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Note

Characterization of a novel lipid A structure isolated from *Azospirillum lipoferum* lipopolysaccharide

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Abstract—The structure of lipid A from *Azospirillum lipoferum*, a plant-growth-promoting rhizobacterium, was investigated. It was determined by chemical analysis, mass spectrometric methods, as well as 1D and 2D NMR spectroscopy. Because of the presence of substituents, the investigated lipid A differs from typical enterobacterial lipid A molecules. Its backbone is composed of a β-(1,6)-linked D-glucosamine disaccharide but lacks phosphate residues. Moreover, the reducing end of the backbone (position C-1) is substituted with α-linked D-galacturonic acid. 3-hydroxypalmitoyl residues are exclusively connected to amino groups of the glucosamine disaccharide. Hydroxyls at positions C-3 and C-3′ are esterified with 3-hydroxymyristic acids. Primary polar fatty acids are partially substituted by nonpolar fatty acids (namely, 18:0, 18:1 or 16:0), forming acyloxyacyl moieties.

Keywords: *Azospirillum lipoferum*; Lipid A structure; Glucosamine

Azospirillum is a genus grouping nitrogen-fixing bacteria closely associated with roots of many wild and cultivated grasses, cereals and food crops from many different regions of the world. Most of these bacteria colonize only the root surface and are not able to penetrate root tissues. They are known as plant-growth-promoting rhizobacteria.[1,2] So far, seven species belonging to this genus have been described, that is, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Azospirillum amazonense*, *Azospirillum halopraeferens*, *Azospirillum irakense*, *Azospirillum largimobile* and *Azospirillum doebereinerae*.[1,3,4] Two of them, *A. lipoferum* and *A. brasilense,* have been well characterized in terms of their genetics, biochemistry and some molecular mechanisms of their interaction with a host plant.[1] It is well known that surface polymers such as lipopolysaccharides and other glycoconjugates play an important role in this interaction.[1,5–10] Studies concerning the chemical characteristics of *A. lipoferum* and *A. brasilense* lipopolysaccharides have been performed by Choma and co-workers as well as by Fedonenko and co-workers.[11–13] In this work, we present a structural investigation of the lipid A backbone from *A. lipoferum* SpBr17, the reference strain deposited with the American Type Culture Collection (ATCC) under number 29709.

Lipid A was isolated from *A. lipoferum* LPS after mild acid hydrolysis. A preliminary chemical analysis of the lipid A preparation revealed that glucosamine, accompanied by galacturonic acid residues, was the only aminosugar present. The presence of GalA was unequivocally confirmed by GC–MS analysis of trimethylsilyl ethers of methyl glycoside methyl ester liberated from the lipid A by methanolysis (2 M HCl in methanol, 85 °C, 16 h). Both components were in the D-absolute configuration. Phosphate residues were not detected by means of ^{31}P NMR spectroscopy of the lipid A.

A fatty acid analysis of the lipid A revealed the presence of a complex set of fatty acids, both ester- and amide-bound. The fatty acid composition had been described earlier by Choma and co-workers.[11,12] It was confirmed that only 3-OH-16:0 is amide-bound. To determine this substitution, trimethysilylated, partly solvolyzed lipid A preparation was analyzed by

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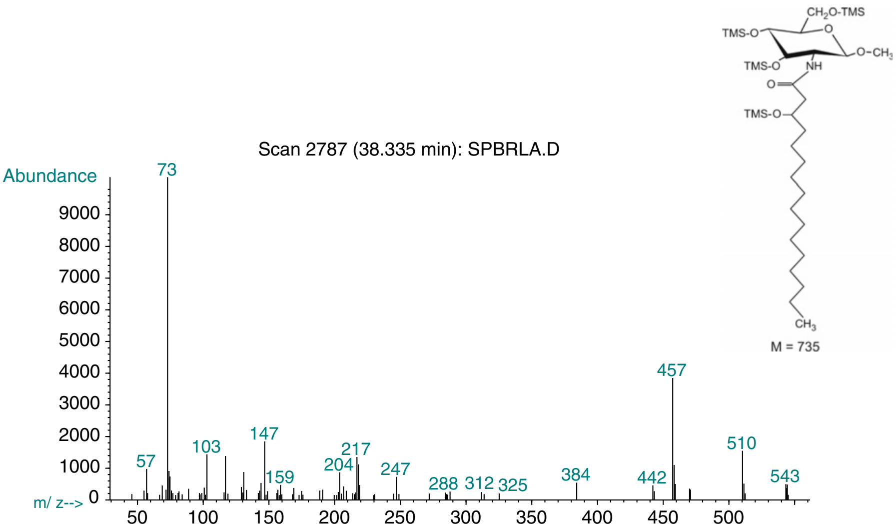

Figure 1. Mass spectrum of a trimethylsilyl derivative of the *N*-acylglucosamine methyl glycoside obtained from *A. lipoferum* lipid A by mild methanolysis and trimethylsilylation. Inset displays structure of the GlcN-(3-OH-16:0) moiety.

GC–MS. The spectrum of a TMS-derivative of *N*-β-hydroxypalmitylglucosamine methyl glycoside is shown in Figure 1. It is compatible with that obtained for *R. leguminosarum* bv. *phaseoli* lipid A.[14] The positions C-3 and C-3′ of glucosamine residues were esterified, and, therefore, the protons H-3 and H-3′ showed the typical downfield shift on the ^{1}H NMR spectrum (Table 2). When 0.25 M NaOH is used the ester-linked acyloxyacyl residues should be released, but this was not the case in our experiment. This procedure allowed us to liberate a large amount of 3-OH-14:0 and substantially less 18:0, 18:1 and 16:0 fatty acids. Acyloxyacyl moieties were isolated from LPS after applying the Kraska procedure, as described by Wollenweber and Rietschel.[15] On GC–MS chromatogram, two peaks with retention times of 39.9 min and 43.4 min were found. The mass spectrum of the first peak showed ions at *m*/*z* 269 and 239. The second peak spectrum contained indicative ions at *m*/*z* 269 and 267. The observed ions allowed us to deduce that 3-(O-16:0)-16:0 and 3-(O-18:0)-16:0 acids were liberated. Based on these experiments, we conclude that 3-OH-14:0 substituted the positions C-3 and C-3′ and the acyloxyacyl residues were bound to amino groups of GlcN.

Samples of intact, HF-treated and base-treated lipid A were investigated by means of MALDI-TOF mass spectrometry. Both positive and negative ion spectra were recorded.

The lipid A and the sample after HF-treatment yielded very similar mass spectra, therefore, only the intact lipid A spectrum is shown here (Fig. 2A). The results confirm the ^{31}P NMR data that the lipid A does not contain any phosphate residues. On the MALDI-TOF mass spectra of the O-deacylated lipid A, the only strong ion was observed at *m*/*z* 1023 $[M-H]^-$ (negative mode) or 1048 $[M+Na]^+$ (positive mode; Fig. 2B). This corresponds to a structure composed of glucosamine disaccharide with GalA and two 3-OH-16:0 residues (calculated mass, 1023.062 Da).

The positive ion MALDI-TOF mass spectrum of the nonmodified lipid A showed two oxonium ions at *m*/*z* 881 and 907, which were formed after laser-induced cleavage of the terminal glucosamine residue. Both of them were composed of one GlcN, one 3-OH-16:0, one 3-OH-14:0, and one nonpolar fatty acid (16:0 and 18:1, respectively). Based on the results described above, we concluded that there is no space for GalA residue in the distal part of the lipid A.

Both positive and negative ion spectra of the intact lipid A sample revealed the presence of three groups of pseudomolecular ions. The main signals $[M-H]^-$ were at *m*/*z* 1288, 1514 and 1741, which correspond to signals at *m*/*z* 1312, 1539 and 1765 on the positive mode spectrum ($[M+Na]^+$ ions) (Fig. 2A). Those signals represent lipid A molecules with a different pattern of O-acylation. The trisaccharide backbone (2 × GlcN + GalA) is N-acylated by two residues of the 3-OH-16:0 fatty acid and can be substituted by various sets of fatty acids (polar and/or nonpolar). The signal on the MALDI-TOF spectrum at *m*/*z* 1312 $[M+Na]^+$ corresponds to a molecule built up of 2 × GlcN, GalA, 2 × 3-OH-16:0 and an ester-linked 18:0 residue (calculated mass, 1290.48 Da). The second signal (at *m*/*z* 1539 $[M+Na]^+$) corresponds to the molecules containing 3-OH-14:0 and 16:0 moieties as ester-linked fatty acids (calculated mass, 1514.60 Da). The signal at *m*/*z* 1765 $[M+Na]^+$ originates from molecules containing two 3-OH-14:0 and one 18:1 fatty acids as ester substituents (calculated mass, 1740.72 Da). Minor fractions with Δ *m*/*z* 28 originate from molecules including one shorter acyl residue. All those signals represent molecules of lipid A containing one nonpolar fatty acid. As it was mentioned before,

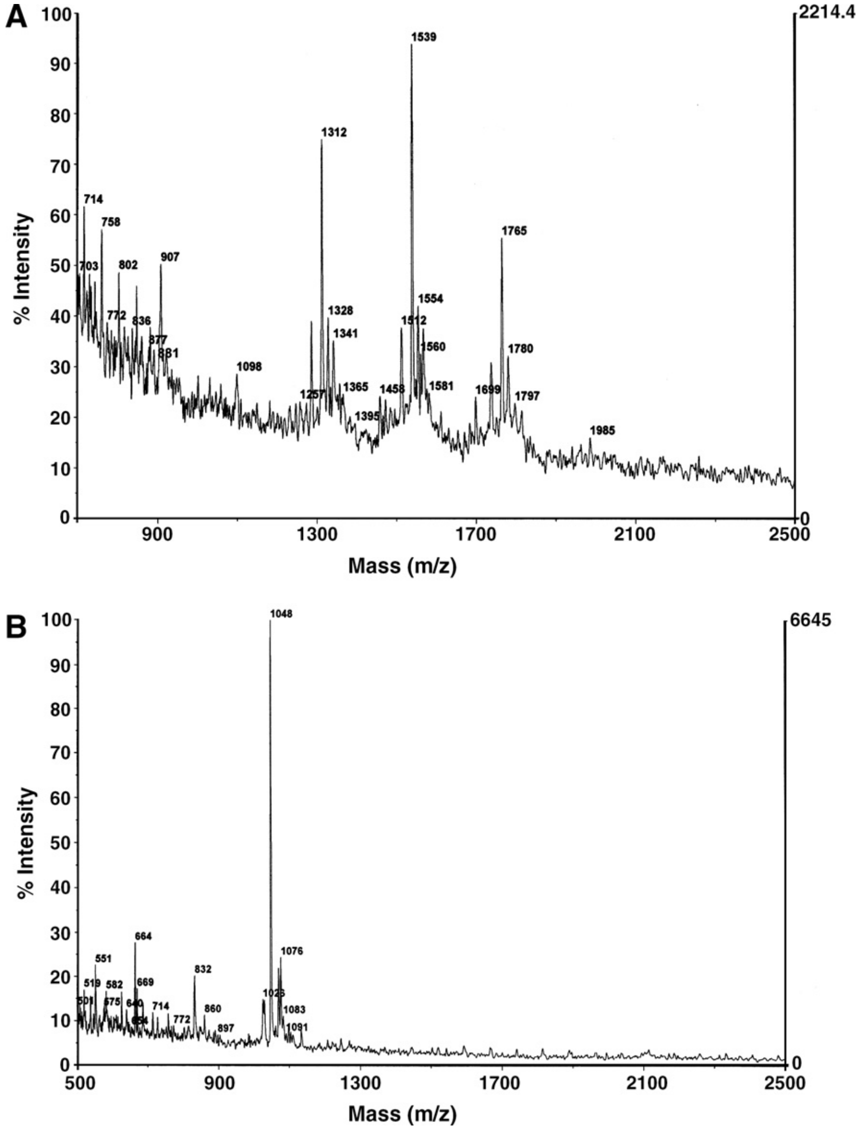

Figure 2. Positive ion MALDI-TOF mass spectra of an intact (A) and an O-deacylated (B) lipid A samples from *Azospirillum lipoferum* SpBr17.

nonpolar fatty residues are components of both B^+ ions and acyloxyacyl moieties. Taken together all those data suggest that acyloxyacyl residues are linked to amino group of terminal glucosamine. The respective ions, their proposed composition and theoretically calculated masses are listed in Table 1. The heaviest lipid A molecule includes five fatty acid residues, where one of them is a secondary acid from an acyloxyacyl moiety.

The proposed structure of the lipid A backbone was fully confirmed by ^{1}H NMR (DQF-COSY, TOCSY and NOESY) analysis, which allowed us to fix the configuration of linkages. Three signals were identified in the anomeric region (Fig. 3, Table 2). One of them was attributed to H-1 of β-linked hexose (**A**), and two of them to H-1 of α-linked residues (marked as **B** and **C**). The chemical shifts and $J_{\text{H-1,H-2}}$ coupling constants were as follows: **A**, 4.52 ppm (~9 Hz), **B**, 5.045 ppm (<3 Hz) and **C**, 5.16 ppm (<3 Hz). The remaining proton signals were found between 3.34 and 5.17 ppm (Table 2).

The sequence of monosaccharides was established by a NOESY experiment (Fig. 4). A strong interresidual NOE signal was observed between the protons H_C-1 and H_B-1, which confirmed the α-(1,1)-linkage between GalA and GlcN residues. The existence of a β-(1,6)-glycosidic linkage between both GlcN residues was proved by the H_A-1 and H_B-6 as well as H_A-1 and H_B-6′ NOE correlation signals. Taken together, these data allow us to present the full structure of the lipid A moiety of *A. lipoferum* (Fig. 5).

In the present study, we demonstrated that *A. lipoferum* forms a variant of a GlcN-type lipid A. The disaccharide backbone has no phosphate residues. The negative charge is provided by the presence of galacturonic acid—the third sugar component found in the backbone. Uptill now, GalA has been considered an

Table 1. Data from MALDI-TOF mass spectrometry of lipid A from *Azospirillum lipoferum* SpBr17 obtained for both, intact and O-deacylated lipid A samples

Ions (*m*/*z*)		Lipid A components				Proposed lipid A composition	Theoretically calculated masses (Da)
$[M-H]^-$	$[M+Na]^+$	GlcN	GalA	3-OH-fatty acids	Nonpolar fatty acids		
1023	1048	2	1	2	0	2 × GlcN + GalA 2 × 3-OH-16:0	1023.062
1250	1274	2	1	3	0	2 × GlcN + GalA 2 × 3-OH-16:0, 3-OH-14:0	1250.43
1262	—	2	1	2	1	2 × GlcN + GalA 2 × 3-OH-16:0, 16:0	1262.46
1288	1312	2	1	2	1	2 × GlcN + GalA 2 × 3-OH-16:0, 18:0	1290.48
1488	1513	2	1	3	1	2 × GlcN + GalA 2 × 3-OH-16:0, 3-OH-14:0, 16:0	1488.58
1515	1539	2	1	3	1	2 × GlcN + GalA 2 × 3-OH-16:0, 3-OH-14:0, 16:0	1514.60
1715	1739	2	1	4	1	2 × GlcN + GalA 2 × 3-OH-16:0, 2 × 3-OH-14:0, 16:0	1714.70
1741	1765	2	1	4	1	2 × GlcN + GalA 2 × 3-OH-16:0, 2 × 3-OH-14:0, 18:1	1740.72
1756	1779	2	1	3	2	2 × GlcN + GalA 2 × 3-OH-16:0, 3-OH-14:0, 16:0, 18:0	1756.77
B_1^+	881	1	0	2	1	GlcN, 3-OH-16:0, 3-OH-14:0, 16:0	881.43
B_1^+	907	1	0	2	1	GlcN, 3-OH-16:0, 3-OH-14:0, 18:1	906.45

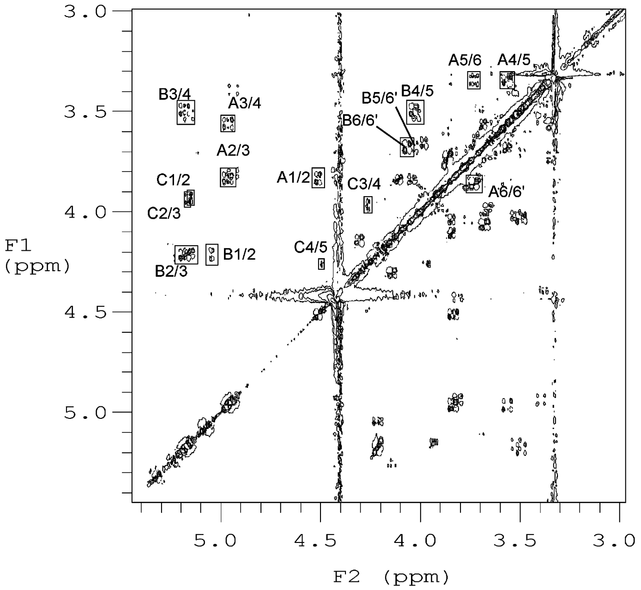

Figure 3. A partial DQF-COSY spectrum of *A. lipoferum* lipid A. The sample was dissolved in $CDCl_3/CD_3OD$ (2:1, v/v). The spectrum was recorded at 500 MHz, at 48 °C. The letters refer to the carbohydrate residues as shown in Table 2 and Figure 5. The numerals refer to the protons in the respective residues.

unusual constituent in lipid A molecules. It has been found only in several strains of bacteria, for example, in the hyperthermophilic bacterium *Aquifex pyrofilus* and the symbiotic bacteria *Rhizobium leguminosarum*, *Rhizobium etli* and *Mesorhizobium huakuii*.[14,16–18] In the cases of *Aquifex* and *Mesorhizobium,* galacturonic acid was a component of the DAG-type lipid A. Moreover, in the lipid A of *Aquifex pyrofilus*, two residues of GalA were detected. The first was linked to C-4′ of distal DAG (2,3-diamino-2,3-dideoxy-D-glucose), and the second to the C-1 of the reducing DAG molecule, forming a tetrasaccharide backbone. ^{1}H NMR analysis confirmed beyond any doubt that GalA from *A. lipoferum* lipid A was linked only to the proximal GlcN residue by an α-(1,1)-glycosidic bond. The position of GalA was the same as in *M. huakuii*.[18]

The biophysical properties of the outer membrane and the endotoxic activity of LPS molecules are strongly correlated with the presence of negative charge phosphate-containing residues.[19] Decrease of biological activity is attributed to lower binding affinity to LPS receptors of human cells caused by an absence of phosphate groups.[19,20] Lipid A from *A. lipoferum* lacks phosphate residues, which are characteristic for enterobacterial lipid A. Therefore, it is possible that *Azospirillum* lipid A is less toxic for eukaryotic cells.

1. Experimental

1.1. Bacteria cultivation, LPS and lipid A isolation

A. lipoferum strain SpBr17 (ATCC 29709) was obtained from D. Kulińska's collection, Warsaw Agricultural

Table 2. 500 MHz ^{1}H NMR data of the sugar backbone of lipid A of *Azospirillum lipoferum* SpBr17 (δ in ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
β-GlcN (A)	4.52	3.84	4.97	3.57	3.34	3.57	3.69
α-GlcN (B)	5.045	4.22	5.17	3.52	4.025	3.68	4.07
α-GalA (C)	5.16	3.93	3.97	4.27	4.48	—	—

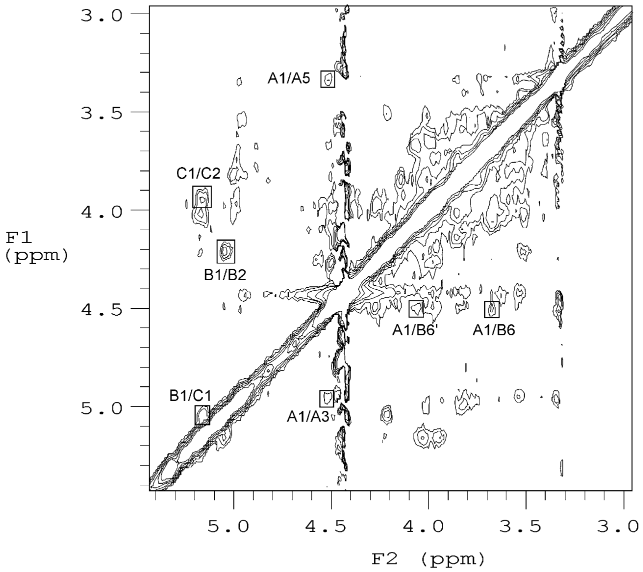

Figure 4. A partial NOESY spectrum of *A. lipoferum* lipid A. The cross-peaks: B1/C1, A1/B6 and A1/B6′, shown NOE signals (intermolecular) are important for structural determination. The cross-peaks: A1/A3, A1/A5, B1/B2 and C1/C2, represent NOE signals (intramolecular) are important for the determination of anomeric configurations of residues A, B, C.

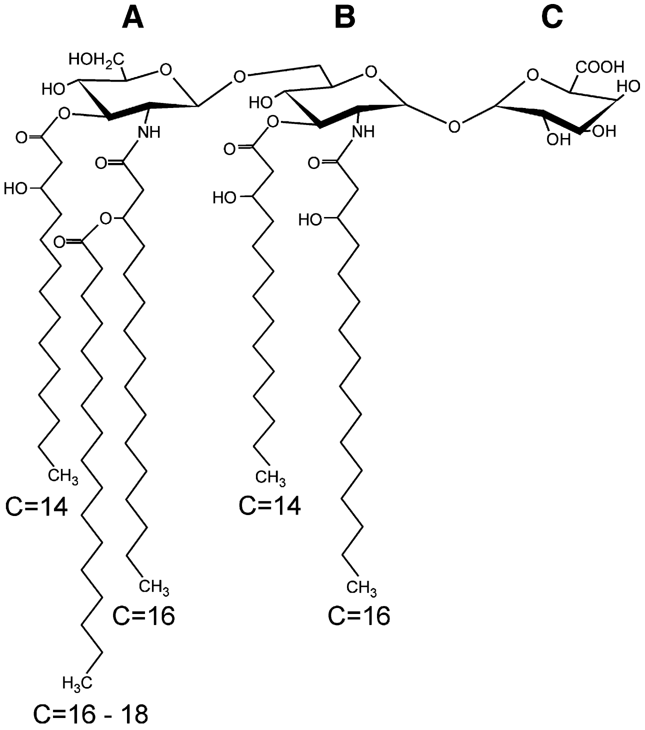

Figure 5. The proposed structure of *Azospirillum lipoferum* SpBr17 pentaacyl lipid A.

University (Warsaw, Poland). This strain was originally isolated by Döbereiner.[21] Bacteria were grown to the early stationary phase of growth at 28 °C in a sodium lactate-containing medium, as previously described by Choma et al.[11] Cells were harvested by centrifugation, washed twice with saline and once with distilled water. To remove the free lipids and membrane phospholipids, the cell pellet was subjected to the Bligh–Dyer delipidation procedure as modified by Que and co-workers.[16] After incubation for 2 h at room temperature with intensive stirring, the mixture was centrifuged at 10,000*g* for 20 min. The delipidated bacterial mass was washed twice with a freshly prepared Bligh–Dyer mixture.

LPS was recovered from the delipidated cells by the classic hot phenol–water method.[22] The water layer containing LPS was dialyzed against tap and distilled water. The crude material was obtained by twofold ultracentrifugation of the water suspension at 105,000*g* for 4 h.

Lipid A was precipitated by hydrolysis of a 150 mg portion of LPS with 1% acetic acid (100 °C, 2 h). The sediment was washed twice with hot water and additionally purified by mixing with two-phase Bligh–Dyer mixture of chloroform, methanol and water (2:2:1.8, v/v/v). The phases were intensively stirred and then separated by centrifugation at 4500*g* (15 min, room temperature). The lower phase containing lipid A was collected and the upper phase was re-extracted by adding another portion of the lower phase from a freshly prepared two-phase Bligh–Dyer mixture. Combined chloroform phases were evaporated to dryness. The preparation of lipid A was stored at −20 °C in $CHCl_3$/MeOH (3:1, v/v).

1.2. Composition analysis

To analyze total fatty acids and sugars, lipid A was subjected to overnight methanolysis (2 M HCl/MeOH, 85 °C, 18 h). Mild methanolysis (1 M HCl/MeOH, 60 °C, 2 h) was performed to liberate ester-linked fatty residues. After cooling, one drop of *tert*-butyl was added, and the sample was vigorously shaken. The reagents were evaporated in a stream of nitrogen. Next, N-acetylation and/or trimethylsilylation was performed.[16] The amide-linked fatty acids were also determined by the preparation and analysis of TMS *N*-acylglucosamine methyl glycosides as described earlier by Bhat and co-workers.[14] Acyloxyacyl residues were liberated according to procedures described by Wollenweber and co-workers.[15,23] For the determination of sugar absolute configurations the methanolyzed (2 M HCl/MeOH, 85 °C, 18 h) lipid A preparation was resuspended in 1 M (*R*)-(−)-2-butanolic HCl and heated (100 °C, 3 h). Sample was N-acetylated, trimethylsilylated and analyzed using GC–MS.[24,25]

1.3. Lipid A modification

Lipid A was subjected to a solvolysis procedure in 48% (v/v) aqueous HF. After incubation at 4 °C for 48 h, the reagent was evaporated in a stream of nitrogen with cooling in an ice bath.[26] Lipid A was O-deacetylated using a two-phase mixture containing methanol/chloroform/1.7 M NaOH (1:2:0.4, v/v/v). Two hour incubation at room temperature with vigorous shaking was performed.[16] The material was centrifuged at 4500*g* for 20 min, and the lower phases containing O-deacylated lipid A were collected and dried under a stream of nitrogen.

1.4. Mass spectrometry

GC–MS (gas chromatography–mass spectrometry) was carried out on a Hewlett–Packard gas chromatograph (HP 5890A) connected to a mass selective detector (MSD HP 5971). The chromatograph was equipped with a capillary column HP-5MS (30 m × 0.25 mm). The carrier gas was helium with the flow rate 0.7 mL/min. The temperature programme for trimethylsilylo derivatives of methyl glycosides and fatty acids was as follows: initially, 80 °C for 2 min, then raised to 310 °C at a ramp rate of 4 °C/min and final time 5 min. The temperature programme for alditol acetates and fatty acid methyl esters was initially 150 °C for 5 min, then raised to 310 °C at a ramp rate of 5 °C/min and final time 20 min.

MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry was performed on a Voyager-Elite (PE Biosystems) instrument. Intact and modified lipid A samples were dissolved in $CHCl_3$/MeOH (2:1, v/v) and mixed with 50% (v/v) 2,5-dihydrobenzoic acid (gentisic acid, DHB) in acetonitrile as a matrix, and dried off. The samples were desorbed with a nitrogen laser ($\lambda = 337$ nm) with extraction voltage of 20 kV. Spectra were obtained as the average of 256 laser shots.

1.5. NMR spectroscopy

^{1}H NMR experiments were performed on a Varian Unity plus 500 instrument at 48 °C using standard Varian software 1D as well as 2D ^{1}H NMR (DQF-COSY, TOCSY and NOESY) were carried out. ^{31}P NMR spectrum was recorded at 40 °C with Bruker Avance 300 spectrometer. An about-5-mg lipid A sample was dissolved in 0.6 ml of $CDCl_3/CD_3OD$ (2:1, v/v) mixture.

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