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# The Structure of a Novel Neutral Lipid A from the Lipopolysaccharide of *Bradyrhizobium elkanii* Containing Three Mannose Units in the Backbone

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**Abstract:** The chemical structure of the lipid A of the lipopolysaccharide (LPS) from *Bradyrhizobium elkanii* USDA 76 (a member of the group of slow-growing rhizobia) has been established. It differed considerably from lipids A of other Gram-negative bacteria, in that it completely lacks negatively charged groups (phosphate or uronic acid residues); the glucosamine (Glc*p*N) disaccharide backbone is replaced by one consisting of 2,3-dideoxy-2,3-diamino-D-glucopyranose (Glc*p*N3N) and it contains two long-chain fatty acids, which is unusual among rhizobia. The Glc*p*N3N disaccharide was further substituted by three D-mannopyranose (D-Man*p*) residues, together forming a pentasaccharide. To establish the structural details of this molecule, 1D and 2D NMR spectroscopy, chemical composition analyses and high-resolution mass spectrometry methods (electrospray ionisation Fourier-transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) and tandem mass spectrometry (MS/MS)) were applied. By using 1D and 2D NMR spectroscopy experiments, it was confirmed that one D-Man*p* was linked to C-1 of the reducing Glc*p*N3N and an α-(1→6)-linked D-Man*p* disaccharide was located at C-4′ of the non-reducing Glc*p*N3N (α-linkage). Fatty acid analysis identified 12:0(3-OH) and 14:0(3-OH), which were amide-linked to Glc*p*N3N. Other lipid A constituents were long (ω-1)-hydroxylated fatty acids with 26–33 carbon atoms, as well as their oxo forms (28:0(27-oxo) and 30:0(29-oxo)). The 28:0(27-OH) was the most abundant acyl residue. As confirmed by high-resolution mass spectrometry techniques, these long-chain fatty acids created two acyloxyacyl residues with the 3-hydroxy fatty acids. Thus, lipid A from *B. elkanii* comprised six acyl residues. It was also shown that one of the acyloxyacyl residues could be further acylated by 3-hydroxybutyric acid (linked to the (ω-1)-hydroxy group).

**Keywords:** bradyrhizobium · fatty acids · glycolipids · structure elucidation

## Introduction

Soil bacteria of the species *Bradyrhizobium elkanii* belong to the group of slow-growing rhizobia. These bacteria are able to induce the development of nitrogen-fixing nodules in the roots of their plant host *Glycine max*, as well as in other wild-growing legumes.[1,2] As a result of the symbiotic relationship, rhizobia inhabit a special micro-niche with access to the products of plant photosynthesis. On the other hand, the plant hosts acquire an easily available nitrogen source. Lipopolysaccharide (LPS), an integral component of the Gram-negative bacteria cell wall, plays an essential role in the symbiosis development.[3] This lipoglycan has, in general, a three-domain architecture and is composed of lipid A (a hydrophobic part that anchors the LPS molecule in the outer membrane) and the core oligosaccharide, which is linked to lipid A and may be substituted by the O-specific polysaccharide (OPS), also known as the O-antigen. Such LPS is called smooth (S) form. The OPS is usually built of oligomeric repeating units that contain from two to eight sugars. LPS, together with outer membrane proteins (OMPs), determine the suitable membrane architecture as well as its semi-permeability, which is very important for the correct morphology and functionality of bacteroids (the endosymbiotic form of rhizobia), in which nitrogen fixation

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takes place. The proper structure of rhizobial LPS is essential for root hair infection, nodule invasion and adaptation to the endosymbiotic conditions.[4,5] The LPS also protects micro-symbiont cells against plant defence responses, that is, the hypersensitivity reaction (HR) and systemic acquired resistance (SAR), by suppressing such reactions during rhizobial infection.[6–8]

Endotoxic active enterobacterial lipid A comprises a β-(1→6)-linked 2-amino-2-deoxy-D-glucopyranose (D-Glc*p*N) disaccharide substituted by two phosphate groups at positions 1 and 4′ as well as six fatty acids, including two acyloxyacyl moieties in a distinct location.[9–11] Naturally occurring lipids A with different structures are usually less or not toxic, such as the lipid A from the LPS of *Rhizobium leguminosarum*,[12] *R. etli*[13] *Rhizobium* Sin-1[14] or *Mesorhizobium loti*.[15] Among rhizobial LPS, the lipid A structures vary. Either the lipid A backbone is composed of the Glc*p*N disaccharide or of one comprising 2,3-diamino-2,3-dideoxy-D-glucopyranose (Glc*p*N3N). Then, the Glc*p*N-containing lipid A backbone can be modified by oxidation of the reducing Glc*p*N to 2-aminogluconate, as found in the LPS of some members of *Rhizobium*. The rhizobial lipid A backbone can be substituted either by phosphate or uronic acid residues. As in other LPS, rhizobial lipid A is always connected to the core region through a residue of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) linked to O-6′.[4] In rhizobial as well as many other lipid A structures, the amino groups of Glc*p*N3N and Glc*p*N, and O-3 and O-3′ of the Glc*p*N are substituted by 3-hydroxy fatty acids, the hydroxy groups of which may be acylated by other non-polar or (in rhizobia) mainly by (ω-1)-hydroxylated long-chain fatty acids, forming acyloxyacyl moieties.[16–18] Also, (ω-1)-OH fatty acids may be esterified by 3-hydroxybutyrate. The most characteristic (ω-1)-OH fatty acid in rhizobial LPS is 28:0(27-OH); one residue of it is present in the lipid A of all members of *Rhizobiaceae* except in *Azorhizobium*, and represents a chemotaxonomical marker of this group of bacteria.[16] Rhizobial lipid A molecules exhibit low biological activity due to their structural peculiarities.[19,20] It was shown that rhizobial lipid A was able to bind to the active site of Toll-like receptor 4 (TLR4), however, no further signalling occurred, and these molecules only blocked the receptor and decreased the defensive reaction.[14]

The detailed structure of lipid A from the LPS of bacteria belonging to the genus *Bradyrhizobium* has not been described so far, apart from some reports concerning the heterogeneity in composition.[21–23] Herein, we present the detailed structure of a novel lipid A isolated from the LPS of *Bradyrhizobium elkanii* USDA 76.

## Results

**Isolation of LPS**: LPS was extracted from *B. elkanii* USDA 76 cells by using hot phenol/water. Almost all material (≈90%) was found in the phenol phase. The LPS preparation was analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 1) and compared to the standard smooth (S) form LPS from *Salmonella enterica* sv. Typhimurium (Sigma). The phenol-soluble LPS occurred mainly as rough (R) or semi-rough (SR) forms with a mass similar to that of the SR-LPS fraction of the standard. Only a small amount of this material was S-form LPS, which gave a typical ladder-like pattern. The OPS contained up to 15 repeating units (as estimated by SDS-PAGE).

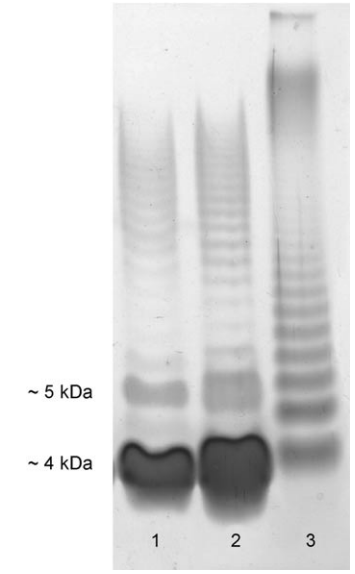


Figure 1. Silver-stained SDS-PAGE of the LPS from *Bradyrhizobium elkanii* (line 1, 5 μg; line 2, 10 μg) and *Salmonella enterica* sv. Typhimurium (line 3, 2 μg).

**Isolation and chemical analysis of *B. elkanii* lipid A**: Lipid A obtained from *B. elkanii* LPS by mild acid hydrolysis was subjected to sugar and fatty acid analyses. D-Mannopyranose (D-Man*p*) and D-Glc*p*N3N (but not Glc*p*N) were identified as components of the lipid A backbone. Fatty acid analysis revealed the presence of two amide-linked 3-hydroxy fatty acids, namely, 12:0(3-OH) and 14:0(3-OH). In addition, ester-linked long (ω-1)-hydroxylated fatty acids with chain lengths in the range of 26–33 carbon atoms were present, two of which occurred also in the oxo form (27-oxo-28:0 and 29-oxo-30:0). The most abundant fatty acid was 27-hydroxyoctacosanoic acid [28:0(27-OH)]. Non-polar long fatty acids, that is, 24:0, 26:0 and 28:0, were also present (Table 1). The amount of 12:0(3-OH) was much lower than that of 14:0(3-OH), even after hydrolysis using a 4 M aqueous solution of HCl. After methanolysis with a 2 M solution of HCl in MeOH, the molecular ratio between both fatty acids was about 1:7. Therefore, it was assumed that a strongly linked unsaturated fatty acid could be present in the lipid A. After hydrogenation using $H_2$ and palladium/active carbon as a catalyst, the amount of 12:0(3-OH) in-

  

Table 1. Fatty acid composition of *B. elkanii* lipid A.

| Fatty acid | Content [nmol mg$^{-1}$] |
|---|---|
| amide linked | |
| 12:0(3-OH) | 1654.7 |
| 14:0(3-OH) | 4263.2 |
| | |
| ester linked | |
| 26:0(25-OH) | 67.8 |
| 28:0(27-OH) | 2606.6 |
| 28:0(27-oxo) | 76.0 |
| 30:0(29-OH) | 969.8 |
| 30:0(29-oxo) | 77.2 |
| 31:0(30-OH) | 446.4 |
| 32:0(31-OH) | 99.4 |
| 33:0(32-OH) | 71.6 |
| 24:0 | 337.6 |
| 26:0 | 178.0 |
| 28:0 | 140.0 |

creased significantly and the proportion of the latter to 14:0-(3-OH) rose to about 1:1.

**NMR spectroscopy of the lipid A backbone**: The purified lipid A was structurally characterised by 1D and 2D NMR spectroscopy. The HMQC spectrum of the lipid A (Figure 2)

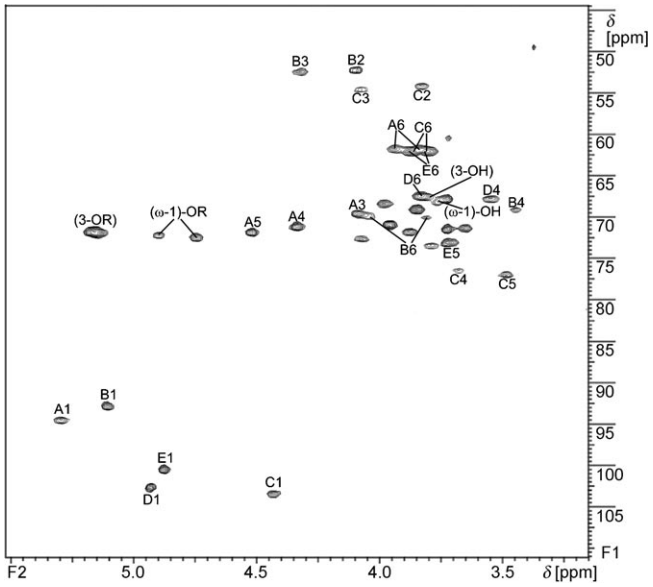


Figure 2. HMQC spectrum (detail) of lipid A from *B. elkanii*. The lipid A sample was dissolved in $CDCl_3/CD_3OD$ (2:1, v/v) with the addition of $D_2O$ (5 μL) and the spectrum was recorded at 300 K. The assigned cross-peaks are labelled according to the text and Tables 2 and 3.

contained signals for five anomeric carbon atoms ($\delta = 92.84$–103.49 ppm), four signals for nitrogen-bearing carbon atoms ($\delta = 51.99$–54.68 ppm), and three groups of signals for unsubstituted ($\delta = 61.78$–62.08 ppm) as well as two for substituted ($\delta = 67.51$ and 69.64 ppm) C-6 carbon atoms. The remaining signals represented sugar ring carbon atoms. Based on $^1$H–$^1$H COSY, TOCSY and $^1$H–$^{13}$C HMBC experiments, five spin systems were identified: **A**, α-D-Man*p*, **B**, α-D-Glc*p*N3N, **C**, β-D-Glc*p*N3N, **D**, α-D-Man*p*, and **E**, α-D-Man*p*. All $^1$H and $^{13}$C NMR chemical shifts could be assigned and are listed in Table 2. The aminosugar spin systems were confirmed by correlation of the protons at the nitrogen-bearing carbon atoms with the corresponding carbon atoms at $\delta =$ 51.99–54.68 ppm. The $^1J$(C1,H1) coupling constants confirmed the anomeric configuration of monosaccharides (Table 2). Relatively large values of $^1J$(C1,H1) (characteristic for α-linked sugars) were found for residues **A**, **B**, **D**, and **E**. Residue **C** was characterised by a small (160.4 Hz) $^1J$(C1,H1) coupling, and, thus, was assumed to be β-configured. A ROESY experiment identified the following interresidual correlations between anomeric and linkage protons: **A1/B1** ($\delta = 5.292/5.110$ ppm), **C1/B6a,b** ($\delta = 4.429/3.811$ and 4.429/4.034 ppm), **D1/C4** ($\delta = 4.932/3.679$ ppm) and **E1/D6** ($\delta = 4.879/3.832$ ppm) (Figure 3). These data were confirmed by an HMBC experiment, in which the following $^1$H–$^{13}$C connectivities were found: **A1/B1** ($\delta = 5.292/94.54$ ppm), **C1/B6** ($\delta = 4.429/62.08$ ppm), **D1/C4** ($\delta = 4.932/76.55$ ppm) and **E1/D6** ($\delta = 4.879/67.51$ ppm). Lipid A was not substituted by phosphate residues, as confirmed by $^{31}$P NMR spectroscopy of the *O*-deacylated lipid A sample.

In summary, the sugar backbone of the lipid A derived from the LPS of *B. elkanii* possessed the following 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-Man*p*

In the $^1$H NMR spectrum of the *O*-deacylated material, four signals ($\delta = 7.45$–7.90 ppm) were assigned to the protons derived from the NH groups of the diaminoglucose residues. Also, signals from olefinic protons were identified at $\delta = 6.580$ and 5.825 ppm ($\delta = 141.74$ and 124.40 ppm, respectively, in the $^{13}$C NMR spectrum). Moreover, the NMR correlations indicated that this unsaturated fatty acid was located at position C-3 of residue **B**. Similar signals had also been observed in the $^1$H NMR spectrum of native lipid A. The *trans* configuration of the double bond was deduced from the chemical shift of the vicinal carbon atom signal ($\delta = 30.82$ ppm) and also from the vicinal coupling constants $^3J$(H,H) (over 15 Hz).

Table 2. $^1$H and $^{13}$C NMR chemical shifts (δ, in ppm) from *B. elkanii* lipid A backbone [$CDCl_3/CD_3OD$ (2:1, v/v) with 5 μL $D_2O$].

| Residue | $^1J$(C1,H1) [Hz] | H1 | H2 | H3 | H4 | H5 | H6 | H6‘ | C1 | C2 | C3 | C4 | C5 | C6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A (α-Man) | 177.5 | 5.292 | 3.956 | 4.072 | 4.338 | 4.519 | 3.841 | 3.940 | 94.58 | 71.03 | 69.77 | 71.22 | 71.90 | 61.78 |
| B (α-GlcN3N) | 177.3 | 5.110 | 4.068 | 4.294 | 3.440 | 4.070 | 3.811 | 4.034 | 92.84 | 51.99 | 52.14 | 69.15 | 72.49 | 69.64 |
| C (β-GlcN3N) | 160.4 | 4.429 | 3.831 | 4.078 | 3.679 | 3.486 | 3.816 | 3.869 | 103.49 | 54.19 | 54.68 | 76.55 | 77.08 | 62.08 |
| D (α-Man) | 171.9 | 4.932 | 3.718 | 3.643 | 3.546 | 3.79 | 3.832 | 3.832 | 102.72 | 73.12 | 71.48 | 67.88 | 73.51 | 67.51 |
| E (α-Man) | 172.4 | 4.879 | 3.958 | 3.872 | 3.723 | 3.723 | 3.813 | 3.876 | 100.54 | 71.04 | 71.81 | 67.89 | 73.17 | 62.08 |

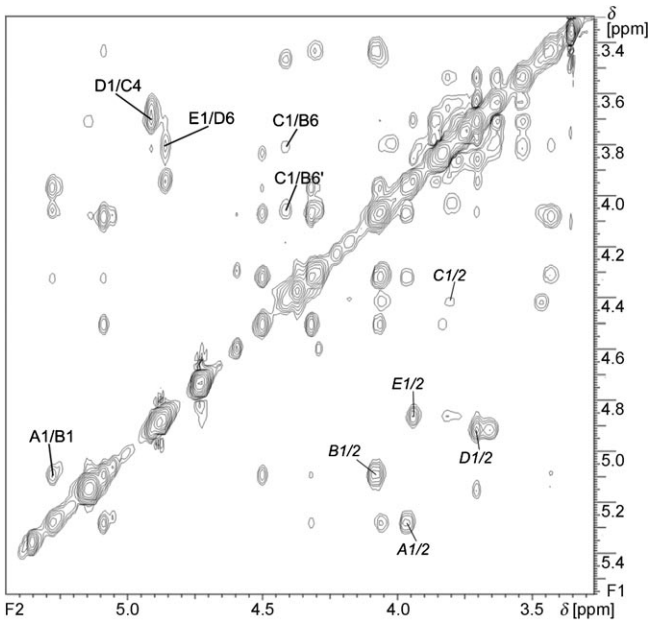


Figure 3. $^{1}H$–$^{1}H$ ROESY spectrum (detail) of lipid A from *B. elkanii*. Lipid A sample was dissolved in $CDCl_3/CD_3OD$ (2:1, v/v) with the addition of $D_2O$ (5 μL), and the spectrum was recorded at 300 K. Interglycosidic connectivities are marked above the diagonal (normal type) and some COSY-type cross-peaks are marked below the diagonal (italic type).

The carbon atom signals at $\delta = 68.53$, 72.79 and 72.60 ppm (Table 3) were attributed to the ω-1 methine carbon atoms of the long chain fatty acids, free and substituted by 3-hydroxybutyric acid, respectively. The chemical shifts for the cross-peaks of α/β and β/γ protons of 3-hydroxybutyroyl group were very similar to those reported for lipid A derived from *R. etli* and NGR234[24,25] (Table 3).

**Mass spectrometry of *B. elkanii* lipid A**: Mass spectrometry experiments on the native lipid A were performed in the positive and negative ion mode. It was found that useful spectra could only be obtained in the positive ion mode, indicating that no negatively charged groups (phosphate, uronic acids) were present in the lipid A molecules. Figure 4 shows the charge-deconvoluted ESI FT-ICR mass spectrum of the native lipid A. Based on the chemical analyses of the

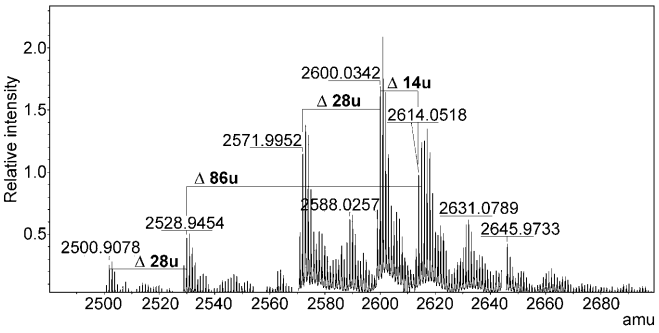


Figure 4. Charge-deconvoluted ESI FT-ICR MS spectrum (positive ion mode) of native *B. elkanii* lipid A. Some mass differences were assigned. The structure corresponding to the signal at 2600.0342 amu is shown in Figure 7.

sugar units and fatty acid residues, the molecules giving the most abundant mass peaks at 2571.9952, 2600.0342 and 2614.0518 amu could be assigned to lipid A species containing three hexose sugars, two GlcN3N units, two 12:0(3-OH) and two 14:0(3-OH) units (probably carrying two double bonds) and also two ester-linked fatty acids, one of which was additionally esterified by 3-hydroxybutyric acid. The mass differences between neighbouring ions were approximately 28 and 14 amu, respectively, due to the different lengths of ester-linked fatty acids. The calculated molecular masses for these three lipid A species were 2571.848, 2599.938 and 2613.954 amu, respectively. The mass signals in the region 2500–2560 amu were derived from lipid A molecules not containing butyric acid residues. Unlabelled clusters of signals originated from molecular ions containing $Na^+$ or $K^+$ adduct ions.

To confirm the compositional details, the MS/MS spectrum of the most prominent peak (at 2600.0342 amu) was obtained using a doubly charged ion at $m/z = 1300.9621$, which was selected as the parent ion. The positive ion electrospray ionisation Fourier-transform ion cyclotron resonance infrared multiphoton dissociation MS/MS (ESI FT-ICR IRMPD MS/MS) spectrum showed intense fragment ions at $m/z = 761.4963$ and at $m/z = 1499.3684$ (Figure 5). The first one could be assigned to be a $Y^+$-fragment peak (according to the nomenclature suggested by Domon and Costello[52]), which is derived from the reducing end of lipid A. The second ion originated from

Table 3. $^{1}H$ and $^{13}C$ NMR chemical shifts (δ, in ppm) from *B. elkanii* lipid A fatty acids [$CDCl_3/CD_3OD$ (2:1, v/v) with 5 μL $D_2O$].

| Fatty acids | $\alpha_1/\alpha_2$ | β | γ | $(CH_2)_n$ | $CH_3$ |
|---|---|---|---|---|---|
| (3-OH)-fatty acids | 2.190/2.297 | 3.825 | 1.360 | 1.284 | 0.889 |
| | 43.94 | 67.82 | 37.72 | 30.16 | 14.10 |
| (3-OR)-fatty acids | 2.503/2.406 | 5.145 | 1.557 | 1.284 | 0.889 |
| | 41.85 | 72.23 | 35.30 | 30.16 | 14.10 |
| (3-OH)-butyrate | 1.803/1.914 | 4.058 | 1.032 | | |
| | n.d. | 66.19 | n.d. | | |
| | | (ω-2) | (ω-1) | ω | |
| long chain (ω-1)-OH fatty acid | | 1.393/1.471 | 3.742 | 1.152 | |
| | | 37.45 | 68.53 | 23.52 | |
| first long chain (ω-1)-OR fatty acid | | 1.461/1.577 | 4.730 | 1.201 | |
| | | 37.21 | 72.79 | 20.70 | |
| second long chain (ω-1)-OR-fatty acid | | 1.500/1.586 | 4.885 | 1.210 | |
| | | 35.25 | 72.60 | 20.70 | |

  

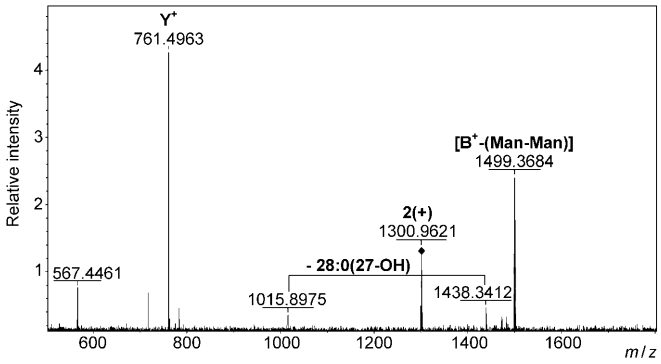


Figure 5. Positive ion ESI FT-ICR IRMPD MS/MS spectrum obtained after fragmentation of the parent, doubly charged ion at 1300.9621 amu from *B. elkanii* lipid A. For details see the text.

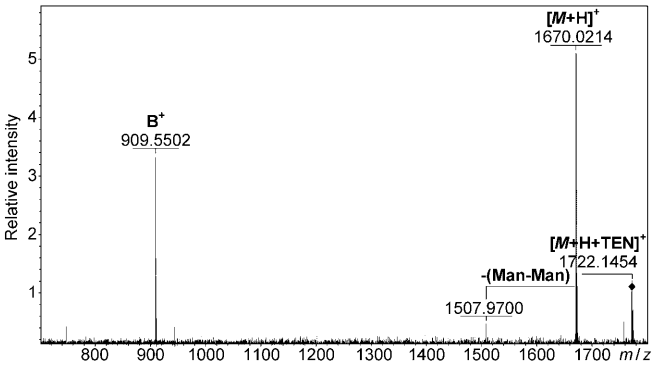


Figure 6. Positive ion ESI FT-ICR IRMPD MS/MS of the triethylammonium (TEN) salt of *O*-deacylated *B. elkanii* lipid A. The molecular ion at 1772.1454 amu $[M+H+TEN]^+$ (the only significant ion on ESIMS spectrum) served as the parent ion.

the $B^+$ fragment (non-reducing end) after additional elimination of the mannosylomannosyl disaccharide (1823.474−364.1056 amu). The reducing fragment ion was composed of one Glc*p*N3N unit, one mannose unit and two fatty acid residues, namely, 12:0(3-OH) and 14:0(3-OH), which probably carry two double bonds (calculated mass: 761.4865 amu). The non-reducing end of the ionised molecule was composed of Glc*p*N3N, two Man*p* units, *N*-linked 12:0(3-OH) and 14:0(3-OH) and two residues of 28:0(27-OH), one of which was further substituted at the (ω-1)-hydroxy group by 3-hydroxybutyric acid. This fragmentation pattern showed that both acyloxyacyl moieties were exclusively bound to the non-reducing Glc*p*N3N residue, confirming the asymmetric acylation of *B. elkanii* lipid A.

The other peaks in the spectrum originated from a double cleavage across the ring, however, they did not give many structural details. The signal at $m/z = 1438.3412$ resulted from the fragmentation of the non-reducing end of lipid A, followed by the consecutive elimination of one 28:0(27-OH) residue (peak at $m/z = 1015.8975$). The fragment ion at $m/z = 567.4461$ was derived from the fragmentation of the reducing end of lipid A.

To obtain more details of the distribution of fatty acids, the positive ion ESI FT-ICR IRMPD MS/MS of the triethylammonium (TEN) salt of *O*-deacylated lipid A was performed (Figure 6), in which the molecular peak at $m/z =$ 1772.1454 ($[M+H+TEN]^+$) served as the parent ion. The spectrum possessed a predominating peak at $m/z =$ 1670.0214 $[M+H]^+$, which was derived from a molecule composed of two Glc*p*N3N units, three Man*p* units and four amide-linked fatty acid residues (two 12:0(3-OH) and two 14:0(3-OH), carrying probably two double bonds, calculated mass: 1669.0178). This tetra-acyl lipid A was further cleaved to a $B^+$-fragment ion at 909.5502 amu, which was derived from the non-reducing end. This fragmentation indicated that the distal Glc*p*N3N unit was substituted by a mannosylomannosyl disaccharide (as proven by NMR spectroscopy analyses (residue **C**)) and bore one 12:0(3-OH) and one 14:0(3-OH) residue (calculated mass: $m/z = 909.559$). The signal at $m/z = 1507.9700$ was derived from the cleavage of one Man*p* residue, probably from the reducing end of lipid A.

In summary, we propose the structure depicted in Figure 7 as the most abundant *B. elkanii* lipid A structure.

## Discussion

In the LPS of *Bradyrhizobium elkanii* USDA 76, a neutral lipid A was present comprising a backbone of three mannose residues linked to a disaccharide of Glc*p*N3N. Two mannose residues furnished an α-(1→6)-linked disaccharide, which α-(1→4)-substituted the C-4′ of the non-reducing Glc*p*N3N residue . The third mannose unit was α-(1→1)-linked to the reducing Glc*p*N3N residue. Such a lipid A structure has not yet been identified in rhizobial LPS, however, it resembles the lipid A isolated from *Bdellovibrio bacteriovorus*,[26] which contained two mannose residues that decorated the backbone at C-4′ and C-1. Another example of mannose-containing lipid A is that derived from the phototrophic bacterium *Rhodomicrobium vanniellii*, in which the C-4′ of the phosphate-free Glc*p*N disaccharide backbone was substituted by a mannose residue.[27]

The presence of Glc*p*N3N in the backbone of lipid A was first described for the LPS of the genus *Rhodopseudomonas*.[28] Apart from *B. elkanii*, lipid A containing Glc*p*N3N as the sole backbone amino sugar was found in two other species of rhizobia (*Mesorhizobium loti*[29] and *M. huakuii*[17]). Also, this sugar was present in lipid A derived from other species outside rhizobia and phototrophic bacteria, for example, in *Thiobacillus* sp.,[30] *Brucella abortus*,[31] *Campylobacter jejuni*[32] and *Aquifex pyrophilus*.[33] Most of these lipids A were negatively charged because either phosphate groups or uronic acid (GalA) were present. The lipid A of the LPS from *B. elkanii* contains neither phosphate nor uronic acid residues and is thus a neutral molecule. Among *Rhizobiales* taxon, phosphate residues in lipid A were also not observed in *R. leguminosarum*,[34] *Rhizobium etli*[18] and *Rhizobium* sp. Sin-1.[35] On the contrary, lipid A from *M. huakuii* was only partly phosphorylated (at C-4′) and con-

  

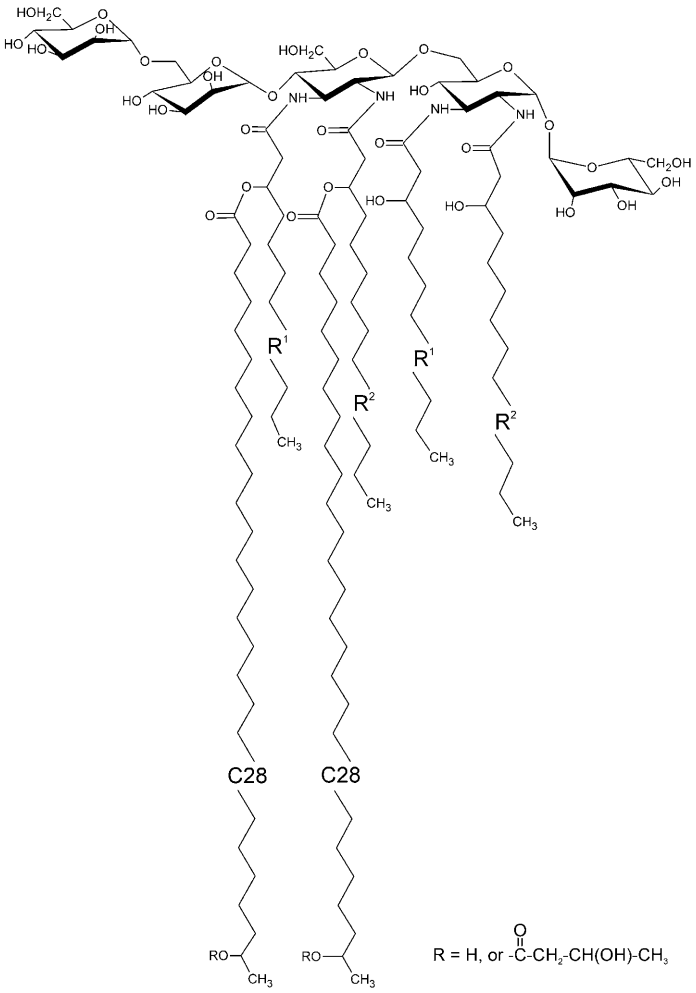


Figure 7. Structure of lipid A from the LPS of *B. elkanii* corresponding to the most prominent mass at 2600.0342 amu. Each GlcN3N residue is substituted by 12:0(3-OH) and 14:0(3-OH) ($R^1$, $R^2$). The exact position (C-2 or C-3) of these primary fatty acids has not been determined and can be interchanged. Fatty acids linked to reducing GlcN3N may be unsaturated.

tained galacturonic acid at C-1 of the reducing Glc*p*N3N residue.[17]

The acylation pattern of lipid A from *B. elkanii* differed to those of other Gram-negative bacteria. It was shown that two 3-hydroxy fatty acids were present (12:0(3-OH) and 14:0(3-OH)), which were exclusively amide-linked to both Glc*p*N3N residues. As shown by NMR spectroscopy, the complete lipid A and the O-deacylated sample probably contain unsaturated fatty acid(s). Amide-bound unsaturated fatty acids are very difficult to liberate by hydrolysis,[36] hence this could be the reason why it could not be identified in fatty acid analysis. Also, the masses assigned in mass spectrometric analyses pointed to the presence of molecules lacking four hydrogen atoms.

Native *B. elkanii* lipid A contained two acyloxyacyl moieties formed by esterification of 3-hydroxy fatty acids with long (ω-1)-hydroxylated fatty acids, with the number of carbon atoms ranging from 26 to 33. One of these fatty acids was further esterified by the 3-hydroxybutyrate moiety as the tertiary substituent. The presence of such long fatty acids is characteristic for rhizobia. All members of this group contain only one residue of 28:0(27-OH) or its oxo form within lipid A.[16,37] The presence of such a wide range of (ω-1)-hydroxy fatty acids in bradyrhizobial LPS has been known for years,[16] however, a lipid A containing two long fatty acyl residues is described here for the first time. Together with other membrane components, such as hopanoids, long-chain fatty acids may have a strong influence on flexibility and permeability of the bradyrhizobial outer membrane and on the viability of bacteroids during symbiotic conditions.[38]

The structure of lipid A is also very important for the integrity of the outer membrane during symbiosis. Mutants with lipid A lacking long (ω-1)-hydroxy fatty acids were more sensitive to changes in pH and environmental osmolarity, grew very slowly, and nodules formed during symbiosis were developed with delay. Also, the effectiveness of symbiosis measured by nitrogen fixation was shown to be reduced.[12,39,40] Thus, it is indispensable to investigate other rhizobial lipids A to better understand the role of bradyrhizobial LPS in nodulation, the molecular mechanisms of symbiosis and the mechanisms of invasion of pathogenic bacteria to eukaryotic cells.

## Conclusion

The chemical structure of the lipid A of the lipopolysaccharide (LPS) from *Bradyrhizobium elkanii* USDA 76 has been determined. In contrast to lipids A derived from other Gram-negative bacteria, lipid A from *B. elkanii* lacks negatively charged groups (phosphate or uronic acid residues), the glucosamine (Glc*p*N) disaccharide backbone is replaced by one consisting of 2,3-dideoxy-2,3-diamino-D-glucopyranose (Glc*p*N3N). In addition, it contains two long-chain fatty acids, which is unusual among rhizobia. The Glc*p*N3N disaccharide was further substituted by three D-mannopyranose (D-Man*p*) residues. 1D and 2D NMR experiments confirmed that one D-Man*p* was linked to C-1 of the reducing Glc*p*N3N and an α-(1→6)-linked D-Man*p* disaccharide was located at C-4′ of the non-reducing Glc*p*N3N (α-linkage). Fatty acid analysis identified 12:0(3-OH) and 14:0(3-OH), which were amide-linked to Glc*p*N3N. Other lipid A constituents were long (ω-1)-hydroxylated fatty acids with 26–33 carbon atoms, as well as their oxo forms (28:0(27-oxo) and 30:0(29-oxo)). The 28:0(27-OH) was the most abundant acyl residue. As confirmed by high-resolution mass spectrometry techniques, these long-chain fatty acids created two acyloxyacyl residues with the 3-hydroxy fatty acids, and it was shown that one of the acyloxyacyl residues could be further acylated by 3-hydroxybutyric acid (linked to the (ω-1)-hydroxy group).

  

## Experimental Section

**Bacterial strain and culture condition**: *Bradyrhizobium elkanii* USDA 76 was grown at 28 °C in 79 CA medium, according to the method of Vincent,[41] for 14 d, with aeration by vigorous shaking.

**Isolation and purification of LPS**: The cell pellet (140 g wet mass) was washed twice with saline solution and once with distilled water, then it was subjected to delipidation according to the method of Que and co-workers.[18] The LPS preparation was obtained from hot 45% phenol/water extraction and reference: [42] as modified by Johnson and Perry.[43] The phenol phase was dialysed extensively against tap and distilled water. Next, enzymatic degradation of nucleic acids and proteins was carried out by using DNase, RNase and proteinase K, respectively, and dialysis was performed again. LPS (approximately 300 mg) was obtained after ultracentrifugation (105 000 *g*, 4 °C, 4 h).

**Isolation and purification of lipid A**: Lipid A was liberated from LPS by mild acid hydrolysis (230 mg LPS, 36 mL of a 1% aqueous solution of acetic acid, 100 °C, 3 h). The free lipid A was purified according to Que and co-workers.[18] Briefly, adequate amounts of chloroform and methanol were added to the hydrolysate to obtain the mixture chloroform/methanol/hydrolysate 2:2:1.8 (v/v/v), and the mixture was vigorously shaken, then centrifuged. The chloroform phase, containing the lipid A, was collected and washed twice with the water phase from a freshly prepared two-phase Bligh/Dyer mixture (chloroform/methanol/water, 2:2:1.8 (v/v/v)). The yield of lipid A was 120 mg. Next, this preparation was purified thoroughly to remove the residual phospholipids using water-saturated *n*-butanol.[44] This method separates, in particular, glycolipids from phospholipids because phospholipids are solubilised in this solvent, but glycolipids precipitate. Briefly, a solution of lipid A (120 mg) in water-saturated butanol (10 mL) was sonicated (10 min, 30 °C), vigorously shaken and centrifuged at 12 000 *g*, 45 min, 4 °C. The supernatant was removed and the pellet containing lipid A was washed again. Finally, pure lipid A (83.5 mg) was obtained, which was stored at −20 °C as a solution in $CHCl_3$/MeOH (2:1, v/v).

**Lipid A *O*-deacylation**: *Bradyrhizobium elkanii* lipid A (40 mg) was *O*-deacylated according to a modified procedure.[45] The lipid A was treated with anhydrous hydrazine (2 mL, 37 °C, 45 min). After cooling, the reaction mixture was poured into cold acetone, and the lipid A precipitate was pelleted by centrifugation (6000 *g*, 4 °C, 15 min). The material was washed twice with acetone and dried in a stream of nitrogen. Finally, *O*-deacylated lipid A was resuspended in water and lyophilised.

**Fatty acid analysis**: For total fatty acid determination, lipid A was subjected to hydrolysis in a 4 M aqueous solution of HCl (100 °C, 4 h). The free fatty acids were extracted with chloroform and converted to their methyl esters with diazomethane. After evaporation, bis-trimethyl silyl trifluoroacetamide (BSTFA) (Sigma) was added and the samples were incubated (65 °C, 4 h). Ester-linked fatty acids were determined after solvolysis of lipid A in a 0.5 M solution of HCl in MeOH (85 °C, 30 min) and derivatisation by using BSTFA.

The unsaturated fatty acids in lipid A were saturated by catalytic hydrogenation in the presence of palladium (Pd 9.94%/active carbon) (Degussa, Germany).[46]

Fatty acids were quantified by GC–MS (Hewlett Packard 5970 instrument equipped with a fused silica capillary column HP-5MS, 30 m × 0.25 mm, film thickness 0.25 μm) with helium as the carrier gas. The temperature program was 120 °C for 3 min, then increased to 320 °C at a rate of 5 °C $min^{-1}$, then 320 °C for 20 min. In the analysis of fatty acid oxidation products the initial temperature was 100 °C.

**Sugar analysis**: For aminosugar analysis, lipid A was hydrolysed in a 4 M aqueous solution of HCl (100 °C, 16 h). After drying, the sample was subjected to *N*-acetylation,[47] followed by reduction with $NaBH_4$ and peracetylation. Neutral sugar analysis was carried out after hydrolysis of lipid A in a 2 M aqueous solution of trifluoroacetic acid (100 °C, 4 h), followed by reduction with $NaBH_4$ and peracetylation.

The absolute configurations of monosaccharides were established by analysis of the acetylated *R*-(−)-2-butylglycosides according to Gerwig and co-workers.[48]

Sugar samples were analysed by GC on a Hewlett Packard gas chromatograph 5890 series II equipped with a HP-5MS column (30 m × 0.25 mm). Helium served as carrier gas and the temperature program was 150 °C for 3 min, then increased to 250 °C at a rate of 3 °C $min^{-1}$, then to 320 °C, 25 °C $min^{-1}$. The final temperature was maintained for 10 min.

**SDS-PAGE**: SDS-PAGE (12.5% acrylamide) was performed as described.[49] The electophorogram was silver-stained.[50]

**Mass spectrometry**: Electrospray ionisation Fourier-transform ion cyclotron resonance mass spectrometry (ESI FT-ICRMS) experiments were performed in negative and positive ion mode by using a hybrid Apex Qe FT-ICRMS instrument (Bruker Daltonics, Billerica, MA, USA), equipped with a 7 tesla superconducting magnet and an Apollo dual ion source. Data were recorded in broadband mode with a 512 k data sampling rate. The mass scale was calibrated externally by using compounds of known structure. For the negative ion mode, samples ($\approx$10 ng $\mu l^{-1}$) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water and triethylamine (pH $\approx$ 8.5). For the positive ion mode, a 30:10:0.4 (v/v/v) mixture of water, acetonitrile and acetic acid (pH $\approx$ 3) was used. Samples were sprayed at a flow rate of 2 μL $min^{-1}$. The capillary entrance voltage was set to 3.8 kV, and the drying gas temperature was set to 150 °C. The spectra, which showed several charge states for each component, were charge deconvoluted and mass numbers given refer to mono-isotopic molecular masses. For MS/MS measurements, infrared multi-photon dissociation (IRMPD) of isolated parent ions was performed with a 25 W, 10.6 μm $CO_2$ laser (Synrad, USA). The duration of laser irradiation was adapted to generate optimal fragmentation and varied between 10 and 80 ms. Details on the structural characterisation of LPS by FT-ICRMS are given elsewhere.[51]

**NMR spectroscopy**: For NMR spectroscopy analysis, *O*-deacylated lipid A (8 mg) was dissolved in [$D_6$]DMSO (0.5 mL) and lipid A (28 mg) was dissolved in $CDCl_3$/$CD_3OD$ (2:1; 0.5 mL) containing 5 μL $D_2O$. 1D and 2D NMR spectra were recorded at 600.1 MHz on an AVANCE DRX-600 spectrometer (Bruker, Rheinstetten, Germany) using Bruker software. All spectra were recorded at 300 K. The following 2D NMR experiments were performed: COSY, DQF-COSY, TOCSY, ROESY, HMQC, DEPT-HSQC and HMBC. The $^1H$ and $^{13}C$ NMR resonances were measured relative to the methyl group signal of [$D_6$]DMSO ($\delta_H$ = 2.50/$\delta_C$ = 39.50 ppm) for *O*-deacylated material and to tetramethylsilane (TMS; $\delta_H$ = 0.0/$\delta_C$ 0.0 ppm) for the lipid A sample.

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[1] L. D. Kuykendall, B. Saxena, T. E. Devine, S. E. Udell, *Can. J. Microbiol.* **1992**, *38*, 501–505.
[2] Y. F. Zhang, E. T. Wang, C. F. Tian, F. Q. Wang, L. L. Han, W. F. Chen, W. X. Chen, *FEMS Microbiol. Lett.* **2008**, *285*, 146–154.
[3] E. L. Kannenberg, N. J. Brewin, *Trends Microbiol.* **1994**, *2*, 277–283.
[4] R. W. Carlson, B. L. Reuhs, L. S. Forsberg, E. L. Kannenberg in *Genetics of Bacterial Polysaccharides* (Ed.: J. B. Goldberg), CRC Press, Boca Raton, Florida, **1999**, pp. 53–90.
[5] N. Fraysse, S. Jabbouri, M. Treilhou, F. Couderc, V. Poinsot, *Glycobiology* **2002**, *12*, 741–748.
[6] U. Albus, R. Baier, O. Holst, A. Pühler, K. Niehaus, *New Phytol.* **2001**, *151*, 597–606.

  

[7] R. Mathis, F. Van Gijsegem, R. De Rycke, W. D'Haeze, E. Van Maelsaeke, E. Anthonio, M. Van Montagu, M. Holsters, D. Vereecke, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 2655–2660.
[8] H. Menezes, C. Jared, *Comp. Biochem. Physiol. Part C* **2002**, *132*, 1–7.
[9] U. Zähringer, B. Lindner, E. T. Rietschel in *Endotoxin in Health and Disease* (Eds.: H. Brade, S. M. Opal, S. N. Vogel, D. C. Morrison), Marcel Dekker, New York, **1999**, pp. 93–114.
[10] A. Aderem, R. J. Ulevitch, *Nature* **2000**, *406*, 782–787.
[11] A. Molinaro, O. Holst in *Microbial Glycobiology: Structures, Relevance and Applications* (Eds.: A. P. Moran, O. Holst, P. J. Brennan, M. von Itzstein), Elsevier, London, **2009**, pp. 29–55.
[12] V. Vedam, J. G. Haynes, E. L. Kannenberg. , R. W. Carlson, D. J. Sherrier, *Mol. Plant-Microbe Interact.* **2004**, *17*, 283–291.
[13] K. D. Noel, D. M. Duelli in *Prokaryotic nitrogen fixation: a model system of analysis of a biological process* (Ed.: E. W. Triplett), Horizon Scientific Press, Wymondham, **2000**, pp. 415–431.
[14] M. L. Vandenplas, R. W. Carlson, B. S. Jeyaretnam, B. McNeill, M. H. Barton, N. Norton, T. F. Murray, J. N. Moore, *J. Biol. Chem.* **2002**, *277*, 41811–41816.
[15] T. Urbanik-Sypniewska, A. Choma, J. Kutkowska, T. Kamińska, M. Kandefer-Szerszeñ, R. Russa, J. Dolecka, *Immunobiology* **2000**, *202*, 408–420.
[16] U. R. Bhat, R. W. Carlson, M. Busch, H. Mayer, *Int. J. Syst. Bacteriol.* **1991**, *41*, 213–217.
[17] A. Choma, P. Sowinski, *Eur. J. Biochem.* **2004**, *271*, 1310–1322.
[18] N. L. S. Que, S. Lin, R. J. Cotter, C. R. H. Raetz, *J. Biol. Chem.* **2000**, *275*, 28006–28016.
[19] E. T. Rietschel, T. Kirikae, F. U. Schade, A. J. Ulmer, O. Holst, H. Brade, G. Schmidt, U. Mamat, H.-D. Grimmecke, S. Kusumoto, U. Zähringer, *Immunobiology* **1993**, *187*, 169–190.
[20] A. B. Schromm, K. Brandenburg, H. Loppnow, U. Zähringer, E. T. Rietschel, S. F. Carroll, M. H. Koch, S. Kusumoto, U. Seydel, *J. Immunol.* **1998**, *161*, 5464–5471.
[21] V. Puvanesarajah, F. M. Schell, D. Gerhold, G. Stacey, *J. Bacteriol.* **1987**, *169*, 137–141.
[22] H. Mayer, J. H. Krauss, T. Urbanik-Sypniewska, V. Puvanesarajah, G. Stacey, G. Auling, *Arch. Microbiol.* **1989**, *151*, 111–116.
[23] M. Carrion, U. R. Bhat, B. Reuhs, R. W. Carlson, *J. Bacteriol.* **1990**, *172*, 1725–1731.
[24] N. L. S. Que, A. A. Ribeiro, C. R. H. Raetz, *J. Biol. Chem.* **2000**, *275*, 28017–28027.
[25] S. K. Gudlavalleti, L. S. Forsberg, *J. Biol. Chem.* **2003**, *278*, 3957–3968.
[26] D. Schwudke, M. Linscheid, E. Strauch, B. Appel, U. Zähringer, H. Moll, M. Müller, L. Brecker, S. Gronow, B. Lindner, *J. Biol. Chem.* **2003**, *278*, 27502–27512.
[27] O. Holst, D. Borowiak, J. Weckesser, H. Mayer, *Eur. J. Biochem.* **1983**, *137*, 325–332.
[28] J. Roppel, H. Mayer, J. Weckesser, *Carbohydr. Res.* **1975**, *40*, 31–40.
[29] R. Russa, T. Urbanik-Sypniewska, K. Lindström, H. Mayer, *Arch. Microbiol.* **1995**, *163*, 345–351.
[30] A. Yokota, M. Rodriguez, Y. Yamada, K. Imai, D. Borowiak, H. Mayer, *Arch. Microbiol.* **1987**, *149*, 106–111.
[31] E. Moreno, E. Stackebrandt, M. Dorsch, J. Wolters, M. Busch, H. Mayer, *J. Bacteriol.* **1990**, *172*, 3569–3576.
[32] A. P. Moran, U. Zähringer, U. Seydel, D. Scholz, P. Stütz, E. T. Rietschel, *Eur. J. Biochem.* **1991**, *198*, 459–469.
[33] B. Plötz, B. Lindner, K. O. Stetter, O. Holst, *J. Biol. Chem.* **2000**, *275*, 11222–11228.
[34] U. R. Bhat, L. S. Forsberg, R. W. Carlson, *J. Biol. Chem.* **1994**, *269*, 14402–14410.
[35] B. Jeyaretnam, J. Glushka, K. Kolli, R. W. Carlson, *J. Biol. Chem.* **2002**, *277*, 41802–41810.
[36] H.-W. Wollenweber, E. T. Rietschel, *J. Microbiol. Methods* **1990**, *11*, 195–211.
[37] A. Choma, T. Urbanik-Sypniewska, R. Russa, J. Kutkowska, H. Mayer, *Syst. Appl. Microbiol.* **2000**, *23*, 185–190.
[38] E. L. Kannenberg, R. W. Carlson, *Mol. Microbiol.* **2001**, *39*, 379–391.
[39] L. A. Sharypova, K. Niehaus, H. Scheidle, O. Holst, A. Becker, *J. Biol. Chem.* **2003**, *278*, 12946–12954.
[40] G. P. Ferguson, A. Datta, R. W. Carlson, G. C. Walker, *Mol. Microbiol.* **2005**, *56*, 68–80.
[41] M. Vincent, *A Manual for the Practical Study of Root-Nodule Bacteria: International Biological Programme Handbook*, Blackwell, Oxford, **1970**.
[42] "Bacterial lipopolysaccharide. Extraction with phenol–water and further application of the procedure": O. Westphal, K. Jann in *Methods in Carbohydrate Chemistry, Vol. 5* (Ed.: R. L. Whistler), Academic Press, New York, **1965**, pp. 83–91.
[43] K. G. Johnson, M. B. Perry, *Can. J. Microbiol.* **1976**, *22*, 29–34.
[44] N. Azzouz, H. Shams-Eldin, R. T. Schwarz, *Anal. Biochem.* **2005**, *343*, 152–158.
[45] Y. Haishima, O. Holst, H. Brade, *Eur. J. Biochem.* **1992**, *203*, 127–134.
[46] E. T. Rietschel, H. Gottert, O. Lüderitz, O. Westphal, *Eur. J. Biochem.* **1972**, *28*, 166–173.
[47] S. Hase, E. T. Rietschel, *Eur. J. Biochem.* **1976**, *63*, 101–107.
[48] G. J. Gerwig, J. P. Kamerling, J. F. Vliegenthart, *Carbohydr. Res.* **1978**, *62*, 349–357.
[49] J. Krauss, U. Seydel, J. Weckesser, H. Mayer, *Eur. J. Biochem.* **1989**, *180*, 519–526.
[50] C. M. Tsai, C. E. Frasch, *Anal. Microbiol.* **1982**, *119*, 115–119.
[51] A. Kondakova, B. Lindner, *Eur. J. Mass Spectrom.* **2005**, *11*, 535–546.
[52] B. Domon, C. E. Costello, *Glycoconjugate* **1988**, *5*, 397–409.

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