Occurrence of an Unusual Hopanoid-containing Lipid A Among Lipopolysaccharides from Bradyrhizobium Species*

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Background: Hopanoids are present in bradyrhizobial lipid A preparations.

Results: Signals from hopanoid carboxyl shows strong correlation with the proton geminal to the hydroxy group of ester-linked long chain fatty acid.

Conclusion: Hopanoids are covalently linked to the lipid A of Bradyrhizobium.

Significance: The presence of such an unusual lipid A substituent may have a strong influence on the membrane properties of Bradyrhizobium.

The chemical structures of the unusual hopanoid-containing lipid A samples of the lipopolysaccharides (LPS) from three strains of Bradyrhizobium (slow-growing rhizobia) have been established. They differed considerably from other Gram-negative bacteria in regards to the backbone structure, the number of ester-linked long chain hydroxylated fatty acids, as well as the presence of a tertiary residue that consisted of at least one molecule of carboxyl-bacteriohopanediol or its 2-methyl derivative. The structural details of this type of lipid A were established using one- and two-dimenional NMR spectroscopy, chemical composition analyses, and mass spectrometry techniques (electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry and MALDI-TOF-MS). In these lipid A samples the glucosamine disaccharide characteristic for enterobacterial lipid A was replaced by a 2,3-diamino-2,3-dideoxy-D-glucopyranosyl-(GlcN3N) disaccharide, deprived of phosphate residues, and substituted by an α-D-Manp-(1→6)-α-D-Manp disaccharide substituting C-4’ of the non-reducing (distal) GlcN3N, and one residue of galacturonic acid (p-GalpA) α-(1→1)-linked to the reducing (proximal) amino sugar residue. Amide-linked 12:0(3-OH) and 14:0(3-OH) were identified. Some hydroxy groups of these fatty acids were further esterified by long (ω-1)-hydroxylated fatty acids comprising 26–34 carbon atoms. As confirmed by mass spectrometry techniques, these long chain fatty acids could form two or three acyloxyacyl residues. The triterpenoid derivatives were identified as 34-carboxy-bacteriohopane-32,33-diol and 34-carboxy-2β-methyl-bacteriohopane-32,33-diol and were covalently linked to the (ω-1)-hydroxy group of very long chain fatty acid in bradyrhizobial lipid A. Bradyrhizobium japonicum possessed lipid A species with two hopanoid residues.

Lipopolysaccharide (LPS) is an integral component of most Gram-negative bacteria cell envelopes. LPS is usually composed of three domains: lipid A, a hydrophobic part that anchors the LPS molecule in the outer membrane and constitutes its outer leaflet, the core oligosaccharide, and very often the O-specific polysaccharide (O-chain). Such LPS is called smooth, found, for example, in Bradyrhizobium japonicum, Bradyrhizobium yuanmengense, and Bradyrhizobium sp. (Lupinus). LPS composed only of lipid A and the core oligosaccharide is called rough. The semi-rough form additionally containing one repeating unit of O-chain was found in Bradyrhizobium elkanii and Bradyrhizobium liaoningense strains (1). Bradyrhizobia are a slow-growing rhizobia forming a beneficial symbiosis with legumes. The endosymbiotic form of rhizobia, in which nitrogen fixation takes place, is called bacteroids. Rhizobial LPS plays an essential role in symbiosis progression. Together with membrane proteins and lipids favors optimal membrane architecture and determine its permeability, important for the morphology and functionality of bacteroids. Several reports demonstrated that the proper structure of rhizobial LPS is essential for root hair infection, nodule invasion, and adaptation to the endosymbiotic conditions (2–5). The LPS also protects microsymbiont cells against plant defense responses, i.e. hyper-sensitivity reaction and systemic acquired resistance, by suppressing such reactions during rhizobial infection (6–8).

LPS isolated from enterobacterial cells is often toxic, which is due to a certain lipid A structure. Toxic enterobacterial lipid A consists of a β-(1→6)-linked glucosaminyl disaccharide substituted by two phosphate groups at positions C-1 and C-4’. Six fatty acid residues, which form two acyloxyacyl moieties, are linked in distinct positions to the sugar backbone (9, 10). The activity of such lipid A results from binding to the TLR4-MD2 receptor complex on macrophages and endothelial cells (11). Activation of TLR4-MD2 initiates a signaling cascade, which results in biosynthesis of pro-inflammatory mediators by macrophages. In severe cases this may lead to septic shock (12). It was proven that, in humans, only such a lipid A structure is able to activate TLR4-MD2, determined by the set of fatty acids, their length, linkages, and spatial conformation (i.e. the presence of two acyloxyacyl residues) (10, 13). In lipid A samples,
the structures of which differ from such enterobacterial ones, are usually less or not toxic in humans (1, 14, 15). The backbone of rhizobial lipid A can be composed either of a glucosaminyl-(D-GlcP) or a 2,3-diamino-2,3-dideoxy-glucosaminyl-(D-GlcP)N3N disaccharide. In some Rhizobium species, the reducing residue of glucosamine-containing lipid A may be oxidized to 2-amino glucuronate (16). The sugar backbone of lipid A can be decorated by phosphate, uronic acid, or mannose residues. The amino groups of D-GlcP3N3N and D-GlcP as well as C-3 and C-3’ of D-GlcP are substituted by 3-hydroxy fatty acids. Hydroxyl groups of the primary fatty acids can be further substituted by nonpolar or very long chain (ω-1)-hydroxy VLCFAs, forming acyloxy-acyl moieties (17–21). The number of carbon atoms in VLCFA differs in each rhizobium strain. The 27-octacosanoic acid is present in LPS of all members of Rhizobia, except of Azorhizobium caulinodans (22–24). Moreover, in bradyrhizobial LPSs several VLCFAs were identified, among them, straight, and monoo, and dimethyl branched-chain fatty acids built up of 26 to 34 carbon atoms (25). VLCFAs can span the entire outer membrane and play a crucial role in its stabilization.

The structure of lipid A isolated from B. elkanii USDA 76 has been described in detail recently (21). It differs considerably not only from other Gram-negative bacteria but also from other rhizobial lipid A samples. The sugar backbone is constituted by five sugar units identified as two dianaminoglucoses and three mannoses. There are four amide-linked fatty acids (two 12:0(3-OH) and two 14:0(3-OH)) in this lipid A. Two of them are substituted by two VLCFA hydroxylated fatty acids, forming acyloxy-acyl residues. Additionally, one of them can be further acylated by 3-hydroxybutyric acid, linked to the (ω-1) hydroxy group. In this article we present the unique lipid A structure from different Bradyrhizobium strains, which form two or three acyloxyacyl moieties with VLCFAs and also contain one or two hopanoid residues linked covalently as tertiary hydrophobic residue(s) to the hydroxy group(s) of (ω-1)-hydroxy fatty acids.

Hopanoid-containing Lipid A of Bradyrhizobium bacteria this lipid component may have additional functions, besides membrane reinforcement. It has been proven that in Frankia, hopanoids can be involved in oxygen protection of the nitrogenase complex by forming of a diffusion barrier (27). In the case of Rh. palustris the bacteriohopane polyols determine membrane integrity and play a role in pH homeostasis (30). Very recently, the first hopanoid-containing lipid A, obtained from LPS of the photosynthetic Bradyrhizobium strain BTaI1, was structurally and functionally characterized (32).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Condition—**Bacteria (B. japonicum USDA 110, B. yuanmingense CCBAU 10071, and Bradyrhizobium sp. (Lupinus) USDA 3045) were grown at 28 °C in 79CA medium according to Vincent (33), for 14 days, with aeration by vigorous shaking.

**Isolation and Purification of LPS and Lipid A Samples—**The cells pellets obtained by centrifugation were washed twice with saline, once with distilled water, and then delipidation was performed according to Que and co-workers (19). The delipidated and dried cell pellets were suspended in 50 mM sodium phosphate buffer (pH 7.0), supplemented with 5 mM EDTA, and digested with lysozyme (6 mg g⁻¹ dry mass, 4 °C, 16 h). The nucleic acids were degraded by treatment with DNase and RNase (0.3 mg g⁻¹ dry mass, 37 °C, 30 min). Cell proteins were digested by incubation with proteinase K (0.3 mg g⁻¹ dry mass, room temperature, for 18 h, followed by incubation for 10 min at 60 °C) (34). The LPS preparations were obtained from hot 45% phenol/water extractions according to Westphal and Jann (35), with further modifications (36). The phenol and water phases, which contained LPS, were dialyzed extensively against tap and distilled water. Pure LPS preparations were obtained by ultracentrifugation (105,000 × g, 4 °C, 4 h). The LPS was obtained from water phase after phenol/water extraction, 820 mg (5.8%) in the case of B. japonicum, 148 mg (1.4%) in the case of B. yuanmingense, and 344 mg (5.7%) in the case of Bradyrhizobium sp. (Lupinus).

Lipid A was liberated from LPS by mild acid hydrolysis (1–2% aqueous acetic acid, 100 °C, 2–3 h). The free lipid A was purified by a two-phase Bligh-Dyer system according to Que et al. (19). Briefly, adequate amounts of chloroform and methanol were added to the hydrolysate to obtain a chloroform/methanol/hydrolysate, 2:2:1.8 (v/v/v), mixture. The mixture was vigorously shaken and then centrifuged. The chloroform phase, containing lipid A, was collected and washed twice with the water phase from the freshly prepared two-phase Bligh-Dyer mixture (chloroform/methanol/water, 2:2:1.8 (v/v/v)). The pure lipid A preparations (B. japonicum, 36 mg; B. yuanmingense, 18 mg; Bradyrhizobium sp. (Lupinus), 12 mg) were stored at −20 °C in CHCl₃/MeOH (3:1, v/v). O-Deacylation of lipid A samples was performed by incubation (1–2 mg) in chloroform, methanol, 0.6 M aqueous NaOH, 2:3:1 (v/v/v), for 1.5 h at room temperature, according to Que-Gewirth and co-workers (37).

**Fatty Acids, Hopanoid Lipids, and Sugars Analysis—**For total fatty acid and hopanoid lipids determination, lipid A preparations were subjected to hydrolysis in 4 M HCl (100 °C, 4 h). Liberated fatty acids and hopanoids were extracted with chloroform and converted to their methyl esters with diazometh-
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An. After evaporation to dryness, hydroxyl groups of fatty acids and hopanoid lipids were derivatized with BSTFA (16 h at room temperature). Neutral and amino sugar analyses were performed according to standard protocols described elsewhere (21).

GC-MS analyses of fatty acids and sugars were performed on a Hewlett Packard gas chromatograph 5890 series II and Agilent Technologies GC System 7890A connected to a mass selective detector EI/CI MSD 5975C, equipped with a HP-5MS column (30 m × 0.25 mm) with helium as a carrier gas (flow rate: 0.7 ml min⁻¹). The temperature program was as follows: 150 °C for 3 min, then raised to 250 °C at 3 °C min⁻¹, then to 320 °C, 25 °C min⁻¹. The final temperature was kept for 10 min for sugar analysis and 20 min for fatty acid analysis.

Mass Spectrometry—Lipid A samples obtained from B. japonicum were analyzed on a high resolution hybrid Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR) instrument (Apex Qe Bruker Daltonics, Billerica, MA) with electrospray ionization (ESI), equipped with a 7 tesla actively shielded magnet. Samples for analysis were prepared as described earlier (21) and measured in the negative ion mode. Mass spectra were charge deconvoluted and mass numbers given refer to the monoisotopic peaks.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed with a Bruker-Daltonics Reflex III instrument (Bruker Daltonics, Bremen, Germany) at an acceleration voltage of 20 kV and delayed ion extraction. Lipid A preparations were dispersed in methanol/water (1:1, v/v) at a concentration of 1 mg/μl with addition of 20 mM EDTA. The matrix solution was prepared from 2,5-dihydroxybenzoic acid in 1% trifluoroacetic acid and the spectra were obtained in positive or negative ion modes.

NMR Spectroscopy—For NMR analysis a sample containing 18 mg of native lipid A from B. japonicum dissolved in 0.6 ml of CDCl₃/CD₂OD (2:1, v/v) with 5 μl of D₂O was used. One- and two-dimensional NMR spectra were recorded at 700 MHz on an AVANCE III spectrometer with Cryoprobe (Bruker) using Bruker software. Spectra were recorded at 27 °C. The following two-dimensional NMR experiments were performed: COSY, DQF-COSY, TOCSY, ROESY, HSQC, HSQC-DEPT, and HMBC. The ¹H and ¹³C resonances were measured relative to TMS (δH/δC 0.0/0.0).

RESULTS

Isolation and Chemical Analysis of Lipid A of Bradyrhizobium—LPS of Bradyrhizobium strains were isolated using hot phenol/water extraction. The material was found mainly in rhizobium—Hopanoid-containing Lipid A of Bradyrhizobium

<Table 1>

| Component | USDA 110 | CCBU 10071 | Bradyrhizobium sp. |
|-----------|---------|----------|-----------------|
| Fatty acids |         |          |                 |
| 12:0(3-OH) | +       | +        | +               |
| 14:0(3-OH) | +       | +        | +               |
| 26:0(25-OH) | +     | +        | +               |
| 27:0(26-OH) | −      | tr.      | tr.             |
| 28:0(27-OH) | +      | +        | +               |
| 30:0(29-OH) | +      | +        | +               |
| 31:0(30-OH) | +      | +        | +               |
| 32:0(31-OH) | +      | +        | +               |
| 33:0(32-OH) | +      | +        | +               |
| 32:0(31-OH) | −      | +        | +               |
| 34:0(33-OH) | −      | +        | +               |
| Hopanoids* |         |          |                 |
| I, R = CH₃ | +       | +        | +               |
| II, R = H | +       | +        | +               |
| Sugars |         |          |                 |
| β-Manp | +       | +        | +               |
| β-Galp | +       | +        | +               |
| β-GlcN3N | +      | +        | +               |

* Branched fatty acid (ω-10) (see Ref. 25).
† Part of fatty acid is present in the dimethyl-branch (ω-10 and ω-11).
‡ For hopanoid structure, see Fig. 1.

In all lipid A preparations a high amount of pentacyclic triterpenoids (hopanoid lipids) was present. Based on GC-MS data of trimethylsilyloxy derivatives of methyl esters obtained from the B. japonicum lipid A preparation two constituents were identified (Fig. 1, A and B). The characteristic fragmentations defined them as 34-carboxyl-bacteriohopane-32,33-diol, the major component of bradyrhizobial lipid A, and 34-carboxyl-2-methyl-bacteriohopane-32,33-diol as the less abundant component. The dominating signal at z/50.645 min, Fig. 1A showed a pseudomolecular ion at z/673, [M+H]⁺, and a prominent ion at z/50.084 min, Fig. 1B) possessed [M+H]⁺ at z/687 and a C-ring fragmentation that yielded an ion at z/205 (38). The presence of the last ion suggested that an additional methyl group could be localized in rings A or B. It is known that 3-methylhopanoids elute later than the non-methylated species, however, in the case of 2-methylhopanoids the retention time is very close to the corresponding nonmethylated ones (39). In B. japonicum lipid A, the TMS derivative of the methylated hopanoid eluted a bit earlier than the non-methylated form, suggesting the presence of a methyl group at C-2 of ring A (Fig. 1).
Mass Spectrometry of Lipid A Preparations—Lipid A preparations were investigated either by ESI FT-ICR-MS or MALDI-TOF-MS.

The charge-deconvoluted ESI FT-ICR mass spectrum of the native lipid A of *B. japonicum* showed lipid A molecules comprising a different acylation pattern, which can be recognized by the mass difference of 14 and 28 Da between neighboring signals (Fig. 2A and Table 2). Monoisotopic masses 2087.390, 2105.422, and 2115.460 Da were assigned to lipid A species containing two Manp, two GlcPN3N, one GalpA, two 12:0(3-OH), two 14:0(3-OH), and one ester-linked fatty acid, forming penta-acyl lipid A. The mass difference of 18 Da originated from a dehydration process, occurring during cleavage of VLCFA. The cluster of low-intensity signals in the 2570–2680 Da region was derived from hexa-acylated lipid A molecules containing two secondary VLCFA substituents. The intensive peaks at 3096.291 and 3110.318 Da could be assigned to the hexa-acylated lipid A molecules containing two secondary VLCFA substituents. The intensive peaks at 3096.291 and 3110.318 Da could be assigned to the hexa-acylated lipid A that contained two ester-linked VLCFA, like 29:0(28-OH) and 32:0(31-OH) or 29:0(28-OH) and 33:0(32-OH). It was postulated that one of those VLCFAs was linked to the hopanoid residue via its hydroxyl group. Such lipid A molecules possess a calculated monoisotopic mass of 3096.343 and 3110.358 Da. Mass differences of 14 Da were due to different lengths of VLCFAs as well as the presence of two hopanoid species. Signals derived from molecules with the highest mass (around 3600 Da) originated from hexa-acyl lipid A containing two hopanoid substituents as tertiary residues, moreover, one of these hopanoid moieties could bear a 2β-methyl group (see Fig. 1).

Mass peaks around 1000 Da originated either from the hopanoid-VLCFA moiety that was cleaved from the native lipid A during mild acid hydrolysis or could be the result of fragmentation during ionization. The mentioned dehydrated form of penta-acylated lipid A (2087.390 Da) likely also resulted from this process. The mass differences between neighboring peaks in this cluster equal 14 Da, originating from both, the different lengths of linked VLCFA and the methylated form of the hopanoid.

The mass spectrum of O-deacylated lipid A of *B. japonicum* USDA 110 contained three sets of signals (Fig. 2B). The peaks at 530.4312 Da were derived from a hopanoid residue, which was cleaved during O-deacylation and was not removed by extraction. The mass peaks at 1651.013 and 1669.030 Da were derived from the tetra-acylated lipid A. The second signal was consistent with a lipid A species composed of two GlcPN3N, two Manp, one GalpA, and four amide-linked fatty acid residues.
(two 12:0(3-OH) and two 14:0(3-OH)). One 3-OH fatty acid was deprived of H$_2$O resulting in an α-unsaturated derivative (see the text above). The signal at 1651.013 Da corresponded to a lipid A built from the same components, which unspecifically lost another water molecule (Δm = 18 Da). The group of peaks at 3320.033 Da was consistent with the ion-cluster of both tetra-acyl lipid A.

Fig. 3, A and B, shows MALDI-TOF mass spectra (positive ion mode) obtained on the native and O-deacylated lipid A preparations isolated from *B. yuanmingense*. Three sets of ions are visible on the spectrum of native lipid A. The main signals at $m/z$ 2669.1, 3133.4, and 3660.6 were consistent with calculated masses of hexa-acylated lipid A, hepta-acylated lipid A, and hepta-acylated-hopanoid containing lipid A, respectively. The mass spectrum of the O-deacylated sample (Fig. 3A) showed a main signal at $m/z$ 1692.3, originating from sodiated tetra-acyl lipid A, composed of two GlcNAc units, two Manp, one GalpA, and four amide-linked fatty acids (two 12:0(3-OH) and two 14:0(3-OH) − H$_2$O). The calculated monoisotopic mass for the [M + Na]$^+$ ion was determined to $m/z$ 1692.0. The signals identified as hexa-acyl lipid A derived from molecules containing two ester-linked VLCFAs (such as 33:0(32-OH) and 34:0(33-OH), with a calculated [M-H$_2$O + H]$^+$ $m/z$ of 2669.02, Fig. 3B). The ion cluster at $m/z$ 3100−3200 was identified as hepta-acyl lipid A containing a third acyl residue (such as 31:0(30-OH), theoretical $m/z$ of [M-H$_2$O + H]$^+$ = 3133.48). The lipid A molecules with mass around 3660 Da additionally contained one hopanoid residue, ester-linked as in lipid A from *B. japonicum* to the (ω-1)-OH-group of one of the VLCFA residues.

Fig. 4 shows the MALDI-TOF mass spectrum (negative ion mode) of the lipid A from *Bradyrhizobium* sp. (Lupinus). Two sets of signals are visible, one around $m/z$ 2583.0 and the other at $m/z$ 3095.2, which were assigned to hexa-acyl lipid A carrying two VLCFAs (e.g. 30:0(29-OH) and 31:0(30-OH), theoretical $m/z$ for [M-H$_2$O − H]$^-$ = 2582.91) and a hexa-acylated lipid A moiety additionally bearing one hopanoid residue (calculated $m/z$ [M-H$_2$O − H]$^-$ = 3095.33).

**NMR Spectroscopy of *B. japonicum*** Lipid A—The native lipid A preparation isolated from *B. japonicum* was dissolved in CDCl$_3$/CD$_3$OD (2:1, v/v) with traces of D$_2$O and fully characterized by one- and two-dimensional NMR spectroscopy. Lipid A was not substituted by phosphate residues, as confirmed by $^{31}$P NMR spectroscopy. The HSQC-DEPT spectrum of the lipid A (Fig. 5, blue and green) contained five signals of anomeric carbons (δ 92.25−103.14), four signals of nitrogen-bear-
ing carbons (δ 52.02–54.49), those of sugar ring carbon atoms (δ 61.47–76.76) and signals for CH-OR as well as for CH-OH groups from fatty acids and hopanoid lipids (δ 68.1–73.2).

Based on 1H/1H COSY, TOCSY, and 1H/13C HMBC experiments five spin systems characterizing sugar pyranoses were identified. Two of them (E and D) were derived from α-D-Manp, C represents β-D-GlcN3N, B represents α-D-GlcN3N, and A was α-D-GalpA. All 1H and 13C chemical shifts for lipid A sugar backbone components were assigned and are listed in Table 3. The anomeric configuration of monosaccharides was confirmed by measuring J(1H,1H) coupling constants. Relatively large values of coupling constants (above 170 Hz) for anomeric signals were found for residues A, B, D, and E, thus identifying their α-configuration. A smaller value of J(1H,1H) (≈164 Hz) was found for residue C, determining its β-configuration. The following connectivities between anomeric and linkage protons were identified on ROESY spectrum: A1/B1 (δ 5.270/5.078), C1/B6a,b (δ 54.407/3.802 and 4.407/3.662), D1/C4 (δ 4.910/3.653), and E1/D6 (δ 4.854/3.816). Taken together, the sugar backbone of B. japonicum lipid A possessed the structure: α-D-Manp(1→6)-α-D-Manp(1→4)-β-D-GlcN3N(1→6)-α-D-GlcN3N(1→1)-α-D-GalpA.

The fine structure of both hopanoid components of bradyrhizobial lipid A was identified. Carbon signals characteristic for the main hopanoid residue in lipid A are listed in Table 4. In the HSQC-DEPT spectrum (Fig. 5, blue and green), the hopanoids’ ring, fatty acid bulk, and terminal signals grouped in the crowded region δ 172.73, and revealed a distinct correlation with the (ω-1) proton of VLCFA (CH2-[(ω-1)-OR]-fragment, δH 4.980). Thus, the hopanoid moiety was a constitutive component of B. japonicum lipid A.

Position of the methyl group in 34-carboxyl-2-methyl-bacteriophepane-32,33-diol was confirmed based on HMBC, TOCSY, and ROESY correlations. A few changes were noticed in chemical shifts of carbons of rings A and B, compared with the non-methylated component. The carbon chemical shifts were as follows: δ 50.22 (C-1), 25.04 (C-2, methine group), 23.15 (2β-C8), 45.45 (C-3), 46.51 (C-4), 50.00 (C-5), 32.87 (C-6), 19.95 (C-7), 41.92 (C-8), 31.23 (C-23), 26.28 (C-24), and 22.30 (C-25). As the carbon atom from the methyl group at C-2 only

| Form of lipid A | Measured mass | Type of molecule | Composition | Calculated monoisotopic mass |
|----------------|---------------|-----------------|-------------|----------------------------|
| Lipid A O-deacetylated | 530.4312 | 1669.030 | H5o | 2 × GlcN3N |
| Lipid A native | 760.4712 | 1008.907 | Lipid A_\text{free-sol} | 1 × GlcN3N |
| | 1651.011 | 2087.390 | Lipid A_\text{free-sol} | 2 × GlcN3N |
| | 2583.951 | 3096.291 | Lipid A_\text{free-sol} | 1 × GlcN3N |
| | 3650.833 | 3650.8126 | Lipid A_\text{free-sol} | 1 × GlcN3N |

**Table 2**: Annotation of mass spectrometric information (main signals) obtained for the O-deacetylated and native lipid A samples from B. japonicum (see Fig. 2, A and B).
correlated with the proton at C-2 in the HMBC spectrum, this group should be in the β position (29). Also, the through-space connectivity of proton at C-2 with the protons from the methyl group confirmed its position as 2β. Moreover, protons from the methyl group showed correlation with protons of methyl groups at position C-24 and C-25 in the ROESY spectrum, but there was no correlation with protons at position C-23 (data not shown). Thus, evidence for β-configuration of this substituent was provided.

All chemical shifts of the α, β, and γ carbon and proton signals of the 3-hydroxy fatty acids (both, 3-O-acylated and those with free OH group) as well as for signals derived from ω, ω-1, ω-2, and ω-3 protons and carbons of substituted and unsubstituted VLCFAs, are summarized in Table 5. Chemical shift data were similar to those reported for B. elkanii lipid A (21). The $^1$H/$^13$C signals of the β-CH group of the unsubstituted 3-hydroxy fatty acid were identified at δ 3.82/68.88, respectively. Two signals derived from β-CH of 3-O-substituted fatty acids were found at δ 5.269/68.10 and 5.145/71.59. The proton/carbon chemical shifts at δ 4.987/73.21 and 4.887/72.07 were derived from the ω-1 methine groups of the VLCFAs, which are connected to the 3-OH group of the amide-linked fatty acids and bore hopanoid residue(s).

Olefinic chemical shifts were identified at δ 6.82/146.19 and 5.88/123.39. HMBC (Fig. 5, red) and ROESY spectra indicated that the double bond was located at the α-C-atom of the amide-linked fatty acid. The formation of such α-unsaturated fatty acids was probably a result of the cleavage of VLCFAs ester-bound to O-3 of an amide-linked 3-OH fatty acid during prolonged mild acid liberation of lipid A from LPS. Thus, these α-unsaturated amide-linked fatty acids were artifacts originating from chemical treatment of the LPS. In addition, a signal characteristic for unsaturated fatty acid possessing a double bond was also identified (δ 5.34/130.26). This signal is characteristic for unsaturated fatty acid possessing a double bond located in the so-called envelope of the acyl chain. Based on MS data it was concluded that presumably this fatty acid was not a component of lipid A and originated from traces of (phospho)lipids co-extracted with LPS. In summary, all data (GC-MS, NMR, and FT-ICR MS) obtained for B. japonicum lipid A identify its structure as depicted in Fig. 6.

**DISCUSSION**

Here we describe unusual hopanoid-containing lipid A samples isolated from LPS of various strains of *Bradyrhizobium*. These lipid A samples had a similar sugar backbone, but differed in the number of ester-linked VLCFAs forming acyloxyacyl moieties. The identified VLCFAs had different acyl chain lengths. Additionally, all studied lipid A preparations contained at least one residue of carboxybacteriohopanediol. The substituent was ester-linked to the hydroxyl group of VLCFA, as a tertiary residue. It had been described earlier that bacteria from the *Bradyrhizobium* genus are able to produce a set of triterpenoids of the hopane series, as well as gammacerane derivatives. Both classes of lipids are also characteristic for *R. palustris*, which is closely related based on 16S rRNA analysis and clusters together in the phylogenetic tree of the α-subgroup of proteobacteria (40). Disregard the fact that hopanoid lipids are widely distributed in the *Bacteria* domain, these components seem to be characteristic only of the slow-growing rhizobia. The other diazotrophic bacteria, which can fix nitrogen in
FIGURE 5. HSQC-DEPT (blue and green) and HMBC (red) spectra of native lipid A from *B. japonicum*. Lipid A sample was dissolved in CDCl₃/CD₃OD (2:1, v/v) with traces of D₂O. The signals were marked as follows: A, α-β-GalpA; B, α-β-GlcP3N; C, β-β-GlcP3N; D, α-β-Manp; E, α-β-Manp; Hop-32 and Hop-33, CH-OH groups at positions of 32 and 33, respectively, of hopanoid lipid side chain (see Fig. 4).

TABLE 3

| Spin system  | 1H Chemical Shifts (ppm) | 13C Chemical Shifts (ppm) |
|--------------|--------------------------|---------------------------|
| H-1/C-1      | 5.270                    | 177.6                     |
| H-2/C-2      | 68.30                    | 94.00                     |
| H-3/C-3      | 69.63                    | 4.077                     |
| H-4/C-4      | 70.96                    | 4.290                     |
| H-5/C-5      | 71.54                    | 3.395                     |
| H-6/C-6      | 169.3                    | 4.055                     |
| H-6'         | 3.802                    | 72.56                     |
| A (α-β-GalpA)| 176.4                    | 69.96                     |
| B (α-β-GlcP3N)| 92.25                    | 72.56                     |
| C (β-β-GlcP3N)| 163.9                    | 72.56                     |
| D (α-β-Manp) | 101.96                   | 72.56                     |
| E (α-β-Manp) | 172.2                    | 67.17                     |

α-β and β-β chemical shifts of atoms involved in glycosidic linkages are bold.
TABLE 4

| Carbon atom | \( \delta \) (ppm) |
|-------------|-----------------|
| 1          | -CH\(_3\)          | 34.06 |
| 2          | -CH\(_2\)         | 19.02 |
| 3          | -CH\(_2\)         | 42.40 |
| 4          | -C                | 33.50 |
| 5          | -CH\(_2\)         | 56.62 |
| 6          | -CH\(_3\)         | 19.01 |
| 7          | -CH\(_2\)         | 33.42 |
| 8          | -C                | 42.00 |
| 9          | -CH\(_3\)         | 50.00 |
| 10         | -C                | 37.78 |
| 11         | -CH\(_2\)         | 20.51 |
| 12         | -CH\(_2\)         | 24.28 |
| 13         | -CH\(_3\)         | 50.10 |
| 14         | -C                | 41.90 |
| 15         | -CH\(_2\)         | 33.98 |
| 16         | -CH\(_3\)         | 23.27 |
| 17         | -CH\(_3\)         | 55.06 |
| 18         | -C                | 44.73 |
| 19         | -CH\(_2\)         | 41.99 |
| 20         | -CH\(_2\)         | 28.06 |
| 21         | -CH\(_3\)         | 46.45 |
| 22         | -C                | 37.22 |
| 23         | -CH\(_3\)         | 33.61 |
| 24         | -CH\(_3\)         | 21.73 |
| 25         | -CH\(_3\)         | 16.16 |
| 26         | -CH\(_3\)         | 16.86 |
| 27         | -CH\(_3\)         | 16.70 |
| 28         | -CH\(_3\)         | 16.28 |
| 29         | -CH\(_3\)         | 20.58 |
| 30         | -CH\(_3\)         | 32.51 |
| 31         | -CH\(_2\)OH        | 28.52 |
| 32         | -CH\(_2\)OH        | 73.99 |
| 33         | -CH\(_3\)OH        | 74.94 |
| 34         | -COOR*             | 172.73 |

*\( R^* \) lipid A (\(-\( \alpha \)-1)-OH-group).
TABLE 5

| No. | Fatty acids | $^1$H  | $^{13}$C  |
|-----|-------------|-------|----------|
| 1.  | Olefinic protons/carbons |       |          |
|     | -CONH-      | 6.823 | 146.19   |
|     | -HC = CH-CONH- | 5.882 | 123.39   |
|     | -OH = CH-CONH- | 2.184 | 32.60    |
|     | -CH$_2$-HC = CH-CONH- | 1.445 | 28.69    |
| 2.  | Olefinic protons/carbons (separated one double bound) |       |          |
|     | -CH$_3$-HC = CH-CONH- | 5.348 | 130.26   |
|     | -CH$_2$-HC = CH-CONH- | 2.020 | 27.41    |
| 3.  | Ist –(3-OR$^*$)-FA$^*$ |       |          |
|     | $\alpha_1/\alpha_2$ | 2.628 | 41.04    |
|     | $\beta_1$ | 5.269 | 68.10    |
|     | $\gamma_1$ | ND$^b$ | ND       |
|     | $\text{CONH-Sug}$ | ND | 169.96 |
|     | R$_{\text{COO}}$- | 1.214 | 19.91    |
| 4.  | IInd – (3-OR$^*$)-FA$^*$ |       |          |
|     | $\alpha_1/\alpha_2$ | 2.413 | 41.03    |
|     | $\beta_1$ | 5.145 | 71.59    |
|     | $\gamma_1$ | 1.571 | 34.69    |
|     | $\text{CONH-Sug}$ | ND | 164.57  |
|     | R$_{\text{COO}}$- | 1.224 | 172.23   |
| 5.  | Ist – [(ω-1)-OR$^*$]$^c$ VLCFA |       |          |
|     | $\omega$ | 1.257 | 20.03    |
|     | $\omega$-1 | 4.980 | 73.21    |
|     | $\omega$-2 | 1.504 | 36.14    |
|     | $\omega$-3 | 1.338 | 25.85    |
|     | $\omega$-4 and next CH$_3$ groups | 1.450 | 28.91    |
|     | R$^a$(-$\text{COO}$-) from hopanoid | 172.82 |
| 6.  | IInd – [(ω-1)-OR$^{***}$]$^c$ VLCFA |       |          |
|     | $\omega$ | 1.213 | 20.03    |
|     | $\omega$-1 | 4.881 | 72.070   |
|     | $\omega$-2 | 1.487 | 36.340   |
|     | $\omega$-3 | 1.308 | 25.67    |
|     | R$^a$(-$\text{COO}$-) from 2nd hopanoid | 172.00 |
| 7.  | (3-OH) FA with unsubstituted OH group |       |          |
|     | $\alpha_1/\alpha_2$ | 2.310 | 43.81    |
|     | $\beta_1$ | 3.825 | 68.88    |
|     | $\gamma_1$ | ND | ND       |
| 8.  | [(ω-1)-OH] VLCFA with unsubstituted OH group |       |          |
|     | $\omega$ | ND | ND       |
|     | $\omega$-1 | 3.746 | 68.45    |
|     | $\omega$-2 | 1.386 | 39.33    |
|     | $\omega$-3 | 1.267 | 26.10    |
| 9.  | [(ω-1)-OH] VLCFA with unsubstituted OH group |       |          |
|     | $\omega$-1 | 3.575 | 67.61    |
|     | $\omega$-2 | 1.232 | 33.19    |
|     | $\omega$-3 | 1.267 | 26.10    |

$^a$ R$^a$ and R$^b$, VLCFA.

$^b$ ND, not determined.

$^c$ R$^a$ and R$^{***}$, hopanoid.

earlier in Mesorhizobium huakuii (20) and Mesorhizobium loti strains (43), which contained a non-reducing trisaccharide backbone comprising a $\beta$-(1→6)-d-GlcP3N3N disaccharide partly substituted with phosphate at C-4′ and an $\alpha$-(1→1)-linked d-GalpA in stoichiometric amounts. Other microorganisms that produce lipid A substituted with $\alpha$-(1→1)-d-GalpA include A. pyrophilus (48), the stalk-forming bacterium Caulobacter crescentus (51), and the plant-growth promoting bacterium Azospirillum lipoferum (52). In the last case, the lipid A backbone constituted a GlcP3N-disaccharide. In Caulobacter and Aquifex the $\beta$-(1→6)-linked d-GlcP3N3N disaccharide was substituted with d-GalpA at C-4′ and C-1.

The genes responsible for this unusual glycosylation of lipid A backbone have been identified recently. A gene cluster that encoded for d-GalA transferase (rgtF, ArnT-like glycosyl transferase), lipid A 1-phosphatase (lpxE), and glycosyl transferase (rgtE), in the relative order: rgtE, duf, rgtF, and lpxE (where duf is a domain with unknown function), was identified in M. loti MAFF303099 genome (43). Similar gene clusters were found also in other mesorhizobial strains (Mesorhizobium ciceri WSM1271 and Mesorhizobium opportunistum WSM2075), as well as in C. crescentus CB15 and A. lipoferum B510 (43). BLAST similarity searches of B. japonicum USDA 110 genome revealed the presence of a similar gene cluster, which contained putative rgtF, lpxE, and rgtE genes, encoding proteins with high sequence identity (37–55%) with respective proteins of M. loti (data not shown), and thus proving the presence of genes necessary for the synthesis of $\alpha$-(1→1)-d-GalpA substituted lipid A in this strain. Brown and co-workers showed that the R. leguminosarum rgtE mutant, which produces LPS completely...
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