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Structural elucidation of a novel core oligosaccharide backbone of the lipopolysaccharide from the new bacterial species *Agrobacterium larrymoorei*

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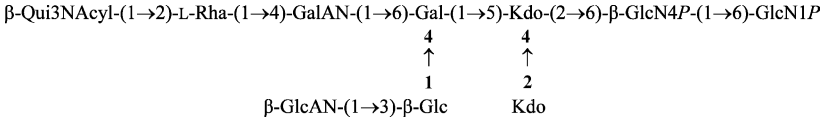
Abstract

Agrobacterium larrymoorei is a Gram-negative phytopathogenic bacterium, which produces tumours on *Ficus benjamina* plants and differs from other *Agrobacteria* both genetically and biochemically. The lipopolysaccharide (LPS) plays an important role in the pathogenesis of *Agrobacteria.* The present paper is the first report on the molecular primary structure of the core region of an *Agrobacterium* LPS. The following structure of the core and lipid A carbohydrate backbone of an R-form LPS of *A. larrymoorei* was determined by chemical degradations and 1D and 2D NMR spectroscopy:



All sugars are α-D-pyranoses if not stated otherwise, Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid, Qui3NAcyl is 3,6-dideoxy-3-(3-hydroxy-2,3-dimethyl-5-oxoprolylamino)glucose, GlcAN and GalAN are amides of GlcA and GalA.

Keywords: Core oligosaccharide; R-form lipopolysaccharide; Phytopathogenic bacteria; 3-Hydroxy-2,3-dimethyl-5-oxoprolyl group; *Agrobacterium larrymoorei*

1. Introduction

The genus *Agrobacterium* includes soil-borne Gram-negative bacteria responsible for the induction of a neoplastic disease (crown gall) or for abnormal root proliferation (hairy root) on many different host plants worldwide. Taxonomy and nomenclature of different *Agrobacterium* species is still under discussion and, most recently, their inclusion in the genus *Rhizobium* has been proposed.[1]

A new bacterial species named *Agrobacterium larrymoorei* was first isolated in 1991 in Florida from tumours developing on *Ficus benjamina* plants and later in Italy and The Netherlands.[2–4] *A. larrymoorei* isolates show an atypical colony morphology different from other known *Agrobacterium* species (*A. tumefaciens*, *A. rhizogenes*, *A. vitis* and *A. rubi*). *Agrobacterium* colonies are usually 2–3 mm in diameter, white-glistening, convex and smooth whereas atypical colonies of *A. larrymoorei* strains are 1–1.5 mm in diameter, flat, transparent and slow-growing.[5] The new bacterium

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differs from the other *Agrobacterium* species also in terms of differential metabolism of carbon substrates and fatty acid content.[3] Moreover, phylogenetic studies based on *rrs* gene (i.e., 16S rRNA gene) analysis revealed that the sequence in the new strains of *Agrobacterium* differed enough to consider it as a new species.[3] DNA–DNA homology studies confirmed this suggestion since the new putative species showed less than 60% homology with other *Agrobacterium* species.[2]

A. larrymoorei is able to survive and move in the fig xylem vessels and to induce tumours on aerial parts of the plants in the presence of wounds; this behaviour is rare among pathogenic *Agrobacterium* species.[4] The event of pathogenesis is modulated by the components of the external membrane of the bacterium, including both proteins and lipopolysaccharide (LPS).[6,7] In the last case, the interaction is based on the recognition of the LPS by particular receptor proteins located on the plant cell wall. It is possible to saturate these receptors with an LPS solution leading to the protection of the plant from the bacterial action. Despite the wealth of information regarding the biological role of the LPS components, there is only one report on the chemical structure of the LPS from *Agrobacterium*,[8] though this information is the first step toward the comprehension of the mechanism of pathogenesis.

2. Results

Extraction of freeze-dried bacterial cells of *A. larrymoorei* with hot phenol/water gave an LPS fraction in the water phase. It was further purified by sequential nuclease and protease treatment and GPC on Sephacryl HR-400. The purified LPS behaved on SDS-PAGE as a typical lipooligosaccharide, running to the end of the gel and giving no ladder-like pattern typical of an O-polysaccharide.

The determination of GlcN and organic phosphate contents of the LPS gave a molar ratio of $\sim$1:1, suggesting that only the lipid A moiety is substituted by phosphate groups in the usual fashion.

Oligosaccharide **1** (Fig. 1) was isolated by GPC after complete deacylation of the LPS. Analytical high-performance anion-exchange chromatography (HPAEC) showed the presence of a single peak. Compositional analysis of **1** revealed D-GlcA, D-Glc, D-Gal, D-GlcN, Kdo and minor not recognized peaks. Methylation analysis showed the presence of terminal GlcA, 3-substituted Glc, 4,6 disubstituted Gal, 6-substituted GlcN, 4,5-disubstituted and terminal Kdo. Additionally, a trace amount of a 4-substituted HexA was found.

The structure of **1** was established by ^{1}H and ^{13}C NMR spectroscopy. Chemical shifts were assigned using COSY, TOCSY, NOESY, ROESY, ^{1}H,^{13}C HSQC and HMBC experiments. Anomeric configurations were established on the basis of the chemical shifts and $J_{1,2}$ values, which were determined from the DQF-COSY-spectrum. The data are presented in Table 1.

The anomeric region of the ^{1}H NMR spectrum (Fig. 2) contained seven signals (**A**–**F**, in order of decreasing chemical shift). One of them appeared at 5.81 ppm, i.e., roughly in the olefin resonance region. The other six signals represented two hexosamine, two hexose and two uronic acid residues. Their identification was achieved by the complete assignment of all signals and the determination of the $^{3}J_{H,H}$ coupling constants. Two hexosamine residues had chemical shifts typical of the lipid A backbone. Particularly, the anomeric signal **A** at 5.65 ppm and the proton signals to which it was correlated in the TOCSY and COSY spectra, were easily identified as belonging to the GlcN I spin system. Similarly, the GlcN II spin system with the anomeric proton **D** resonance at 4.85 ppm, was recognized. One hexose spin system, for residue **C**, with the anomeric proton signal at 5.27 ppm possessed the α-*galacto* configuration as inferred from the chemical shifts and the $^{3}J_{3,4}$ and $^{3}J_{4,5}$ values (3 and 1 Hz, respectively).

Two other anomeric resonances at 4.58 and 4.52 ppm (**E** and **F**, respectively) possessed the β-*gluco* configuration. The residue **E** was identified as β-GlcA because in the TOCSY spectrum correlations within its spin system ended at H-5 (d, *J* 10 Hz). The **F** residue was identified as β-glucose from the TOCSY and COSY spectra. The β-*gluco* configuration of residues **D**, **E** and **F** was supported by a NOESY experiment, which showed intraresidual NOE connectivities from H-1 to H-3 and H-5.

The characteristic signals of H-3 of two Kdo residues were present at 1.82 (H-3ax) and 2.17 ppm (H-3eq, residue **G**); 1.84 (H-3ax) and 2.23 ppm (H-3eq, residue **H**). Their α-configuration was established on the basis of the chemical shift of H-3eq as well as $^{3}J_{H7,H8a}$ and $^{3}J_{H7,H8b}$ values.[9,10]

The two remaining resonances in the anomeric region, **B** and **B′**, belonged to the same spin system since they were correlated in the TOCSY and COSY spectra via two other proton resonances. As demonstrated below, **B** is the anomeric proton of a hex-4-enuronic residue, which resulted from β-elimination in a 4-substituted uronic acid residue under alkaline conditions.[11] Thus, the **B′** resonance should belong to H-4 of residue **B**.

The ^{13}C NMR chemical shifts were assigned by an ^{1}H,^{13}C HSQC experiment, using the interpreted ^{1}H NMR spectrum. Seven signals were identified in the anomeric region and labelled according to decreasing chemical shift of the anomeric protons (Table 1). The C-1 and C-2 signals of both Kdo residues **G** and **H** were not detected in this experiment but they were revealed by a HMBC experiment (see below). Signals shifted to low-field indicated substitution at O-6 of residues **A**, **C**

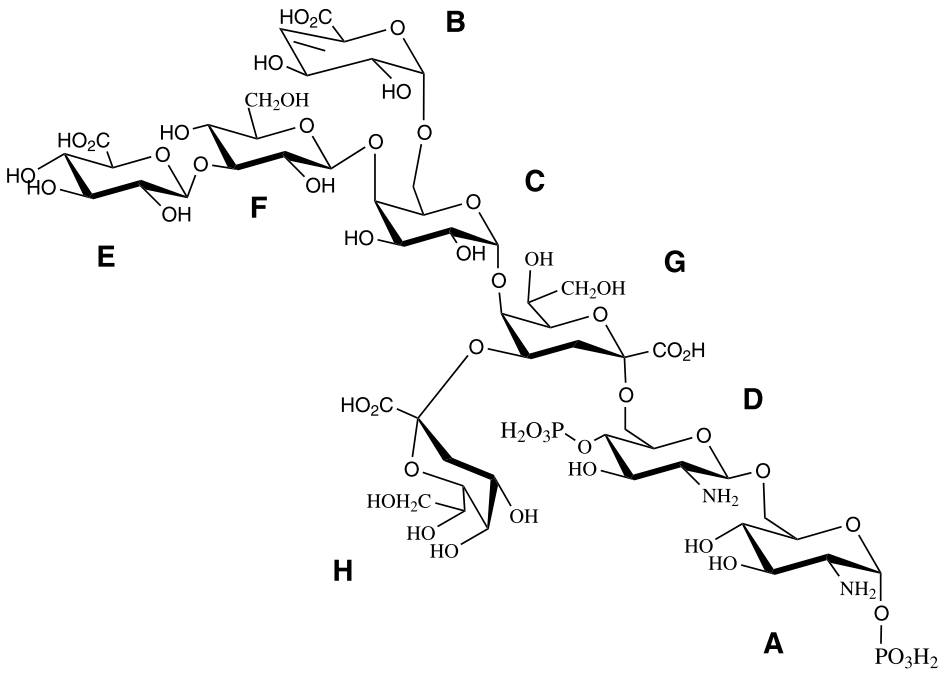

Fig. 1. Structure of oligosaccharide **1** obtained by alkaline treatment from the LPS from *A. larrymoorei*.

and **D**, O-5 (**G**), O-4 (**C** and **G**), O-3 (**F**), whereas residues **E** and **H** are terminal sugars. The ^{13}C NMR chemical shifts of the spin system for **B** confirmed the previous suggestion of a hex-4-enuronic acid residue. Moreover, in the $^{1}H,^{13}C$ HMBC spectrum signals for H-1, H-4 (**B′**) and H-3 of residue **B** correlated to a double-bond carbon resonance at 149.2 ppm, which was identified as C-5 of residue **B**, whereas only proton H-4 correlated to a carbonyl carbon at 171.2 ppm, which was recognised as C-6.

The NMR data were in accord with methylation data, and, therefore, the oligosaccharide **1** comprises a lipid A backbone, two Kdo residues and a small oligosaccharide following the Kdo residues. This oligosaccharide is built up of terminal GlcA, 3-subsituted Glc, 4,6-disubstituted Gal and a terminal hex-4-enuronic residue, which in the

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of sugar residues in oligosaccharide **1** from LPS of *A. larrymoorei*

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
A	5.65	3.40	3.89	3.63	4.15	4.28/3.75
GlcN I	91.9	55.8	71.0	71.0	73.6	71.0
B	5.42	3.86	4.34	5.81		
Δ4,5 GalA	100.7	71.6	67.2	108.3	149.2	171.2
C	5.27	3.57	3.93	4.36	4.21	4.19/4.11
Gal	100.5	72.9	72.0	76.8	69.6	68.3
D	4.85	3.03	3.94	3.81	3.71	3.46/3.71
GlcN II	101.0	56.9	71.5	73.8	75.6	63.9
E	4.58	3.38	3.49	3.47	3.84	
GlcA	103.3	73.1	73.0	73.3	74.0	
F	4.52	3.30	3.52	3.39	3.44	3.72/3.90
Glc	103.6	74.5	81.0	71.1	76.9	61.9
	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8/C-8
G	1.82/2.17	4.23	4.30	3.73	3.91	3.90/3.67
Kdo I	35.0	69.5	70.2	73.8	70.8	64.6
H	1.84/2.23	4.15	4.30	3.67	3.98	3.88/3.66
Kdo II	35.0	66.5	68.4	74.0	70.5	64.7

Residues are labelled according to decreasing chemical shifts of the anomeric signals. Resonances at 177.0 and 102.0 ppm were tentatively assigned C-1 and C-2 of two Kdo residues, respectively.

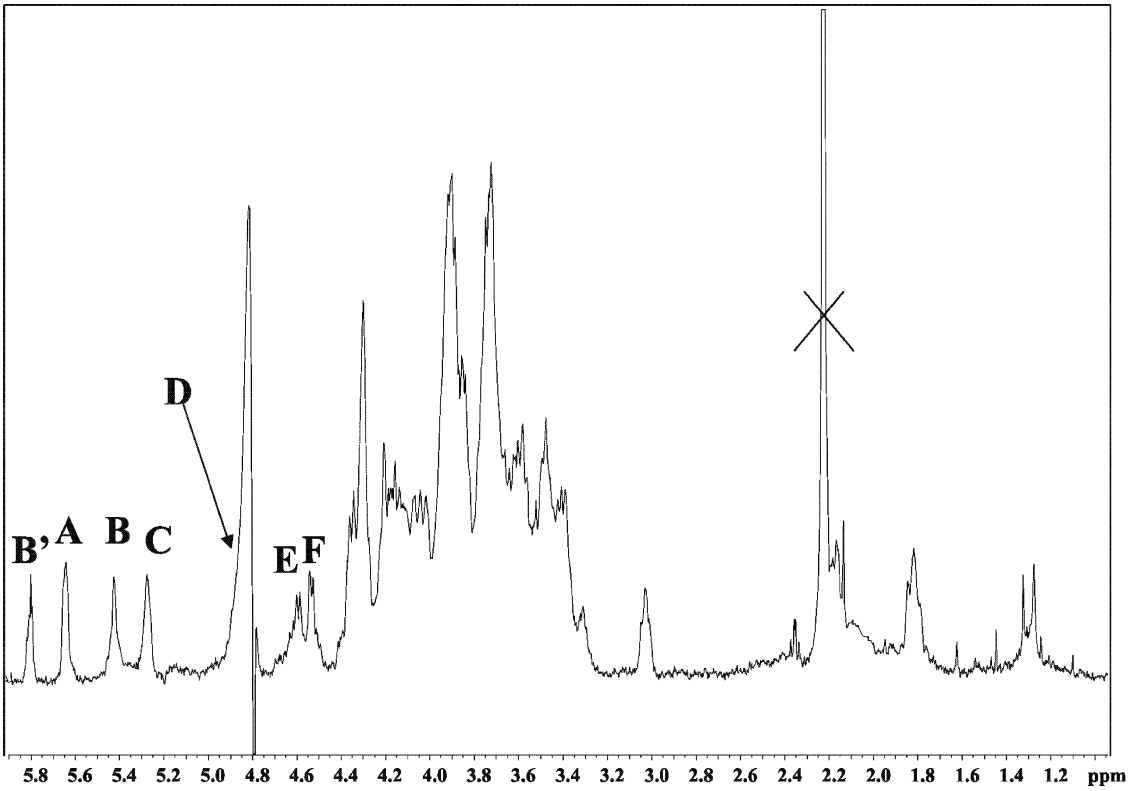

Fig. 2. ^{1}H NMR spectrum of oligosaccharide **1**. Anomeric signals are designated by capital letters; the anomeric peak **D** is covered by the HOD signal.

initial LPS should bear a substituent at O-4 eliminated by alkaline treatment.

The monosaccharide sequence in the oligosaccharide **1** was inferred using interresidual NOE correlations measured by 2D NOESY (Fig. 3) and long range scalar connectivities measured by HMBC. An interresidual NOE contact was observed between H-1 of GlcN **D** and H-6 of GlcN **A** (not shown in Fig. 3), thus establishing the (1→6)-linkage in the lipid A backbone.

The deoxy-protons H-3ax and H-3eq of Kdo **G** showed an NOE contact to H-6 of Kdo **H**, and H-3ax of **G** showed a contact to H-5 of Gal **C**. These interresidual NOE connectivities are characteristic for the sequence α-Gal-(1→5)-[α-Kdo-(2→4)]-α-Kdo.[12] Accordingly, a strong NOE contact between H-1 of **C** and H-5 of **G**, together with weak contacts to H-7 and H-8a, were observed. According to its ^{13}C chemical shifts, residue **C** should be 4,6-disubstituted. Indeed, the anomeric proton of the terminal hex-4-enuronic acid residue **B** gave an NOE with H-6a,b of residue **C**, whereas the β-anomeric proton of glucose **F** gave an NOE contact with H-4 of Gal **C**.

The connection between the terminal GlcA **E** and Glc **F** was inferred by an NOE contact between the anomeric proton of residue **E** and H-3 of residue **F**. Since Kdo possesses no anomeric proton, it was not possible to confirm the linkage of Kdo **G** to GlcN **D** by NOE. However, since all other linkages in oligosaccharide **1** were identified, the (2→6)-linkage between **G** to **D** could be established by a medium-sized downfield displacement of the C-6 signal of residue **D** to 63.9 ppm, that is characteristic of a ketosidic linkage (Table 1).

The HMBC spectrum confirmed the structure of oligosaccharide **1** since it contained most required long-range correlations to demonstrate the linkage pattern. Particularly, interresidual connectivities between H-1/C-1 of **C** and C-5/H-5 of Kdo **G**, H-1/C-1 of **F** and C-4/H-4 of **C**, H-1/C-1 of **B** and C-6/H-6 of **C**, H-1/C-1 of **E** and C-3/H-3 of **F** were observed.

Two signals were found in the ^{31}P NMR spectrum of oligosaccharide **1** at 1.5 and 4.2 ppm, which were assigned by a ^{1}H,^{31}P HMQC experiment to phosphate groups linked to O-4 of residue **D** and O-1 of residue **A**, respectively. This assignment was in good agreement with the expected chemical shifts of the signals of the carbons linked to the phosphate groups and of the corresponding protons.[13] Thus, the core-lipid A carbohydrate backbone structure of the LPS was established (Fig. 1).

In order to establish the structure of the oligosaccharide component that was lost by β-elimination in the LPS under strong alkaline conditions, the LPS was subjected to mild acid hydrolysis with aq 1% AcOH to remove

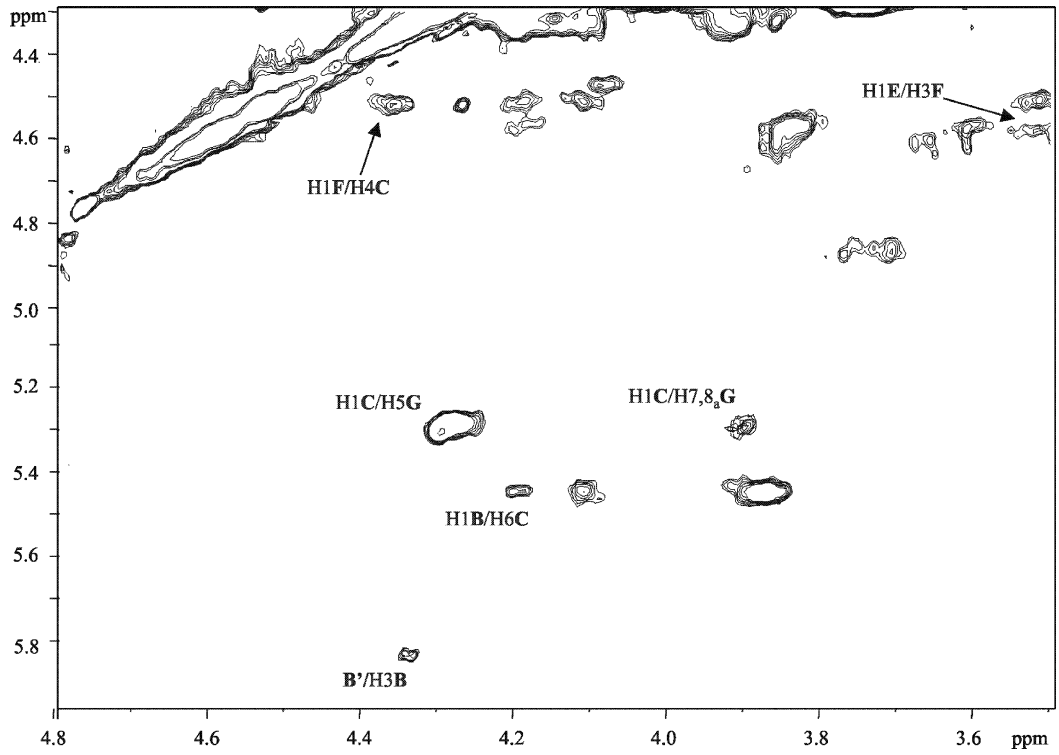

Fig. 3. Part of a NOESY spectrum of oligosaccharide **1**. Annotations refer to interresidual cross-peaks. Monosaccharides are as shown in Fig. 2. The capital letters refer to residues as denoted in Table 1.

terminal Kdo and lipid A. The residual material had no organic phosphate as indicated by a colorimetric assay and ^{31}P NMR spectroscopy. Hence, the core region is free of phosphate.

The oligosaccharide product obtained, **2**, was studied by compositional/methylation analyses and 2D NMR spectroscopy. Compositional analysis of oligosaccharide **2** showed D-GlcA, D-GalA, D-Glc, D-Gal, L-Rha and D-Qui3N, the last sugar being detected in a minor amount. Methylation analysis of oligosaccharide **2** revealed terminal GlcA, 4-substituted GalA, 2-substituted Rha, 4,6 di-substituted Gal, 3-substituted Glc and small amounts of 5-substituted Kdo and terminal Qui3N. Despite the presence of an amino function in oligosaccharide **2**, no acetyl signal was present in the ^{1}H NMR spectrum. However, in the aliphatic region of the spectrum there were two singlets at 1.37 and 1.49 ppm for methyl groups and two doublets at 2.45 and 2.67 ppm for an AB system of a diastereotopic methylene group (Fig. 4). A large *geminal* coupling (J 17.2 Hz) in the AB system was distinctive of a five-membered ring. Therefore, it was suggested that Qui3N bears an unusual acyl group at N-3.

2D NMR spectroscopic analysis was performed using COSY, TOCSY, NOESY, HSQC and HMBC experiments (Table 2). Several sets of signals were visible in the deoxy region of the ^{1}H NMR spectrum, which belonged to the multiple forms of the Kdo residue at the reducing end of oligosaccharide **2**. Six signals in the anomeric region were assigned to residues **L**–**R**.

The spin system **L** was assigned to GalA using TOCSY and COSY spectra by chemical shifts and $^{3}J_{3,4}$ and $^{3}J_{4,5}$ values (3.6 and 2 Hz, respectively). The anomeric proton at 5.66 ppm (**M**) was correlated by the TOCSY experiment to a methyl signal at 1.21 ppm and, consequently, the spin system **M** belongs to rhamnose, which was confirmed by low $^{3}J_{1,2}$ and $^{3}J_{2,3}$ values (2 and 3 Hz, respectively). A signal at 5.13 ppm was recognised as the anomeric proton of galactose **N** by the $^{3}J_{3,4}$ and $^{3}J_{4,5}$ values.

Two anomeric proton resonances appeared at 4.61 ppm, **P** deriving from β-glucose and **Q** from Qui3N, as evident from the COSY and TOCSY spectra. Particularly, the TOCSY spectrum showed a cross-peak between the anomeric signal **Q** and a methyl signal at 1.26 ppm, and all sugar ring $^{3}J_{H,H}$ values were $\sim$10 Hz. Finally, residue **R** was identified as GlcA, as followed from the chemical shifts and coupling constant values. The β configuration of residues **P**–**R** was corroborated by $^{3}J_{1,2}$ values of $\sim$8 Hz and NOE contacts of H-1 to H-3 and H-5.

Six signals for anomeric carbons were identified by an ^{1}H,^{13}C HMQC experiment (Table 2). Downfield displacements of carbon resonances were identified for residue **L** (C-4), **M** (C-2), **N** (C-4 and C-6) and **P** (C-3), whereas residues **Q** and **R** were recognised as terminal sugars. Moreover, C-3 of **Q** was identified as a nitrogen-

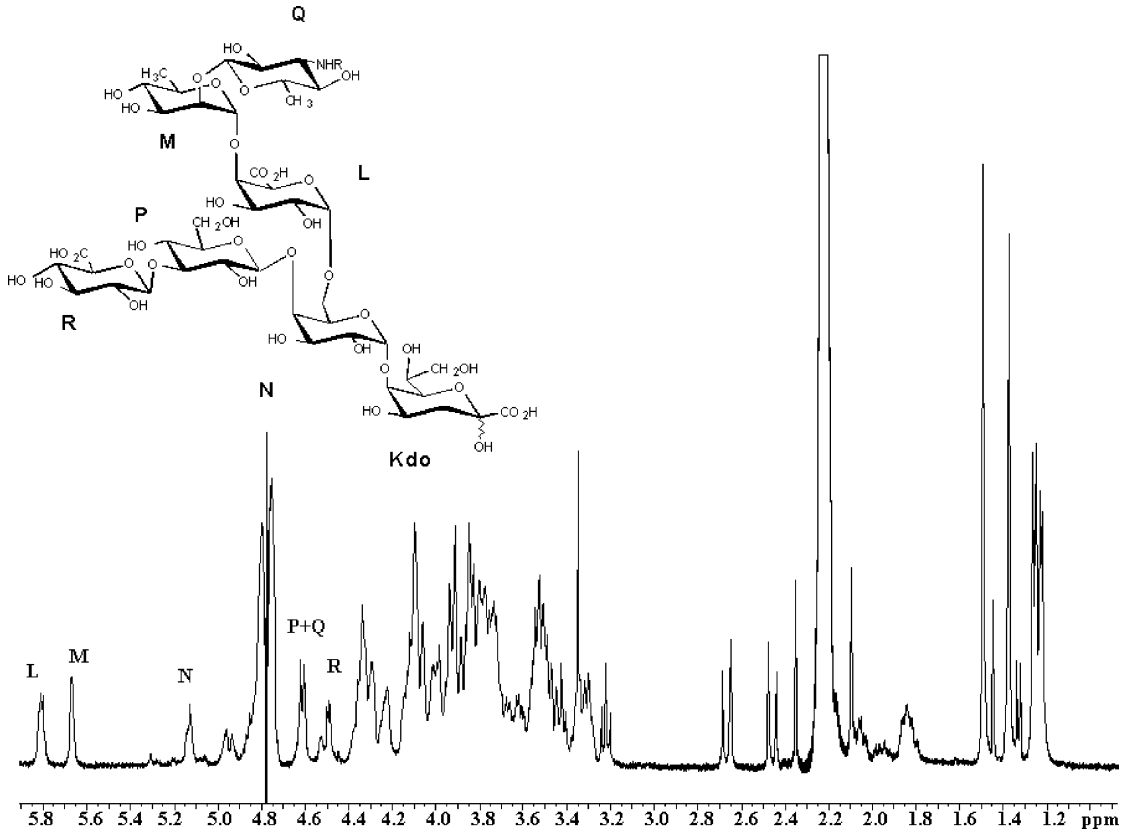

Fig. 4. ^{1}H NMR spectrum and structure of oligosaccharide **2** obtained by mild acid hydrolysis from the LPS from *A. larrymoorei*. The significant peaks in the anomeric region are designated by capital letters.

bearing carbon on the basis of its chemical shift of 58.2 ppm. The reducing α-Kdo*p* was tentatively identified and its C-5 found to have a glycosylation shift. These data were in agreement with methylation analysis data.

Table 2
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of sugar residues in oligosaccharide **2** from LPS of *A. larrymoorei*

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
L	5.81	4.09	4.00	4.50	4.97	
4-GalA	99.9	67.5	71.5	82.5	72.0	175.3
M	5.66	4.10	3.97	3.41	3.77	1.21
2-Rha	99.7	81.8	71.2	73.4	69.2	17.9
N	5.13	3.54	3.86	4.28	4.35	4.09
4,6-Gal	101.0	73.1	71.0	76.4	70.0	68.8
P	4.61	3.29	3.67	3.52	3.49	3.91/3.74
3-Glc	106.0	72.3	81.3	72.4	76.9	61.2
Q	4.61	3.46	3.90	3.23	3.52	1.26
Qui3NAcyl	105.0	72.8	58.2	74.3	74.6	18.3
R	4.34	3.79	3.73	3.91	4.29	
GlcA	105.1	69.8	71.2	71.0	75.0	173.6
	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8/C-8
	1.84/2.21	4.13	4.08	3.61	3.98	3.94/3.61
5-Kdo I	35.0	66.1	73.1	72.1	70.1	64.0

Residues are labelled according to decreasing chemical shifts of their anomeric signals. Chemical shifts for the 3-hydroxy-2,3-dimethyl-5-oxoprolyl group (Acyl): C-1 to C-5 at 176.0, 71.0, 79.9, 44.0 (δ_H 2.45, 2.67) and 180.0 ppm, respectively; two methyl groups at δ_C/δ_H 18.8/1.49 (CH_3-2) and 23.3/1.37 (CH_3-3).

Therefore, the main difference between **2** and **1** is the presence in the former of an additional 2-substituted rhamnose, a terminal Qui3N and, evidently, the predicted 4-substituted GalA residue, which was unequivocally identified in **2**. The remaining sugar residues, except for the terminal Kdo and GlcN of lipid A, were identical in both oligosaccharides.

It was reasonable to arrange the three additional residues in the oligosaccharide **2** as **Q**→2-**M**→4-**L**→6-**N**, and this sequence was confirmed by the ROESY and HMBC spectra. In the NOESY spectrum (Fig. 5), cross-peaks between H-1 of GalA **L** and H-6a,b of Gal **N**, H-1 of Rha **M** and H-4 of GalA **L**, and H-1 of Qui3N **Q** and H-2 of Rha **M** were of particular importance. During the alkaline treatment, the LPS underwent β-elimination and lost a Qui3N-(1→2)-Rha disaccharide, leaving a terminal hex-4-eneuronic acid.

In the HMBC spectrum, the methylene signals of the *N*-acyl group showed a long range correlation with three carbons, viz., a carbonyl carbon C-5, a tertiary oxygen-bearing carbon C-3 and a quaternary carbon C-2, both C-2 and C-3 correlated to the methyl signals present as singlet in the ^{1}H NMR spectrum. Since one of the two carbons bears oxygen, the two methyl groups are located on the two different carbons, C-2 and C-3. Indeed, only one of two methyl groups (CH_3-2) correlated with another carbonyl signal (C-1), which in its turn correlated with proton H-3 of residue **Q**. The NOESY spectrum showed a cross-peak of CH_3-3 with both CH_3-2 and methylene protons H-4. Taking account of these and literature data,[14] the N-acyl substituent of Qui3N was identified as a 3-hydroxy-2,3-dimethyl-5-oxoprolyl group. This conclusion was confirmed by GC-MS in methylation analysis, which showed a peak for a carbohydrate derivative having a high retention time. Based on its MS fragmentation, this was recognised as a partially methylated derivative of Qui3N retaining the *N*-acyl group (Fig. 6).

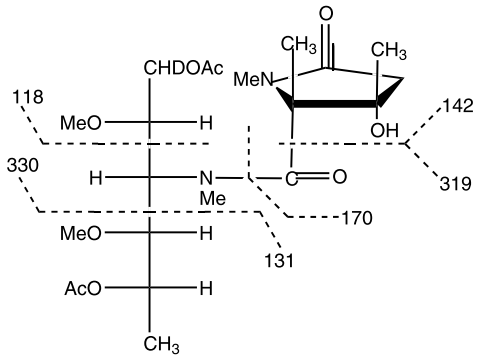

Fig. 6. The permethylated and acetylated derivative of Qui3N bearing the 3-hydroxy-2,3-dimethyl-5-oxoprolyl group as found in GC-MS. Shown are the main fragments found in the MS spectrum.

In addition, both uronic acid **L** and **R** in oligosaccharide **2**, should be amidated at their carboxyl function since the chemical shifts of the H-5 signal showed no pD dependence in the ^{1}H NMR spectrum and because residue **L** easily underwent β-elimination under alkaline conditions.[15] Since no other additional signals were present in the NMR spectra, we propose that simple amides of uronic acids are present.

In summary, combining the data from the alkaline and acidic degradations together, we established the complete structure of the carbohydrate backbone of the R-form LPS of the Gram-negative bacterium *Agrobacterium larrymoorei*, which is shown in Fig. 7.

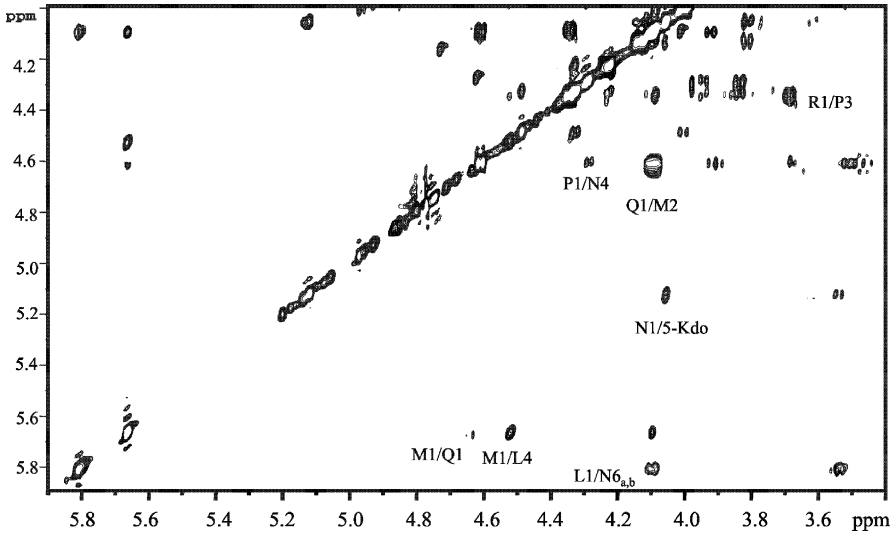

Fig. 5. Part of a NOESY spectrum of oligosaccharide **2**. Annotations refer to interresidual cross-peaks. The capital letters refer to residues as defined in Table 2.

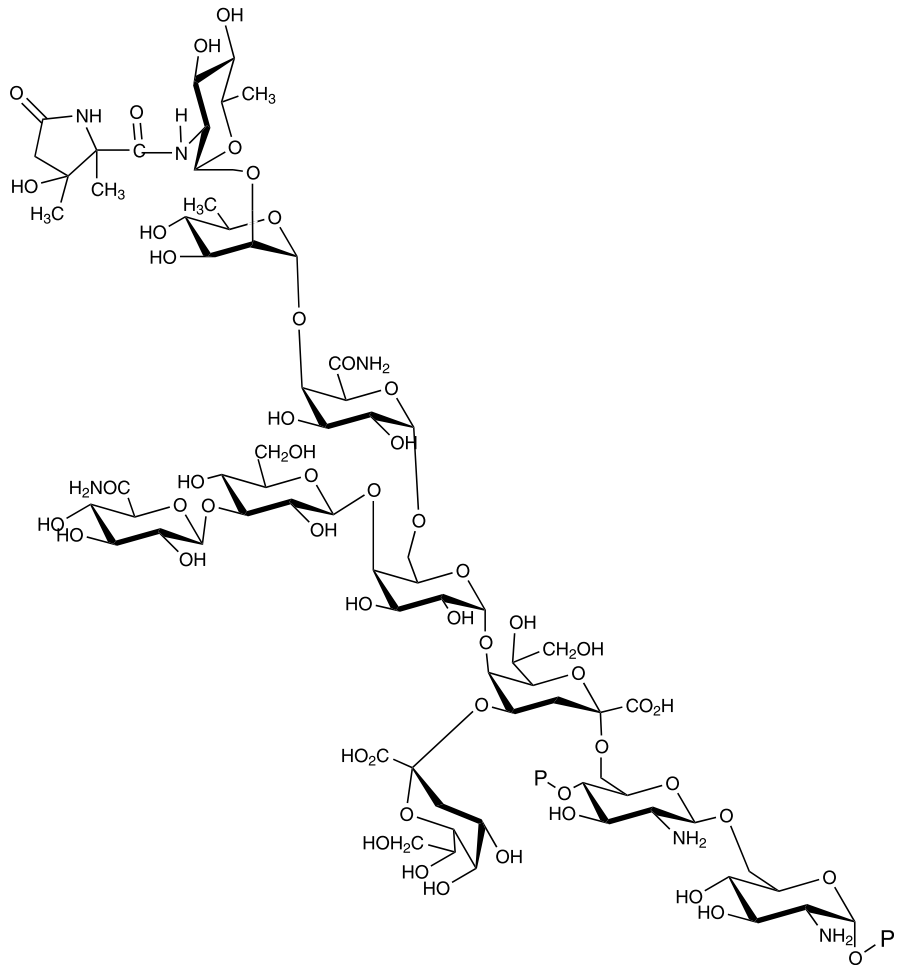

Fig. 7. The complete oligosaccharide backbone of the LPS from *A. larrymoorei*.

3. Discussion

Depending on the saccharide size, there are two kinds of lipopolysaccharides, deriving from smooth and rough bacteria (S-LPS and R-LPS). Both structures are built up of lipid A and covalently bonded core, and the core region comprises up to 15 monosaccharides. In S-LPS, the core region is covalently linked to an O-specific polysaccharide chain. Both LPS forms are present in wild-type Gram-negative bacteria, a core-lipid A moiety being the minimum structural unit required for the life of bacteria.

The LPS plays a key role in the interaction with, and defence reactions of, host plants, and, therefore, the knowledge on the LPS structure is of great relevance. Despite the wealth of information in the biology of *Agrobacterium* genus and in the chemistry and biochemistry of the LPS of Gram-negative bacteria, there are no data available in literature on the chemical structure of the *Agrobacterium* LPS, apart from a paper by our group.[8]

A. larrymoorei produces only an R-form LPS, a lipooligosaccharide, and in this work the first core structure was elucidated for an *Agrobacterium* species. From a biochemical point of view, this core is unusual since it lacks heptose residues and phosphate groups. Heptoses are absent from the LPS of a few bacteria studied so far (e.g., in *Chlamydia*, *Branhamella*, *Acinetobacter* or *Rhizobium*).[16,17] It is particularly interesting that this chemical peculiarity is now found in *Agrobacterium*, a *Rhizobium* correlated bacterium, confirming the suggested close taxonomical relationship of these two genera.

The absence of phosphate groups is more frequent, and uronic acids are introduced instead to provide a negative charge.[16,17] It is thought that these negatively charged groups with bivalent ions as counterparts assemble ionic bridges between LPS molecules, which contribute to the rigidity and stability of the Gram-negative cell wall. The LPS of *A. larrymoorei* is unusual in this respect since uronic acids are present as uronamides and, hence, do not provide any negative charge.

Remarkable is also the finding of an unusual *N*-acyl group, which is a derivative pyroglutamic acid. This group has been identified in the core region only once as a component of the LPS of *Campylobacter coli*.[18] In

both bacteria, it is linked as amide to Qui3N, which often bears unusual substituents in the LPS. The same *N*-acyl group has also been identified in the O-chain of *Pseudomonas fluorescens*[14] and *Pseudomonas putida* (C. de Castro, unpublished data) and similar pyroglutamic acid derivatives in *Vibrio cholerae* and *Vibrio anguillarum*.[19,20]

4. Experimental

4.1. Bacteria and bacterial LPS

Agrobacterium larrymoorei was cultivated as described.[5] The LPS was obtained from lyophilised bacteria (3 g) by the phenol/water extraction method as described.[21] For further purification, the LPS fraction was subjected to sequential nuclease and protease treatment followed by GPC on Sephacryl HR-400 (Pharmacia) using a column (1.5×90 cm) eluted with 50 mM NH_4HCO_3 at 0.4 mL min^{-1}. The yield of the purified LPS was 84 mg or 2.8% of the bacterial dry mass.

4.2. Isolation of oligosaccharides 1 and 2

The LPS (16 mg) was O-de-acylated with anhyd hydrazine (1 mL) at 37 °C for 30 min, cooled, poured into ice-cold acetone (20 mL), and allowed to precipitate. The precipitate was centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone, dried, dissolved in water and lyophilised (12 mg, 75% of the LPS mass). The sample was subsequently N-deacylated with 4 M KOH as described.[15] After desalting using a column (50×1.5 cm) of Sephadex G-10 (Pharmacia), the resulting oligosaccharide fraction (7 mg, 43% of the LPS mass) was further fractionated by GPC on a column (50×3 cm) of TSK HW-40 (E. Merck) in 2:5:250 pyridine–acetic acid–water to give oligosaccharide **1** (5 mg, 31% of the LPS mass). This fraction contained only one carbohydrate component as determined by analytical HPAEC on a CarboPac PA1 column (4×250 mm) using a linear gradient of sodium acetate (0.2–0.9 M) in 0.1 M NaOH over 50 min.

Another portion of the LPS (20 mg) was hydrolysed in aq 1% AcOH (100 °C, 2 h), and the precipitate (lipid A) was removed by centrifugation (8000*g*, 30 min). The supernatant was fractionated by GPC on a column (50×3 cm) of Sephadex G-50 (Pharmacia). One main fraction was obtained (11 mg, oligosaccharide **2**) as shown by HPAEC.

4.3. General and analytical methods

The LPS was analysed by discontinuous SDS-PAGE (12% separating gel) utilizing a miniprotean gel system (Bio-Rad). A sample (4 μg) was run at constant voltage (150 V) and stained with silver nitrate.[22]

Neutral sugars and their absolute configurations, as well as organic phosphate and Kdo were determined as reported.[23–26] For methylation analysis of Kdo and uronic acids,[27] oligosaccharides were N-acetylated with acetic anhydride (15 μL) in 0.5 M NaOH (150 μL) for 10 min, then carboxyl-methylated with methanolic HCl (0.1 M, 5 min) and consecutively with diazomethane in order to improve the solubility in Me_2SO. The methylated product[28] was hydrolysed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxyl-methylated as above, carboxyl-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analysed by GLC-MS. Methylation analysis of the complete core region was carried out as described above, and the sample was hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxyl-methylated, carboxyl-reduced, acetylated and analysed by GLC-MS.

4.4. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 instrument, and ^{31}P NMR spectra on a Bruker DRX-400 spectrometer. All experiments were carried out at 30 °C in D_2O solution at pD 14 and 7 (uncorrected values) for oligosaccharides **1** and **2**, respectively. Chemical shifts were measured relative to internal acetone (δ_H 2.225, δ_C 31.5). Aq 85% phosphoric acid was used as reference (0.00 ppm) for ^{31}P NMR spectroscopy. Coupling constants were determined on a first order basis from 2D phase sensitive DQF-COSY.[29,30]

Phase-sensitive DQF-COSY was performed using the standard Varian tndqcosy program (with water suppression) with an acquisition time 0.258 s and 4096 data points in the *F*2 dimension. The data matrix was zero-filled in the *F*1 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. Similarly, NOE experiments were performed using the Varian standard tnnoesy and tnroesy programs (both with water suppression) with a mixing time of 200 ms. The TOCSY experiment was performed using standard Varian tntocsy program (with water suppression) with a spinlock time of 80 ms. The heteronuclear experiments were performed using pulse field gradient programs gHSQC and gHMBC. ^1H detected $^1H,^{13}C$ gHMBC was optimised for 6 Hz coupling constant, and ^1H detected $^1H,^{31}P$ gHSQC for 8 Hz coupling constant.

The spectra were assigned using the computer program PRONTO, which enables a simultaneous display of different 2D NMR spectra and the individual labelling of cross-peaks.[31]

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