Structural Determination of a Novel O-Chain Polysaccharide of the Lipopolysaccharide from the Bacterium *Xanthomonas campestris* pv. *pruni*

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In this paper, we report the structure of the O-specific polysaccharide of the LPS fraction of the strain type NCPPB416 of *X. campestris* pv. *pruni*. It is built up of three different monosaccharides – glucose, rhamnose and xylose – in an intricate block-wise polymer. Herein, the primary structure is elucidated by means of chemical degradation and 2D NMR spectroscopy.

Introduction

Lipopolysaccharides (LPSs) are vital and exclusive structural components of the outer membranes of all Gram-negative bacterial hosts of both animal and vegetal organisms. They play an important role in the communication between the pathogen and host cells.[1] Structurally, they comprise three regions in their smooth (S) form, namely the O-specific polysaccharide (or O-antigen), the core region and the lipid A (the endotoxic active moiety). Rough-form (R) LPSs lack the O-specific polysaccharide. Even though the role of LPS against animal organisms is well acknowledged, not much is known about their action against vegetal organisms. The use of mutants of plant pathogenic bacteria lacking LPSs or defective in their biosynthesis lead to an understanding of their role in virulence expression and in the recognition processes that take place in the first steps of the interaction between the pathogen and plant cells.[2]

Xanthomonas campestris pv. *pruni*[3] (sin. *Xanthomonas arboricola* pv. *pruni*) is the causal agent of bacterial leaf spots and cancers of several cultivated plants belonging to the genus *Prunus* (i.e., peach, apricot, plum). These diseases are present in most areas where the fruit plants are cultivated and very often they represent the limiting factors for their cultivation, which causes crop loss and, in the presence of heavy infections and high rates of disease, also plant dieback and death. It has not been excluded that LPSs of *X. campestris.* pv. *pruni* may play an important role in pathogenesis, as has been reported for other pathovars of *X. campestris*.

The recognised economic importance of the diseases caused by *X. campestris.* pv. *pruni*, as well as the above considerations, prompted us to characterise the LPSs of this pathogen. In this paper, we report the structure of the O-specific polysaccharide of the LPS fraction of the strain type NCPPB416 of *X. campestris* pv. *pruni*.

Results and Discussion

Freeze-dried bacterial cells were subjected to the sequential extraction procedure as outlined in the Exp. Sect. The LPS fraction was found in the water phase of the hot phenol/water extraction and was further purified by GPC. The LPS did not show the typical ladder pattern when studied by SDS page electrophoresis, but instead displayed a pattern indicating a wide continuous distribution of molecular masses. This migration upon electrophoresis suggested a non-regular O-chain structure and/or a mixture of LPSs with different O-chain structures. A mild hydrolysis with acetic acid allowed the lipid A moiety to be removed by precipitation, leaving the polysaccharide moiety in solution. This solution was purified further several times by GPC. Compositional analysis of the resulting O-polysaccharide fraction revealed the presence of three monosaccharides: L-xylose, L-rhamnose and D-glucose.

Despite the presence of only three monosaccharide residues, the methylation analysis showed a complex mixture of derivatives, namely terminal xylose, 3-substituted and 2-substituted rhamnose, 2,4-disubstituted and 2,4-disubsti-

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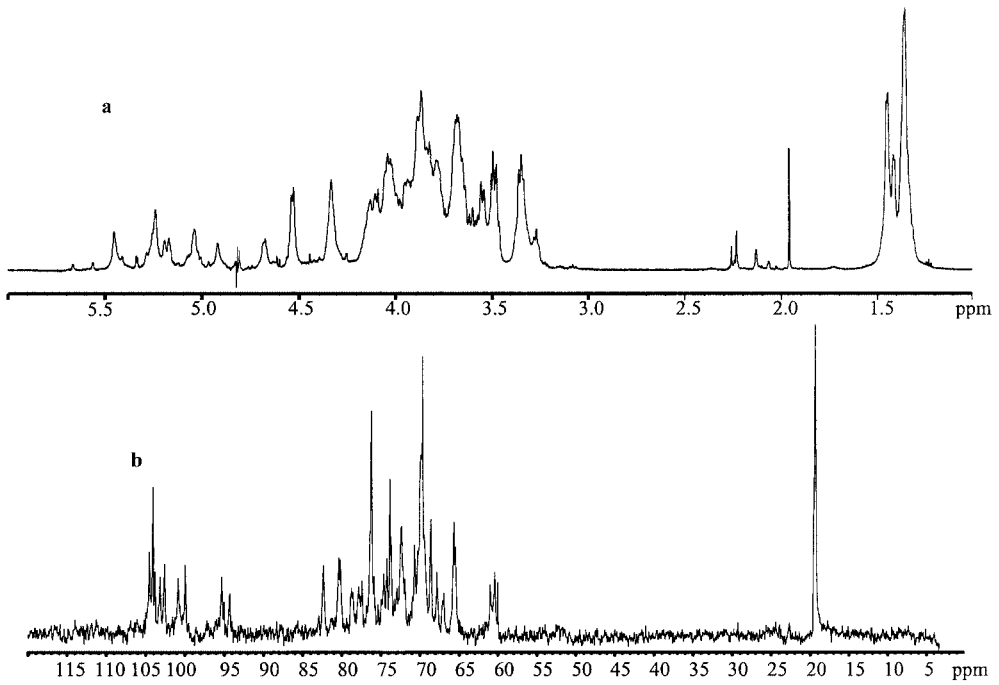


Figure 1. The ^1H NMR and ^{13}C NMR spectra of the O-chain of the LPS from *Xanthomonas campestris* pv. *pruni;* both spectra were measured at 30 °C in D_2O

tuted rhamnose, 2-substituted glucose and 2,3-disubstituted glucose.

Both the ^1H and ^{13}C NMR spectra of the O-chain (Figure 1, part a and b) suggest a very irregular structure and/or a mixture of products. Actually, several signals of anomeric protons are present in the ^1H NMR spectrum in the region $\delta = 5.50-4.40$ ppm and large methyl group signals occur at $\delta = 1.2-1.4$ ppm (6-H of Rha). The ^{13}C NMR spectrum contains at least ten signals in the anomeric region ($\delta = 105.5-94.9$ ppm) and many glycosylated carbon signals in the range $\delta = 82.1-75.8$ ppm, and a very intense methyl group signal at $\delta = 17.9$ ppm (C-6 of rhamnose).

Even with this puzzling situation, a full 2D NMR analysis was attempted at 600 MHz. Most of the ^1H and ^{13}C NMR spectral resonances of the O-polysaccharide were assigned tentatively by COSY, TOCSY, NOESY, gHSQC and gHMBC (Table 1), which identified seven types of monosaccharide residues. The configurations of the α-anomeric centres of the glucose and rhamnose residues was established by a coupled HSQC experiment, which gave values of 173 Hz for all residues, whereas xylose showed a β configuration in agreement with a value of 165 Hz.[4] A further indication of this β configuration is the observation of nuclear Overhauser effect between the 1-H, 3-H and 5-H_{ax} atoms of the xylose ring. In all cases, the configurations at the anomeric centres are in agreement with the expected values of chemical shifts in the ^1H and ^{13}C NMR spectra.

Using COSY and TOCSY experiments, starting from the signals of the anomeric resonances, we identified the majority of the *gluco* and *xylo* spin systems; the spin systems of rhamnose were assigned starting from signals of both the anomeric and methyl group resonances.

Table 1. Assignment of ^1H and ^{13}C NMR spectroscopic chemical shifts (ppm) of the main spin systems in the O-chain polysaccharide; the spectra were measured in D_2O at 303 K

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
3,4-Rha	5.04	4.17	3.98	3.72	3.81	1.27
	102.5	71.0	76.1	82.1	69.9	17.9
2,4-Rha	4.96	4.05	3.99	3.81	3.79	1.32
	100.8	77.1	73.2	80.7	69.9	17.9
3-Rha	5.43	4.20	3.88	3.69	3.78	1.39
	102.9	71.8	77.0	72.5	70.1	17.9
2-Rha	5.17	4.01	3.93	3.55	3.96	1.32
	102.3	78.9	71.0	73.0	70.4	17.9
2-Glc	5.31	3.71	3.93	3.53	3.94	3.70
	94.9	77.8	73.0	69.5	72.3	60.9
2,3-Glc	5.31	3.65	3.99	3.59	3.94	3.78
	95.1	75.8	77.0	69.0	72.1	61.0
t-Xyl	4.55	3.33	3.45	3.69	3.37/3.91	
	105.5	75.6	77.8	71.5	65.5	

The complexity of the NMR spectra prevented us from surmising any structure and, thus, a selective degradation was planned.

On the basis of the NMR spectroscopic results and the methylation data, Smith degradation was chosen as a suitable approach to depolymerise the O-chain and, hence, to obtain a simpler product. Thus, a complete degradation of the OPS, including mild acid hydrolysis of the periodate-oxidised and borohydride-reduced polysaccharide, resulted in two main fractions that were fractionated by GPC on BioGel P-2. One of the two fractions was eluted in the void volume (fraction **1**) whereas the other ones were eluted in the oligosaccharide region (fractions **2a**−**c**). Compositional

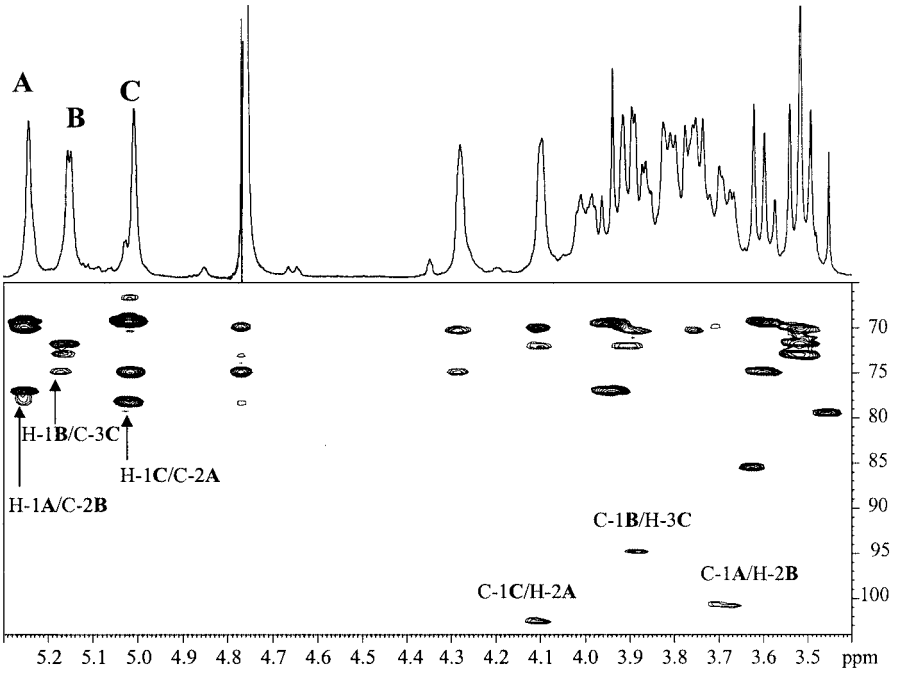


Figure 2. Expansion of the ^{1}H NMR and HMBC spectra of fraction **1**; the relevant inter-residual HMBC correlations of the anomeric region are indicated

and methylation analyses of fraction **1** revealed the presence of 3-substituted rhamnose, 2-substituted rhamnose and 2-substituted glucose units. The ^{1}H NMR spectrum (Figure 2, a) was very straightforward, indicating a regular polysaccharide with a trisaccharide repeating unit (**A**−**C**).

The α configuration at the anomeric centres of all of these three residues was established by a coupled HSQC spectrum that showed values of 173 Hz.[4]

By performing COSY, TOCSY and gHSQC experiments, we assigned all of the ^{1}H and ^{13}C NMR spectral resonances for residues **A**−**C** (Table 2), while by means of NOESY and the gHMBC (Figure 2, b) spectra it was possible to gather the sequence of the three residues. Actually, in the HMBC spectrum, C-1**A** shows an inter-residual scalar connectivity with H-2**B**, while C-1**B** has a cross peak with H-3**C** and C-1**C** shows a correlation with H-2**A**. All these data univocally assigned the structure below to fraction **1**.

Table 2. ^{1}H and ^{13}C NMR spectroscopic chemical shifts (ppm) of fraction **1**; the spectra were measured in D_2O at 303 K; chemical shifts of glycosylated carbon atoms are in bold

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	5.250	4.105	3.908	3.520	3.752	1.323
2-α-Rha	100.5	**78.3**	70.1	72.1	69.6	17.1
B	5.159	3.688	3.947	3.518	3.998	3.815
2-α-Glc	94.8	**77.1**	73.2	69.7	72.1	60.5
C	5.019	4.287	3.882	3.604	3.756	1.323
3-α-Rha	102.5	66.8	**75.0**	70.5	69.6	17.1

→2)-α-L-Rha*p*-(1→2)-α- D-Glc*p*-(1→3)-α- L -Rha*p*-(1→

A **B** **C**

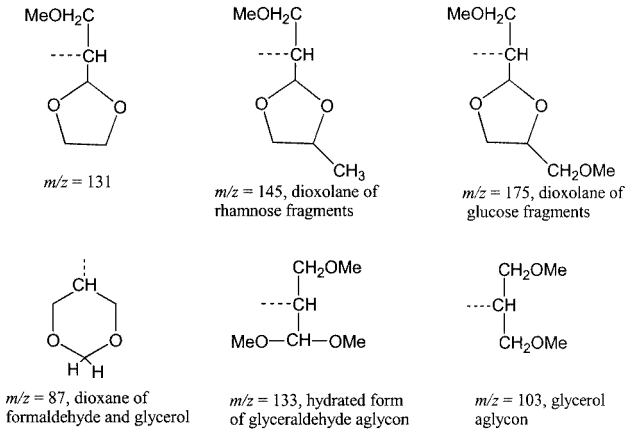
On the contrary, the analysis of fractions **2a**−**c** was more intricate because of the heterogeneity of the oligosaccharide mixture. Compositional and methylation analyses of the mixture of the three fractions showed the presence of terminal rhamnose, 3-substituted rhamnose, 2-substituted rhamnose and terminal glucose units. An aliquot of each fraction **2** was analysed by GC-MS as its permethylated oligosaccharide derivative to establish the nature of the oligomers it contained. This analysis showed, inter alia, peaks at m/z = 393 relating to the glycosidic cation of the disaccharide Rha−Glc (fraction **2a**), at m/z = 363 for the disaccharide glycosidic cation of rhamnose (fraction **2b**), at m/z = 189 for the glycosidic cation of rhamnose (fraction **2c**). Combining these analysis and methylation data, we ascertained that fractions **2a** and **2b** contained a disaccharide and fraction **2c** was rhamnose. Nevertheless, each of the three fractions was heterogeneous with respect to the aglycon units attached to the saccharide moiety as deduced by GC-MS and NMR spectra (see below).

2a: α-D-Glc-(1→3)-α-L-Rha-*O*-R

2b: α- L-Rha-(1→2)-α- L-Rha-*O*-R

2c: α- L-Rha-*O*-R

The nature of the aglycon moieties was elucidated with the help of GC-MS (Scheme 1) and 2D NMR spectroscopy. In the EI-MS spectra, besides the ions of the saccharide moieties, two different types of ions were found, one relating to the glycerol aglycon and the other relating to the 1,3-dioxolane/dioxane aglycon. Both kinds of aglycon are



Scheme 1. Aglycon fragments identified after permethylation of the oligosaccharide contained in fractions **2a**−**2c**; the value of *m*/*z* is relative to that of the diagnostic cation found in GC-MS analysis

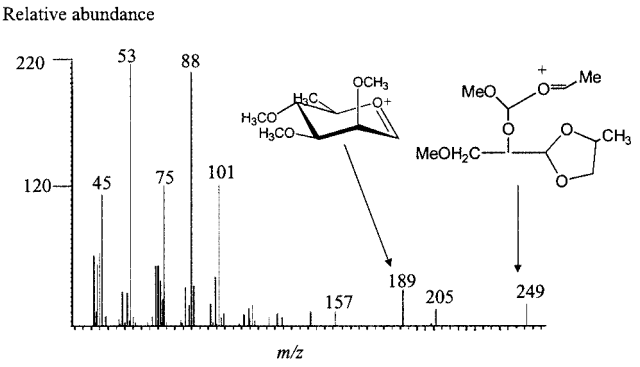


Figure 3. Expanded region of the EI-MS of fraction **2c**; the ion at *m*/*z* = 249 is related to the whole aglycon moiety bearing a carbohydrate fragment, while the ion at *m*/*z* = 189 is that of the rhamnose oxonium cation

expected to form by periodate oxidation. If the glycerol-type aglycon is a primary product of Smith degradation, the 1,3-dioxolane/dioxane fragments derive from a trans-acetalation of the aglycon moiety with the complementary fragments obtained as Smith degradation products.[5,6]

Peaks diagnostic of the aglycon groups were found in the GC-MS at *m*/*z* = 87, 103, 131, 133, 145 and 175, and, in addition, several fragments of the complete aglycon bearing a carbohydrate fragment were found, e.g. at *m*/*z* = 249, which confirmed the MS analysis above (Figure 3).

The NMR spectroscopic data are in agreement with these assumptions. Actually, we found all the signals in the spectra that are congruent with the presence of a disaccharide of rhamnose and of a disaccharide built up of rhamnose and glucose and, in addition, a rhamnose glycoside. Moreover, we also recognised some of the aglycon fragments. For instance, the hydrated-carbonyl form of the glyceraldehyde aglycon has a typical chemical shift at δ = 90.3/5.17 ppm (Figure 4).

All these data taken together suggest a glucorhamnan backbone with a trisaccharide repeating unit whose residues

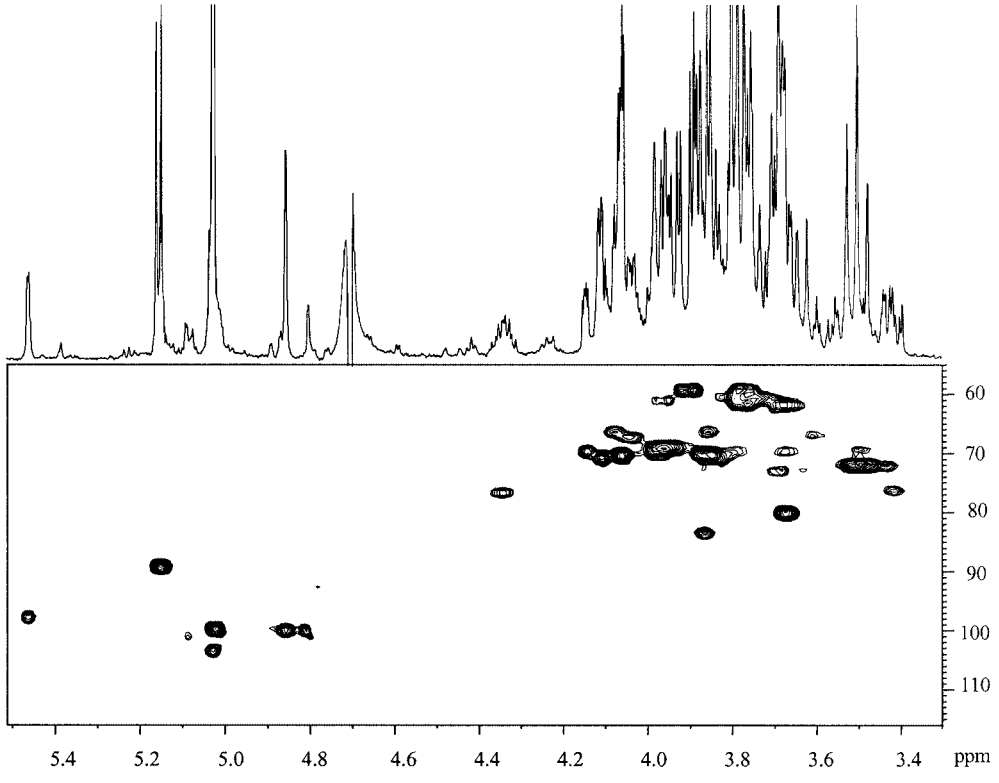


Figure 4. Expansion of the ^{1}H NMR and HMBC spectra of the mixture **2a**−**2c**; a cross peak is detectable at δ = 90.3/5.17 ppm. that corresponds to the hydrated carbonyl form of the glyceraldehyde; the other peaks in the anomeric region belong to carbohydrate residues

bear branches built of a single xylose unit. Some indications on the xylose distribution were deduced by the sizes of the Smith degradation products, taking into account that no information can be obtained on the xylosilation of the 3-linked Rha residue (**C**), which is, in any case, inert to the Smith reaction. Besides fraction **1**, which eluted in the void volume, only disaccharide and monosaccharide components were found in fractions **2a**−**c**, and no higher oligosaccharides were found in any significant amount. In particular, the polysaccharide of fraction **1**, for which a molecular mass of roughly 6000 Da was measured by GPC, can be derived only by sequences of the backbone trisaccharide repeating units (**A**−**B**−**C**) where both **A** and **B** are xylosilated residues. On the other hand, sequences where these two residues are not xylosilated give only monosaccharide fragments (Rha) and sequences where only **A** or only **B** are xylosilated give only disaccharide Glc−Rha or Rha−Rha fragments, respectively. Combinations of these three sequences always give the above products, except when two repeating units singly branched at 2-Glc and 2-Rha, respectively, are connected, in which case trisaccharides are obtained. On the contrary, their combination with the sequence where **A** and **B** are both xylosilated should always yield higher oligosaccharide fragments.

These considerations suggest a non-regular, but not random, xylose distribution.

An alternative hypothesis is that the mixture comprises at least two polysaccharides where, in one, the backbone trisaccharide repeating units contain **A**, **B** and, eventually, **C** residues all xylosilated, and the other with one of the other three sequences or with a combination of them, but not with the one forming the trisaccharide. Since all attempts to separate the eventual polysaccharide mixture were unsuccessful, we suggest a unique blockwise O-chain structure where structural features are present with different branch densities in analogy to that found for many other natural O-chain polysaccharides.[6−11]

Further information on the xylose distribution is gleaned from the ratios of the methylated products.

2,4-Rha/2-Rha = 3:2

2,3-Glc/2-Glc = 3:2

3,4-Rha/3-Rha = 3:7

These ratios suggests that both 2-linked Rha and Glc residues are mainly branched whereas the opposite situation exists for the 3-substituted Rha units that are found as non-nodal residues more than two-thirds of the time. This observation might suggest that, for steric reasons, the 3-linked Rha is not branched when one or both of the other residues of the repeating unit are xylosilated.

In conclusion, we suggest that the O-chain polysaccharide of *X.campestris.* pv. *pruni* has a complex structure where the following repeating units (**types 1−5**), possibly in amounts very similar to those found in the methylation data, are mainly present.

	A	**B**	**C**
	→2)-α-L-Rha*p*-(1→2)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→		
	4	3	4
	↑	↑	↑
type 1	β-L-Xyl*p*	β-L-Xyl*p*	
type 2	β-L-Xyl*p*		
type 3		β-L-Xyl*p*	
type 4			β-L-Xyl*p*
type 5	-	-	-

The assembly of O-chain polysaccharide structures has been studied extensively in animal-associated bacteria, in which they are, for the most of cases, have rigorously repetitive structures. On the other hand, structural heterogeneity has been found previously in plant pathogenic bacteria in a few OPSs of *Xanthomonas campestris* pvs. *campestris,*[6,7] *begoniae,*[8] *vignicola,*[9] *Xanthomonas fragrariae*,[10] and *Pseudomonas fluorescens.*[11] In all these polymers, the lack of regularity is due to a monosaccharide present in a non-stoichiometric amount in the side chain, as was found in this case. The non-stoichiometric glycosylation of the side branches by xylose units can be considered as a post-polymerisation "decoration" that the bacterium applies as a strategy to evade the host immune response.

Therefore, biosynthetic studies are needed to comprehend this biosynthesis.

Experimental Section

Bacterial Growth, Isolation and Purification of the LPS Fraction: Strain type NCPPB416 of *Xanthomonas campestris* pv. *pruni* was maintained for long-term storage at −70 °C in 30% glycerol or lyophilised, and were grown routinely on Wilbrink's medium[12] slants at 25 °C. The bacterium was grown in the above liquid medium (200 mL) on a rotary shaker at 150 rpm at 25 °C for 96 h. Cultures were centrifuged (20000 *g* × 15 min), the pellets were washed twice with brine (0.8% NaCl) and the bacterial cells were freeze-dried.

The dried cells (5.6 g) from *Xanthomonas campestris* pv. *pruni* cultures (2.5 L) were suspended in ultrapure water (200 mL) and extracted with hot phenol/water according to the conventional procedure.[13] Both phases were separately dialysed against distilled water, freeze-dried and screened by 12% SDS-PAGE on a miniprotean gel system from Bio-Rad. Samples (4 μg) where run at constant voltage (150 V) and stained according to the published procedure.[14] The lipopolysaccharide fraction (458 mg) was found exclusively in the water phase.

LPS fraction was further purified from other contaminant material on a Sephacryl HR 400 column (Pharmacia, 1.5 × 90 cm, eluent NH_4HCO_3 50 mM, flow rate of 0.4 mL/min). Eluate was monitored with an R.I. refractometer (R410 Waters) and the fractions corresponding to distinctive peaks were collected and screened by SDS-PAGE to obtain an LPS fraction (270 mg, 4.8% of dry cell weight).

Compositional and Methylation Analysis: Monosaccharides were analysed as acetylated *O*-methyl glycoside derivatives and lipids as methyl esters, according the following procedure.

LPS (1 mg) was dried in a desiccator over P_2O_5 for 1 h under vacuum and then treated with 1 M methanolic HCl at 80 °C for 18 h.

The methyl esters of the fatty acids were recovered by extraction with *n*-hexane and analysed by GC-MS.

The methanolic phase was dried and the methyl glycosides were treated with acetic anhydride (100 μL) and pyridine (200 μL) at 80 °C for 30 min. After workup, the mixture of peracetylated derivatives was analysed by GC-MS. Absolute configurations were deduced by analysis of the chiral 2-octyl derivatives according to the published procedure.[15]

GS-MS analysis for fatty acids and methyl and octyl glycoside derivatives were run on a Hewlett−Packard 5970 instrument, with an SPB-5 capillary column (Supelco, 30 m × 0.25 mm i.d., flow rate of 0.8 mL/min; He as carrier gas), with the following temperature programme: 150 °C for 5 min, 150 to 300 °C at 5.0 °C/min, 300 °C for 5 min. Mass spectra were recorded by using an ionisation energy of 70 eV and an ionising current of 0.2 mA.

Glycosyl-linkage analyses of LPS and fraction **1** were performed according to the Sandford's procedure.[16] The permethylated lipopolysaccharide was recovered in the organic layer of the water/chloroform extraction and converted into its partially methylated alditol acetates, which were analysed by GC-MS with the temperature programme above. The oligosaccharides **2a**−**2c** were permethylated as above and then analysed by GC-MS using the following conditions: 150 °C for 5 min, 150 → 300 °C at 5.0 °C/min, 300 °C for 25 min.

Isolation of the O-Specific Polysaccharide Fraction: The LPS fraction (70 mg) was dissolved in 1% AcOH (1 mL) and heated at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000 rpm for 20 min and the clear supernatant freeze-dried. A further purification of this sample was performed by GPC on Sephacryl HR 300 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow rate of 0.4 mL/min). The eluate was monitored by refractive index as above mentioned. The O-chain was isolated in approx. 90% yield from LPS.

NMR Spectroscopy: The NMR spectroscopy experiments were carried out at 30 °C on Bruker DRX 400 and Bruker DRX 600 spectrometers, both equipped with reverse multinuclear probes. Chemical shifts in the spectra recorded in D_2O are expressed on the δ scale and were referenced to the methyl signal of internal acetone (δ_H = 2.225 ppm and δ_C = 31.4 ppm). Two-dimensional spectra (DQF-COSY, TOCSY, NOESY, and phase-sensitive gradient-HSQC and HMBC) were measured using standard Bruker software.

For DQF-COSY, 1024 FIDs of 4096 complex data points were collected, with 40 scans per FID. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and resolution was enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. For other homonuclear experiments, typically 512 FIDs of 2048 complex data points were collected with 40 scans per FID and then elaborated as above. In all cases, the spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak. A mixing time of 200 ms was used in the NOESY experiment. For the HSQC spectrum, 256 FIDs of 1024 complex points were acquired with 50 scans per FID, and the GARP sequence was used for ^{13}C NMR decoupling during acquisition. Processing and plotting was performed with the standard Bruker Xwin NMR 1.3 programme.

Smith Degradation: An aliquot of O-chain polysaccharide (20 mg) was submitted to Smith degradation.[17] Briefly, it was treated with 50 mM $NaIO_4$ at 4 °C for 7 d, followed by addition of ethane-1,2-diol, reduction ($NaBH_4$), acidification (2 M acetic acid), dialysis and freeze-drying. The oxidised polymer was hydrolysed with 1% acetic acid at 100 °C for 1.5 h and then the acid was removed by freeze-drying. The product was purified on a Bio-Gel P2 column (2 × 100 cm) and was eluted in the void volume with 50 mM ammonium bicarbonate buffer (pH = 5), monitored with a Waters differential refractometer, and dried (15 mg).

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