (Fig. 4, left arrow). This kind of signal had not been seen previously in lipid A from diverse sources (47–50).

**NMR Evidence for a Methylated 1-Phosphate Moiety**—The 3.61 ppm doublet in the $^1$H NMR spectrum of L. interrogans lipid A does not show any cross-peaks in the $^3$H–$^1$H COSY analysis (Fig. 4). To determine whether or not this signal arises from a methylated phosphate group, selective inverse decoupling difference spectroscopy was used to detect possible heteronuclear coupling of the phosphorus atom to the 3.61 ppm doublet (49, 50). The difference spectrum (Fig. 5C) of the off and on resonance $^{31}$P-decoupled $^1$H NMR spectra (Figs. 5, A and B) demonstrated the collapse of the 3.61 ppm doublet to a singlet during phosphorus irradiation. In addition, the difference spectrum showed simplification of a proton signal at 5.44 ppm to a doublet and of another proton at 4.05 ppm to a doublet-doublet (Fig. 5C, inset). The latter chemical shifts and peak shapes are consistent with phosphorus coupling to the H-1 and H-2 atoms, respectively, of a proximal α-linked glucopyranoside unit (49, 50). The heteronuclear coupling data provide strong evidence that L. interrogans lipid A contains an unprecedented monophosphodiester unit bridging the C-1 atom of the proximal sugar and a methyl group.

**Confirmation of a Methyl Group on the 1-Phosphate Moiety by MALDI-TOF Mass Spectrometry**—The negative ion MALDI-TOF mass spectra of intact L. interrogans lipid A (Figs. 2 and 6A) show an intense peak with m/z near 1726 atomic mass units, interpreted as the molecular ion [M + H]$^-$ . Additional structural information is contained in the positive ion mode spectrum (Fig. 6B). The B$_1^+$ oxonium ion (Fig. 6B) forms during fragmentation of the disaccharide glycosidic linkage (Fig. 7) (51) and reflects the mass of the distal sugar unit. The B$_2^0$ ion (Fig. 7) is generated by loss of the substituent attached to the 1-position in the proximal sugar (51). The mass of the group attached to the anomeric carbon is determined by comparing the m/z of the B$_1^+$ ion to the molecular weight derived from the [M + H]$^-$ ion (Fig. 6A). The mass of the proximal unit (without the substituent at the 1-position) is determined from the difference of B$_1^+$ and B$_2^0$.

Fig. 6B shows prominent peaks at m/z 1003.0 and 1614.1, which are interpreted as the B$_1^+$ and B$_2^0$ ions, respectively, of L. interrogans lipid A (Fig. 7). Under the ionization conditions employed, the molecular ion [M + H]$^+$ (m/z = 1727.4) is not detected (Fig. 6B). The B$_1^+$ ion at m/z 1614.1 is 112.4 atomic mass units less than the molecular weight deduced from the negative mode spectrum in Fig. 6A (1726.5). If L. interrogans lipid A contained an unsubstituted phosphate group at the 1-position, as is present in E. coli lipid A, the B$_2^0$ ion should be 97.0 atomic mass units smaller than the molecular weight predicted from the negative mode spectrum (52). Given the experimental error of the MALDI-TOF measurements, the discrepancy of 15.4 atomic mass units (112.4 - 97.0) is consistent with the presence of a methyl substituent on the 1-phosphate group of L. interrogans lipid A, as deduced from the above NMR experiments (Fig. 5).

The difference in mass between the B$_1^+$ and B$_2^0$ ions is 611.1 atomic mass units (Fig. 6B), suggesting the presence of two acyl...
chains on the proximal unit of \textit{L. interrogans} lipid A. Given the absolute selectivity of \textit{L. interrogans} LpxA for 3-hydroxylauryl-ACP and UDP-GlcNAc3N (28), the GlcN3N 3-position must be acylated with 3-hydroxylaurate. To account for the 611.1 atomic mass units difference in the B1/H11001 and B2/H11001 ions (Fig. 6B), the acyl chain at the GlcN3N 2-position could be 3-hydroxypalmitate (Fig. 7). In fact, 3-hydroxylaurate and 3-hydroxypalmitate were predominant components in the fatty acid analysis (data not shown). The size of the B1/H11001 ion at \( m/z 1003.0 \) atomic mass units suggests that four acyl chains are attached to the distal unit (Fig. 7).

Mild Alkaline Hydrolysis of \textit{L. interrogans} Lipid A—Exposure of lipid A or lipid A precursors to aqueous triethylamine at \( 37 \, ^\circ \text{C} \) releases unsubstituted \( O \)-linked 3-hydroxyacyl chains (42, 53). Mild triethylamine does not remove \( O \)-linked acyloxyacyl moieties or \( O \)-linked normal fatty acids under standard conditions. Exposure of \textit{L. interrogans} lipid A to aqueous triethylamine at \( 37 \, ^\circ \text{C} \) did not alter its molecular weight, as judged by mass spectrometry (not shown), demonstrating the absence of unsubstituted, \( O \)-linked 3-hydroxyacyl chains.

Treatment of \textit{L. interrogans} lipid A with 0.1 M NaOH for 30 min shifted \([M+H]^+\) from \( m/z \) 1725.5 to 1336.5 atomic mass units (Fig. 6, A and C), suggesting the release of two ester-linked acyl chains with the masses of C12:1 and C14:1 (Fig. 7A). A 5-min exposure to 0.1 M NaOH yielded partially \( O \)-deacylated intermediates, as judged by the appearance of peaks at \( m/z \) 1545.2 and 1517.4 atomic mass units (Fig. 6B), consistent with the presence of ester-linked C12:1 and C14:1 moieties.

The MALDI-TOF analyses of the partially and completely hydrolyzed \textit{L. interrogans} lipid A samples in the positive mode (Fig. 6, D and F, respectively) are in accord with the negative mode data (Fig. 6, C and E). Importantly, the \( B_1^+ \) ion is shifted from \( m/z \) 1003.0 atomic mass units to \( m/z \) 614.1 atomic mass units after complete hydrolysis (Fig. 6F), demonstrating conclusively that both ester-linked acyl chains must be located on the distal unit of \textit{L. interrogans} lipid A. The difference in mass between the \( B_1^+ \) and \( B_2^+ \) ions following complete hydrolysis is 613.2 atomic mass units (Fig. 6F), which is the essentially same as observed for the untreated material (Fig. 6B) and in good agreement with the expected value of 612.9 for the structure shown in Fig. 7.

The molecular weight of the dilute NaOH treated \textit{L. interrogans} lipid A is 1337.5 (Fig. 6E). Given that the corresponding \( B_2^+ \) ion is observed at \( m/z \) 1227.3 atomic mass units (Fig. 6F), the molecular mass of the substituent present at the 1-position of this substance is 110.2 (i.e. \( 1337.5 \) - \( 1227.3 \)). This result is in good agreement with what is expected for the loss of a methylated phosphate residue (111.0 atomic mass units) (Fig. 7).

Two-dimensional \(^1\text{H}\) NMR Analysis of \textit{L. interrogans} Lipid A—All of the chemical shifts and coupling constants for \textit{L. interrogans} lipid A are summarized in Table I, using the proposed structure and numbering scheme shown in Fig. 7A. Many of the protons assigned in the \(^1\text{H}-^1\text{H}\) COSY of \textit{L. inter-
Fig. 7. Proposed structures of intact and O-deacylated L. interrogans serovar Pomona lipid A. The numbering scheme for the intact lipid A in A is used for the NMR assignments. The position of the cis-double bond is inferred based on the mechanism by which unsaturated fatty acids are synthesized in E. coli (58–60). The fully O-deacylated material is shown in B. Fatty acid analysis of L. interrogans lipid A shows equal amounts of 3-hydroxylaurate and 3-hydroxypalmitate; 3-hydroxylaurate is attached at positions 3 and 3'-configuration (Fig. 7). NOE spectroscopy (not shown) data permit the sequential identification of H-2 through H-6a and H-6b for each hexose ring (Fig. 4 and Table I). The small J_{1,2} coupling (3.2 Hz) and the large J_{2,3}, J_{3,4} and J_{4,5} couplings (9 to 11 Hz) suggest that the proximal pyranose ring is in the α-anomeric configuration with axially disposed H-2, H-3, H-4, and H-5 protons (Fig. 7). The large J_{1,2} coupling (7.1 Hz) shows that the distal sugar is in the β-configuration (Fig. 7). NOE spectroscopy analysis (not shown) demonstrates the following NOE dipolar interactions: 1) from the resolved H-1' to H-3' and to H-5' within the distal pyranose unit and to the H-6a and H-6b of the proximal sugar; 2) from H-1 to H-2 and from H-2 to H-4 in the proximal sugar; and 3) from H-5 to H-3 and to H-6a and H-6b in the proximal sugar. The NOE from H-1' to H-6a and H-6b is diagnostic for the β-1',6 linkage (49, 50). The multiple 1,3 diaxial and single axial-equatorial (H-1 to H-2) intramolecular NOEs confirm that both sugar rings adopt the chair conformations with a β-linkage between the proximal α-glucopyranose and the distal β-glucopyranose rings.

The low field position of H-1 at 5.44 ppm (Fig. 4) is characteristic of an anomeric proton of a sugar 1-phosphate, similar to H-1 in E. coli lipid A, which resonates at 5.46 ppm (49, 50). However, H-4' of L. interrogans lipid A (3.40 ppm) resonates at significantly higher field than the H-4' of E. coli lipid A (4.17 ppm), suggesting that the 4'-position of L. interrogans lipid A is not phosphorylated. The B{+}_1 ion detected by mass spectrometry in the positive ion mode (Fig. 6B) likewise suggests the absence of a phosphate group at position 4'. The 31P NMR data (Fig. 3) conclusively demonstrate only a single phosphate group in L. interrogans lipid A with heteronuclear coupling between the phosphorus atom and H-1 of the proximal sugar (Fig. 5).

The 31P NMR data (Fig. 3) show that L. interrogans lipid A resonate near 4.2 ppm and 3.9 ppm, respectively (Fig. 4 and Table I), significantly upfield of the H-3 and H-3' signals in E. coli lipid A at 5.25 and 5.18 ppm (49, 50). Both the 3- and 3'-positions of E. coli lipid A are substituted with ester-linked acyl chains, and therefore

| Position | δC | δH (mult., J/Hz) | δP |
|----------|----|-----------------|----|
| Proximal GlcN3N | 1 | 95.3 | 5.44 [dd, J_{3,4} = 3.2]^a | +0.676 |
| | 2 | 53.52 | 4.05 [ddd, J_{2,3} = 11.2]^a |  |
| | 3 | 53.46 | 4.21 [dd, J_{2,3} = 9.8] |  |
| | 4 | 69.00 | 3.57 [dd, J_{2,3} = 8.9] |  |
| | 5 | 73.63 | 3.98 [m] |  |
| | 6a | 69.29 | 4.04 [m] |  |
| | 6b | 69.75 | 3.85 [m] |  |
| Distal GlcN3N | 1' | 101.9 | 4.47 [dd, J_{1,2} = 7.1] |  |
| | 2' | 55.08 | 3.75 [m] |  |
| | 3' | 56.71 | 3.90 [m] |  |
| | 4' | 70.31 | 3.39 [m] |  |
| | 5' | 79.05 | 3.40 [m] |  |
| | 6'a | 62.66 | 3.87 [m] |  |
| | 6'b | 62.73 | 3.73 [m] |  |
| 3-Hydroxyacyl | α2 | 45.0 | -2.35, 2.30 |  |
| | β2 | 68.8 | -3.93 [m] |  |
| | γ2 | 38.5 | -1.44 [m] |  |
| | α3 | 42.3 | -2.40 |  |
| | β3 | 70.0 | -3.95 [m] |  |
| | γ3 | 38.5 | -1.44 [m] |  |
| | α'3 | 42.4 | -2.58, 2.45 |  |
| | β'3 | 73.3 | -5.13 [m] |  |
| | γ'3 | 62.6 | -1.63 [m] |  |
| | α3' | 42.3 | -2.32 |  |
| | β3' | 73.5 | -5.22 [m] |  |
| | γ3' | 62.6 | -1.63 [m] |  |

a Measured from the resolved H-2 double-doublet in the 31P-decoupled 1H NMR difference spectrum (Fig. 5C).
H-3 and H-3′ of E. coli lipid A are shifted considerably downfield relative to typical nonesterified sugar oxymethines groups 
(49, 50). L. interrogans lipid A does not contain esterified sugar oxymethines groups at positions 3 and 3′.

13C NMR Evidence for a GlcN3N Disaccharide in L. interro-
gans Lipid A—In lipid A disaccharides consisting of two glu-
osamine units (49, 50), two cross-peaks (originating from C-2
and C-2′) are observed in the 52–57-ppm region of the HMRC
spectrum. As shown in Fig. 8, the HMRC spectrum of L. interro-
gans lipid A reveals four sugar resonances between 52 and 58
ppm. Two of these cross-peaks are attributed to C-2 (53.5 ppm)
and C-2′ (55.1 ppm), because they correlate to H-2 at 4.05 ppm
and H-2′ at 3.75 ppm, respectively. The cross-peaks at 53.5
ppm and 56.7 ppm correlate with H-3 (4.21 ppm) and H-3′ (3.90
ppm), demonstrating unequivocally that both C-3 and C-3′
are substituted with nitrogen atoms in L. interrogans lipid A. The
presence of aminomethine compared with oxymethines groups
accounts for the large differences in the chemical shifts ob-
erved for H-3 and H-3′ of L. interrogans lipid A versus E. coli
lipid A. Moreover, the C-2 and C-3 shifts of L. interrogans lipid
A agree with those reported for the α form of 2,3-diamino-2,3-
dideoxyglucose in a 2:1 benzene-dimethyl sulfoxide mixture
(54), whereas the C-2′ and C-3′ shifts are close to those of the
β form of 2,3-diamino-2,3-dideoxyglucose (54).

The HMRC spectrum (Fig. 8) confirms two anomic protons.
H-1 of the proximal sugar at 5.44 ppm correlates with C-1 at 96
ppm, whereas H-1′ of the distal sugar at 4.48 ppm connects to
C-1′ at 105 ppm. These C-1 and C-1′ chemical shifts are charac-
teristic of the α- and β-anomeric configurations, respectively
(54), and are consistent with the 1H NMR data (Fig. 4). The
prominent three-proton doublet of L. interrogans lipid A at 3.61
ppm correlates to a carbon signal at 54.34 ppm, close to that of the
CD3HOD signal from the methanol solvent and in accord
with the proposal that the methyl doublet arises from a meth-
ylated phosphate group (Fig. 7). The striking cross-peaks
within the olefinic carbon region near 132 ppm (Fig. 8) corre-
late with proton signals at 5.35 and 5.40 ppm (Fig. 4), diagno-
sis for the presence of unsaturated acyl chains.

1H NMR Analysis of the Acyloxyacyl Residues and Mono-
unsaturated Acyl Chains in L. interrogans Lipid A—The R-3-
hydroxyacyl chains that are the hallmark of all lipid A mole-
cules are readily detected in L. interrogans lipid A by 1H NMR
(Fig. 4). The β-oxymethine protons of these acyl chains (Fig.
7A) resonate between 3.7 and 4.2 ppm when the β-OH group is
not substituted, but they are shifted to about 5.2 ppm when a
secondary acyl chain is present (48–50, 55–57) (Fig. 4). The
four α/β and four γ/β cross-peaks (Fig. 4) confirm that there are
four β-hydroxyacyl chains in L. interrogans lipid A. Two of the
four α/β cross-peaks overlap near 2.4 and 3.95 ppm (α2,β2 and
α3,β3). Two of the four γ/β cross-peaks are detected near 1.5
and 3.95 ppm (γ2,β2 and γ3,β3). These signals are character-
istic of α- and γ-methylene protons adjacent to β-oxymethines of
unsubstituted β-hydroxyacyl chains (48–50). The two re-
mainin sets of α/β and γ/β cross-peaks (Fig. 4) are detected
near 2.4–2.6 and 5.2 ppm and near 1.6 and 5.2 ppm, respec-
tively. The downfield shift of the β2′ and β3′ protons versus the
β2 and β3 protons (Fig. 4) confirms the presence of two acy-
loxyacyl moieties in L. interrogans lipid A (Fig. 7A).

Prominent cross-peaks are also observed near 5.38 and 2.1
ppm and near 5.42 and 2.05 ppm (Fig. 4). These signals arise
from the spin coupling of olefinic protons to adjacent vinylic
methylene in the secondary acyl chains (Fig. 7A). The COSY
analysis is therefore consistent with both the HMRC and the
mass spectrometry in demonstrating the presence of unsat-
urated secondary acyl chains in L. interrogans lipid A. The
exact location and stereochemistry of the double bonds re-
 mains to be determined. However, if L. interrogans gen-
erates fatty acid cis-double bonds by the same anaerobic pathway as
E. coli (58–60), one would expect the double bonds of both the
C12:1 and the C14:1 chains to be located at position ω-7 (Fig.
The COSY analysis supports this idea, because it shows a cross-peak between at least one vinylic methylene and one \( \beta \)-methylene group (Fig. 4), as expected for the proposed structure of the C12:1 chain (Fig. 7A). The total correlation spectroscopy data (not shown) confirm the connectivity of olefinic protons to a subset of \( \alpha \) - and \( \beta \)-methylene within the secondary acyl chains, as well as showing the expected strong connectivity to vinylic and aliphatic methylenes.

**DISCUSSION**

The studies presented above document for the first time the existence of a lipid A molecule in *L. interrogans* with the proposed structure shown in Fig. 7A. The identification of this substance is consistent with the fact that the *L. interrogans* genome encodes a complete set of Lpx proteins (13), which catalyze the biosynthesis of the lipid A anchor of LPS in virtually all other Gram-negative bacteria (Fig. 9) (2). *L. interrogans* is the first spirochete shown to possess the lpx genes, in contrast to *T. pallidum, T. denticola, and B. burgdorferi*, which do not make lipid A (3, 6, 7). The absence of lipid A and LPS in the latter organisms (3–5) may explain their restricted ability to grow outside of their mammalian hosts. It would be of great interest to inactivate the lpxA gene (2, 61–63) in *L. interrogans* to determine whether or not this organism is viable in the absence of its LPS. However, at present no mutagenesis system is available for pathogenic *Leptospira* spp. Lipid A is essential for growth in all Gram-negative bacteria examined to date (2, 64) with the exception of *Neisseria meningitidis* strains containing a polysialic acid capsule (65, 66). In the latter, the lpxA gene can be deleted with the consequence that the bacteria grow slowly and now require their polysialic acid capsule for viability (66).

The NMR studies shown in Figs. 4 and 8, in conjunction with the mass spectrometry of native and NaOH treated lipid A (Fig. 6), demonstrate unequivocally that *L. interrogans* lipid A consists of a hexa-acylated \( \beta \)-1,6-linked disaccharide in which the usual glucosamine residues are replaced with the more stable GlcN3N analog (28, 29, 67). The selectivity of *L. interrogans* LpxA for UDP-GlcNAc3N and 3-hydroxyauroyl-acyl carrier protein, documented in the preceding manuscript (28), independently supports this structural assignment. The two secondary acyl chains of *L. interrogans* lipid A appear to be unsaturated (Figs. 4, 6, 7A, and 8), which is unusual but not without precedent (68–70). Additional structural studies will be required to determine the exact location of the double bonds in the secondary acyl chains.

The 4'-position of *L. interrogans* lipid A is not phosphorylated. The latter finding indicates that a 4'-phosphatase must be present in this organism, as in *R. etli* and *R. leguminosarum* in which the 4'-phosphate group is also missing (42, 48, 71, 72). Removal of the 4'-phosphate group, when it occurs, appears to be a late step in lipid A biosynthesis, given that the 4'-phosphate residue is actually necessary for the attachment of the Kdo sugars (71, 73, 74). All bacteria with a 4'-phosphatase, including *L. interrogans*, retain the 4'-kinase encoded by *lpxK* (13, 75) (Fig. 9). In preliminary studies, 4'-phosphatase activity was observed using washed *L. interrogans* membranes (not shown) with the hexa-acylated substrate \([4'-\text{32P}]\text{Kdo}_2\text{-lipid A}\) from *E. coli* (76). No dephosphorylation of the tetra-acylated precursors \([4'-\text{32P}]\text{lipid IV}_A\) or \([4'-\text{32P}]\text{Kdo}_2\text{-lipid IV}_A\) (77) was detected.

The most unique aspect of *L. interrogans* lipid A is the finding that its 1-phosphate group is methylated (Figs. 4–6). This structural feature is without precedent in lipid A biochemistry (2, 33). In fact, the enzymatic methylation of phosphate groups appears to be very rare in all of biology. To our knowledge, Kates *et al.* (31) have reported the only other example of a methylated lipid phosphate residue, found in the halophile *Halobacterium salinarium*, which synthesizes a methylated
against human THP-1 cells, indicating that it is not contaminated by impurities. An initial survey of the lipid A described above demonstrates that it is inactive in the limulus lysate assay and a-factor mating pheromone of yeast (84) is essential for membrane association and signaling. In these well-documented examples of membrane lipid and membrane protein methylation, S-adenosyl-methionine serves as the methyl donor. We have recently found that membranes of L. interrogans catalyze the S-adenosyl-methionine-dependent methylation of Kdo-lipid A (85).

The characterization of the structure of L. interrogans lipid A sets the stage for the analysis of its biosynthesis and bioactivity. An initial survey of the lipid A described above demonstrates that it is inactive in the limulus lysate assay and against human THP-1 cells, indicating that it is not contaminated with a classical endotoxin, such as E. coli lipid A. However, when assayed with mouse RAW 264.7 cells, L. interrogans lipid A induces tumor necrosis factor with about one-tenth the potency of E. coli lipid A. We are currently evaluating L. interrogans lipid A with macrophages derived from various mouse TLR2 knockout strains. It may be that the robust TLR2 activating activity seen with intact L. interrogans LPS (22) requires more than just the lipid A moiety. Isolation of LPS from L. interrogans mutants blocked in defined steps of O-antigen and/or core biosynthesis (12) might address this question. In addition, chemically synthesized versions of L. interrogans lipid A need to be prepared to validate our proposed structure and to determine whether or not the activity seen with L. interrogans lipid A is real or is due to other biologically active impurities.

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A Methylated Phosphate Group and Four Amide-linked Acyl Chains in *Leptospira interrogans* Lipid A: THE MEMBRANE ANCHOR OF AN UNUSUAL LIPOPOLYSACCHARIDE THAT ACTIVATES TLR2

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