CHEMBIOCHEM

DOI: 10.1002/cbic.200700693

The Acylation and Phosphorylation Pattern of Lipid A from *Xanthomonas Campestris* Strongly Influence its Ability to Trigger the Innate Immune Response in Arabidopsis

Alba Silipo,*[a] Luisa Sturiale,[b] Domenico Garozzo,[b] Gitte Erbs,[c] Tina Tandrup Jensen,[c] Rosa Lanzetta,[a] J. Maxwell Dow,[d] Michelangelo Parrilli,[a] Mari-Anne Newman,[c] and Antonio Molinaro*[a]

Lipopolysaccharides (LPSs) are major components of the cell surface of Gram-negative bacteria. LPSs comprise a hydrophilic heteropolysaccharide (formed by the core oligosaccharide and the O-specific polysaccharide) that is covalently linked to the glycolipid moiety lipid A, which anchors these macromolecules to the external membrane. LPSs are one of a group of molecules called pathogen-associated molecular patterns (PAMPs) that are indispensable for bacterial growth and viability, and act to trigger innate defense responses in eukaryotes. We have previously shown that LPS from the plant pathogen Xanthomonas campestris *pv.* campestris *(*Xcc*) can elicit defense responses in the model plant* Arabidopsis thaliana*. Here we have extended these studies by analysis of the structure and biological activity of LPS from a nonpathogenic* Xcc *mutant, strain 8530. We show that this* Xcc *strain is defective in core completion and introduces significant modification in the lipid A region, which involves the degree of acylation and nonstoichiometric substitution of the phosphate groups with phosphoethanolamine. Lipid A that was isolated from* Xcc *strain 8530 did not have the ability to induce the defense-related gene PR1 in* Arabidopsis*, or to prevent the hypersensitive response (HR) that is caused by avirulent bacteria as the lipid A from the wild-type could. This suggests that* Xcc *has the capacity to modify the structure of the lipid A to reduce its activity as a PAMP. We speculate that such effects might occur in wild-type bacteria that are exposed to stresses such as those that might be encountered during plant colonization and disease.*

Introduction

Lipopolysaccharides (LPSs) are ubiquitous and vital components of the cell surface of the majority of Gram-negative bacteria.[1–2] They are amphiphilic macromolecules composed of a hydrophilic heteropolysaccharide (which comprises the core oligosaccharide and O-specific polysaccharide or O-chain) that are covalently linked to a lipophilic moiety, termed lipid A, which anchors these macromolecules to the outer membrane. LPSs that do not possess the O-chain are termed rough LPSs or lipo-oligosaccharides (LOSs). LPSs are involved in membrane functions that are essential for the survival of the Gram-negative bacteria, that is, the formation of a rigid and efficient barrier to antimicrobial substances, and the ability to resist harsh environments that might include those that are encountered by pathogens during disease in eukaryotes. LPSs are also key molecules in the recognition process of the host defense system of eukaryotes during infection. LPSs represent one of a group of molecules termed pathogen-associated molecular patterns (PAMPs); these are conserved and are generally indispensable microbial structures that are able to elicit innate immune responses in diverse eukaryotes. In animal and insect cells, the recognition of PAMPs is often mediated by LRR (leucine-rich repeat) proteins such as Toll in *Drosophila* and the Toll-like receptors (TLR) in mammals.[3–6] Recognition of LPSs in mammalian cells occurs through the lipid A moiety, which is responsible for most of the biological effects of LPS in animals. Lipid A toxicity in animals strongly depends on its structure, and is also influenced by the covalently linked core region, which possesses immunogenic properties.[1–2]

LPSs can be recognized by plants to elicit or potentiate a range of plant defense-related responses that include induction of pathogenesis-related (PR) proteins, production of reactive oxygen and nitrogen species and prevention of the hypersensitive response (HR), a programmed cell death that is triggered by avirulent bacteria.[7–9] In comparison with animal and

[a] *Dr. A. Silipo, Prof. R. Lanzetta, Prof. M. Parrilli, A. Molinaro*
Dipartimento di Chimica Organica e Biochimica
Università di Napoli "Federico II"
Complesso Universitario Monte S. Angelo
Via Cintia 4, 80126 Napoli (Italy)
Fax: (+39) 081-674393
E-mail: molinaro@unina.it
silipo@unina.it

[b] *Dr. L. Sturiale, Dr. D. Garozzo*
Istituto per la Chimica e la Tecnologia dei Materiali Polimerici–ICTMP–CNR
95123 Catania (Italy)

[c] *Dr. G. Erbs, Dr. T. T. Jensen, Prof. M.-A. Newman*
Faculty of Life Sciences, Department of Plant Biology
University of Copenhagen, 1871 Frederiksberg (Denmark)

[d] *Prof. J. M. Dow*
BIOMERIT Research Centre, Department of Microbiology
BioSciences Institute, National University of Ireland
Cork (Ireland)

human cells, little is known about the mechanisms of LPS perception by plants and the cognate signal transduction pathways that lead to these responses. Recent findings have suggested that the lipid A moiety might be at least partially responsible for LPS perception by *Arabidopsis thaliana*, which leads to a rapid burst of NO, a hallmark of innate immunity in animals.[8] By using synthetic O-antigen polysaccharides (oligorhamnans) it has been shown that the O-chain of LPS is recognized by *Arabidopsis*, and that this recognition leads to elicitation of a specific gene transcription response that is associated with defense.[10] We have previously addressed the issue of the molecular basis of elicitation of plant defenses by LPS through determination of the structure of the LOS of the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) wild-type strain 8004[11] and examination of the effects of LOS and fragments that were obtained by chemical treatments on the immune response in *Arabidopsis thaliana*. Intact LOS and the lipid A and core oligosaccharides that are derived from it were all able to induce the defense-related genes *PR1* and *PR2* in *Arabidopsis*, and to prevent the hypersensitive response (HR) caused by avirulent bacteria.[11]

We have extended our studies on the molecular basis of recognition and induction of LOS-mediated plant defense responses by analysis of the structure and function of LOS from the nonpathogenic mutant strain 8530 of *Xcc*.[12] We show that LOS from *Xcc* mutant strain 8530 is defective in core completion, and introduces chemical modification to the lipid A region. Importantly these alterations in the lipid A structure render it inactive in elicitation of *PR1* gene transcription and the prevention of HR. This suggests that *Xcc* has the capacity to modify lipid A to affect its activity as a PAMP. Such effects might occur in wild-type bacteria that are exposed to stresses such as those that might be encountered during plant colonization and disease.

Results

Structural characterization of the fully deacylated LOS fraction

LOS was isolated from *Xcc* strain 8530 as outlined in the Experimental Section. The fatty acid composition, compositional and linkage analysis of carbohydrates that was obtained by GLC–MS for isolated LOS are reported in Table 1. All monosaccharides are in the D configuration, and fatty acids are in the *R* configuration. The approach to define the primary structure of LOS from *Xcc* 8530 mutant was to employ two chemical degradations followed by compositional, 2D NMR spectroscopic and MS analyses of the obtained compounds.

Compositional and MALDI-TOF analysis of completely deacylated LOS

The LOS fraction was completely deacylated by anhydrous hydrazine followed by hot KOH. The compositional analysis (Table 1) of the obtained product, **OS1** revealed the presence of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and 6-Glc*p*N (1:2 ratio); no uronic acids were detected. Phosphate assays gave positive results.

Table 1. Monosaccharides and fatty acids components of LOS, OS1 and OS2. All monosaccharides are in the D configuration and fatty acids with *R* configuration. The diagnostic ion fragments observable by GLC/MS analysis of carbohydrate (as partially methylated alditol acetate derivatives) and of fatty acids (as O-methyl ester derivatives) are also present in the table.

Assignment	Characteristic ion peaks (*m/z*)	LOS	OS1	OS2
6-substituted-Glc*N*p	117, 159, 189, 233	x	x	x
terminal-Kdo*p*	72, 161, 206, 205, 250,294	x	x	x
terminal-Gal*p*A	118, 162, 163, 207	x	–	x
10:0 (3-OH)	103, 184, 201	x	–	–
iso and *iso-ante* 11:0 (3-OH)	103, 198, 215	x	–	–
12:0 (3-OH)	103, 212, 229	x	–	x
iso and *iso-ante* 13:0 (3-OH)	103, 226, 243	x	–	x
10:0	74, 87, 155, 186	x	–	–
11:0	74, 87, 169, 200	x	–	–

The isolated oligosaccharide **OS1** was analyzed by MALDI-TOF mass spectrometry (Figure 1). The negative-ion mass spectrum showed two peaks at *m/z* 719.4 and 799.4 (Δ*m/z* 80).

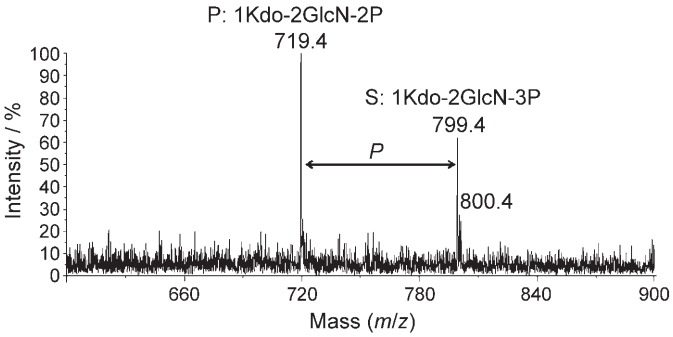


Figure 1. Negative-ion MALDI-TOF mass spectrum, acquired in reflector mode, of **OS1** that was obtained by full de-acylation of LOS from *Xcc* 8530 mutant.

Species **P** at *m/z* 719.4 matched with a bis-phosphorylated trisaccharide with two hexosamine and a Kdo residues while species **S** at *m/z* 799.4 (Δ*m/z* 80) differed from the previous one by the presence of an additional phosphate group.

NMR characterization of OS1

In agreement with the above MS data, the NMR analysis (DQF-COSY, TOCSY, NOESY, ROESY, ^{31}P,^{1}H and ^{13}C,^{1}H HSQC, ^{13}C,^{1}H HMBC) of **OS1** showed the existence of a mixture of two oligosaccharides that differ by the phosphorylation pattern. The anomeric region of the ^{1}H NMR spectrum showed two anomeric signals, each split into two spin systems; this accounts for a different magnetic environment (**A**/**A**′, **B**/**B**′, Figure 2, Table 2),

Spin systems **A, A′, B** and **B**′ were all identified as 2-deoxy-2-amino-glucose residues (GlcN), as indicated by their high $^{3}J_{H,H}$ ring proton values (all around 8–10 Hz), which are diagnostic

Table 2. ^{1}H, ^{13}C (italic) and ^{31}P (bold) NMR chemical shifts (ppm) of deacylated core-lipid A backbone (**OS1**) of LOS from *Xcc* 8530 mutant. Chemical shifts are relative to internal acetone and external aq. 85% phosphoric acid.

	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)							
Unit	1	2	3	4	5	6	7	8
A	5.56	3.29	3.81	3.38	4.08	4.22/3.77		
6-α-GlcN I	*91.1*	*55.1*	*70.3*	*70.16*	*73.0*	*69.5*		
	2.97							
A′	5.55	3.23	3.82	3.38	4.08	4.22/3.77		
6-α-GlcN I	*91.1*	*55.1*	*70.3*	*70.16*	*73.0*	*69.5*		
	2.97							
B	4.81	2.99	3.77	3.68	3.64	3.54		
6-β-GlcN II	*100.0*	*56.1*	*73.2*	*74.1*	*74.6*	*62.2*		
				5.17				
B′	4.89	2.95	3.75	3.90	3.64	3.54		
6-β-GlcN II	*100.0*	*56.1*	*73.2*	*74.1*	*74.6*	*62.2*		
				5.17				
C			1.84/2.07	4.39	4.15	3.75	3.89	3.87/3.66
t-α-Kdo	*174.9*	*100.3*	*36.4*	*69.5*	*69.9*	*71.8*	*70.1*	*63.9*
				6.90				
C′			1.80/1.97	4.06	4.05	3.74	3.89	3.87/3.66
t-α-Kdo	*174.9*	*100.3*	*36.4*	*66.6*	*67.2*	*71.8*	*70.1*	*63.9*

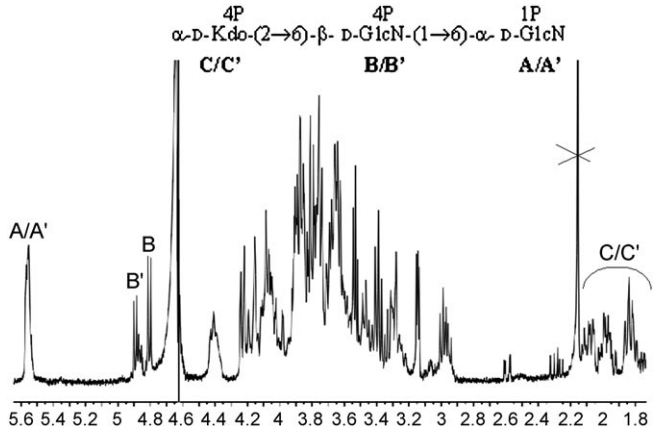


Figure 2. The ^{1}H NMR spectrum of **OS1** that was obtained by full de-acylation of LOS from *Xcc* 8530 mutant. Capital letters refer to each identified spin system as described in the text and denoted as in Table 2.

of *gluco*-configurations. The $^{1}H,^{13}C$ HSQC spectrum showed the correlation of H2**A/A′** and **B/B′** with nitrogen-bearing carbon signals. The high-field shift of the proton resonances of H2 was indicative of the absence of acylation at these positions. The chemical shifts of H1 and C1 of residue **A** and **A′**, the $^{3}J_{H1,H2}$ coupling constant (3.2 Hz) and the *intra*-residual NOE contact of H1 with H2 were all in agreement with an α-anomeric configuration of both residues **A** and **A′**. Residues **B** and **B′** were both identified as β-configured residues as indicated by the chemical shifts of H1 and C1, the $^{3}J_{H1,H2}$ value (8.4 Hz) and the *intra*-residual NOE contact of H1 with H3 and H5.

Because of the absence of the anomeric proton signal, the spin system of Kdo **C/C′** (Figure 2) was identified by starting from the diastereotopic H3 methylene protons that were found in a shielded region at $\delta = 1.84$ ppm **C**/$\delta = 1.80$ ppm **C′** and $\delta = 2.07$ ppm **C**/1.97 **C′** ($H3_{ax}$ and $H3_{eq}$, respectively). The α configuration at C2 was assigned by the chemical shift values of $H3_{eq}$, and by the values of $^{3}J_{H7,\,H8a}$ and $^{3}J_{H7,\,H8b}$.[13]

The downfield shift of the carbon resonances allowed us to locate the positions of the glycosylation at O6 of **A/A′** and **B/B′**; **C/C′** were terminal residues.

The *inter*-residual NOE contacts of the NOESY and the scalar correlations that are present in the HMBC spectra were used to infer the sequence of residues in the oligosaccharide chain and to confirm the glycosylation positions. The *inter*-residual NOE contacts of H1 **B** with $H6_{a,b}$ **A** and of H1 **B′** with $H6_{a,b}$ **A′** allowed us to identify the β-(1→6) linkage between the GlcN residues of the lipid A. The weak downfield shift of C6 of **B** and **B′** ($\delta = 62.2$ ppm) was in agreement with the α-(2→6) ketosidic linkage of Kdo **C** with residue **B** and of Kdo **C′** with residue **B′**.

The $^{31}P,^{1}H$ HSQC spectrum (Figure 3) showed three cross peaks, whose ^{31}P chemical shifts were in accordance with the presence of phosphate groups. Two of these signals, at $\delta =$ 2.97 and 5.17 ppm that correlated with the proton signals at $\delta = 5.65/5.55$ and 3.68 ppm were identified as H1 of α-GlcN **A/**

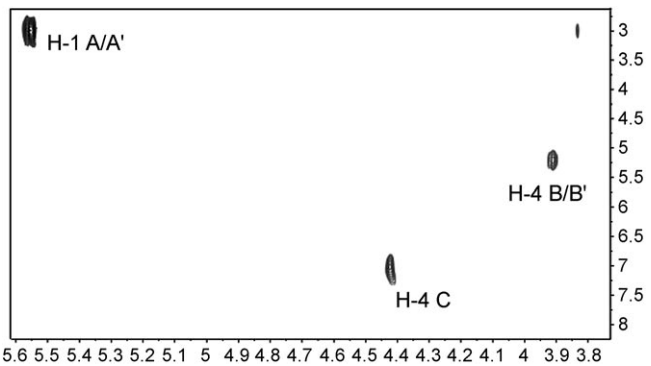
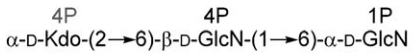


Figure 3. The $^{31}P,^{1}H$ HSQC spectrum of **OS1**. Capital letters refer to each identified spin system as described in the text and denoted as in Table 2.

A′ and H4 of β-GlcN **B/B′, which** compose the lipid A backbone. The third phosphate group was linked at O4 of one of the two α-Kdo residues, namely residue **C**, as suggested by both H4 and C4 **C** resonances that were downfield shifted by phosphorylation ($\delta = 4.39$ and 69.5 ppm, respectively, Table 2). In agreement, a cross peak between the ^{31}P signal at $\delta =$ 6.90 ppm and H4 **C** at $\delta = 4.39$ ppm was found in the $^{31}P,^{1}H$ HSQC spectrum (Figure 3).

Thus, these results, in accordance with the MALDI-MS characterization, can be summarized in a mixture of two oligosaccharides [Eq. (1)] that differ in the nonstoichiometric phosphorylation of the terminal Kdo residue (gray-colored phosphate group):



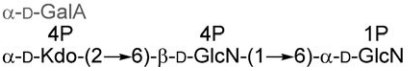
(1)

Structural characterization of the de-O-acylated LOS fraction

To detect the presence of alkaline-labile substituents (i.e., phosphate residues) that were likely lost by harsh alkaline treatment, the LOS was only de-O-acylated by mild hydrazinolysis and the obtained product (**OS2**) underwent complete chemical and NMR investigations, which revealed the presence of a mixture of oligosaccharides.[11] The NMR spectra were recorded on a solution of 1% deuterated SDS with 5 μL of 32% NH_4OH (298 K, pD 9.5).

In full accordance with the GLC-MS analysis of **OS2** (Table 1), the ^{1}H NMR spectrum (Figure 4) showed an additional spin system with respect to **OS1**, residue **D**. It was identified as 2-deoxy-2-amino-galacturonic acid (GalNA) as indicated by its $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ values (3 Hz and 1 Hz, respectively), which are diagnostic of a *galacto*-configuration. Moreover, the chemical shifts of H1 and C1, the $^{3}J_{H1,H2}$ coupling constant (3.2 Hz) and the *intra*-residual NOE contact of H1 with H2 were all in agreement with an α-anomeric configuration of residue **D**; the long-range correlation in the HMBC spectrum of both H4 and H5 with a carboxylic group revealed the nature of uronic acid of this residue. The chemical shifts and multiplicity of the H1 signal suggested that it was coupled to a phosphate signal. The assignment of the other spin systems that were already detected in the previous analysis was straightforward (Table 3). A complete 2D NMR analysis led to the identification of an oligosaccharide that contained the same saccharidic backbone as previously described, which differed by the phosphorylation pattern of residue **G** (α-Kdo). In the ^{31}P NMR spectrum, signals could be recognized in two different regions of the spectrum. One signal was found in the chemical shift region that is typical of a monophosphate di-ester group, at $\delta = -0.9$ ppm, whereas three signals, at $\delta = 3.3$, 5.3 and 6.9 ppm, were recognized as monophosphate monoester groups. The location of these phosphorus-containing groups was deduced from the $^{31}P,^{1}H$ HSQC spectrum (Figure 5). The phosphate groups at $\delta =$ 3.3 and 5.3 ppm, which were correlated to proton resonances at $\delta = 5.33$ and 3.77 ppm were attributed to H1 of **E** and H4 of **F**, respectively. Thus, as expected, two phosphate groups could be assigned to the di-acylated lipid A, and were linked to O1 of α-GlcN of **E** and to O4 of β-GlcN of **F**. The signal at $\delta =$ -0.9 ppm correlated to two protons at $\delta = 5.55$ ppm and 4.76 ppm, H1 of **D** and H4 of **G**, respectively. Thus, the phosphodiester group cross-linked the anomeric position of α-galacturonic acid at the O4 of Kdo residue **G**. Furthermore, an alternative Kdo residue, **G′**, was identified in a lesser amount and carried a simple monophosphate monoester group at position 4 (Table 2 and Figure 5).

From the above data, the following oligosaccharide [Eq. (2)] was identified (the α-D-GalA residue is gray colored to indicate its nonstoichiometric presence):



(2)

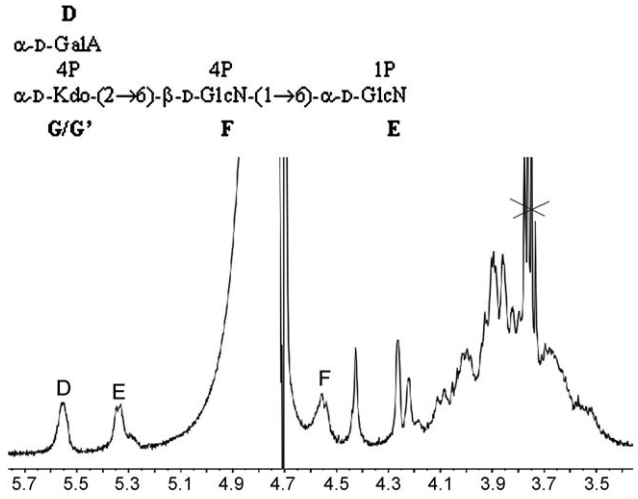


Figure 4. Section of the ^{1}H NMR spectrum of **OS2** that was obtained by de-O-acylation of LOS. Capital letters refer to each identified spin system as described in the text and denoted as in Table 3.

To obtain information on the intact molecule and on the primary structure of lipid A, the intact LOS was analyzed by MALDI-MS (Figure 6). The negative-ion MALDI mass spectrum showed peaks that corresponded to molecular ions in the mass range of *m/z* 1900–2400 Th (**L1**–**L4**), and peaks that were related to fragments that arose from the glycoside bond cleavage between the Kdo and the lipid A moiety[14] and were attributable to the lipid A (**LA1**–**LA2**). The lipid A portion, analogously to wild-type LPS lipid A, showed a remarkable heterogeneity due to the fatty acid variability. The group of peaks around *m/z* 1477.6 (**LA1**), matched with penta-acylated Lipid A species that carried two GlcN and two phosphate groups with acyl chains of different length (Δ*m/z* 14), which were distributed on the disaccharide backbone. These ions were identified as penta-acylated lipid A molecules that carried (*R*)-12:0 (3-OH) and/or (*R*)-13:0 (3-OH) in amide linkage, different ester-linked 3-hydroxy fatty acids [(*R*)-10:0 (3-OH), (*R*)-11:0 (3-OH), (*R*)-12:0 (3-OH), (*R*)-13:0 (3-OH)] and one secondary fatty acid, 11:0 or

Table 3. ^{1}H, ^{13}C (italic) and ^{31}P (bold) chemical shifts (ppm) of de-O-acylated core-lipid A backbone (**OS2**) of LOS from *Xcc* 8530 mutant. The sample was solved in 1% deuterated SDS solution with 32% NH_4OH (5 μL, pD 9.5). Chemical shifts are relative to internal acetone and external aq. 85% phosphoric acid.

	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)							
Unit	1	2	3	4	5	6	7	8
E	5.33	3.77	3.90	3.66	3.93	4.07/3.80		
6-α-GlcN	*92.7*	*54.9*	*71.1*	*69.3*	*71.3*	*68.3*		
	3.3							
F	4.55	3.84	3.76	3.77	3.65	3.88/3.69		
6-β-GlcN	*102.9*	*55.2*	*74.5*	*71.0*	*74.2*	*63.4*		
				5.3				
G			2.17/1.80	4.76	4.22	3.88	3.93	3.89
t-α-Kdo	*174.1*	*100.9*	*32.3*	*72.0*	*71.4*	*70.9*	*70.1*	*63.3*
				-0.9				
G′			2.26/n.d.	4.46	4.00	n.d.	n.d.	n.d.
t-α-Kdo	*174.1*	*100.9*	*32.1*	*73.3*	*70.7*			
				6.92				
D	5.55	3.84	3.91	4.26	4.42	-		
t-α-GalA	*96.0*	*69.5*	*70.0*	*71.1*	*73.8*	*170.9*		
	−0.9							

n.d. = not determined.

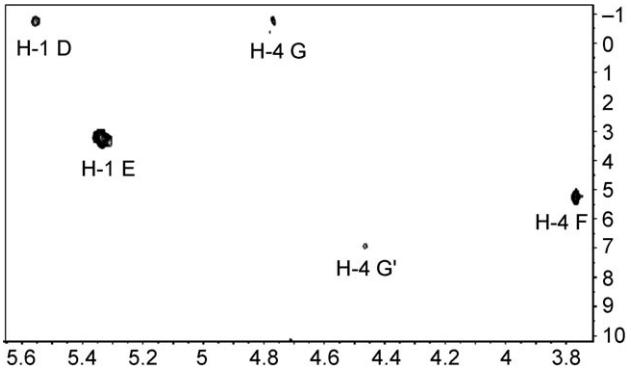


Figure 5. The $^{31}P,^{1}H$ HSQC spectrum of **OS2**.

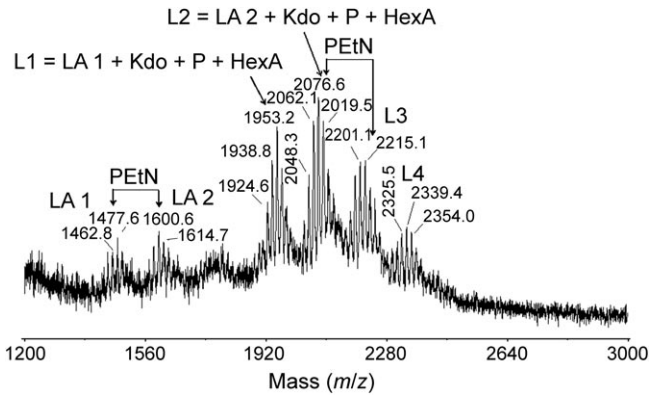


Figure 6. Negative-ion linear MALDI-TOF mass spectrum of intact LOS from *Xanthomonas campestris* pv. *campestris* 8530 mutant strain.

10:0. The ion group **LA2** around *m/z* 1600.6 (Δ*m/z* 123) was attributable to the presence of an additional residue of 2-aminoethyl phosphate (PEtN) and to a small amount of a hexa-acylated lipid A species. The lipid A from the *Xcc* 8530 mutant had a sugar and fatty acid composition that was identical to the *Xcc* wild-type,[11,15] but differed by the degree of acylation and phosphorylation. Thus, while the *Xcc* wild-type's lipid A was mainly hexa-acylated, *Xcc* 8530 mutant's lipid A species were mainly penta-acylated, and both polar heads were nonstoichiometrically substituted by PEtN groups (see below).

The molecular ions of the intact LOS were also identified. The group of ions labeled **L1** was consistent with a LOS that is composed of a bis-phosphorylated penta-acylated lipid A (**LA1**), one Kdo, one phosphate and one hexuronic acid; species **L2** carried a PEtN on the lipid A backbone (**LA2**). Species **L3** differed by the presence of a second PEtN moiety that is likely present on the polar heads of the lipid A. Small amounts of hexa-acylated species were also present (**L4**).

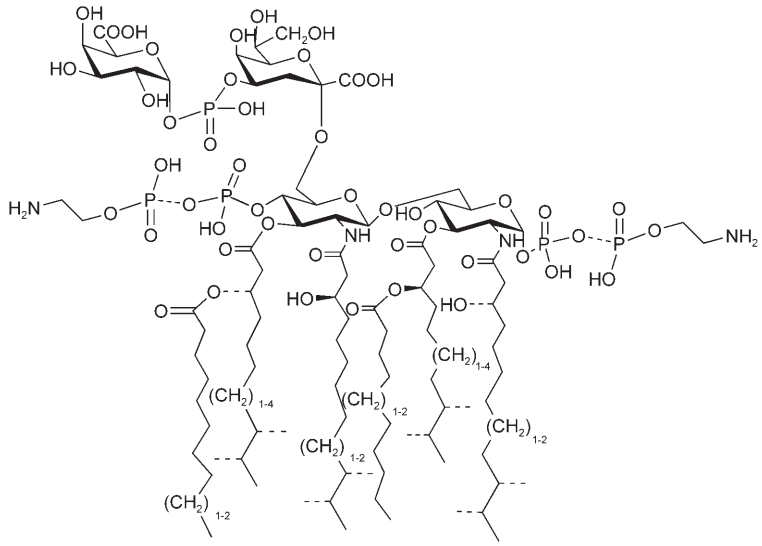
On the basis of compositional analysis, NMR spectroscopy and MALDI-MS measurements, the complete structure of the LOS from *Xcc* was determined (Scheme 1).

The effects of lipid A on induction of the defense-related gene *PR1*

Induction of *PR1* gene expression in the treated leaves was analyzed by real-time RT-PCR on extracted RNA (Table 4). Accumulation of the *PR1* transcript was observed in response to treatment with *Xcc* lipid A 20 hours after treatment. The level of transcript decreased over time, and no accumulation was evident at 24 h. In contrast, no *PR1* transcript accumulation was detected in response to *Xcc* strain 8530 lipid A alone.

The effect of lipid A on the hypersensitive response

In many different plants, pre-treatment with LPS, LOS or lipid A can prevent the hypersensitive response (HR) that is induced by avirulent bacteria, a phenomenon that has been termed localized induced resistance (LIR).[9] The lipid A from *Xcc* 8530 did



Scheme 1. The complete structure of the LOS from *Xanthomonas campestris* pv. *campestris* 8530 mutant strain. The dotted lines indicate nonstoichiometric substitutions. The penta-acylated species lacks one of the two secondary fatty acids; the hexa-acylated species was present in a nonstoichiometric amount. Dotted methyl groups on fatty acids are present as a possible single substitution. A mild acid hydrolysis allowed the separation of the lipid A from the core region, and the detailed lipid A analysis via MS. The sugar sequence was obtained by completely de-acylating the sample; a fine analysis of the phosphorylation pattern and the location of the galacturonyl-phophate was obtained by MS and NMR spectroscopic analysis of the de-O-acylated sample. A detailed MS analysis of the intact LOS confirmed the lipid A and the core structure.

Table 4. PR1 gene induction in *Arabidopsis* ecotype Col-0 after lipid treatment.

Time after treatment [h]	*Xcc* 8004 (wt) lipid A	*Xcc* 8530(mutant) lipid A
4	0	0
12	+12***	+1 (ns)
20	+213***	+1 (ns)
24	+5***	0

+: fold upregulated compared to water-treated tissue, after normalization to 18SrRNA; ns: not significant; *** = $p < 0.001$. The experiment was repeated three times.

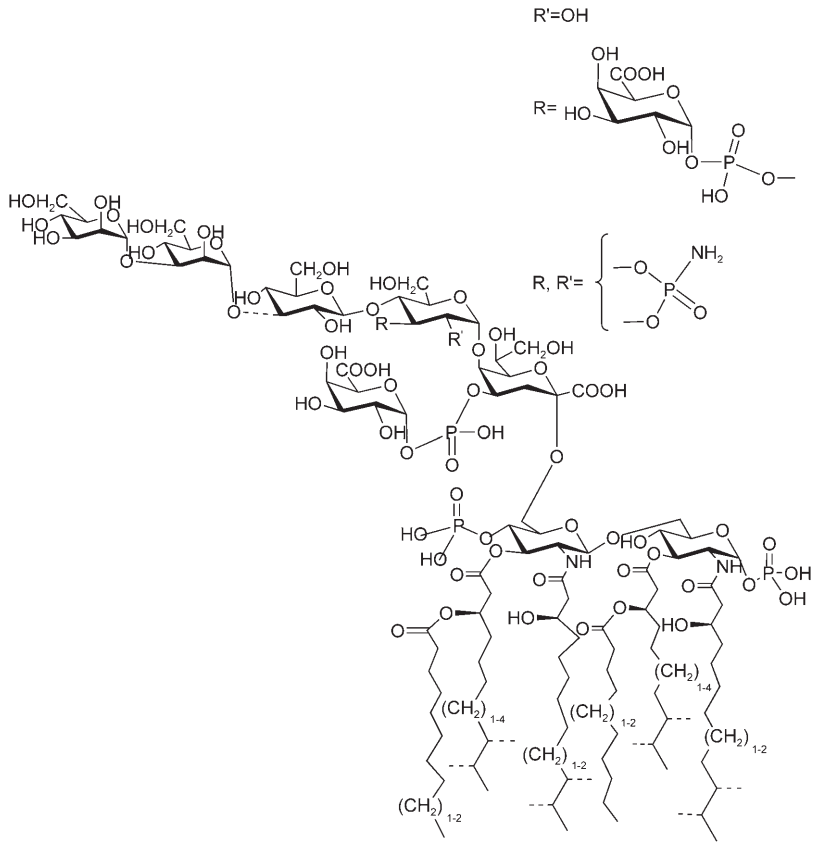
not prevent the hypersensitive response in *Arabidopsis*, while lipid A from the wild-type *Xcc* strain 8004 did.

Discussion

This paper reports for the first time the characterization of the complete structure of an LPS molecule with a deep rough phenotype from a plant pathogenic bacterium. The *Xcc* mutant strain 8530 produces a deep-rough LOS (Scheme 1) in which the core region is substantially truncated to contain a single Kdo residue to which a galacturonyl phosphate is attached. Furthermore, the lipid A moiety of the LOS from the mutant shows considerable alteration with decreases in the degree of acylation and non stoichiometric substitution of both polar heads by phosphoethanolamine groups. *Xcc* strain 8530 (Scheme 1) is derived from the wild-type strain 8004 (Scheme 2), and carries a Tn5 insertion in *rfaX*, a gene of unknown function.[12] Comparison of the structure of the core oligosaccharides of *Xcc* strain 8530 with the wild-type *Xcc* strain (described in ref. [11]), suggests that strain 8530 is defective in the transfer of a mannosyl residue to the Kdo present in the inner core (compare Schemes 1 and 2). However RfaX has no amino acid similarity to glycosyl transferases and the identity of the transferase involved and the role of RfaX in this process remains obscure.

The mechanism of plant defense activation in response to general elicitors is suggested to be analogoues to the innate immune system in vertebrate and invertebrate organisms, where the term pathogen-associated molecular patterns (PAMPs) was introduced to describe the molecules recognized by host pattern recognition receptors (PRRs). PAMPs are usually indispensable for microbial fitness and have molecular structures that are shared by many related microbes but not with their host. The recognition of microbial PAMPs allows the host to distinguish between self and nonself, and to signal unequivocally the presence of infection. Bacteria, viruses and other microbes are thus recognized as invading pathogens through the recognition of molecular signatures for a given pathogen class. Plants have evolved and maintained a capacity to recognize several general elicitors, which are pathogen surface molecules and can be considered to be PAMPs, these bind to PRRs and trigger the expression of immune response genes and the production of antimicrobial compounds. LPS, peptidoglycan, glucan, mannan and some proteins are all surface-derived compounds that can act as general elicitors of immunity in both plants and animals.[9] and refs therein The minimal structural requirement of a PAMP for elicitor activity can be different between plant and mammalian hosts. In order to pursue the study on innate immunity mechanisms in plants, and to clarify the structure–activity relationship of *Xcc* LOS, we have already demonstrated that both lipid A and core oligosaccharide were able to up-regulate, in two temporal phases, *PR-1* and *PR-2* genes that are related to innate immune response in *Arabidopsis*.[11] In this work, we have continued the chemical dissection of the LOS molecule by exploiting our expertise in molecular biology.

Our results show for the first time that the acylation and phosphoethanolamine substitution of lipid A influences its ability to trigger the innate immune response also in plants and thus to act as a PAMP. When introduced into host plants, bacterial populations of *Xcc* strain 8530 decline by two to three orders of magnitude over 24 h, whereas those of the wild-type increase.[7] This *in planta* behavior of the mutant might be due in part to the increased sensitivity of the strain to plant-released antimicrobial compounds; increased sensitivity to a range of chemicals can be demonstrated in vitro. As has been already demonstrated for a number of Gram-negative bacteria,[16] modification in the lipid A–core region can be very variable, can be regulated by environmental conditions, and can modulate bacterial virulence. It has been established for mam-



Scheme 2. The complete structure of the LOS from *Xanthomonas campestris* pv. *campestris* wild-type strain 8004.

malian pathogens that the addition of phosphoethanolamine groups as well as changes in the acylation pattern of lipid A can provide increased resistance to some antimicrobial compounds,[16] and attenuate, or eventually antagonize the endotoxic properties of the lipid A. It is tempting to speculate that similar alterations in lipid A in the *Xcc* deep rough mutant represent an adaptive response of *Xcc* to increased stresses that are experienced because of the absence of the outer core. This raises the possibility that similar alterations might occur in the wild-type lipid A during plant disease, under the stress conditions that are encountered and/or in response to specific environmental cues. In animals, the activation of the innate immune system is performed by the natural mixture of variously acylated lipid A species produced by each bacterium. *Porphyromonas gingivalis,* for example, produces a LPS with significant heterogeneity in the acylation pattern of lipid A, which contains both penta-acylated and tetra-acylated structures.[17] These differently acylated structures have opposing effects on the activation of Toll-like receptor 4 (TLR4).[18] The acylation pattern of *P. gingivalis* lipid A can be influenced by hemin, a component that is found in the microenvironment of the gingival cervicular fluid.[19] Thus, by altering the relative amount of the different lipid A structures in response to an environmental cue, *P. gingivalis* is able to modulate the triggering of innate host response. Reduction of the inflammatory potency of bacterial LPS in mammalian tissues can also involve the activity of deacylating enzymes of host or bacterial origin that cleave acyl chains,[16] and refs therein which greatly reduces its sensing via TLR4. Whether alterations in the acylation pattern or phosphoethanolamine substitution of *Xcc* lipid A occur in the host and are triggered by specific plant environmental cues is unknown. The different ability of lipid A from *Xcc* wild-type and *Xcc* 8530 mutant strains to act as PAMPs may reflect differences in the lipid A molecular conformation, which is strongly influenced by both the net negative charge, its distribution within the hydrophilic headgroup and by the degree of acylation.[20–21] These differences in shape have consequences for the biological activity of lipid A and derivatives in human cells; only conical molecules exhibit endotoxicity, whereas cylindrical molecules are inactive.[20–22] A similar relationship between conformation and biological activity might occur for plant systems, and can then influence the capacity to bind and trigger the activation of plant immune receptors. With respect to the wild-type *Xcc* lipid A, one of the main structural differences reside in the presence of species with both phosphate groups substituted by PEtN groups. The charged groups in *Xcc* LOS have already appeared to have a key role in recognition of both lipid A and core oligosaccharide by putative plant receptors.[11] In fact, fully de-phosphorylated *Xcc* LOS from the wild-type strain 8004 gives rise to a molecule with a single negative charge on the Kdo residue of the inner core that is unable to induce any tested defense response in *A. thaliana*.[11] In a similar fashion, the substitution of both phosphate groups in *Xcc* strain 8530 with phosphoethanolamine groups would amend the net charge of the lipid A with possible consequences for binding of the molecule to putative plant receptors.

Experimental Section

Bacterial growth and LOS extraction: The *Xcc* strains used were strain 8004, a rifampicin-resistant mutant of a wild-type isolate of *Xcc*,[23] and strain 8530, a Tn5 mutant of strain 8004.[7] Both strains were cultured in peptone yeast extract-glycerol medium (NYGB) at 28 °C.[24] Freeze-dried cells were extracted three times with a mixture of aqueous 90% phenol/chloroform/petroleum ether (2:5:8, *v*/*v*/*v*).[25] After removal of the organic solvents under vacuum, the LOS fraction was precipitated from phenol with water, washed first with aqueous 80% phenol, and then three times with cold acetone, and lyophilized to 4.3% of the dry mass. To get rid of all the cell contaminants, the LOS fraction was further subjected to enzy-

matic hydrolysis with RNase, DNase, and proteinase K, followed by size-exclusion chromatography on Sephacryl S-300 in 50 mM NH_4CO_3 (yield: 3% of dried cells). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 13.5%) was performed as described; gels were stained with silver nitrate.[26–27]

Chemical degradation of LOS for structural analysis: For isolation of **OS1** and **OS2**, LOS (30 mg) was treated with anhydrous hydrazine (2 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (20 mL), and allowed to precipitate. The precipitate was then centrifuged (3000 *g*, 30 min), washed twice with ice-cold acetone, dried, dissolved in water and lyophilized (oligosaccharide **OS2**, 32 mg, 80% of the LOS). An aliquot of product (15 mg) was de-*N*-acylated with 4 M KOH as described,[12] to give **OS1**. Salts were removed by gel permeation chromatography with Sephadex G-10 (Pharmacia) column (50×1.5 cm) to yield the resulting oligosaccharide **OS1**.

Chemical degradation of LOS for plant tests: Free lipid A was obtained by hydrolysis of the LOS (20 mg) with 10 mM sodium acetate buffer pH 4.4, (100 °C, 3 h). The solution was extracted three times with $CHCl_3$/MeOH/H_2O (100:100:30, *v/v/v*) and centrifuged (4 °C, 5000 *g*, 15 min). The organic phase contained the lipid A, and the water phase contained the core oligosaccharide.

General analytical methods: Determination of sugars residues and of their absolute configuration, GLC and GLC-MS were all carried out as described.[28–29] Monosaccharides were identified as acetylated *O*-methyl glycosides derivatives. After methanolysis (2 M HCl/MeOH, 85 °C, 24 h) and acetylation with acetic anhydride in pyridine (85 °C, 30 min) the sample was analyzed by GLC-MS. Linkage analysis was carried out by methylation of the complete core region as described.[30–31] The sample was hydrolyzed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxyl-reduced, acetylated and analyzed by GLC-MS.

Total fatty acid content was obtained by acid hydrolysis. LOS was first treated with 4 M HCl (4 h, 100 °C) and then neutralized with 5 M NaOH (30 min, 100 °C). Fatty acids were then extracted in $CHCl_3$, methylated with diazomethane, and analyzed by GLC-MS. The ester-bound fatty acids were selectively released by base-catalyzed hydrolysis with 0.5 M NaOH /MeOH (1:1, *v/v*, 85°, 2 h), then the product was acidified, extracted in $CHCl_3$, methylated with diazomethane, and analyzed by GLC-MS. The absolute configuration of the fatty acids was determined as previously described.[32,15]

NMR spectroscopy: For structural assignments of **OS1**, 1D and 2D ^{1}H NMR spectra were recorded in D_2O (0.5 mL) at 300 K, pD 7 (uncorrected value) on a Bruker 400-DRX and on a Varian INOVA 500 spectrometer. For structural assignments of **OS2**, 1D and 2D ^{1}H NMR spectra were recorded in a solution of 1% deuterated SDS (sodium dodecyl sulfate) with 32% NH_4OH (5 μL) at 298 K, pD 9.5 (uncorrected value). Spectra were calibrated with internal acetone [$\delta_H = 2.225$ ppm, $\delta_C = 31.45$ ppm]. ^{31}P NMR experiments were carried out by using a Bruker DRX-400 spectrometer; aqueous 85% phosphoric acid was used as an external reference ($\delta = 0.00$ ppm). ROESY was measured by using data sets ($t1 \times t2$) of 4096×256 points with mixing times between 200 ms and 700 ms. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, by using data sets of 4096×256 points. TOCSY were performed with spinlock times from 20 to 100 ms, by using data sets ($t1 \times t2$) of 4096×256 points. In all homonuclear experiments, 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 cosine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase-sensitive DQF-COSY.[33–34] HSQC and HMBC experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain by using data sets of 2048×256 points. Experiments were carried out in the phase-sensitive mode. A 60 ms delay was used for the evolution of long-range connectivities in the HMBC experiment. In all heteronuclear experiments, the data matrix was extended to 2048×1024 points by using forward linear prediction extrapolation.[35]

Mass spectrometry: MALDI mass spectra were acquired in negative polarity with a Voyager STR instrument (Applied Biosystems) that was equipped with nitrogen laser ($\lambda = 337$ nm) and provided with delayed extraction technology. Ions that were generated by the pulsed laser beam were accelerated through 24 kV. The mass spectra that are reported were the result of 256 laser shots.

The MALDI spectrum of native LOS was measured in linear mode, and sample preparation was performed according to the *thin layer* procedure that has been widely described[36] by using 2,4,6-trihydroxyacetophenone (THAP) as a matrix; MS analysis of the oligosaccharide sample was performed in reflector mode by using a matrix solution of dihydroxybenzoic acid (DHB; 50 mg mL^{-1}) in 0.1% TFA/ACN (8:2), by the classic dried drop method: a sample/matrix solution mixture (1 μL, 1:1, *v/v*) was deposited onto a stainless-steel MALDI sample plate and left to dry at room temperature.

Plant tests: Lipid A from both *Xcc* wild-type and the mutant *Xcc* strain 8530 were tested for their ability to suppress HR and induce *PR1* gene expression in *A. thaliana* accession Columbia (Col-0). The HR suppression test was carried out exactly as described in Bedini et al. (2005).[11] For the defense-gene-induction test, six-week-old *Arabidopsis* leaves were inoculated with Lipid A (50 μg mL^{-1}) that had been dissolved in water. Control leaves were infiltrated with water. Plants were placed at 25 °C with 16 h of light, and leaves were harvested 4, 12, 20 and 24 h after the inoculation.

Total RNA extraction and cDNA synthesis: Total RNA was extracted from treated plant material by using RNAwiz™ (Ambion, Huntingdon, UK), any contaminating genomic DNA was removed by the DNA-free™ Kit (Ambion) by following the manufacturers instructions. cDNA synthesis was performed by using iScript™ cDNA Synthesis Kit (Bio-Rad). A final concentration of 35 ng μL^{-1} of reversely transcribed total RNA was used. A nonRT-control (without reverse transcriptase added) was made for each sample.

Quantitative Real time RT-PCR and statistical evaluations: The induction of *PR1* gene expression was analyzed by quantitative real time RT-PCR. Primer design, quantitative real time RT-PCR and statistical evaluations were performed as described in ref. [11]. The experiment was repeated three times.

Acknowledgements

The work at the University of Copenhagen is supported by a grant from The Danish Council for Technology and Innovation (Copenhagen, Denmark). Financial support from the Research Project ""Innovazione e Tecnologie per il Miglioramento della Sostenibilità Agroindustriale, della Sicurezza e Qualità Alimentare" financed in the framework of the agreement between "Regione Sicilia", MIUR and MEF (APQ, project code RS-20) is gratefully aknowledged.

Keywords: lipopolysaccharides · mass spectrometry · NMR spectroscopy · structure–activity relationships · *Xanthomonas campestris* 8530

[1] C. R. H. Raetz, C. Whitfield, *Annu. Rev. Biochem.* **2002**, *71*, 635–700.
[2] C. Alexander, E. T. Rietschel, *J. Endotoxin Res.* **2001**, *7*, 167–202.
[3] K. Miyake, *Trends Microbiol.* **2004**, *12*, 186–192.
[4] R. Medzhitov, *Nat. Rev. Immunol.* **2001**, *1*, 135–145.
[5] S. Akira, K. Takeda, T. Kaisho, *Nat. Immunol.* **2001**, *2*, 675–680.
[6] M. Triantafilou, K. Triantafilou, *Trends Immunol.* **2002**, *23*, 301–304. .
[7] U. Conrath, O. Thulke, V. Katz, S. Schwindling, A. Kohler, *Eur. J. Plant Pathol.* **2001**, *107*, 113–119.
[8] D. Zeidler, U. Zahringer, I. Gerber, I. Dubery, T. Hartung, W. Bors, P. Hutzler, J. Durner, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 15811–15816.
[9] M. A. Newman, J. M. Dow, A. Molinaro, M. Parrilli, *J. Endotoxin Res.* **2007**, *13*, 69–84.
[10] E. Bedini, C. De Castro, G. Erbs, L. Mangoni, J. M. Dow, M. A. Newman, M. Parrilli, C. Unverzagt, *J. Am. Chem. Soc.* **2005**, *127*, 2414–2416.
[11] A. Silipo, A. Molinaro, L. Sturiale, J. M. Dow, G. Erbs, R. Lanzetta, M. A. Newman, M. Parrilli, *J. Biol. Chem.* **2005**, *280*, 33660–33668.
[12] J. M. Dow, A. E. Osbourn, T. J. G. Wilson, M. J. Daniels, *Mol. Plant–Microbe Interact.* **1995**, *8*, 768–777.
[13] G. I. Birnbaum, R. Roy, J. R. Brisson, H. J. Jennings, *J. Carbohydr. Chem.* **1987**, *6*, 17–39.
[14] L. Sturiale, D. Garozzo, A. Silipo, R. Lanzetta, M. Parrilli, A. Molinaro, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1829–1834.
[15] A. Silipo, A. Molinaro, R. Lanzetta, M. Parrilli, B. Lindner, O. Holst, *Eur. J. Org. Chem.* **2004**, 1336–1343.
[16] C. R. Raetz, C. M. Reynolds, M. S. Trent, R. E. Bishop, *Annu. Rev. Biochem.* **2007**, *76*, 295–329.
[17] R. A. Reife, S. R. Coats, M. Al-Qutub, D. M. Dixon, P. A. Braham, R. J. Billharz, W. N. Howald, R. P. Darveau, *Cell. Microbiol.* **2006**, *8*, 857–868.
[18] S. R. Coats, C. T. Do, L. M. Karimi-Naser, P. H. Braham, R. P. Darveau, *Cell Microbiol.* **2007**, *9*, 1191–1202.
[19] M. N. Al-Qutub, P. H. Braham, L. M. Karimi-Naser, X. Liu, C. A. Genco, R. P. Darveau, *Infect. Immun.* **2006**, *74*, 4474–4485.
[20] A. B. Schromm, K. Brandenburg, H. Loppnow, U. Zähringer, E. T. Rietschel, S. F. Carroll, M. H. J. Koch, S. Kusumoto, U. Seydel, *J. Immunol.* **1998**, *161*, 5464–5471.
[21] A. B. Schromm, K. Brandenburg, H. Loppnow, A. P. Moran, M. H. J. Koch, E. T. Rietschel, U. Seydel, *Eur. J. Biochem.* **2000**, *267*, 2008–2013.
[22] M. G. Netea, M. van Deuren, B. J. Kullberg, J.-M. Cavaillon, J. W. M. Van der Meer, *Trends Immunol.* **2002**, *23*, 135–139.
[23] M. J. Daniels, C. E. Barber, P. C. Turner, W. G. Cleary, M. K. Sawczyc, *J. Gen. Microbiol.* **1984**, *130*, 2447–2455.
[24] M.-A. Newman, E. von Roepenack-Lahaye, A. Parr, M. J. Daniels, J. M. Dow, *Plant J.* **2002**, *29*, 487–495.
[25] C. Galanos, O. Luderitz, O. Westphal, *Eur. J. Biochem.* **1969**, *9*, 245–249.
[26] O. Westphal, K. Jann, *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
[27] R. Kittelberger, F. Hilbink, *J. Biochem. Biophys. Methods* **1993**, *26*, 81–86.
[28] K. Leontein, J. Lönngren, *Methods Carbohydr. Chem.* **1978**, *62*, 359–362.
[29] A. Molinaro, C. De Castro, R. Lanzetta, A. Evidente, M. Parrilli, O. Holst, *J. Biol. Chem.* **2002**, *277*, 10058–10063.
[30] S. Hakomori, *J. Biochem.* **1964**, *55*, 205–208.
[31] O. Holst, *Methods in Molecular Biology, Vol. 145: Bacterial Toxins: Methods and Protocols*, Humana, Totowa, **2000**.
[32] E. T. Rietschel, *Eur. J. Biochem.* **1976**, *64*, 423–428.
[33] U. Piantini, O. W. Sørensen, R. R. Ernst, *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
[34] M. Rance, O. W. Sørensen, G. Bodenhausen, G. Wagner, R. R. Ernst, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479–485.
[35] D. J. States, R. A. Haberkorn, D. J. Ruben, *J. Magn. Reson.* **1982**, *48*, 286–292.
[36] L. Sturiale, D. Garozzo, A. Silipo, R. Lanzetta, M. Parrilli, A. Molinaro, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1829–1344.

Received: November 14, 2007
Published online on March 18, 2008

