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

Iwona Komaniecka[‡1]**, Adam Choma**[‡]**, Andrzej Mazur**[‡]**, Katarzyna A. Duda**[§]**, Buko Lindner**[¶]**, Dominik Schwudke**[¶]**, and Otto Holst**[§]

From the [‡]*Department of Genetics and Microbiology, Maria Curie-Sklodowska University, 20-033 Lublin and the Divisions of* [§]*Structural Biochemistry and* [¶]*Bioanalytical Chemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, 23845 Borstel, Poland*

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-dimensional 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-(GlcpN3N) 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) GlcpN3N, and one residue of galacturonic acid (D-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-carboxyl-bacteriohopane-32,33-diol and 34-carboxyl-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 com-posed of three domains: lipid A, a hydrophobic part that anchors the LPS molecule in the outer membrane and consti-tutes their 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 yuanmingense*, and *Bradyrhizobium* sp. (Lupi-nus). LPS composed only of lipid A and the core oligosaccha-ride is called rough. The semi-rough form additionally contain-ing one repeating unit of O-chain was found in *Bradyrhizobium elkanii* and *Bradyrhizobium liaoningense* strains (1). Bradyrhi-zobia are a slow-growing rhizobia forming a beneficial symbio-sis 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 archi-tecture and determine its permeability, important for the mor-phology and functionality of bacteroids. Several reports dem-onstrated 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 sup-pressing 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 substi-tuted 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 pres-ence 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-GlcpN) or a 2,3-diamino-2,3-dideoxy-glucosaminyl-(D-GlcpN3N) disaccharide. In some *Rhizobium* species, the reducing residue of glucosamine-containing lipid A may be oxidized to 2-aminogluconate (16). The sugar backbone of lipid A can be decorated by phosphate, uronic acid, or mannose residues. The amino groups of D-GlcpN3N and D-GlcpN as well as C-3 and C-3′ of D-GlcpN 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,[2] 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 *Rhizobiales*, except of *Azorhizobium caulinodans* (22–24). Moreover, in bradyrhizobial LPSs several VLCFAs were identified, among them straight, and mono, 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 diaminoglucoses 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.

Hopanoids are a class of pentacyclic triterpenoid lipids occurring in a wide range of Gram-negative and Gram-positive bacteria. Squalene and different species of hopanoid derivatives were discovered in *Streptomyces* spp., ethanol-tolerant bacterium *Zymomonas mobilis*, purple non-sulfur bacterium *Rhodopseudomonas palustris*, and *Rhodomicrobium vanniellii*, *Methylococcus capsulatus*, nitrogen-fixing *Azotobacter* and *Beijerinckia*, in symbiotic nitrogen-fixing *Frankia* and a wide group of *Bradyrhizobium* strains (26–30). Hopanoid lipids are thought to stabilize the phospholipid plasma membranes, sharing this function with eukaryotic sterols (31). In nitrogen-fixing

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 cell 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. yaunmingense*, 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 CHCl$_3$/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-

---

ane. 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 μg/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 recorded 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 CDCl$_3$/CD$_3$OD (2:1, v/v) with 5 μl of D$_2$O, 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, HSQCnd, HSQC-DEPT, and HMBC. The ^{1}H and ^{13}C resonances were measured relative to TMS (δ_H 0.0/δ_C 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 the water phase (for yields see "Experimental Procedures"). Lipid A was liberated from LPS by mild acid hydrolysis and subsequently subjected to sugar and fatty acid analyses. D-GlcpN3N was the only amino sugar component. A high amount of mannose (D-Manp) was also present in all lipid A preparations, whereas only small amounts of galacturonic acid (D-GalpA) were detected. Fatty acid analysis revealed the presence of two amide-linked 3-hydroxy fatty acids, *i.e.* 12:0(3-OH) and 14:0(3-OH). Ester-linked VLCFAs with chain lengths of 26–34 carbon atoms were identified as well (Table 1). The most abundant VLCFAs of strain *B. japonicum* were 26:0(25-OH),

TABLE 1

Fatty acid, hopanoids, and sugar components of lipid A isolated from LPS of *Bradyrhizobium* strains

The symbols represent: +, present; ++, the main fatty acid/sugar component; −, lack of component; tr, traces.

Component	*B. japonicum* USDA 110	*B. yuanmingense* CCBAU 10071	*Bradyrhizobium* sp. (Lupinus) USDA 3045
Fatty acids			
12:0(3-OH)	++	++	++
14:0(3-OH)	++	++	++
26:0(25-OH)	++	+	+
27:0(26-OH)[a]	−	tr.	tr.
28:0(27-OH)	+	+	+
29:0(28-OH)[a]	+	−	+
30:0(29-OH)	+	+	+
31:0(30-OH)[a]	++	++	++
32:0(31-OH)[b]	++	−	+
33:0(32-OH)[a]	++	+	++
34:0(33-OH)	−	+	−
Hopanoids[c]			
I, R = CH$_3$	+	+	+
II, R = H	++	++	++
Sugars			
D-Manp	++	++	++
D-GalpA	+	+	+
D-GlcpN3N	+	+	+

[a] Branched fatty acid (ω-10) (see Ref. 25).
[b] Part of fatty acid is present in the dimethyl-branched forms ((ω-10) and (ω-11)) (see Ref. 25).
[c] For hopanoid structure, see Fig. 1.

32:0(31-OH), and 33:0(32-OH). Lipid A from *B. yuanmingense* and *Bradyrhizobium* sp. (Lupinus) contained mainly 31:0(30-OH).

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 *m/z* 234 visible on both mass spectra derived from the cleavage between C-33 and C-32 and corresponded to the fragment bearing a carboxyl group. This ion resulted from a rearrangement followed by transferring the TMS group from position C-32 to the carboxyl group. The mass spectrum of the TMS derivative of 34-carboxyl-bacteriohopane-32,33-diol methyl ester (t_R = 50.645 min, Fig. 1*A*) showed a pseudomolecular ion at *m/z* 673, [M-15]$^+$, and a prominent ion at *m/z* 191, which were derived from fragmentation of the third (C) ring of the molecule. The mass spectrum of the TMS derivative of 34-carboxyl-2-methyl-bacteriohopane-32,33-diol methyl ester (t_R = 50.084 min, Fig. 1*B*) possessed [M-15]$^+$ at *m/z* 687 and a C-ring fragmentation that yielded an ion at *m/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).
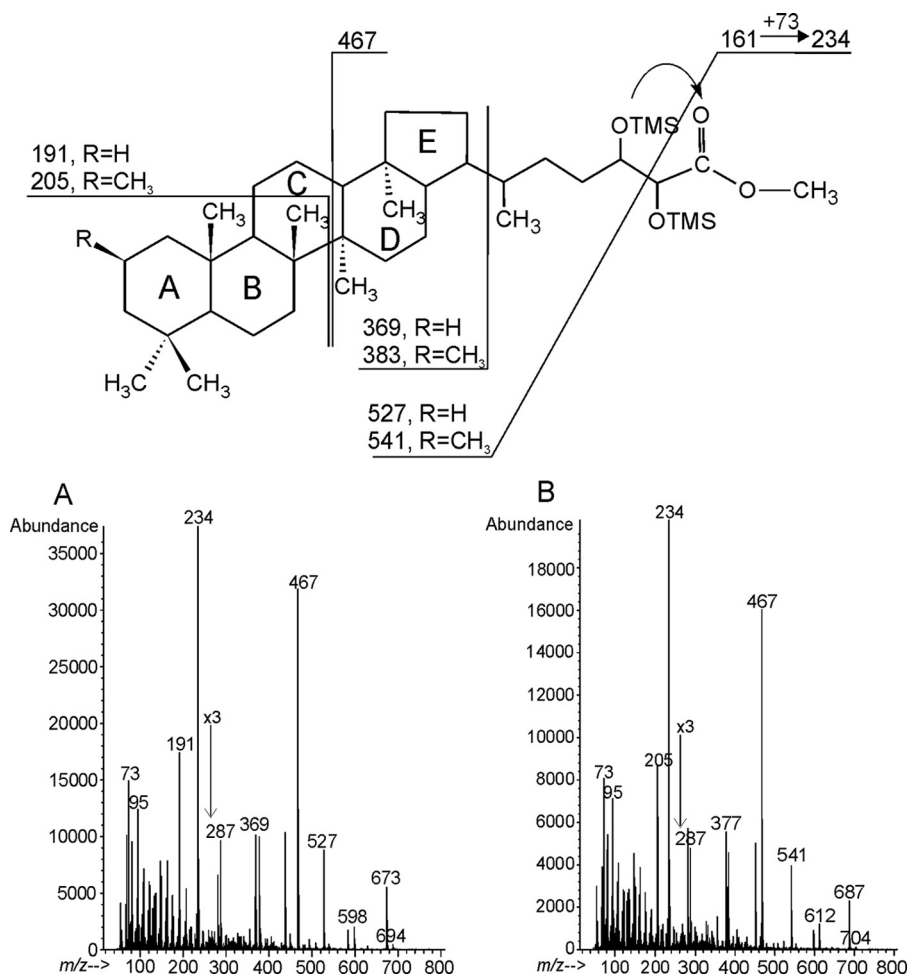
FIGURE 1. **Mass spectra (GC-MS) of hopanoid components of *B. japonicum* lipid A (acid hydrolyzed, carboxymethylated, and trimethylsilylated preparation).** *A,* TMS derivative of 34-carboxyl-bacteriohopane-32,33-diol methyl ester; *B,* TMS derivative of 34-carboxyl-2β-methyl-bacteriohopane-32,33-diol methyl ester.

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. 2*A* and Table 2). Monoisotopic masses 2087.390, 2105.422, and 2115.460 Da were assigned to lipid A species containing two Man*p*, two Glc*p*N3N, one Gal*p*A, 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 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 (Δm = 512.418 Da) via its hydroxyl group. Such lipid A molecules possess a calculated monoisotopic mass of 3096.343 and 3110.358 Da. Mass differ-

ences 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. 2*B*). 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 Glc*p*N3N, two Man*p*, one Gal*p*A, and four amide-linked fatty acid residues
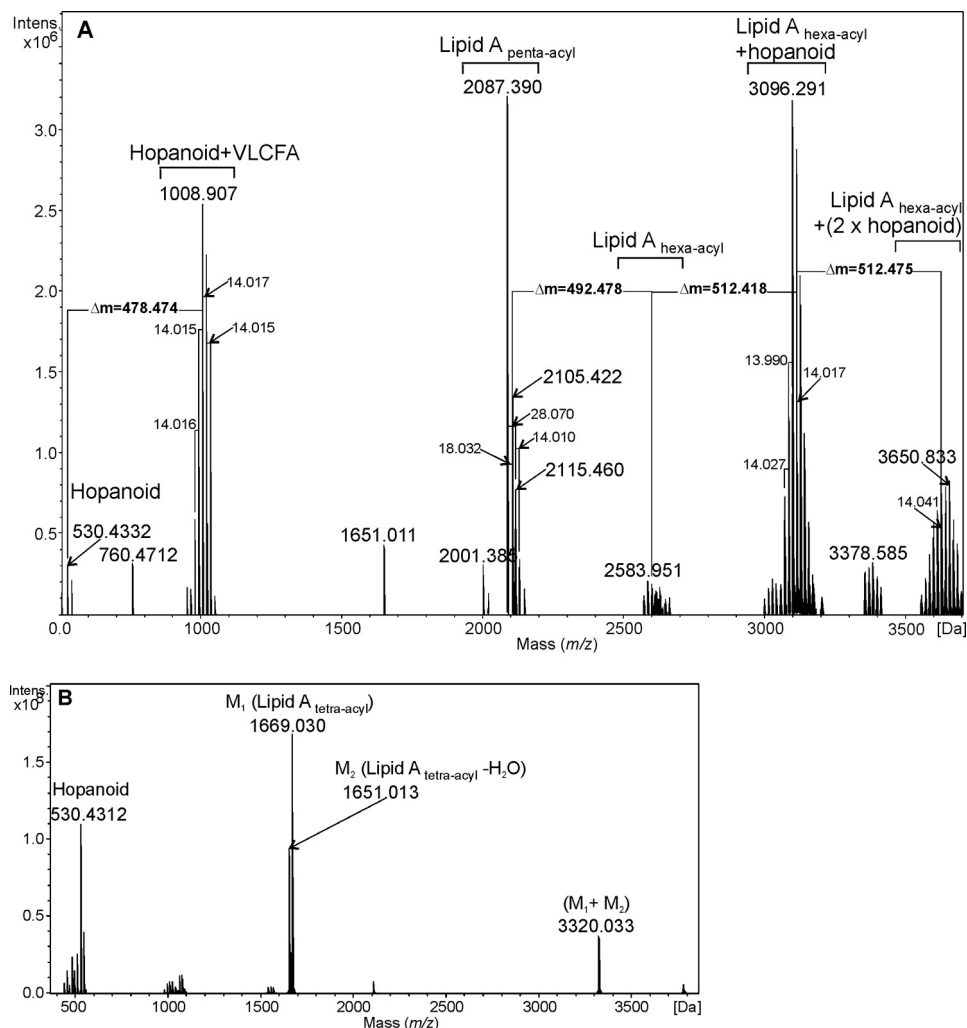
FIGURE 2. **Charge-deconvoluted ESI FT-ICR mass spectrum of the native (A) and O-deacylated (B) lipid A isolated from B. japonicum.**

(two 12:0(3-OH) and two 14:0(3-OH)). One 3-OH fatty acid was deprived of H_2O 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 ($\Delta m = 18$ Da). The group of peaks at 3320.033 Da was consistent with the ion-cluster of both forms of 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 GlcpN3N units, two Manp, one GalpA, and four amide-linked fatty acids (two 12:0(3-OH) and two 14:0(3-OH)) $-H_2O$). 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 $[M-H_2O + H]^+$ 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 $[M-H_2O + H]^+$ = 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 $[M-H_2O - H]^-$ = 2582.91) and a hexa-acylated lipid A moiety additionally bearing one hopanoid residue (calculated m/z $[M-H_2O - H]^-$ = 3095.33).

NMR Spectroscopy of B. japonicum Lipid A—The native lipid A preparation isolated from *B. japonicum* was dissolved in $CDCl_3/CD_3OD$ (2:1, v/v) with traces of D_2O 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-

TABLE 2

Annotation of mass spectrometric information (main signals) obtained for the *O*-deacylated and native lipid A samples from *B. japonicum* (see Fig. 2, *A* and *B*)

Form of lipid A	Measured mass	Type of molecule	Composition	Calculated monoisotopic mass
	Da			*Da*
Lipid A *O*-deacylated	530.4312	Hopanoid		530.4335
	1669.030	Lipid A$_{\text{tetra-acyl}}$	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) -H$_2$O	1669.0174
Lipid A native	760.4712	[Y]-ion type	$1 \times$ Glc$p$N3N $1 \times$ Gal$p$A $1 \times$ 14:0 (3-OH), $1 \times$ 12:0 (3-OH)	760.4726
	1008.907	Hopanoid + VLCFA	Hopanoid 530.4335 u $1 \times$ 32:0 (31-OH)	1008.9084
	1651.011	Lipid A$_{\text{tetra-acyl}}$ -H$_2$O	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) $-2 \times$ H$_2$O	1651.0068
	2087.390	Lipid A$_{\text{penta-acyl}}$ -H$_2$O	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) $1 \times$ 29:0 (28-OH) $- 2 \times$ H$_2$O	2087.4353
	2583.951	Lipid A$_{\text{hexa-acyl}}$	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) $1 \times$ 29:0 (28-OH), $1 \times$ 32:0 (31-OH) -H$_2$O	2583.920
	3096.291	Lipid A$_{\text{hexa-acyl}}$ **+ hopanoid**	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) $1 \times$ 29:0 (28-OH), $1 \times$ 32:0 (31-OH) **1 $\times$ hopanoid 530.4335** -H$_2$O	3096.3428
	3650.833	Lipid A$_{\text{hexa-acyl}}$ **+ (2 $\times$ hopanoid)**	$2 \times$ Glc$p$N3N $2 \times$ Man$p$ $1 \times$ Gal$p$A $2 \times$ 14:0 (3-OH), $2 \times$ 12:0 (3-OH) $1 \times$ 31:0 (30-OH), $1 \times$ 32:0 (31-OH) **1 $\times$ hopanoid 530.4335** **1 $\times$ hopanoid 544.4491** -H$_2$O	3650.8126

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 ^{1}H/^{1}H COSY, TOCSY, and ^{1}H/^{13}C HMBC experiments five spin systems characterizing sugar pyranoses were identified. Two of them (E and D) were derived from α-D-Man$p$, C represented β-D-Glc$p$N3N, B represents α-D-Glc$p$N3N, and A was α-D-Gal$p$A. All ^{1}H and ^{13}C 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 $^1J_{(C1,H1)}$ 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 $^1J_{(C1,H1)}$ (~164 Hz) was found for residue C, determining its β-configuration. The following connectivities between anomeric and linkage protons were identified on ROESY spectrum: **A**1/**B**1 (δ 5.270/5.078), **C**1/**B**6a,b (δ 54.407/3.802 and 4.407/3.662), **D**1/**C**4 (δ 4.910/3.653), and **E**1/**D**6 (δ 4.854/3.816). Taken together, the sugar backbone of *B. japonicum* lipid A possessed the structure: α-D-Man$p$-(1→6)-α-D-Man$p$-(1→4)-β-D-Glc$p$N3N-(1→6)-α-D-Glc$p$N3N-(1→1)-α-D-Gal$p$A.

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_H 0.7–2.8 and δ_C 16–57 ppm. Signals for *CH*-OH groups from positions 32 and 33 of the hopanoid side chains were located in the glycosidic region, at δ 3.800/73.99 and δ 4.200/74.94, respectively. The signal of the carboxyl group of the hopanoid was assigned at δ_C 172.73, and revealed a distinct correlation with the (ω-1) proton of VLCFA (*CH*-[(ω-1)-OR]-fragment, δ_H 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-bacteriohopane-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-CH$_3$**), 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
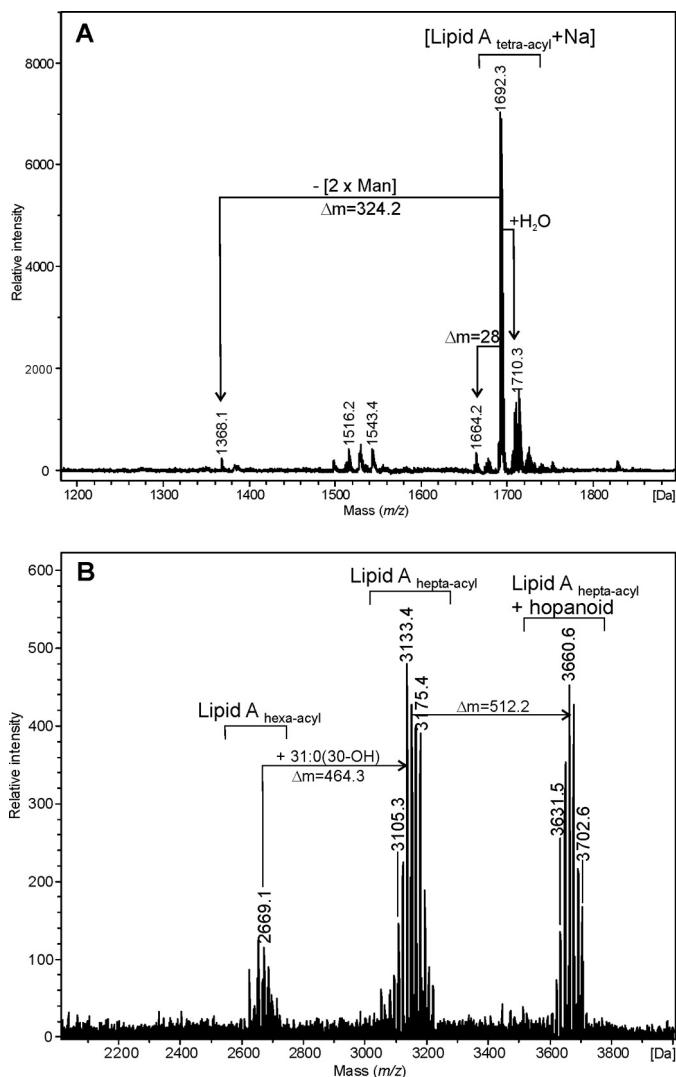
FIGURE 3. **MALDI-TOF mass spectrum (positive-ion mode) of the *O*-deacylated (*A*) and native (*B*) lipid A from *B. yuanmingense*.**
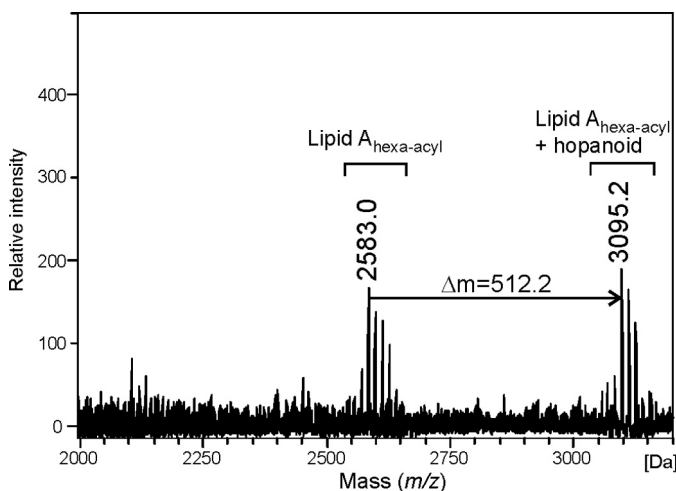

FIGURE 4. **MALDI-TOF mass spectrum (negative-ion mode) of the native lipid A sample from *Bradyrhizobium sp.* (Lupinus).**

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 VLCFA, 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 4.98/73.21 and 4.88/72.07 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 esterbound 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 olefinic proton/carbon belonging to another 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 16 S 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
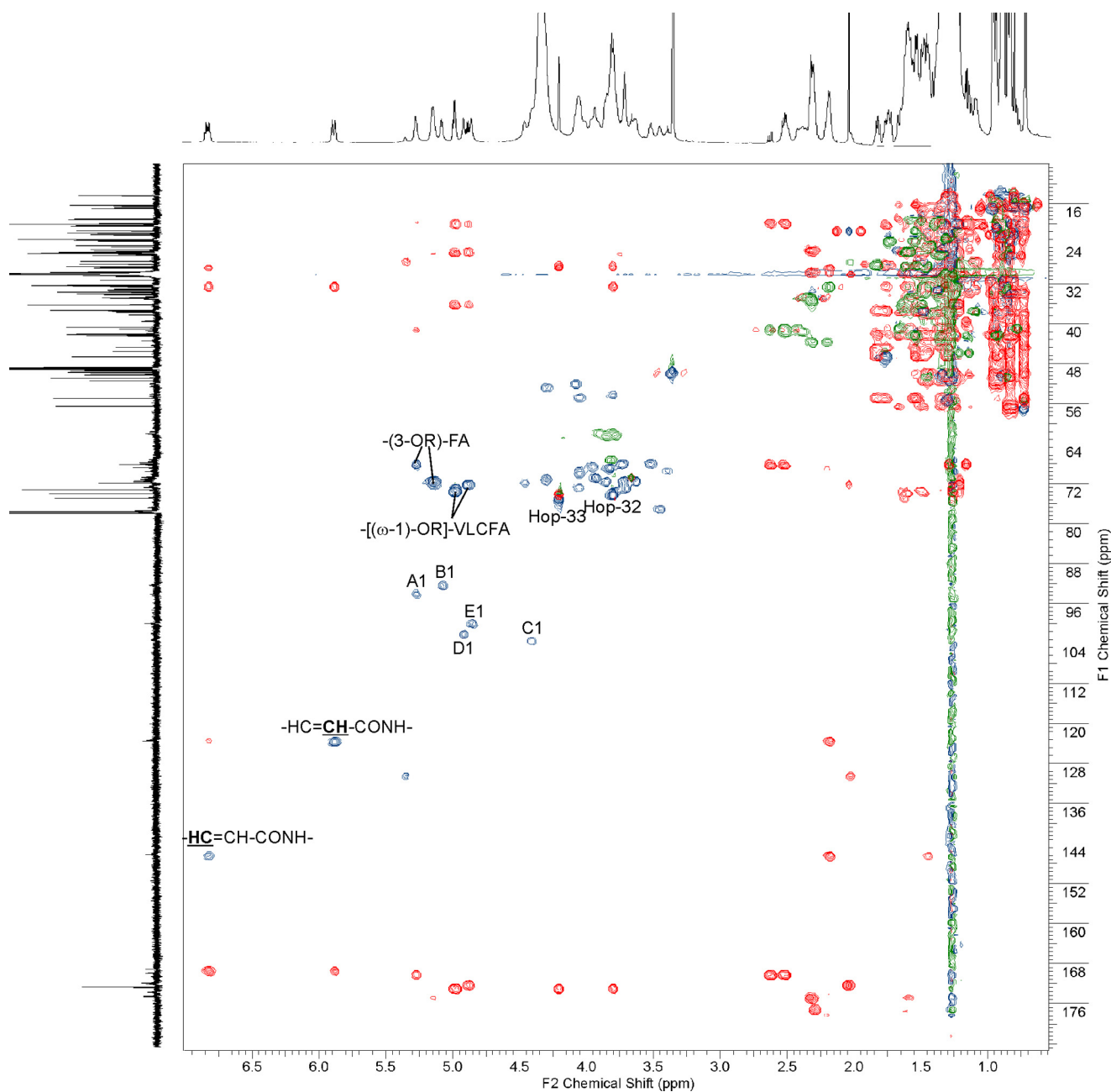
FIGURE 5. **HSQC-DEPT (*blue* and *green*) and HMBC (*red*) spectra of native lipid A from *B. japonicum*.** Lipid A sample was dissolved in CDCl₃/CD₃OD (2:1, v/v) with traces of D₂O. The signals were marked as follows: *A*, α-D-Gal*p*A; *B*, α-D-Glc*p*N3N; *C*, β-D-Glc*p*N3N; *D*, α-D-Man*p*; *E*, α-D-Man*p*; 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

¹H and ¹³C NMR signals (δ; ppm) from *B. japonicum* lipid A backbone (in CDCl₃/CD₃OD (2:1) with traces of D₂O)

¹³C chemical shifts of atoms involved in glycosidic linkages are bold.

Spin system	¹J(C-1,H-1)	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-6′
	Hz							
A (α-D-Gal*p*A)	177.6	5.270	3.966	4.050	4.291	4.459		
		94.00	68.30	69.63	70.96	71.54	169.3	
B (α-D-Glc*p*N3N)	176.4	5.078	4.077	4.290	3.395	4.055	3.802	3.662
		92.25	52.02	52.54	69.22	72.56	**69.96**	
C (β-D-Glc*p*N3N)	163.9	4.407	3.805	4.046	3.653	3.447	3.788	3.900
		103.14	54.09	54.49	**75.86**	76.76	61.47	
D (α-D-Man*p*)	172.0	4.910	3.707	3.628	3.515	3.712	3.816	3.816
		101.96	71.17	71.27	67.66	72.83	**67.17**	
E (α-D-Man*p*)	172.2	4.854	3.934	3.858	3.715	3.779	3.861	3.786
		99.94	70.68	71.54	67.62	73.19	61.98	

TABLE 4
^{13}C NMR chemical shifts of the hopanoid residue (34-carboxyl-bacteriohopane-32,33-diol) of *B. japonicum* lipid A

Carbon atom		δ
		ppm
1	-CH$_2$	40.66
2	-CH$_2$	19.02
3	-CH$_2$	42.40
4	-C	33.50
5	-CH	56.62
6	-CH$_2$	19.01
7	-CH$_2$	33.42
8	-C	42.00
9	-CH	50.00
10	-C	37.78
11	-CH$_2$	20.51
12	-CH$_2$	24.28
13	-CH	50.10
14	-C	41.90
15	-CH$_2$	33.98
16	-CH$_2$	23.27
17	-CH	55.06
18	-C	44.73
19	-CH$_2$	41.99
20	-CH$_2$	28.06
21	-CH	46.45
22	-CH	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$_2$	32.51
31	-CH$_2$	28.52
32	-CH-OH	73.99
33	-CH-OH	74.94
34	-COOR*[a]	172.73

[a] R*, lipid A (-(ω-1)-OH-group).

endosymbiotic conditions, *e.g. Rhizobium*, do not contain detectable amounts of triterpenoid lipids (28, 41). Diploptene, diplopterol, 2β-methyldiplopterol, aminobacteriohopanetriol, and adenosylhopane, accompanied by tetrahymanol and their methylated homologues: 2β-methyltetrahymanol, 20α-methyltetrahymanol, and 2β,20α-dimethyltetrahymanol, were identified in cells of *B. japonicum* USDA 110 (28, 29). The hopanoid content in bradyrhizobial cells is usually high, and in some strains accounts for more than 40% of the total lipid fraction (28). Apart of their presence in bradyrhizobial cells, the 2-methylbacteriohopanepolyols are frequently found in cyanobacteria and are even proposed to be a biomarker of these prokaryotes (41, 42).

Very recently, the first hopanoid-containing lipid A, obtained from LPS of the photosynthetic *Bradyrhizobium* strain BTAi1, was structurally and functionally characterized (32). In this lipid A, one of the two ester-linked fatty acids was further substituted by one hopanoid residue.

In our work we identified two hopanoid lipids, *i.e.* 34-carboxyl-bacteriohopane-32,33-diol and its methylated derivative 34-carboxyl-2β-methyl-bacteriohopane-32,33-diol. They were present in LPS preparations obtained from *B. japonicum*, *B. yuanmingense*, and *Bradyrhizobium sp.* (Lupinus) strains. MS and NMR data proved that these hopanoid residues were covalently linked to the lipid A, forming a tertiary acyl substituent. The hydrophobic character of hopanoid rings together with the presence of two hydroxyl groups in the side chain, determined their particular position in the membrane. The VLCFAs, which

were found to be components of all bradyrhizobial lipid A samples, contained between 26 and 34 carbon atoms, and were present in mono- and dimethyl branched and straight forms. These fatty acids were parts of two or, in case of *B. yuanmingense* lipid A, even three acyloxyacyl residues and may span the entire outer membrane. When one or two are additionally substituted by a hopanoid residue, their hydroxy groups could be placed at the boundary of the double leaflet, whereas the hydrophobic rings could be hidden inside the membrane. Thus, the presence of such unusual lipid A substituents is thought to have a strong influence on the membrane properties and act as a stabilizer of the membrane bilayer. However, additional studies are needed to establish the biophysical properties of such macromolecules and enlighten their possible function in the bacterial outer membrane. In case of lipid A from the photosynthetic *Bradyrhizobium* strain it was proven, by biophysical analysis of reconstituted asymmetric liposomes, that the architecture of this unusual lipid A was optimally suited to induce a high ordering of the outer membrane, reinforcing its stability and rigidity (32). Furthermore, hopanoid lipids of nitrogen-fixing bacteria (*Frankia*) are proposed to form a kind of diffusion barrier to protect the oxygen-sensitive nitrogrenase-hydrogenase complex from oxidative damage (27). This may also hold true for *Bradyrhizobium*, which, in contrast to *Rhizobium*, are able to fix nitrogen also in the free-living state (non-symbiotically). Our studies proved that the lipid A backbone of LPS from all examined strains were composed of a D-GlcpN3N-disaccharide, substituted at position C-4′ by an α-D-Manp-(1→6)-α-D-Manp disaccharide, whereas the position C-1 was occupied by α-(1→1)-linked D-GalpA.

The presence of D-GlcpN3N in the lipid A backbone of the LPS of nitrogen-fixing bacteria is rather common. This amino sugar was reported for lipid A of the LPS from *Mesorhizobium loti* (18, 43), *M. huakuii* (20), *A. caulinodans* (24), and other symbiotic bacteria belonging to the genera *Ochrobactrum* and *Phyllobacterium*.[3] D-GlcpN3N was also found in lipid A derived from other, non-rhizobial bacteria, *e.g. Rhodopseudomonas* (where the presence of this amino sugar was described for the first time) (44), *Thiobacillus* sp. (45), pathogenic *Brucella abortus* (46), and *Campylobacter jejuni* (47), and also in the hyperthermophilic bacterium *Aquifex pyrophilus* (48).

Mannose-containing lipid A samples were identified earlier in the predatory bacterium *Bdellovibrio bacteriovorus*, where mannose residues occupied positions C-1 and C-4′ of the D-GlcpN3N-disaccharide (49), and in phototrophic bacterium *Rhodomicrobium vannielli* (50), in which the C-4′ of the glucosaminyl disaccharide backbone was occupied by one mannose residue. Recently, we reported the presence of a neutral mannose-containing lipid A in LPS of *B. elkanii* USDA 76 (21). In this bacterium it was demonstrated that two mannose residues forming a disaccharide were linked to C-4′ and one residue to C-1 of the D-GlcpN3N-disaccharide.

In *B. japonicum* USDA 110 position C-1 of the lipid A backbone was substituted by an α-(1→1)-linked D-GalpA. This unique substitution of the lipid A backbone had been noticed

---

[3] A. Choma, personal communication.

TABLE 5
^{1}H and ^{13}C NMR chemical shifts of fatty acids from *B. japonicum* lipid A

No.	Fatty acids signals	^{1}H	^{13}C
		ppm	
1.	Olefinic protons/carbons		
	-**C**ONH-		169.28
	-**HC** = CH-CONH-	6.823	146.19
	-HC = **CH**-CONH-	5.882	123.39
	-CH$_2$-**CH$_2$**-HC = CH-CONH-	2.184	32.60
	-**CH$_2$**-CH$_2$-HC = CH-CONH-	1.445	28.69
2.	Olefinic protons/carbons (separated one double bound)		
	-CH$_2$-**HC = CH**-	5.348	130.26
	-**CH$_2$**-HC = CH-	2.020	27.41
3.	Ist –(3-OR′)-FA[a]		
	α_1/α_2	2.628/2.532	41.04
	β	5.269	68.10
	γ	ND[b]	ND
	CONH-Sug		169.96
	R-**C**OO-		164.57
	ω	1.214	19.91
4.	IInd – (3-OR″-FA[a]		
	α_1/α_2	2.413/2.525	41.03
	β	5.145	71.59
	γ	1.571	34.69
	-**C**ONH-Sug		172.23
	R-**C**OO-		174.66
5.	Ist – [(ω-1)-OR*][c] VLCFA		
	ω	1.257	20.03
	ω-1	4.980	73.21
	ω-2	1.504; 1.623	36.14
	ω-3	1.338	25.85
	ω-4 and next CH$_2$ groups	1.450	28.91
	R*(-**C**OO-) from hopanoid		172.82
6.	IInd – [(ω-1)-OR**][c] VLCFA		
	ω	1.213	20.03
	ω-1	4.881	72.070
	ω-2	1.487; 1.588	36.340
	ω-3	1.308	25.67
	R**(-**C**OO-) from 2nd hopanoid		172.00
7.	(3-OH) FA with unsubstituted OH group		
	α_1/α_2	2.310/2.210	43.81
	β	3.825	68.88
	γ	ND	ND
8.	[(ω-1)-OH] VLCFA with unsubstituted OH group		
	ω	ND	ND
	ω-1	3.746	68.45
	ω-2	1.386; 1.473	39.33
	ω-3	1.267	26.10
9.	[(ω-1)-OH] VLCFA with unsubstituted OH group		
	ω-1	3.575	67.61
	ω-2	1.323; 1.435	33.19
	ω-3	1.267	26.10

[a] R′ and R″, VLCFA.
[b] ND, not determined.
[c] R* and R**, hopanoid.

earlier in *Mesorhizobium huakuii* (20) and *Mesorhizobium loti* strains (43), which contained a non-reducing trisaccharide backbone comprising a β-(1→6)-D-Glc$p$N3N disaccharide partly substituted with phosphate at C-4′ and an α-(1→1)-linked D-Gal$p$A in stoichiometric amounts. Other microorganisms that produce lipid A substituted with α-(1→1)-D-Gal$p$A 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 Glc$p$N-disaccharide. In *Caulobacter* and *Aquifex* the β-(1→6)-linked D-Glc$p$N3N disaccharide was substituted with D-Gal$p$A 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 trans-

ferase), 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 α-(1→1)-D-Gal$p$A substituted lipid A in this strain. Brown and co-workers showed that the *R. leguminosarum rgtE⁻* mutant, which produces LPS completely
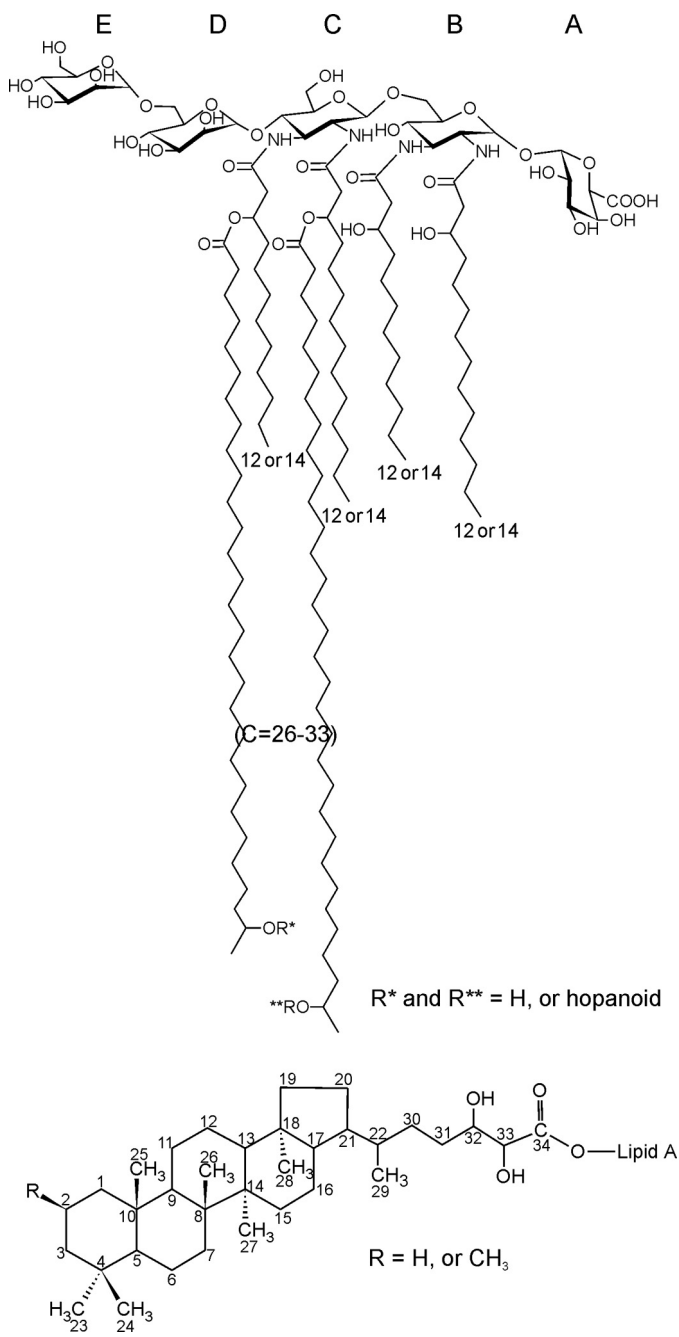
FIGURE 6. **Proposed structure of *B. japonicum* lipid A.**

devoid of D-Gal*p*A, was severely compromised in membrane stability (43). Thus, we assume that the synthesis of D-Gal*p*A-containing lipid A by *Bradyrhizobium* bacteria, apart from the presence of the hopanoid substituent, may cause an additional stabilization of the outer membrane, most probably by the presence of divalent cationic bridging, which is similar to mammalian pathogens that produce phosphate-containing LPS (lipid A) (10, 43).

REFERENCES

1. Komaniecka, I., Zdzisinska, B., Kandefer-Szerszen, M., and Choma, A. (2010) Endotoxic activity of lipopolysaccharides isolated from *Bradyrhizobium*, *Mesorhizobium* and *Azospirillum* strains. *Microbiol. Immunol.* **54,** 717–725
2. de Maagd, R. A., Rao, A. S., Mulders, I. H., Goosen-de Roo, L., van Loosdrecht, M. C., Wijffelman, C. A., and Lugtenberg, B. J. (1989) Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with altered lipopolysaccharides: possible role of surface charge or hydrophobicity in bacterial release from the infection thread. *J. Bacteriol.* **171,** 1143–1150
3. Brewin, N. J., Perotto, S., Kannenberg, E. L., Rae, A. L., Rathbun, E. A., Lucas, M. M., Kardailsky, I., Gunder, A., Bolanos, L., Donovan, N., Drobak, B. K., Nester, E. W., and Verma, D. P. (1993) Mechanisms of cell and tissue invasion by *Rhizobium leguminosarum*: the role of cell surface interactions. in *Current Plant Science and Biotechnology in Agriculture, Advances in Molecular Genetics of Plant-Microbe Interactions* (Nestery, E. W., and Verma, D. P. S., eds) Vol. 2, pp. 369–380, Kluwer Academic Publishers, Dordrecht/Boston/London
4. Fraysse, N., Jabbouri, S., Treilhou, M., Couderc, F., and Poinsot, V. (2002) Symbiotic conditions induce structural modifications of *Sinorhizobium* sp. NGR234 surface polysaccharides. *Glycobiology* **12,** 741–748
5. Carlson, R. W., Forsberg, L. S., and Kannenberg, E. L. (2010) Lipopolysaccharides in *Rhizobium*-legume symbioses. in *Endotoxins: structure, function and recognition* (Wang, X., and Quinn, P. J., eds) pp. 339–386, Springer, The Netherlands
6. Dow, M., Newman, M. A., and von Roepenack, E. (2000) The induction and modulation of plant defense responses by bacterial lipopolysaccharides. *Annu. Rev. Phytopathol.* **38,** 241–261
7. Albus, U., Baier, R., Holst, O., Pühler, A., and Niehaus, K. (2001) Suppression of an elicitor-induced oxidative burst in *Medicago sativa* cell cultures by *Sinorhizobium meliloti* lipopolysaccharides. *New Phytologist* **151,** 597–606
8. Mathis, R., Van Gijsegem, F., De Rycke, R., D'Haeze, W., Van Maelsaeke, E., Anthonio, E., Van Montagu, M., Holsters, M., and Vereecke, D. (2005) Lipopolysaccharides as a communication signal for progression of legume endosymbiosis. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 2655–2660
9. Zähringer, U., Lindner, B., and Rietschel, E. Th. (1999) Chemical structure of lipid A: recent advances in structural analysis of biologically active molecules. in *Endotoxin in Health and Disease* (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds) pp. 93–114, Marcel Dekker, New York
10. Raetz, C. R., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71,** 635–700
11. Park, B. S., Song, D. H., Kim, H. M., Choi, B.-S., Lee, H., and Lee, J.-O. (2009) The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex. *Nature* **458,** 1191–1195
12. Beutler, B., and Rietschel, E. T. (2003) Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **3,** 169–176
13. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282,** 2085–2088
14. Urbanik-Sypniewska, T., Choma, A., Kutkowska, J., Kamińska, T., Kandefer-Szerszeń, M., Russa, R., and Dolecka, J. (2000) Cytokine inducing activities of rhizobial and mesorhizobial lipopolysaccharides of different lethal toxicity. *Immunobiology* **202,** 408–420
15. Tsukushi, Y., Kido, N., Saeki, K., Sugiyama, T., Koide, N., Mori, I., Yoshida, T., and Yokochi, T. (2004) Characteristic biological activities of lipopolysaccharides from *Sinorhizobium* and *Mesorhizobium*. *J. Endotoxin Res.* **10,** 25–31
16. Bhat, U. R., Forsberg, L. S., and Carlson, R. W. (1994) Structure of lipid A component of *Rhizobium leguminosarum* bv. *phaseoli* lipopolysaccharide. *J. Biol. Chem.* **269,** 14402–14410
17. Gil-Serrano, A. M., González-Jiménez, I., Tejero-Mateo, P., Megías, M., and Romero-Vazquez, M. J. (1994) Analysis of the lipid moiety of lipopolysaccharide from *Rhizobium tropici* CIAT899: identification of 29-hy-

droxytriacontanoic acid. *J. Bacteriol.* **176,** 2454–2457

18. Russa, R., Urbanik-Sypniewska, T., Lindström, K., and Mayer, H. (1995) Chemical characterization of two lipopolysaccharide species isolated from *Rhizobium loti* NZP2213. *Arch. Microbiol.* **163,** 345–351

19. Que, N. L., Lin, S., Cotter, R. J., and Raetz, C. R. (2000) Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli. J. Biol. Chem.* **275,** 28006–28016

20. Choma, A., and Sowinski, P. (2004) Characterization of *Mesorhizobium huakuii* lipid A containing both D-galacturonic acid and phosphate residues. *Eur. J. Biochem.* **271,** 1310–1322

21. Komaniecka, I., Choma, A., Lindner, B., and Holst, O. (2010) The structure of a novel neutral lipid A from the lipopolysaccharide of *Bradyrhizobium elkanii* containing three mannoses units in the backbone. *Chem.-Eur. J.* **16,** 2922–2929

22. Bhat, U. R., Carlson, R.W., Busch, M., and Mayer, H. (1991) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipolysaccharides from bacteria belonging to the α-2 subgroup of *Proteobacteria. Int. J. Syst. Bacteriol.* **41,** 213–217

23. Bhat, U. R., Mayer, H., Yokota, A., Hollingsworth, R. I., and Carlson, R. W. (1991) Occurrence of lipid A variants with 27-hydroxyoctacosanoic acid in lipopolysaccharides from members of the family *Rhizobiaceae. J. Bacteriol.* **173,** 2155–2159

24. Choma, A., Komaniecka, I., Turska-Szewczuk, A., Danikiewicz, W., and Spolnik, G. (2012) Structure of lipid A from a stem-nodulating bacterium *Azorhizobium caulinodans. Carbohydr. Res.* **352,** 126–136

25. Choma, A., and Komaniecka, I. (2011) Straight and branched ω-1 hydroxylated very long chain fatty acids are components of *Bradyrhizobium* lipid A. *Acta Biochim. Pol.* **58,** 51–58

26. Neunlist, S., Holst, O., and Rohmer, M. (1985) Prokaryotic triterpenoids. The hopanoids of the purple non-sulphur bacterium *Rhodomicrobium vannielii*: an aminotriol and its aminoacyl derivatives, *N*-tryptophanyl and *N*-ornithinyl aminotriol. *Eur. J. Biochem.* **15,** 561–568

27. Berry, A. M., Harriott, O. T., Moreau, R.A., Osman, S. F., Benson, D. R., and Jones, A. D. (1993) Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6091–6094

28. Kannenberg, E. L., Perzl, M., and Härtner, T. (1995) The occurrence of hopanoid lipids in *Bradyrhizobium* bacteria. *FEMS Microbiol. Lett.* **127,** 255–262

29. Bravo, J.-M., Perzl, M., Härtner, T., Kannenberg, E. L., and Rohmer, M. (2001) Novel methylated triterpenoids of the gammacerane series from the nitrogen-fixing bacterium *Bradyrhizobium japonicum* USDA 110. *Eur. J. Biochem.* **268,** 1323–1331

30. Welander, P. V., Hunter, R. C., Zhang, L., Sessions, A. L., Summons, R. E., and Newman, D. K. (2009) Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1. *J. Bacteriol.* **191,** 6145–6156

31. Rohmer, M., Bouvier, P., and Ourisson, G. (1979) Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 847–851

32. Silipo, A., Vitiello, G., Gully, D., Sturiale, L., Chaintreuil, C., Fardoux, J., Gargani, D., Lee, H. I., Kulkarni, G., Busset, N., Marchetti, R., Palmigiano, A., Moll, H., Engel, R., Lanzetta, R., Paduano, L., Parrilli, M., Chang, W. S., Holst, O., Newman, D. K., Garozzo, D., D'Errico, G., Giraud, E., and Molinaro, A. (2014) Covalently linked hopanoid-lipid A improves outer-membrane resistance of a *Bradyrhizobium* symbiont of legumes. *Nat. Commun.* **5,** 5106, 10.1038/ncomms6106

33. Vincent, M. (1970) A manual for the practical study of root-nodule bacteria. *International Biological Programme, Handbook No. 15.* Blackwell, Oxford

34. Reitz, M., Rudolph, K., Schröder, I., Hoffmann-Hergarten, S., Hallmann, J., and Sikora, R. A. (2000) Lipopolysaccharides of *Rhizobium etli* strain G12 act in potato roots as an inducing agent of systemic resistance to infection

by the cyst nematode *Globodera pallida. Appl. Environ. Microbiol.* **66,** 3515–3518

35. Westphal, O., and Jann, K. (1965) Bacterial lipopolysaccharide: extraction with phenol-water and further application of the procedure. in *Methods in Carbohydrate Chemistry* (Whistler, R. L., ed) Vol. 5, pp. 83–91, Academic Press, New York

36. Johnson, K. G., and Perry, M. B. (1976) Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* **22,** 29–34

37. Que-Gewirth, N. L., Ribeiro, A. A., Kalb, S. R., Cotter, R. J., Bulach, D. M., Adler, B., Girons, I. S., Werts, C., and Raetz, C. R. (2004) A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. *J. Biol. Chem.* **279,** 25420–25429

38. Simonin, P., Tindall, B., and Rohmer, M. (1994) Structure elucidation and biosynthesis of 31-methylhopanoids from *Acetobacter europaeus. Eur. J. Biochem.* **225,** 765–771

39. Simonin, P., Jürgens, U. J., and Rohmer, M. (1996) Bacterial triterpenoids of the hopane series from the *Prochlorothrix hollandica* and their intracellular localization. *Eur. J. Biochem.* **241,** 865–871

40. Young, J. P., Downer, H. L., and Eardly, B. D. (1991) Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16 S rRNA gene segment. *J. Bacteriol.* **173,** 2271–2277

41. Rohmer, M., Bouvier-Nave, P., and Ourisson, G. (1984) Distribution of hopanoid triterpenes in prokaryotes. *J. Gen. Microbiol.* **130,** 1137–1150

42. Summons, R. E., Jahnke, L. L., Hope, J. M., and Logan, G. A. (1999) 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. *Nature* **400,** 554–557

43. Brown, D. B., Muszynski, A., and Carlson, R. W. (2013) Elucidation of a novel lipid A α-(1,1)-GalA transferase gene (rgtF) from *Mesorhizobium loti*: heterologous expression of *rgtF* causes *Rhizobium etli* to synthesize lipid A with α-(1,1)-GalA. *Glycobiology* **23,** 546–558

44. Roppel, J., and Mayer, H. (1975) Identification of a 2,3-diamino-2,3-dideoxyhexose in the lipid A component of lipopolysaccharides of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris. Carbohydr. Res.* **40,** 31–40

45. Yokota, A., Rodriguez, M., Yamada, Y., Imai, K., Borowiak, D., and Mayer, H. (1987) Lipopolysaccharides of *Thiobacillus* species containing lipid A with 2,3-diamino-2,3-dideoxyglucose. *Arch. Microbiol.* **149,** 106–111

46. Moreno, E., Stackebrandt, E., Dorsch, M., Wolters, J., Busch, M., and Mayer, H. (1990) *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the α-2 subdivision of the class *Proteobacteria. J. Bacteriol.* **172,** 3569–3576

47. Moran, A. P., Zähringer, U., Seydel, U., Scholz, D., Stütz, P., and Rietschel, E. T. (1991) Structural analysis of lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. *Eur. J. Biochem.* **198,** 459–469

48. Plötz, B. M., Lindner, B., Stetter, K. O., and Holst, O. (2000) Characterization of a novel lipid A containing D-galacturonic acid that replaces phosphate residues. *J. Biol. Chem.* **275,** 11222–11228

49. Schwudke, D., Linscheid, M., Strauch, E., Appel, B., Zahringer, U., Moll, H., Muller, M., Brecker, L., Gronow, S., and Lindner, B. (2003) The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing α-D-mannoses that replace phosphate residues. *J. Biol. Chem.* **278,** 27502–27512

50. Holst, O., Borowiak, D., Weckesser, J., and Mayer, H. (1983) Structural studies on the phosphate-free lipid A of *Rhodomicrobium vannielii* ATCC 17100. *Eur. J. Biochem.* **137,** 325–332

51. Smit, J., Kaltashov, I. A., Cotter, R. J., Vinogradov, E., Perry, M. B., Haider, H., and Qureshi, N. (2008) Structure of a novel lipid A obtained from the lipopolysaccharide of *Caulobacter crescentus. Innate Immun.* **14,** 25–37

52. Choma, A., and Komaniecka, I. (2008) Characterization of a novel lipid A structure isolated from *Azospirillum lipoferum* lipopolysaccharide. *Carbohydr. Res.* **343,** 799–804