Characterization of a Novel Lipid-A from *Rhizobium* Species Sin-1

A UNIQUE LIPID-A STRUCTURE THAT IS DEVOID OF PHOSPHATE AND HAS A GLYCOSYL BACKBONE CONSISTING OF GLUCOSAMINE AND 2-AMINOGLUCONIC ACID

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The structure of the lipid-A from *Rhizobium* species Sin-1, a nitrogen-fixing Gram-negative bacterial symbiont of Sesbania, was determined by composition, nuclear magnetic resonance spectroscopy, and mass spectrometric analyses. The lipid-A preparation consisted of a mixture of structures due to differences in fatty acylation and in the glycosyl backbone. There were two different disaccharide backbones. One disaccharide consisted of a distal glucosaminosyl residue β-linked to position 6 of a proximal 2-aminoglucono-1,5-lactonosyl residue, and in the second disaccharide, the proximal residue was 2-amino-2,3-dideoxy-o-erythro-hex-2-enono-1,5-lactone. For both disaccharides, the distal glucosamine was acylated at C-2‘ primarily with β-hydroxy-palmitate (β-OHC16:0) which, in turn, was O-acylated with 27-hydroxyoctacosanoic acid. For some of the lipid-A molecules, the distal glucosaminosyl residue was also acylated at C-3’ with β-hydroxy-myristate (β-OHC14:0). Whereas other molecules were devoid of this acyl substituent. Both the 2-amino-2,3-dideoxy-o-erythro-hex-2-enono-1,5-lactonosyl residues were acylated at C-2, primarily with β-OHC16:0. Minor amounts of lipid-A molecules contained β-OHC14:0 at C-3 and/or β-hydroxy stearate (β-OHC18:0) or β-hydroxyoctadecenoate (β-OHC18:1) as the C-2 and C-2‘ N-acyl substituents.

Rhizobia refer collectively to the group of Gram-negative bacteria that belong to the Rhizobiaceae family and form nitrogen-fixing symbioses with legume plants. The major constituent of the Gram-negative bacterial cell wall is lipopolysaccharide (LPS).† The LPS molecule has three structural regions as follows: the O-chain polysaccharide, core oligosaccharide, and the hydrophobic lipid-A. The LPS has been shown to be important in the symbiotic infection process (1–4). Structural changes to both the O-chain polysaccharide and to the lipid-A appear to be important for symbiotic infection (5). These changes include methylation of the O-chain glycosyl residues and increased fatty acylation of the lipid-A with 27-hydroxyoctacosanoic acid (27-OHC28:0) (6), a long-chain fatty acyl component that is common to the lipid-A isolated from members of the Rhizobiaceae (7–9). As with other Gram-negative bacteria, the LPS of rhizobia most likely have important roles that enable these bacteria to adapt to different environments; in this case, the intracellular environment of the legume host cell. These roles probably include acting as a permeation barrier toward potential toxins (e.g. defense response molecules from the host) as well as other structural adaptations that allow survival within the host cell. Lipid-A is considered the least variable region in the LPS molecule. The lipid-A structure from enteric bacteria is largely conserved, consisting of a β(1–6)-linked glucosamine disaccharide backbone with phosphate groups at C-1 and C-4 and β-hydroxy fatty acyl groups and acyloxyacyl residues at positions 2 and 3, and 2’ and 3’, respectively (10, 11). Modifications to this structure that are thought to contribute to the virulence of enteric pathogens (e.g. *Salmonella typhimurium*) occur under certain physiological conditions. These modifications can include the addition of a palmitoyl residue, hydroxylation of a myristoyl substituent, and the addition of aminoa ribinosyl and phosphoethanol amine moieties (12). The LPS from other bacterial species show some variability in the glycosyl backbone and fatty acylation patterns of their lipid-A structures. Some of these lipid-A structures have 2,3-diaminoglucosamine replacing one or both of the glucosaminosyl residues (13, 14). Fatty acyl components can be present that have shorter chain lengths, sites of unsaturation, or keto functional groups (15). A structurally unusual lipid-A from the hyperthermophilic bacterium, *Aquifex pyrophilus*, has been reported recently (16) in which both the glycosidic and 4’-phosphate groups are replaced by galacturonosyl residues.

Several reports indicate variation in the glycosyl components of lipid-A from *Rhizobium* species. The structure of *Sinorhizobium meliloti* lipid-A is similar to enteric bacterial lipid-A in that it contains an acylated and bis-phosphorylated glucosamine disaccharide (17). Other rhizobial lipid-A from *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium lupini*, and *Mesorhizobium loti*) have 2,3-diaminoglucosine as a constituent of the lipid-A backbone (13, 14). Perhaps one of the more unusual lipid-A structures is that reported for *Rhizobium etli* and *Rhizobium leguminosarum* (9, 18, 19). Briefly, this unusual lipid-A contains a fatty acylated glycosyl backbone of β-glucosamine linked to C-6 of 2-aminogluconate in which...
the glucosamine 4′-position is substituted with galacturonic acid. Unlike the lipid-A of enteric bacteria, this rhizobial lipid-A is devoid of phosphate and contains 27-OHC28:0 as the only acyloxyacyl residue. Despite the unusual structure of this rhizobial lipid-A, its biosynthesis is reported to occur via the same pathway as that of Escherichia coli from UDP-GlcNAc to the lipid-A precursor two residues of 3-deoxy-d-manno-2-octulosonic acid lipid-IVα (20). However, at this biosynthetic stage both R. etli and R. leguminosarum contain novel enzymes that process this precursor into the unique rhizobial structure. Several of these enzymes have been reported. These include phosphatase activities that remove the 1- and 4′-phosphates (21, 22), a unique acyl carrier protein (Acp-XL), and transferase for 27-OHC28:0 (21), and an oxidase that converts the proximal glucosamine into 2-aminogluconate (19). The transferase that adds galacturonic acid to the C-4′ position has not yet been reported.

The LPS from Gram-negative bacterial pathogens are known as endotoxins and cause several pathophysiological symptoms such as fever, leukopenia, hypertension, disseminated intravascular organ failure, multiple organ failure, etc. The LPS from enteric bacteria (such as E. coli and Salmonella) are extremely potent molecules with regard to their biological activity. The lipid-A portion of these enteric LPS induces the toxic biological activities of LPS (11, 23). Development of variant lipid-A-like structures that can act as antagonists for the toxic lipid-A of enteric bacterial LPS has important implications for the prevention of septicemia as well as for the development of vaccines (24–27). Modification of the lipid-A backbone or the fatty acyl components (e.g., deletion, addition, and/or change in length or position of fatty acids) results in partial or total loss of toxicity (27, 28). In fact, the lipid-A from Rhodobacter sphaeroides and Rhodobacter capsulatus, which have the enteric-like bio-phosphorylated glucosamine disaccharide backbone but contain shorter fatty acyl components, one of which is unsaturated and another has a 3-keto group, are not toxic and inhibit the toxic activity of enteric LPS (26, 29). Thus, unusual lipid-A structures are a potential resource for molecules that can act as antagonists for sepsis and as non-toxic adjuvants for vaccines.

In this paper we describe the structural elucidation of another unusual rhizobial lipid-A, that from Rhizobium sp. Sin-1. This lipid-A, as with the R. etli and R. leguminosarum lipid-A, is completely devoid of phosphate and contains glucosamine and 2-amino glucuronate. However, unlike the R. leguminosarum lipid-A, it is devoid of galacturonic acid and any other acidic components and has a different fatty acylation pattern. This structure was determined by a combination of both NMR and mass spectrometric techniques. The companion paper (48) describes the ability of the Rhizobium sp. Sin-1 LPS to prevent enteric LPS-induced cytokine production.

### EXPERIMENTAL PROCEDURES

**Bacterial Strain and Growth Conditions—**Rhizobium sp. (Sesbania) Sin-1 (Rhizobium sp. Sin-1), was isolated from root nodules of Sesbania aculeata (a tropical legume) grown in Tamil Nadu, India, and was provided to us by Hari Krishnan, University of Missouri, Columbia (30). Rhizobium sp. Sin-1 is closely related to Rhizobium galegae that nodulates the temperate legumes, Galega orientalis and Galega officinalis (30), but its species has not yet been assigned. Bacteria were grown on yeast extract mannitol (YEM) medium (30) at the fermentation plant, University of Georgia.

**Isolation of LPS—**Crude LPS was isolated by the hot phenol/water extraction method (31). The crude LPS preparation was treated with RNase, DNase, and proteinase K and further purified by gel filtration on a Sephadex G-150 (Amer sham Biosciences) column using a solution of 0.25% deoxycholic acid buffer having 0.2 mM NaCl, 1 mM EDTA, 10 mM Tris base (final pH 9.25) as the eluant (32).

**Glycosyl Composition Analysis—**The compositions of the lipid-A samples were determined by gas-liquid chromatography-mass spectrometry (GLC-MS). Samples were subjected to methanolysis (in 1 M HCl at 80 °C for 18 h), followed by N-acetylation and trimethylsilylation (TMS) (34). Analysis of the resulting TMS methylglycosides and fatty acid methyl esters was performed by combined GLC-MS using a 50-m methyl silyl column with a temperature program of 80 °C for 2 min, then 20 °C/min to 160 °C and holding for 2 min, 2 °C/min to 200 °C, 10 °C/min to 260 °C and holding for 11 min.

**Mass Spectrometry—**Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectra were acquired on an HP-MALDI instrument equipped with a nitrogen laser (337 nm) and using a 20-kV extraction voltage. The lipid-A were dissolved in chloroform/methanol (1:1, v/v). Equal volumes of lipid-A preparation and MALDI matrix (30 mg of 2,5-dihydroxybenzoic acid, in 1 ml of water mixed with 1 ml of acetoneitrile, followed by addition of 8.7 mg of 1-hydroxyisoquinone and sonication) were mixed on the MALDI probe and vacuum-dried. Spectra were acquired in the positive mode, and each spectrum is an average of 100 laser shots.

**Q-TOF Mass Spectroscopy—**Mass spectra were run on a Q-TOF hybrid mass spectrometer (Q-TOF II, Micromass, UK) equipped with an electrospray ion source (Z-spray). The samples were dissolved in methanol/methanol (1:1 v/v) and infused into the mass spectrometer with a syringe pump (Harvard Apparatus Cambridge, MA) at a flow rate of 5 μl/min. A 3-kV potential was applied to the capillary, and nitrogen was employed as both the drying and nebulization gas. Glu-fibrinopeptide B was used as the calibration standard in the positive mode. For the MS analysis the quadrupole (Q) is operated in R-only mode with all ions transmitted into the pusher region of the TOF analyzer, and the MS spectra was recorded from m/z 400 to 2000 with a 1-s integration time. For MS/MS spectra, the transmission window of the quadrupole was set to allow 1 atomic mass unit, and the selected precursor ions were allowed to fragment in the hexapole collision cell. The collision energies (30–55 eV) were optimized for maximized product yield, and argon was used as collision gas. The MS/MS data were integrated over a period of 4–5 min for each precursor ion.

**NMR Analysis—**The lipid-A preparation that was recovered as ethanol-washed precipitate (2–5 mg) was dissolved in CDCl3/CD3OD (1:1, v/v) and transferred into a 5-mm NMR tube. NMR spectra were obtained at 25 °C using a Varian Unity HD NMR spectrometer. Two-dimensional NMR spectra were referenced to tetramethylsilane (δ = 0.00 ppm). Two-dimensional NMR spectra, except COSY experiments, were acquired in the phase-sensitive mode. COSY spectra were recorded with 2K data points, 1 s relaxation delay, and 16 scans/increment. One COSY experiment was recorded with a spectral width of 4 kHz with 800 increments, and a t-DMAX mode was employed with a spectral width of 2 kHz and 720 increments. Data were zero-filled to 2K points with square sine-bell weighting in both dimensions before Fourier transformation. A TOCSY spectrum was recorded with a relaxation delay of 1.0 s, 16 scans per increment, 256 increments, and a mixing time of 80 ms. A proton-
detected single bond $^1$H, $^{13}$C two-dimensional chemical shift correlation spectrum was recorded using the heteronuclear single quantum correlation spectroscopy (HSQC) method. Sixteen scans per increment and 256 increments were recorded. The two-dimensional data were processed using Gaussian functions and zero-filled to a final size of $2 \times 2K$. The heteronuclear multiple bond correlation (HMBC) spectrum was recorded with 2K points, a relaxation delay of 1.0 s, and 96 scans at 600 increments. The NOESY data were collected with two sets of 256 time-incremented spectra, 48 scans/increment, 1-s relaxation delay, and a mixing time of 500 ms. The data were processed with Gaussian weighing in both dimensions and zero-filled to $2 \times 2K$.

RESULTS

Lipid-A Composition—The GLC-MS chromatogram of TMS methylglycosides from *Rhizobium* sp. Sin-1 lipid-A is shown in Fig. 1. The carbohydrate portion of *Rhizobium* sp. Sin-1 lipid-A consists of glucosamine and 2-aminoglucuronate. These results are similar to those reported for the lipid-A from *R. leguminosarum* and *R. etli* (9). However, unlike *R. leguminosarum* and *R. etli* lipid-A, which contains galacturonic acid, the *Rhizobium* sp. Sin-1 lipid-A does not have this or any other acidic glycosyl residue. The trace amount of glucose seen in the GLC profile is due to a contaminant because subsequent structural analysis (see below) showed that glucose is not part of this lipid-A. The *Rhizobium* sp. Sin-1 lipid-A also differs from that of *R. etli* and *R. leguminosarum* in its fatty acylation pattern. The major fatty acyl components of the lipid-As from all three species are similar; $\beta$-hydroxyoxymyristate ($\beta$-OHC14:0), $\beta$-hydroxypalmitoleate ($\beta$-OH18:0), $\beta$-hydroxyoctadec-2-enate ($\beta$-OH18:1), and 27-OHC28:0. However, the ratio of $\beta$-OHC16:0 to $\beta$-OHC14:0 is greater in the *Rhizobium* sp. Sin-1 lipid-A than in that from *R. etli* or *R. leguminosarum*. Also, as will be described further below, the major lipid-A species from *Rhizobium* sp. Sin-1 contains four rather than the five fatty acyl residues observed in the lipid-A from *R. leguminosarum* or *R. etli*. The presence of 27-OHC28:0 as a major fatty acyl component is consistent with the previous reports (37) that show that this is a typical fatty acyl component of the lipid-A from members of the Rhizobiaceae even though there is variability in other structural aspects, such as the glycosyl backbone structures.

Mass Spectrometry—The lipid-A recovered after mild acid hydrolysis of the LPS was evaluated by MALDI-TOF mass spectrometry in the positive mode. Mass spectrometry of this lipid-A preparation (spectrum not shown) showed three clusters of pseudo-molecular ions. Each of these ion clusters contained [M + H]$^+$, [M + Na]$^+$, and [M + K]$^+$ ions of molecules that varied in the fatty acyl chain length. The [M + Na]$^+$ ions for each ion cluster were $m/z$ 1291, 1517, and 1743. These ions are separated from one another by a mass difference of $m/z$ 226, the incremental mass for $\beta$-OHC14:0. The intensity of the $m/z$ 1743 ion, and the other ions in that cluster, was much less than the $m/z$ 1291 or 1517 ion intensities indicating that this higher molecular weight lipid-A may be a minor component.

Fig. 1. GLC-MS profile of TMS methylglycosides of lipid-A from *Rhizobium* sp. Sin-1. GlcN-onate, 2-aminoglucuronate, and Glc, glucose; and GlcN, glucosamine. The fatty acids are indicated as defined in the text.

Initially, separation of the different lipid-As was attempted using DEAE-cellulose ion-exchange chromatography. It was expected that the lipid-A would bind to DEAE-cellulose due to the presence of 2-aminoglucuronate. However, most of the lipid-A eluted without binding to the DEAE column, with a small portion eluting at 60 mM ammonium acetate. Analysis (GLC-MS) of the bound and non-bound fractions did not reveal any glycosyl or fatty acyl differences. The fact that the vast majority of the lipid-A did not bind to DEAE-cellulose suggested that it did not carry a negative charge even though it contained 2-aminoglucuronate. It was, therefore, possible that the 2-aminoglucuronate was largely present as a 1,5-lactone resulting in a lipid-A that does not carry a charge. In fact, the molecular ion species at $m/z$ 1291, 1517, and 1743 are all consistent with structures in which the 2-aminoglucuronate residue would be present as a lactone (discussed further below). This lactone form of 2-aminoglucuronate has been reported for *R. etli* CE3 lipid-A (9).

The various lipid-A species were further separated by TLC. This procedure resolved the lipid-A into three spots having $R_f$ (relative factor) values of 0.89, 0.62, and 0.42. Although the yield was low, preparative TLC allowed the purification of the lipid-A with an $R_f$ value of 0.62. This lipid-A had an [M + Na]$^+$ ion of $m/z$ 1291. Both composition and MS analyses of this TLC-purified lipid-A ($m/z$ 1291) indicated that it consisted of one glucosamine, one 2-aminoglucuno-1,5-lactone, one 27-OHC:280, and either a combination of one $\beta$-OHC14:0 and one $\beta$-hydroxyoctadec-2-enate ($\beta$-OH18:0), or two $\beta$-hydroxyoctadec-2-enate ($\beta$-OH16:0) residues. Tandem MS-MS analysis of the $m/z$ 1291 ion (spectrum not shown) gave B1 and C1 ions of $m/z$ 839 and 856, due to cleavage on either side of the glucosaminosyl glycosidic oxygen. These ions are due to fragments in which 27-OHC28:0 and $\beta$-OHC16:0 are both attached to the distal glucosaminosyl residue. Thus, the third fatty acyl substituent attached to the proximal 2-aminoglucuronolactone must be $\beta$-OHC16:0 in order for the mass to be $m/z$ 1291. There were

Fig. 2. Mass spectrum (MALDI-TOF) of the *Rhizobium* sp. Sin-1 lipid-A preparations recovered as ethanol-soluble (top) and ethanol-insoluble (bottom) material. The insets show the spectra of the predominant lipid-A species. The proposed compositions for the various ions are given in Table 1.
Novel Rhizobium Lipid-A Structure

Proposed compositions of the various Rhizobium sp. Sin-1 lipid-A molecules observed by MALDI-TOF MS analysis

Ions of \( m/z \) 1291, 1499, 1527, and 1555 were also observed (see Fig. 2). These ions are probably due to molecules with the same compositions less water (i.e., \(-18\) mass units from ions \( m/z \) 1347, 1517, 1545, and 1573, respectively). Other additional ions (Fig. 2) observed were \( m/z \) 1269, which is the \([M + H]^+\) ion that corresponds to the \([M + Na]^+\) ion of \( m/z \) 1291, and \( m/z \) 1307, which is the \([M + K]^+\) ion of this same molecular species.

| \([(M + Na)^+\)] | GlcN | GlcN-\(\beta\)OH14:0 | \(\beta\)OH14:0 | \(\beta\)OH16:0 | \(\beta\)OH18:0 | 2\(\beta\)OH28:0 |
|-----------------|------|---------------------|-----------------|-----------------|-----------------|-----------------|
| 1291            | 1    | 1                   | 0               | 2               | 0               | 1               |
| 1347            | 1    | 1                   | 1               | 0               | 1               | 1               |
| 1517            | 1    | 1                   | 1               | 2               | 0               | 1               |
| 1545            | 1    | 1                   | 0               | 0               | 1               | 1               |
| 1573            | 1    | 1                   | 1               | 0               | 2               | 1               |
| 1743            | 1    | 1                   | 3               | 0               | 1               | 1               |

These ions of \( m/z \) 1329, 1499, 1527, and 1555 were also observed (see Fig. 2). These ions are probably due to molecules with the same compositions less \( m/z \) 18 mass units (Fig. 2) from ions \( m/z \) 1447, whereas the ethanol-insoluble fraction contained largely \( m/z \) 1573 cluster of ions (1499, 1517, 1527, 1545, 1555, and 1573), which is the \([M + Na]^+\) ion of this same molecular species.

Lipid-A species 1

H-1: 4.51 C-1: 103.7
H-2: 3.82 C-2: 56.1
H-3: 4.98 C-3: 78.1
H-4: 3.57 C-4: 70.8
H-5: 3.38 C-5: 78.5
H-6a: 3.73 C-6: 63.7
H-6b: 3.87 C-6: 63.8

Lipid-A species 2

H-1: 4.45 C-1: 104.1
H-2: 3.86 C-2: 56.2
H-3: 4.95 C-3: 78.1
H-4: 3.53 C-4: 70.9
H-5: 3.38 C-5: 78.6
H-6a: 3.73 C-6: 63.8
H-6b: 3.87 C-6: 63.8

Proposed compositions of the various Rhizobium sp. Sin-1 lipid-A molecules observed by MALDI-TOF MS analysis

| Residue spin system | Glucosamine (A) | 2-Aminoglucono-2-ene-1,5-lactone (B) |
|--------------------|-----------------|-------------------------------------|
| \( ^1H \) \( ^{13}C \) | \( ^1H \) \( ^{13}C \) |
| Lipid-A species 1 | H-1 4.51 C-1 103.7 | H-1 4.96 C-1 104.1 |
| Lipid-A species 2 | H-1 4.45 C-1 104.1 | (C-1) \( ^{13}C \) 174.0 |

NMR Analysis—Detailed NMR analysis was performed on the ethanol-insoluble lipid-A fraction. NMR analysis readily showed that this fraction consisted of a mixture of several molecules due, in addition to variation in the types of the four fatty acids, to variation in the glycosyl backbone structure.

Through a combination of COSY, TOCSY, and HSQC NMR spectra, a complete proton and carbon assignment could be made for each of four glycosyl ring spin systems (labeled A, B, C, and D) present in this lipid-A fraction. These assignments are given in Table II, and the COSY, TOCSY, and HSQC spectra are shown in Figs. 3, 4, and 5, respectively. Spin systems A and C are very similar to one another and, in fact, partially overlap. The H1/C1 resonances for spin system A are at 8.45/103.7, whereas those for system C are at 8.45/104.1 and show that these residues are \( \beta \)-linked. The connectivities from H1 through H3 for both A and C are slightly offset from one another and can be traced (see Fig. 3), whereas the H4, H5, and H6 resonances are more remote from one another.

1H and \( ^{13}C \) NMR chemical shifts of sugar backbone and selected acyl moieties of Rhizobium sp. Sin-1 lipid-A

| Residue spin system | Glucosamine (C) | 2-Aminoglucono-1,5-lactone (D) |
|--------------------|-----------------|--------------------------------|
| \( ^1H \) \( ^{13}C \) | \( ^1H \) \( ^{13}C \) |
| Lipid-A species 1 | H-1 4.45 C-1 104.1 | H-1 4.96 C-1 104.1 |
| Lipid-A species 2 | H-1 4.45 C-1 104.1 | (C-1) \( ^{13}C \) 174.0 |

Hydroxy fatty acids

| \( ^1H \) \( ^{13}C \) | \( ^1H \) \( ^{13}C \) |
|---------------------|---------------------|
| \( \beta \)-Hydroxy fatty acids | \( \beta \)-H 4.02 C-3 70.8 |
| \( \beta \)-Acylated fatty acids | \( \beta \)-H 5.08 C-3 73.5 |
| 27-OH28:0 fatty acids | (\( \omega \)-1)H 3.75 C-27 70.1 |

* Deduced from the HMBC experiment.

Insufficient amounts of the other lipid-A species isolated from the TLC plates for MS and composition analyses.

It was discovered that washing the lipid-A with ethanol resulted in two different, relatively pure, lipid-A fractions; one fraction was insoluble in ethanol and a second was soluble. These two fractions were analyzed by MALDI-TOF, and their spectra are shown in Fig. 2. The ethanol-soluble lipid-A contained the \( m/z \) 1291 cluster of ions (1269, 1291, 1307, 1329, and 1347), whereas the ethanol-insoluble fraction contained largely the \( m/z \) 1517 cluster of ions (1499, 1517, 1527, 1545, 1555, and 1573) with a small amount of the 1291 cluster. Based on the lipid-A components described above (see Fig. 2), the proposed compositions of these ions are given in Table I. Except for the highly heterogeneous variation in fatty acylation, the lipid-A in each of these fractions is relatively pure. The lipid-A in the ethanol-insoluble fraction were all tetraacylated with \( \beta \)-OH14:0, \( \beta \)-OH16:0, and \( \beta \)-OH18:0. This was also true of the lipid-A molecules in the ethanol-soluble fraction. There is also micro-heterogeneity in the glycosyl backbone structure in that some molecules contain 2-amino-1,5-lactone as one of the glycosyl backbone components, and other molecules are present that are 18 mass units less than the 2-amino-1,5-lactone-containing molecules, i.e., 1329 (1347–18), 1499 (1517–18), 1527 (1545–18), and 1555 (1573–18). The structural basis for these latter ions was deduced from the NMR data and is described below.

NMR Analysis—Detailed NMR analysis was performed on the ethanol-insoluble lipid-A fraction. NMR analysis readily showed that this fraction consisted of a mixture of several molecules due, in addition to variation in the types of the four fatty acids, to variation in the glycosyl backbone structure.

Through a combination of COSY, TOCSY, and HSQC NMR analyses, a complete proton and carbon assignment could be made for each of four glycosyl ring spin systems (labeled A, B, C, and D) present in this lipid-A fraction. These assignments are given in Table II, and the COSY, TOCSY, and HSQC spectra are shown in Figs. 3, 4, and 5, respectively. Spin systems A and C are very similar to one another and, in fact, partially overlap. The H1/C1 resonances for spin system A are at 8.45/103.7, whereas those for system C are at 8.45/104.1 and show that these residues are \( \beta \)-linked. The connectivities from H1 through H3 for both A and C are slightly offset from one another and can be traced (see Fig. 3), whereas the H4, H5,
H6a, and H6b resonances for both spin systems overlap. Both A and C glycosyl spin systems are consistent with those reported for the distal β-glucosaminosyl residue of R. etli lipid-A (19). The downfield H2/C2 and H3/C3 chemical shifts are consistent with both A and C glucosaminosyl residues having N- and O-fatty acyl substituents at these respective positions. The H6/C6 (3.73, 3.87) chemical shift for both A and C glucosaminosyl residues shows that, in both cases, there is no substitution at this position.

The presence of two distal A/D glucosaminosyl residues, A and C, indicates that each one is linked to a different proximal residue. The B and D spin systems are due to these two different proximal residues. As described above, MS analysis indicated that all of the lipid-A molecules contain 2-aminogluconic acid as a 1,5-lactone. Spin system D is consistent with such a residue. This spin system does not have an H1/C1 resonance that would be typical of a glycosyl anomeric center. Instead, there is an H2/C2 resonance at 4.96/56.7 that is consistent with an N-acylated 2-aminogluconate residue as reported for R. etli lipid-A (19). The H3 chemical shift at 5.55 in spin system D is upfield from that reported (19) for 2-aminogluconate of R. etli lipid-A (5.03) and indicates that residue D does not have a fatty acyl moiety at O-3. The chemical shifts of H4/C4 through H6/C6 for residue D are somewhat different from those reported for the R. etli lipid-A 2-aminogluconate residue (19). For example, the H5 (6.07) of residue D is shifted downfield from the reported value (6.57) (19), a result that supports the conclusion that residue D is the lactone form of 2-aminogluconate.
The downfield C6 chemical shift (δ73.3) indicates that residue D is substituted at this position. The fourth spin system, due to residue B, is assigned to an unsaturated 3-deoxy form of 2-amino-2,3-dideoxy-D-erythro-hex-2-enono-1,5-lactone, i.e. 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone. For residue B, there is no H2, and therefore, the C2 resonance could not be detected from the HSQC experiment. The connectivities of this spin system are from H3/C3 (δ77.55/131.0) to H4/C4 (δ5.06/84.0) to H5/C5 (δ3.79/73.3) and to H6/C6 (δ3.96, 3.73/72.6). The downfield chemical shift of H3/C3 (δ77.55/131.0) shows that this is a vinyl H/C center and supports the presence of a 2,3-unsaturation site in this residue. The presence of this unsaturated residue is consistent with those molecular ions that are 18 mass units less than the larger boldface type 18), ions in the ethanol-insoluble fraction. In summary these NMR results show that this lipid-A fraction contains molecules with two different glycosyl disaccharide backbones. In one case a β-glucosaminosyl residue is linked to the 6-position of 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone, and in the second case a β-glucosaminosyl residue is linked to the 6-position of 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone (i.e. A→B). Integration of the CH1 resonances indicate that the relative ratio of these two lipid-A species is 3:2 (A→B; C→D). The presence of these two disaccharides was confirmed by an HMBC experiment, Fig. 7, which clearly showed connectivity from C-H1 to C-D6, and from A-H1 to B-C6, as well as from B-H6 to C-C1, and from B-H6 to A-C1.

The presence of four glycosyl residues in this lipid-A fraction is consistent with the existence of lipid-A molecules with two different disaccharide backbones. The sequences of these two disaccharides were determined by NOESY and HMBC NMR experiments. The NOESY spectrum, Fig. 6, shows that the H1 of glucosaminosyl residue C (δ64.45) has intra-residue NOEs to H3 and H5 and an inter-residue NOE to H6 of residue D, 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone. The H1 of glucosaminosyl residue A (δ64.51) also has intra-residue NOEs to its H3 and H5 and an inter-residue NOE to H6 of residue B, 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone. With regard to residues B and D, intra-residue NOEs were observed from H4 to H3 and H5 and from H4 to H2 and H6 for residue D and from H4 to H5 in residue B (the spectral width in this experiment did not allow detection of NOEs to H3). These NOEs would be somewhat unexpected for a normal glycosyl ring structure due to the fact that the protons involved on are opposite sides of the ring. However, such NOEs are reasonable for residues B and D due to the long mixing time (500 ms) required for this experiment and to the C3 half-chair conformation of these lactone rings rather than the normal glycosyl C3 chair conformation.

In summary these NMR results show that this lipid-A fraction contains molecules with two different glycosyl disaccharide backbones. In one case a β-glucosaminosyl residue is linked to the 6-position of 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone (i.e. C→D), and in the second case a β-glucosaminosyl residue is linked to the 6-position of 2-amino-2,3-dideoxy-β-erythro-hex-2-enono-1,5-lactone (i.e. A→B). Integration of the AH1 and CH1 resonances indicate that the relative ratio of these two lipid-A species is 3:2 (A→B; C→D). The presence of these two disaccharides was confirmed by an HMBC experiment, Fig. 7, which clearly showed connectivity from C-H1 to C-D6, and from A-H1 to B-C6, as well as from B-H6 to C-C1, and from B-H6 to A-C1.

The downfield chemical shifts for H2/C2, H2/C2', and H3'/C3' of the two glycosyl backbone structures (see Table II) are consistent with these positions being fatty-acylated. Fig. 8 shows the region of the COSY spectrum pertaining to the fatty acyl substituents. The two cross-peaks at δ4.02/2.42–2.49 (designated as α2/β2, α3'/β3') and at δ4.04/1.45–1.6 (designated as γ2/β2, γ3'/β3') are due to the α (δ2.42–2.49), β (δ4.04), and γ (δ1.45–1.60) protons of β-hydroxy fatty acyl residues that are not substituted at their β-OH group, i.e. they are not acyloxy-acylated. The cross-peaks at δ5.08/2.35–2.44 and δ5.08/1.56 are
due to the \( \alpha \) (82.35–2.44), \( \beta \) (55.10), and \( \gamma \) (61.56) protons of a \( \beta \)-hydroxy fatty acid in which the \( \beta \)-OH group is substituted with another acyl residue (i.e. it contains an acyloxyacyl residue) as indicated by the downfield shift of the \( \beta \)-proton. The cross-peaks at 83.75/1.39–1.55 and 83.75/1.17 are due to the to \( \omega \)-2' (61.17), \( \omega \)-1'2' (83.75), and \( \omega \)-2'2' (61.39–1.55) protons of the 27-OHC28:0 residue. All of these fatty acyl chemical shifts are consistent with those reported for the lipid-A from \( R. \) etli CE3 (19) with the exception of the \( \omega \)-1'2' proton which is upfield compared with that reported for \( R. \) etli CE3 lipid-A (83.75 versus 84.92). This indicates that the 27-OH position of the very long-chain fatty acid in \( Rhizobium \) sp. Sin-1 lipid-A, unlike \( R. \) etli CE3 lipid-A, is not substituted by \( \beta \)-hydroxybutyric acid. Furthermore, no cross-peaks near 84.25/1.25 were observed that would account for the terminal methyl group and \( \beta \)-oxyethylenic proton of the \( \beta \)-hydroxybutyrate residue. These NMR results support the MS data described above and confirm that the \( Rhizobium \) sp. Sin-1 lipid-A molecules are not \( \beta \)-hydroxybutyrate. The cross-peaks observed at 65.23 and 2.60, 2.49, and 1.27 are very likely due to contaminating polyhydroxybutyrate which is reported to have very similar chemical shifts (65.3, 2.65, 2.5, and 1.27) (38). Polyhydroxybutyrate is synthesized by bacteria, including rhizobia, as a carbon and energy source. Furthermore, the method used to isolate polyhydroxybutyrate, i.e. precipitation of with methanol, is similar to the extraction and ethanol precipitation method used to prepare this lipid-A preparation (38). Another cross-peak at 65.32/1.99 can be assigned to the chemical shift of the methine and adjacent methylene protons of the double bond in \( \beta \)-hydroxyoctadecenoic acid (\( \beta \)-OHCl18:1), a minor component of this lipid-A preparation (Fig. 1).

The above NMR and MS data show that the lipid-A molecules in this ethanol-insoluble lipid-A preparation contain three primary acyl fatty acids and one acyloxyacyl residue on two different disaccharide backbones. These structures, 3 and 4, are shown in Fig. 9. The proximal 2-aminoglucono-1,5-lactonsy1 and 2-amino-2,3-dideoxy-\( \alpha \)-erythro-hex-2-enono-1,5-lactonsy1 residues on these structures are not acylated at position 3. The inability to separate structure 3 from structure 4 was most likely due to the fact that these structures primarily differ only by the fact that structure 4 contains a 2,3-double bond. In addition to structures 3 and 4, two other corresponding structures (not shown), found in the ethanol-soluble fraction, are present that lack the \( \beta \)-OHC14:0 substituent on C-3'. These latter structures account for the \( m/z \) 1291, 1329, and 1347 ions. The 27-OHC28:0 substituent is on the distal glucosaminosy1 residue as shown by the MS/MS results for the \( m/z \) 1291 ion described above. It is likely present as the acyloxyacyl residue as reported for the lipid-A of \( R. \) etli CE3 (19).

In summarizing the above results, it has been shown that mild acid hydrolysis of \( Rhizobium \) sp. Sin-1 LPS gave a lipid-A preparation containing three groups of molecules that differed from one another in their level of \( \beta \)-hydroxymyristoylation. The structures of the major ion cluster, represented by \([M + Na]^+\) \( m/z \) of 1517, are shown in Fig. 9 as structures 3 and 4. The lower molecular weight lipid-A ion cluster, represented by the \( m/z \) 1291 ion, differ from structures 3 and 4 in that they lack a \( \beta \)-hydroxymyristoyl moiety at position C-3', whereas the small

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**Fig. 8.** A partial 60 MHz 1H-1H COSY spectrum of the ethanol-insoluble lipid-A fraction from \( Rhizobium \) sp. Sin-1 lipid-A showing the key fatty acyl chain assignments as indicated and described in the text. Those cross-resonances marked "**" are due to contaminating polyhydroxybutyrate as described in the text. Those marked "**" are also present but not shown in this figure.

**Fig. 9.** Structures of major lipid-A species found in the LPS from \( Rhizobium \) sp. Sin-1. Structures 1 and 2 are those hypothesized to be present in the intact LPS prior to mild acid hydrolysis. Structures 3 and 4 are those produced from 1 and 2, respectively, by mild acid hydrolysis and isolated in the ethanol-insoluble lipid-A fraction. Additional corresponding structures lacking a \( \beta \)-OHC14:0 residue at C-3' are also present but not shown in this figure.
amount of the higher molecular weight molecules, represented by the m/z 1743 ion, probably contain an additional β-hydroxymyristoyl moiety at C-3.

**DISCUSSION**

The glycosyl backbone structures for the lipid-A from several rhizobial species have been reported to consist of more “normal” enteric-like structures such as bis-phosphorylated glucosamine disaccharides or phosphorylated 2,3-diaminoglucose disaccharides (13, 39, 40). Thus, the *Rhizobium* sp. Sin-1 lipid-A described here is only the second structure reported that contains 2-amino-glucurate, the first being the lipid-A from *R. etli* lipid-A (9, 18, 19). However, although similar to the *R. etli* CE3 lipid-A, the *Rhizobium* sp. Sin-1 lipid-A is structurally unique with regard to both its glycosyl backbone structure and its fatty acylation pattern. It does not have any acidic components, i.e. it is devoid of both phosphate and acidic glycosyl residues such as galacturonic acid. In addition, the lipid-A preparation from *R. etli* has molecules with either glucosamine or 2-amino-glucuronic acid as the proximal residue (19); however, all of the *Rhizobium* sp. Sin-1 lipid-A molecules have 2-amino-glucurono-1,5-lactone as the proximal residue. In a portion of the isolated lipid-A molecules, this proximal residue exists as a 2,3-unsaturated version of 2-amino-glucurono-1,5-lactone, 2-amino-2,3-dideoxy-o-erythro-hex-2-eno-1,5-lactone. This unsaturated residue was also hypothesized for one of the lipid-A structures reported for *R. etli* (19). The fatty acylation pattern of *Rhizobium* sp. Sin-1 lipid-A molecules is unlike that for *R. etli* lipid-A in that some molecules are devoid of fatty acyl substituents at both C-3' and C-3 instead of just at C-3, whereas other molecules lack a fatty acyl substituent only at C-3. Because the variation in the structures of the different lipid-A ion clusters observed by MALDI-TOF analysis are due to the presence or absence of a fatty acyl residue at C-3’ and/or C-3 and also to a mass difference of 226 mass units, it is likely that when these positions are fatty acylated, it is with β-OHIC14:0.

As mentioned in the previous paragraph, several of the structural features of the *Rhizobium* sp. Sin-1 lipid-A preparation are probably the result of the mild acid hydrolysis procedure that releases lipid-A from the LPS. These features include the lactonization of 2-amino-glucuronic acid as well as the acid-catalyzed elimination of a C-3 fatty acyl residue from this lactone resulting in a proximal 2-amino-2,3-dideoxy-o-erythro-hex-2-eno-1,5-lactonosyl residue. Therefore, the lipid-A structures that would be expected to be present in the intact LPS are structures 1 and 2 as shown in Fig. 9. Also shown in Fig. 9 are the observed structures, 3 and 4, that result from mild acid hydrolysis of the LPS.

The biosynthetic mechanism for the synthesis of *Rhizobium* sp. Sin-1 lipid-A is not known. However, the similarities of its structure with *R. etli* and *R. leguminosarum* lipid-A suggests that the biosynthetic steps of *Rhizobium* sp. Sin-1 lipid-A synthesis would be similar to those reported for *R. etli* and *R. leguminosarum*. These steps would include all of the enzyme activities that convert UDP-N-acetylglucosamine into two residues of 3-deoxy-d-manno-2-octulosonic acid lipid-IVα as well as specific enzymes that process this common lipid-A precursor into the mature lipid-A structures (i.e. 1 and 2 in Fig. 9, and the corresponding structures lacking β-OHIC14:0 at C-3’). These processing enzymes would be the 4’- and 1-phosphatases, the glucosamine oxidase, the acyl carrier protein and transferase for 27-OHC28:0, and the acylase that removes the fatty acyl group from C-3. However, unlike *R. etli*, the *Rhizobium* sp. Sin-1 lipid-A structures suggest that this organism would lack the UDP-galacturonosytransferase that adds galacturonic acid to C-4’ and would possibly contain an additional acylase that removes, from a portion of the molecules, the fatty acyl group from C-3’.

As described in the Introduction, the lipid-A from enteric bacteria is a very toxic molecule, and therefore, there is interest in structural analogs that would act as non-toxic antagonists of endotoxins. An example of one such structure is the lipid-A from *R. spheroides* (41) or *R. capsulatus* (26, 29). Synthetic analogs of this structure have been synthesized and are being evaluated for use as endotoxin antagonists for the treatment of sepsis (42–45). Recently, two reports presented results on the biological activities of rhizobial LPS in mice. One report (46) showed that *S. typhimurium* LPS and LPS from *R. etli*, *R. leguminosarum*, and *Rhizobium* sp. Sin-1 stimulated the production of an inducible LPS receptor, CD14, in the bone marrow cells of a normal mouse. *S. typhimurium* LPS failed to induce CD14 production in bone marrow cells from mutant mouse cell lines that are defective in the toll-like receptor 4 gene (tlr-4), which is the expected result since the signal transduction pathway with regard to LPS involves the transmembrane Tlr4 protein. However, the rhizobial LPS still stimulated CD14 production in the mutant cells suggesting that, in mice, the rhizobial LPS may be acting via an alternative mechanism that does not involve Tlr4. The second report (47) showed that the *R. leguminosarum* LPS was toxic to galactosamine-sensitized mice but at an LD$_{50}$ that was 178 times greater than that for *S. typhimurium* LPS. In addition, that report showed that the rhizobial LPS could stimulate the production of several cytokines in murine blood. The laboratory of Dr. James Moore (University of Georgia, Veterinary Medicine), in collaboration with our laboratory, is currently investigating the biological activities of the LPS from *R. etli*, *R. leguminosarum*, and *Rhizobium* sp. Sin-1 in equine blood, and in human mononc 6 cells. These data (48) show that these LPS do not effectively stimulate the production of TNF in equine blood or in the mononc 6 cells and that *Rhizobium* sp. Sin-1 LPS is particularly effective at inhibiting the ability of enteric LPS to stimulate TNF in human mononc 6 cells. Because the *Rhizobium* sp. Sin-1 lipid-A preparation consisted of several structural variations, the exact structure(s) that is optimal for its inability to stimulate TNF production and its ability to act as an endotoxin antagonist remains to be determined. Work is currently in progress to determine these structural features.

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