The Structure of Lipid A of the Lipopolysaccharide from *Burkholderia caryophylli* with a 4-Amino-4-deoxy-L-arabinopyranose 1-Phosphate Residue Exclusively in Glycosidic Linkage

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Abstract: From the lipopolysaccharides (LPSs) of the plant-pathogenic bacterium *Burkholderia caryophylli*, the complete structure of lipid A has been characterized. For the first time, a 4-amino-4-deoxy-L-arabinopyranose 1-phosphate residue was proven to be exclusively linked to the reducing end of lipid A from a wild-type LPS. The LPSs of *B. caryophylli* were degraded by mild acetate buffer hydrolysis at pH 4.4. The obtained lipid A was analyzed as such, and also after de-O-acylation or dephosphorylation. The structure of lipid A was identified mainly by means of matrix-assisted laser desorption/ionisation mass spectrometry, and by various 1D and 2D ^1H and ^{13}C NMR spectroscopic measurements.

Keywords: lipid A · lipopolysaccharides · mass spectrometry · NMR spectroscopy

Introduction

Burkholderia caryophylli (*Pseudomonadaceae*, RNA group II)[1] is a phytopathogenic gram-negative bacterium that is responsible for the wilting of carnation.[2] One characteristic feature of its lipopolysaccharides (LPSs), consistent with several others from bacteria of the genus *Burkholderia*, is the occurrence of two different O-specific polysaccharides. In the case of LPSs from *B. caryophylli*, two linear homopolysaccharides were identified as O-specific polysaccharides, that is, caryophyllan, which is furnished from α-1 $\rightarrow$ 7-linked caryophyllose, and caryan, which is built up from β-1 $\rightarrow$ 7-linked caryose units.[3–6] The caryan is acetylated in a nonstoichiometric but systematic pattern, which leads to the establishment of repeating units in a homopolymer,[7] while only the side chain of the caryophyllan is randomly acetylated, and no chemical repeating unit was possible to define.[8] The core region of the LPSs contains a structural element that commonly occurs in the *Salmonella* type core regions of enterobacterial LPS,[9] namely α-D-Glc*p*-(1 $\rightarrow$ 3)-[L-α-D-Hep*p*-(1 $\rightarrow$ 7)-]-L-α-D-Hep*p*-(1 $\rightarrow$ 3)-L-α-D-Hep*p*-(1 $\rightarrow$ 5)-[α-Kdo-2 $\rightarrow$ 4)]-α-Kdo-(2 $\rightarrow$).[10] The Glc*p* residue of this moiety is substituted by two hexoses, that is, Glc*p* and Gal*p*. The core region from LPSs of *B. caryophylli* is free of phosphate and contains a Glc*p* residue that is β-(1 $\rightarrow$ 4)-linked to the first Hep residue and which is substituted at O6 by another α-Glc*p* residue. Most strikingly, and identified for the first time, the core region of *B. caryophylli* LPSs possesses two L-α-D-Hep*p*-(1 $\rightarrow$ 5)-α-Kdo-2 $\rightarrow$ moieties, one of which is linked to lipid A, and the other to the branching Kdo residue.

In LPSs, the O-specific polysaccharide is linked to the core region, which in turn is bound to the lipid A.[11] Endotoxic active lipid A, for example, enterobacterial lipid A,[12, 13] is known to possess a rather conserved structure that is characterized by a β-(1 $\rightarrow$ 6)-linked 2-amino-2-deoxy-D-glucopyranose (D-Glc*p*N) disaccharide backbone with phosphate groups attached to O1 and O4′. (*R*)-3-Hydroxy fatty acids and (*R*)-3-acyloxyacyl residues are linked at positions 2 and 3, and 2′ and 3′, respectively. In many LPSs, lipid A is further substituted by compounds that reduce its net negative charge, for example, by 2-aminoethanol phosphate (PE) and 4-amino-4-deoxy-L-arabinopyranose (L-Ara*p*4N).[14, 15] The latter constituent was in most of these cases identified to substitute the phosphate residue at O4′ (e.g., in LPS of *Proteus mirabilis*,

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Salmonella enterica). There is good evidence that this particular substitution, together with extensive esterification of diphosphates in LPS by 2-aminoethanol, renders gram-negative bacteria resistant against the antibiotic polymyxin B.[14, 15] To date, only lipid A from LPS of a polymyxin-resistant mutant of *Klebsiella pneumoniae* O3 was shown to possess two L-Ara*p*4N residues, one substituting the glycosidic and the other the 4′-phosphate groups.[16] It is thought that the glycosidic L-Ara*p*4N substitution of lipid A does not contribute to resistance against polymyxin B. Two lipid A precursors were identified in 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo) deficient mutants of *Salmonella enterica* sv. Typhimurium, namely, precursors I_A and II_A (both are tetraacylated D-Glc*p*N disaccharides that differ in the phosphate substitution), representing the sole lipid A species known with only one L-Ara*p*4N residue exclusively substituting the glycosidic phosphate group.[17, 18] In this study we report the first case of a wild-type lipid A in which such a substitution pattern occurs.

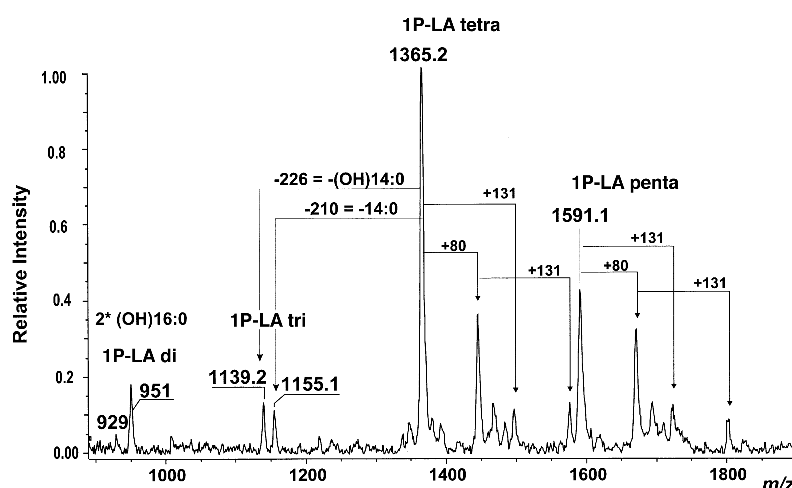

Figure 1. Negative ion MALDI mass spectrum of the lipid A of LPSs from *B. caryophylli*.

Results

Isolation and compositional analysis of lipid A: Lipid A was obtained by mild hydrolysis of the LPSs in a yield of 5% of LPSs dry mass. Compositional analysis revealed the presence of D-GlcN, a 4-amino-4-deoxy-pentose, and phosphate. The 4-amino-4-deoxy-pentose was identified as 4-amino-4-deoxy-L-arabinose (Ara4N) by comparison of its acetylated *O*-methyl and *O*-octyl glycosides with those of the authentic compound isolated from LPSs of *Burkholderia cepacia*.[19]

Fatty acid analysis revealed the presence of (*R*)-3-hydroxyhexadecanoic (16:0(3OH)) (amide-linked), (*R*)-3-hydroxytetradecanoic (14:0(3OH)), and tetradecanoic acid (14:0) (ester-linked) in molar ratios of ~2.0:1.6:1.0.

Analysis of lipid A: The negative ion MALDI-TOF mass spectrum of the isolated native lipid A (Figure 1) composed of a complex pattern of molecular ion peaks, indicating that the isolated lipid A consists of a heterogeneous mixture. In accordance with results obtained from the compositional analysis and NMR measurements (see below), the mass peaks could be assigned in a straight forward manner. There are three series of ion peaks starting at *m/z* 1139.2, 1365.2, and 1591.1, representing tri-, tetra-, and pentaacylated lipid A species respectively. The ion at *m/z* 1591.4 is consistent with a monophosphorylated diglucosamine backbone possessing two amide-linked 16:0(3-OH), two ester-linked 14:0(3-OH), and one 14:0 residuess (calculated average mass 1591.4 Da). The prominent tetraacylated species lack one and the low abundant triacetylated species two 14:0(3-OH) fatty acid residues ($\Delta m/z$ 226 and 452, respectively). The differently acylated series exhibited the same heterogeneity with respect to the nonstoichiometric substitution with phosphate ($\Delta m/z$ +80) and/or Ara4N ($\Delta m/z$ +131).

Analysis of de-O-acylated lipid A: The amide-bound acyloxyacyl group was inferred from MALDI-TOF MS of the partial de-O-acylated product obtained from treatment with NH_4OH. It was found that this mild procedure selectively splits acyl and acyloxyacyl esters, leaving the acyl and acyloxyacyl amides unaffected.[20] Analysis of the product by MALDI-TOF (Figure 2) revealed ions corresponding to

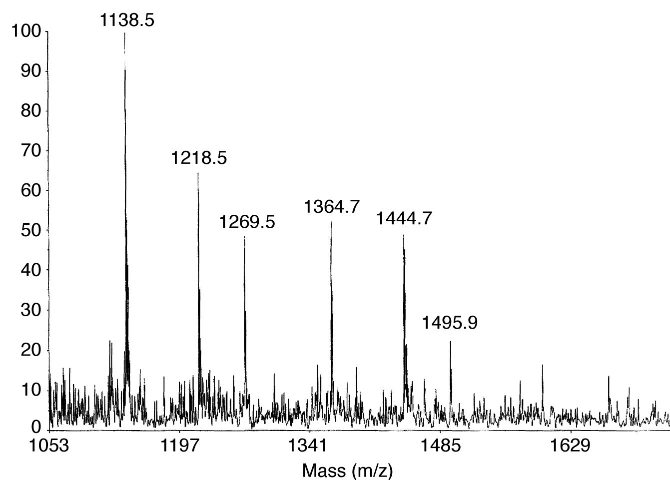

Figure 2. Negative ion MALDI mass spectrum of de-O-acylated lipid A of LPSs from *B. caryophylli*.

triacyl species at *m/z* 1138.5, 1218.5, 1269.5, and ions corresponding to tetraacyl species at *m/z* 1364.7, 1444.7, and 1495.9. The ions at *m/z* 1138.5, 1218.5, and 1269.6 were of particular significance. The ion at *m/z* 1218.5 was consistent with a bis-phosphorylated lipid A species possessing two amide-linked 16:0(3-OH) residues, one of which was esterified by a 14:0 residue. The other two ions both lacked a phosphate residue; moreover, the one at *m/z* 1269.5 carried an

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L-Ara4N residue ($\Delta m/z$ +131). The ion at *m/z* 1444.7 was assigned to a lipid A species possessing two amide-linked 16:0(3-OH) residues, one of which was esterified by a 14:0 residue, one ester-linked 14:0(3-OH) residue and two phosphate residues. The ions at *m/z* 1364.7 and 1495.9 both lacked one phosphate, and the second one carried an L-Ara4N residue ($\Delta m/z$ +131).

Analysis of dephosphorylated lipid A: The distribution of fatty acids in lipid A was determined by analysis of the dephosphorylated lipid A. An aliquot of lipid A was treated with 48% aqueous HF, and the product obtained was analyzed by mass in the positive ion mode. The spectrum (Figure 3) shows prominent sodium and potassium adduct ions of two molecular species at *m/z* 1551.4, 1535.4, and *m/z* 1325.2, 1309.0, which can be attributed to penta- and tetraacyl species, respectively. The absence of ions with $\Delta m/z$ +131 indicated that the L-Ara4N residue must have been attached through a phosphate group. Furthermore, the spectrum comprises laser-induced in-source fragment ions resulting from the cleavage of the glycosidic linkage of the disaccharide backbone. The formation of oxonium ions (*m/z* 853.901 and 623.402) is exclusively observed from nonreducing distal GlcN.[21] Thus, the oxonium ion at *m/z* 853.9 was consistent with a lipid A molecule containing one residue each of 14:0(3-OH), 16:0(3-OH), and 14:0. The presence of the 14:0 residue proved that the nonreducing GlcN carried an acyloxyacyl group. The oxonium ion at *m/z* 623.4 was consistent with a lipid A molecule that carried one 16:0(3-OH) and one 14:0 residue, confirming that the 14:0 residue was esterifying the 3-hydroxy group of 16:0(3-OH) residue.

NMR spectroscopy of lipid A: As lipid A was well soluble in Me_2SO, it was possible to analyze it in $[D_6]Me_2SO$ by NMR spectroscopy. The ^1H and ^{13}C chemical shift assignments (Table 1) are based on two-dimensional correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame nuclear Overhauser effect spectroscopy (ROESY), and ^1H-^{13}C and ^1H-^{31}P heteronuclear single quantum coherence (HSQC) experiments. The determination of chemical shifts and coupling constants (characteristic of a *gluco*-configuration) revealed that both GlcN residues of the lipid A backbone were pyranoses in $^4C_1$ conformation. Starting from the anomeric and/or amide proton signals in the COSY and TOCSY spectra, it was possible to assign the chemical shifts of each sugar residue. In the anomeric region of the ^1HNMR spectrum (Figure 4), three signals at δ 5.41, 5.33, and 4.72 ppm were attributed to H1 of β-L-Ara4N **A**, α-GlcN **B**, and β-GlcN **C**, respectively. The signals of the remaining ring protons were in the region of $\delta = 3.1-5.0$ ppm (Figure 5). Most chemical shifts of the fatty acids were between $\delta = 0.8$ and 2.4 ppm. The H3 protons of 16:0(3-O-(14:0)), and 16:0(3-OH)/14:0(3-OH) were at δ5.04 and 3.80, respectively. In agreement with the results of other experiments, the lipid A was found to be composed of three sugars substituted with fatty acids.

The ^{13}CNMR spectrum was assigned by a ^1H-^{13}C HSQC experiment (Figure 5, Table 1). Three

Table 1. ^1H and ^{13}C NMR chemical shifts [ppm] of sugar residues of lipid A of LPS from *B. caryophylli* NCPP 2151.[a]

Unit	Chemical shift δ ($^1H/^{13}C$) 1	2	3	4	5	6
A, β-	5.41	3.55	3.80	3.26	4.01	
Ara*p*4N	(95.5)	(69.0)	(74.0)	(54.0)	(n. d.)	
B, α	5.33	3.90	4.98	3.56	4.06	3.66/3.83
Glc*p*N	(93.9)	(52.2)	(73.9)	(69.6)	(71.1)	(67.3)
C, β-	4.72	3.68	3.79	4.04	3.17	3.55/3.80
Glc*p*N	(99.0)	(54.1)	(68.0)	(71.0)	(77.0)	(60.7)

[a] The spectra were recorded at 400 MHz (^1H) and 100 MHz (^{13}C) in $[D_6]Me_2SO$ at 60 °C. The ^1H NMR data are given for each unit in the first row, the ^{13}C NMR data in the second (in parentheses). Chemical shifts are expressed relative to Me_2SO (at 60 °C), δ_H 2.49, δ_C 39.7. Characteristic chemical shifts for the 3-CHO- of 3-hydroxy fatty acids were at ($^1H/^{13}C$) $\delta = 5.04/71.1$ {of 16:0[3-O(14:0)]} and at 3.80/68.5 ppm [of 16:0(3-OH)/14:0(3-OH)].

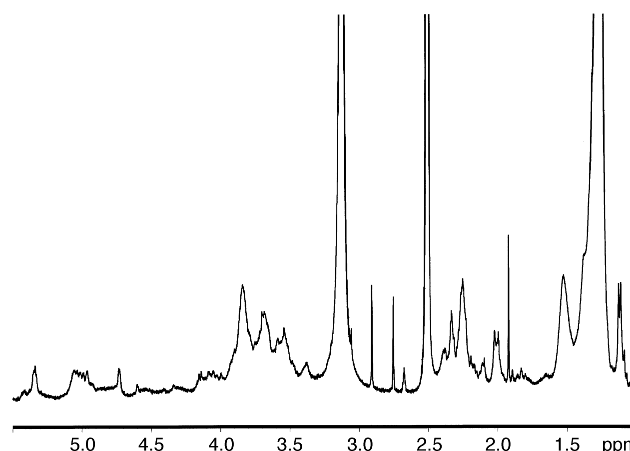

Figure 4. The 1D ^1H NMR spectrum of lipid A of LPSs from *B. caryophylli*. The spectrum was recorded in $[D_6]Me_2SO$ at 400 MHz and 60 °C.

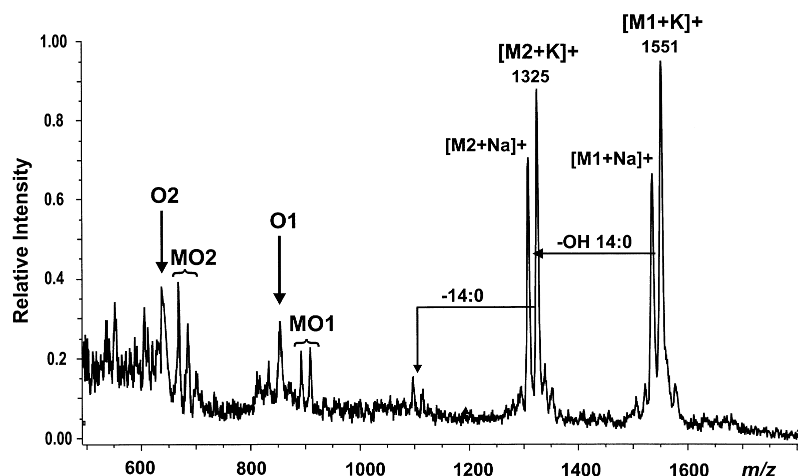

Figure 3. Positive ion MALDI mass spectrum of dephosphorylated lipid A of LPSs from *B. caryophylli*.

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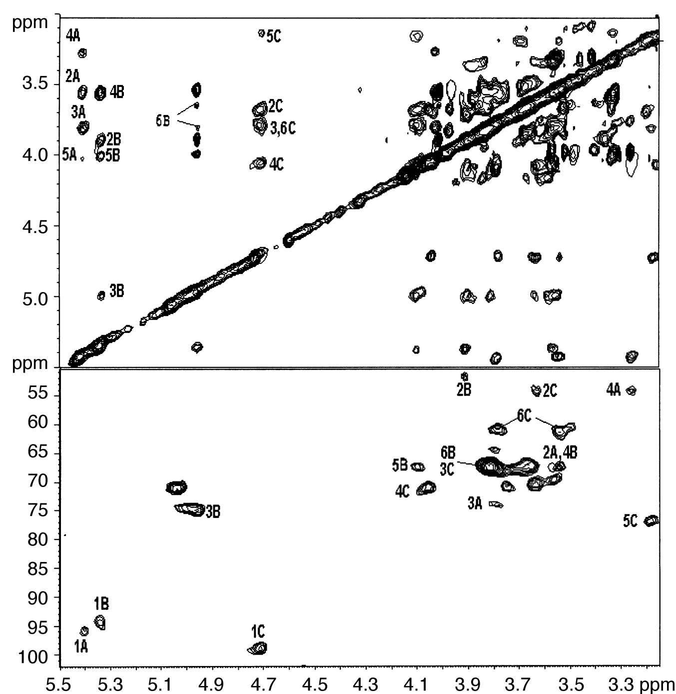

Figure 5. Sections of the TOCSY (top) and ^{1}H-^{13}C HSQC (bottom) spectra of lipid A of LPSs from *B. caryophylli*. Both spectra were recorded in $[D_6]Me_2SO$ at 400 MHz and 60 °C. The *letters* refer to the carbohydrate residues, and the *arabic numerals* to the protons/carbons in the respective residues.

resonances (of the two GlcN and the Ara4N) were identified in the anomeric region, that is, at $\delta = 95.5$ (**A**), 93.9 (**B**), and 99.0 ppm(**C**). Of the ring sugar signals in the region $\delta = 50$—77 ppm, those of **A**4, **B**2 and **C**2 at $\delta = 3.26$, 3.90, 3.68 ppm respectively, correlated to signals at $\delta = 54.0$, 52.2, and 54.1 ppm, respectively, and thus the carbon atoms that were substituted by amino groups were identified. The proton resonance of B3 was shifted downfield to $\delta = 4.98$ ppm, which is typical for protons located geminal to an *O*-acylated carbon. An alternative resonance for the proton resonance of **C**3′ was identified at $\delta = 3.79$ ppm, proving that O3 of this residue was not always acylated and thus confirming the partial lack of 14:0(3-OH) in this position as revealed by MALDI-TOF-MS. The **B**6 resonance at $\delta = 67.3$ ppm indicated the substitution of O6 of GlcN I. A cross peak between the resonances $\delta = 71.1$ ppm and 5.04 ppm identified H3 of the 16:0(3-OH) moiety of 16:0(3-O-(14:0)). In the TOCSY and COSY spectra, the signal at 5.04 ppm correlated with the two diastereotopic α-methylene protons at $\delta = 2.32$ and 2.25 ppm (H2a and H2b), and with the γ-methylene protons of the acyl chain at $\delta = 1.47$ ppm. In the HSQC spectrum, the cross-peak between the signals at $\delta = 68.5$ and 3.80 ppm was attributed to H3 of the 14:0(3-OH) and 16:0(3-OH) residues, and this signal correlated with the α-methylene protons at $\delta = 2.27$ ppm, and the γ-methylene protons at $\delta = 1.46$ ppm of the acyl chains.

The ROESY spectrum established the sequence of the two GlcN residues. The proton **C**1 gave strong intraresidual ROE contacts to protons **C**3 and **C**5, validating the β-configuration of GlcN **C**. In addition, **C**1 possessed a strong interresidual ROE contact to proton **B**6a, and weak ROE contacts to protons **B**4 and **B**6b, establishing the β-1,6 linkage between the GlcN residues **C** and **B**. The ROE contacts found in the spectrum were in agreement with the assumption that a rigid glycosidic bond was present in the disaccharide, thus allowing the fatty acid chains to be parallel; this results in a highly densely packed conformation.[22]

^{1}H-^{31}P HSQC and 1D ROESY experiments were applied to establish the positions of the phosphate and Ara4N residues. Three cross peaks were observed in the ^{1}H-^{31}P HSQC spectrum (Figure 6), one of which was a weak signal in F1

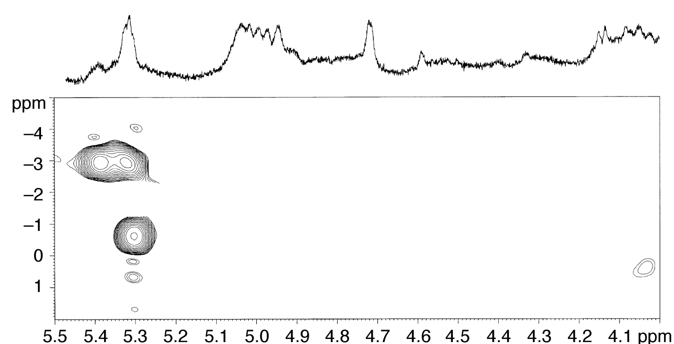

Figure 6. Section of the ^{1}H-^{31}P HSQC spectrum.

dimension at $\delta = 0.4$ ppm, representative of a monophosphate monoester group and correlated with a proton resonance at $\delta = 4.04$ ppm which was identified as proton **C**4. Thus, if present, one phosphate group was linked to O4 of GlcN **C**. The other two cross-peaks correlated with resonances in the anomeric region in F2 dimension. The first of these, at $\delta = -0.58$ ppm, correlated with the proton **B**1 and, thus, indicated a second phosphate group α-linked to C1 of **B**. The other, at $\delta = -2.9$ ppm, showed a double correlation of the ^{31}P resonance with two protons at $\delta = 5.33$ ppm (proton **B**1) and $\delta = 5.41$ ppm (proton **A**1). This result established that the Ara4N residue, when present, was linked to the anomeric position of GlcN **B** by a phosphodiester. Additional evidence for that was provided by the selective 1D ROESY spectrum (Figure 7) in which a selective excitation of proton **A**1

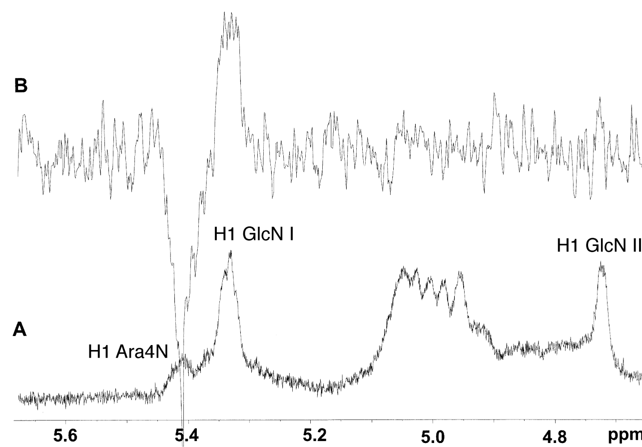

Figure 7. Section of the 1D ROESY spectrum. A selective excitation of proton **A**1 resulted in an increased intensity of the **B**1 proton resonance, proving an intraresidual NOE contact between these protons.

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resulted in an increased intensity of the **B**1 proton resonance, proving an intraresidual NOE contact, and, thus, a spatial proximity between these protons.

On the basis of these data, the weak intensity of the monophosphate resonance that correlated with the proton **C**4 resonance at $\delta = 4.04$ ppm can be explained as a result of a nonstoichiometric substitution of this position by phosphate. This interpretation is in agreement with the lipid A species isolated from TLC, which lacked one phosphate group, but still contained Ara4N (see Figure 1).

Taken together, the lipid A fraction isolated from LPS of *B. caryophylli* consisted of molecular species with structures depicted in Figure 8.

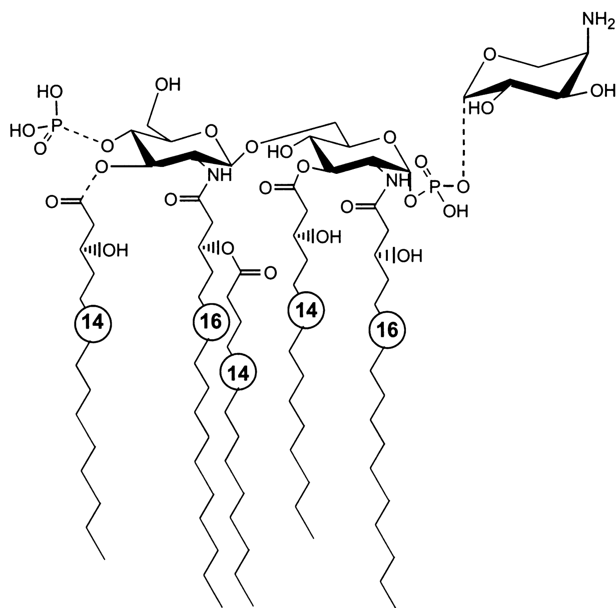

Figure 8. The structure of isolated lipid A of LPSs from *B. caryophylli.* Dotted lines indicate nonstoichiometric substitutions.

Discussion

For the first time, a mixture of lipid A components was isolated from a wild type LPS that possesses (in nonstoichiometric amounts) one L-Ara*p*4N residue that is exclusively linked by its phosphate group to the reducing end of the glucosamine disaccharide backbone. Although the analysis of the presence of GlcN, phosphate, and L-Ara*p*4N in the LPSs (data not shown) suggested that the last constituent was present in nonstoichiometric amounts, it is likely that another portion of this substituent was lost during the mild acetic acid hydrolysis necessary to isolate the lipid A moiety, since the linkage of L-Ara*p*4N is expected to be quite sensitive to acidic conditions. Earlier, the two lipid A precursors I_A and II_A had been identified in Kdo-deficient mutants of *Salmonella enterica* sv. Typhimurium, both of which possessed only one L-Ara*p*4N residue substituted exclusively by the glycosidic phosphate group.[17, 18]

MALDI-MS experiments revealed that the structure (Figure 8) of the carbohydrate backbone β-D-Glc*p*N-(1 $\rightarrow$ 6)-α-D-Glc*p*N-1 $\rightarrow$ P $\leftarrow$ 1-β-L-Ara*p*4N was substituted by two amide-linked (*R*)-16:0(3-OH) fatty acids, of which one at the nonreducing GlcN (residue **C**) was further substituted at its 3-hydroxy group by a 14:0 residue. Furthermore, two unsubstituted ester linked (*R*)-14:0(3-OH) fatty acids were present at positions O3 of both GlcN **B** and **C**, and a monophosphate residue was linked to O4 of **C**. To date, amide-linked (*R*)-16:0(3-OH) was identified only in few other lipid A, for example, LPSs from *Helicobacter pylori*, *Rhodospirillum fulvum*, and *Porphyromonas fragilis*.[13]

Lipid A is considered as a valuable chemotaxonomic and phylogenetic marker. *Burkholderia caryophylli* is a phytopathogenic gram-negative bacterium that had earlier been included in the genus *Pseudomonas*. Application of ribosomal RNA (rRNA) similarity studies showed that this original genus *Pseudomonas* is diverse and contains five distantly related groups, of which, the new genus *Burkholderia* was classified to RNA group II. The comparison of the structures lipid A from LPSs of *B. caryophylli* and *Ps. aeruginosa*, the latter of which possesses amide-linked (*R*)-12:0(3-OH) and differs in fatty acid composition in general, and does not possess L-Ara*p*4N at all,[13] confirms such separation.

The biosynthesis of the attachment of L-Ara*p*4N to lipid A in LPSs of *Salmonella enterica* and *Escherichia coli* has been elucidated only recently.[23, 24] An inner membrane enzyme (ArnT) was identified to be expressed in polymyxin-resistant mutants that add one or two L-Ara*p*4N residue to lipid A or its precursors. In *E. coli*, biosynthesis of the core region begins with the attachment of two Kdo residues to precursor IV_A, the tetraacylated, and bisphosphorylated GlcN-disaccharide, which is performed by one Kdo-transferase WaaA (KdtA), and which results in Kdo_2-lipid IV_A.[25–27] Completion of lipid A employs two additional acylation steps of Kdo_2-lipid IV_A, and then the Kdo residue that is attached to lipid A is substituted at O5 by L,D-Hep*p*, followed by further steps of core biosynthesis. Most interestingly, ArnT adds two L-Ara*p*4N residues to Kdo_2-lipid IV_A, and to Kdo_2-lipid A (Re-LPS).[23] To precursor IV_A it adds only one L-Ara*p*4N residue to the glycosidic phosphate group, a finding which explains very well why lipid A precursors of Kdo-deficient mutant strains exclusively possess L-Ara*p*4N at the glycosidic phosphate. Although the biosynthesis of the LPSs of *B. caryophylli* has not yet been elucidated, it may be speculated that the transfer of the L-Ara*p*4N residue to a lipid A precursor occurs prior to the attachment of core sugars. A further substitution of the 4′-phosphate in later steps of LPSs biosynthesis in *B. caryophylli* evidently does not take place; this may be due to a substrate specificity of a L-Ara*p*4N transferase that is different to that of ArnT, or to peculiarities in the conformation of the lipid A precursor possessing two Kdo residues, or of an Re-LPS species. It may well be the substrate specificity of an Re-LPS species, since the lipid A possesses an acyl pattern different to that of lipid A of LPS from *E. coli*.

Resistance of gram-negative enterobacteria to the polycationic antibiotic polymyxin B is believed to be associated with particular alterations in the LPS, that is, with a shift towards a less anionic net charge achieved by a higher level of substitution of phosphate residues by 2-aminoethanol phosphate and L-Ara*p*4N.[14, 15, 28–31] Evidently, in particular, the

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esterification of the 4′-phosphate group of lipid A with L-Ara*p*4N leads to resistance against polymyxin B, whereas such substitution of the glycosidic phosphate plays a less significant role. Thus, a phosphate monoester at O4′ of lipid A should represent a part of the receptor site for polymyxin B. Unfortunately, it was not possible to isolate the pure lipid A fraction with L-Ara*p*4N from the lipid A precipitate of the LPSs of *B. caryophylli*. Thus we could not test whether such lipid A possesses any reactivity with polymyxin B.

Experimental Section

Bacteria and bacterial LPSs: *Burkholderia caryophylli* strain NCPP 2151 was cultivated as described.[3] The LPSs were obtained from lyophilized bacteria by the phenol/water extraction method as described (yield: 6% of the bacterial dry mass).[3]

Preparation of lipid A , de-O-acylated lipid A, and dephosphorylated lipid A: Free lipid A was obtained by treatment of LPS (100 mg) with sodium acetate buffer (0.1M) (pH 4.4) containing 1% sodium dodecylsulfate (SDS) (100 °C, 2 h). The solution was then lyophilized, treated with 2M HCl/EtOH (1:100, v:v) to remove the SDS, evaporated, dissolved in water, and ultracentrifuged (100.000 × g, 4 °C, 90 min). The obtained precipitate (free lipid A) was washed with water (yield: 5 mg, 5% of the LPS). Thin-layer chromatography (TLC) of lipid A was carried out on silica gel 60 TLC plates (20 × 20 cm, 0.25 µm thickness), and eluted with $CHCl_3$/MeOH/H_2O (100:75:15, by vol). Compounds were visualized by spraying the plate with 10% ethanolic H_2SO_4 and charring. Lipid A was de-O-acylated either by mild hydrazinolysis (37 °C, 1.5 h) with anhydrous hydrazine in tetrahydrofuran, followed by precipitation with acetone at 4 °C and centrifugation (2.750 × g, 15 min, yield: 2.5 mg, 50% of lipid A), or by treatment with 11% NH_4OH at 20–22 °C for 18 h. Lipid A was dephosphorylated by treatment with 48% aqueous HF (4 °C, 48 h), then neutralization, dialysis, and lyphilization.

General and analytical methods: The content of organic bound phosphate was determined as described previously.[32] The sugar composition was identified by GLC and combined GLC mass spectrometry (MS) after methanolysis (80 °C, 16 h) with 2M methanolic HCl and acetylation (80 °C, 30 min) with acetic anhydride in pyridine. The absolute configuration of the monosaccharides was obtained according to the method published by Leontein et al.[33] Fatty acid analysis (determination of ester-, amide-bound fatty acids and determination of the absolute configuration) were performed as described previously,[34, 35] as were GLC and GLC-MS analysis.[34, 35] The temperature program for the determination of the absolute configuration of sugars was 150 °C for 8 min and then 2 °C min^{-1} to 200 °C, followed by 6 °C min^{-1} to 260 °C, and 260 °C for 5 min. For the analysis of fatty acids, the temperature program was 150 °C for 3 min and then 10 °C min^{-1} to 280 °C over 20 min.

Mass spectrometric analysis: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of native and TLC-separated fractions of lipid A, de-*O*-acylated, and dephosphorylated lipid A was performed with a Bruker-Reflex II instrument (Bruker-Daltonik, Bremen, Germany) in the positive and negative ion mode at an acceleration voltage of 20 kV. In general, aqueous suspensions of the compounds were prepared at a concentration of 5 µg µL^{-1} and treated with an ion exchanger (Amberlite IR-120, Merck) to remove disturbing cations as described.[21] One µL of the sample was then mixed with 1 µl of a saturated solution of re-crystallized 2,5-dihydroxybenzoic acid (Aldrich) in methanol containing 30% acetonitrile and 0.1% trifluoroacetic acid, and 0.5µL aliquots were deposited on the metallic sample surface. The mass spectra shown are the average of at least 50 single analyses. Mass scale calibration was performed externally with similar compounds of known chemical structure.

NMR spectroscopy: ^{1}H, ^{13}C, and ^{31}PNMR spectra of a solution of lipid A (5 mg in 0.6 mL) in [D$_6$]Me_2SO was recored at operating frequencies of 400, 100, and 162 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe at 60 °C in the fourier transformed mode, by using Bruker standard software. ^{13}C and ^{1}H chemical shifts are expressed relative to Me_2SO (δ_H 2.49, δ_C 39.7). The assignment of the proton chemical shifts was achieved by COSY, TOCSY, and ROESY experiments. The assignment of carbon chemical shifts was achieved by a ^{1}H-^{13}C HSQC experiment. The homonuclear experiments were performed with 4096 data points in the F2 dimension and 512 experiments in F1. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution-enhanced in both dimensions by a shifted sine-bell function before fourier transformation. The TOCSY and ROESY experiments were performed with a mixing time of 80 ms and 300 ms, respectively. The 1D ROESY experiment was performed by using selective excitation with a shaped pulse. The program was furnished by Bruker software. The heteronuclear experiment was performed as a gHSQC experiment by using a pulse field gradient program.

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