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Structural Characterisation of the Core Oligosaccharides Isolated from the Lipooligosaccharide Fraction of *Agrobacterium tumefaciens* A1

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Abstract: Three different oligosaccharide structures from the lipooligosaccharide fraction of *Agrobacterium tumefaciens* strain A1 were determined by means of chemical and spectrometrical methods. The peculiar feature of this oligosaccharide family consisted of its unusual length, that was very close to the that minimal requested for the external membrane functionality as exemplified from oligosaccharide **3**, where the inner core is glycosylated from only one sugar moiety onwards.

Keywords: mass spectrometry · NMR spectrosocopy · oligosaccharide · structure elucidation

Introduction

Agrobacterium tumefaciens gains in importance as it causes crown gall disease in a wide range of dicotyledonous plant species. The disease characterized by neoplastic transformation at the site of infection results from the transfer and expression of oncogenes from the bacterium to susceptible plant cells. Most of the genes required for tumorgenesis are found on large extrachromosomal elements called Ti plasmids as demonstrated by the transformation of nonpathogenic bacteria in plant pathogenic bacteria, after transferring of such plasmid.[1]

The infection itself is a complex process and it is depending on the recognition and absorption of the bacterium on the host: according to the accepted mechanism, *A. tumefaciens* is attracted to wound sites of the root surfaces by chemotaxis; the presence of phenolic compounds in synergy with monosaccharides then triggers the activation of the virulence genes.[2] In order to transfer its T-DNA into the plant cell, the bacterium has to be adsorbed on the wounded area; this event is modulated by the components of the external membrane of the bacterium, both proteins and lipopolysaccharides (LPS).[3] In the last case, the interaction is based on the recognition of a portion of the lipopolysaccharide, defined by the term *epitope*, by particular receptor proteins[4] situated on the plant cell wall. Further studies showed that the epitope recognized by the plant is located in the O-specific polysaccharide (OPS) of the LPS, as demonstrated by a reduced virulence of bacteria mutated in genes encoding the biosynthesis of the OPS.[4,5]

Despite the wealth of information regarding the biological role of the different LPS regions of these bacterial species, that is, lipid A, core region and OPS, only few data are available on their chemical structures so far.[6–8] However, such information is a prerequisite for the understanding of the pathogenesis mechanism. In this context, the present strain DSM 30150 (equivalent to strain ATCC 4720, here referred as A1) has been studied. A classification based on biochemical, physiological and nutritional characters places this strain in the TT111 group,[9] but differently to the representative strain of this and to all other groups, A1 possesses very low oncogenic properties.[10] Therefore, a punctual structural investigation of the membrane carbohydrate components of *A. tumefaciens* A1 was promoted in order to clarify whether its pathogenic profile can be correlated to structural features of its LPS.

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Results and Discussion

Bacteria and bacterial LPS: Dry cells were recovered by centrifugation from the culture medium (120 mg L^{-1} yield) and sequentially extracted with phenol/chloroform/light petroleum (PCP) and the hot phenol/water methods. The two organic and the water phases were screened for the presence of LPS by SDS-PAGE which was found only in the PCP extract (yield 2%, g_{LPS} per g_{cells}) and showed the typical mobility of low molecular mass molecules characteristic for lipooligosaccharide (LOS).

Compositional analyses: Combining the information from sugar compositional and methylation analyses, this LOS fraction contained terminal- and 6-substituted Man, terminal Gal, 6-linked GlcN, 4,5- and 8-linked 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, and as a minor component terminal Kdo. The D-configuration was assigned to the mannose, glucosamine and galactose residues by analysing the 2-(+)-octyl derivatives and 2-(+)-butyl derivatives whereas for the Kdo it was assumed as D, in analogy to the configuration of Kdo in other LPS.

Fatty acid analysis identified C14:0(3-OH), C16:0(3-OH), C18:1(3-OH) and C28:0(27-OH).

ESIFT-Mass spectrometry: The charge deconvoluted negative ion ESIFT-MS spectrum (see also Experimental Section) of the native LOS revealed, besides some contaminations with phospholipids, two abundant molecular species at 2759.609 and 2921.664 u differing by one hexose unit. Both species show satellite peaks corresponding to molecules carrying an additional phosphate group (marked by $+P$) and to molecules with a different fatty acid composition (marked by -26 u). According to the fatty acid analysis this mass difference originates from the exchange of a C18:1(3-OH) by a C16:0(3-OH) residues. Mass peaks marked by $+22$ u represent sodium adduct ions. Capillary skimmer dissociation (CSD) of the native LPS (Figure 1a) induces the cleavage between lipid A and core oligosaccharide.[11] Due to an accompanying fragment ion induced by the decarboxylation of Kdo ($\Delta m = -44$ u) two core oligosaccharides could be identified at m/z 925.268 and 763.217. Based on the component analyses these ions were in agreement with oligosaccharides consisting of two Kdo and three or two Hex (calculated masses of the B-fragment ions 925.2678 and 763.2150, respectively). The corresponding Y-fragment ion at m/z 1994.374 represents the most abundant lipid A species. Taking into account the fatty acid component analysis and literature data that β-hydroxybutyric acid was found in LOS of other members of this species,[12] its composition could be identified consisting of two GlcN, two C14:0(3-OH), one C16:0(3-OH), one C18:1(3-OH), one C28:0 (27-OH), one C4(3-OH) and two *P* (calculated mass 1994.374). Further lipid A species comprising an additional phosphate group and the exchange of C18:1(3-OH) by C16:0(3-OH) are also visible in the spectrum. Combining the respective lipid A and core oligosaccharides add up to the various intact LPS species shown in the Figure 1b. The CSD spectrum comprises further fragmentation of the lipid A species, for example, the loss of phosphate group and successive cleavage of the two ester-linked C14:0(3-OH) fatty acid residues at m/z 1670.200 and 1426.015, respectively (Figure 1a).

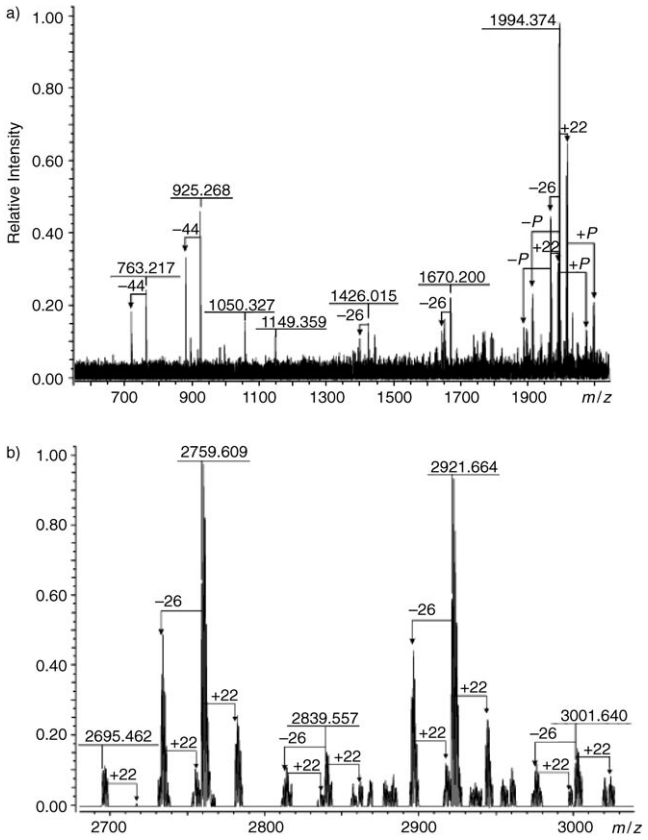


Figure 1. Negative ion ESIFT mass spectra of the native LOS from *Agrobacterium tumefaciens* A1. a) Capillary skimmer dissociation (CSD) mass spectrum; b) charge deconvoluted mass spectrum obtained under normal conditions. Mass numbers given refer to the monoisotopic molecular mass.

Isolation of oligosaccharide phosphates and NMR spectroscopy: Mild hydrazinolysis of the LOS mixture (50 mg) led to the de-O-acylated products (35 mg, 70%) that were further subjected to strong alkaline treatment in order to remove the amide-linked acyl residues. From this mixture of oligosaccharide phosphates (7 mg, 14% of the LPS), oligosaccharides **1, 2** and **3** were isolated by high-performance anion-exchange chromatography (HPAEC) (1.2, 1.0 and 0.7 mg, 2.4, 2.0 and 1.4% of the LPS, with retention time of 67.73, 90.60 and 123.07 min, respectively), representing the complete carbohydrate backbone of three different LOS molecules. Their structures were elucidated by NMR spectroscopy.

The complete assignment of ^{1}H and ^{13}C NMR resonances of each oligosaccharide was achieved (Tables 1–3), combining the information obtained from DQF-COSY, TOCSY, ROESY, gHSQC and gHMBC NMR experiments according to the general strategy shown below in detail for the more

complex oligosaccharide **1**. Anomeric protons were sequentially labelled with a capital letter reflecting their decreasing order of chemical shifts. Five anomeric protons were present (Figure 2a) in a 1:1 ratio at δ 5.67, 5.25, 4.93, 4.84 and 4.50 ppm (**A**–**E**, respectively), whereas in the high-field region two sets of the diastereotopic methylene signals were observed, due to the presence of two Kdo residues. Thus, a heptasaccharide structure was identified in the deacylated LOS.

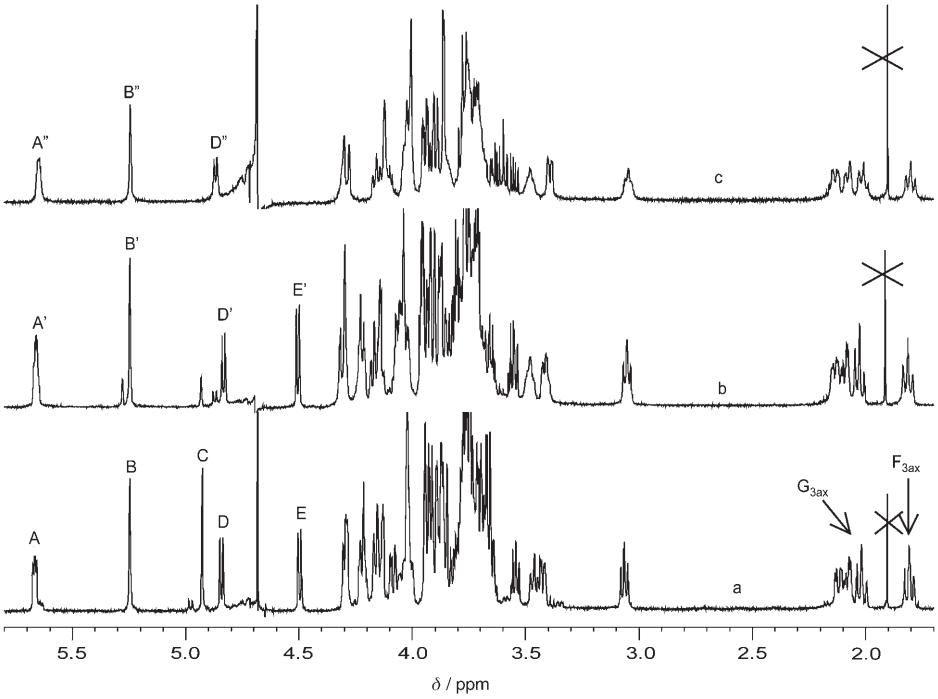


Figure 2. ^{1}H NMR spectra recorded at 600 MHz and 303 K of the three different HPAEC fractions a) **1**, b) **2** and c) **3**.

The stereochemistry of each residue was immediately recognized from the analysis of the TOCSY spectrum of the anomeric region (Figure 3). In this experiment, the efficiency of the magnetisation transfer is ruled by the vicinal coupling constants ^3J(H,H) and hence by the stereochemistry of monosaccharides investigated. Small ^3J(H,H) values, characteristics of axial/equatorial- or equatorial/equatorial-arranged protons on a pyranose ring, hinder the magnetisation transfer, thus decreasing the intensity of the successive correlations in the spectrum or, in some cases, preventing them completely. Residues with large coupling values such as *gluco*-configured residues, show a complete correlation pattern as found for both, **A** and **D**. Their H-1 protons showed six different correlations, however, for unit **D** some of them were partially overlapped. A different scenario appeared for the anomeric protons of **B** and **C**. Each residue showed one main correlation (with H-2) and a less intense one (H-3), which is typical for *manno*-configured residues. H-1 of **E** correlated only with three densities, a pattern typical for a *galacto*-configured residue.

Combining the information of the TOCSY experiment with those arising from the COSY spectrum allowed the complete assignment of all protons of residues **A, B, C** and **D**. Those of residue **E** and the two Kdo units could be completely assigned only after analysis of the ROESY spectrum.

The two *gluco*-configured residues **A** and **D** were attributed to GlcN units due to the correlations of their H-2 protons at 3.41 and 3.06, respectively, to the nitrogen-bearing carbons at 55.6 and 56.5 ppm, respectively. **A** was identified as GlcN I of lipid A, being phosphorylated at O-1 in accordance with the double doublet multiplicity of its anomeric proton (^3J(H-1,H-2) = 3.2 Hz; ^3J(H-1,P) = 8.1 Hz) and its anomeric carbon value at 91.7 ppm. Its α-configuration was indicated by the rather small ^3J(H-1,H-2) value of 3.2 Hz. The complete assignment of all the proton and carbon resonances identified a clear low field displacement of the C-6 resonance,[12] due to the glycosylation effect of an aldose residue.

Residue **D** could be attributed to the second sugar (GlcN II) of the lipid A backbone, being β-configured, as inferred by the value of 8.1 Hz of its ^3J(H-1,H-2), and linked to O-6 of GlcN I. This structural feature was indicated in the

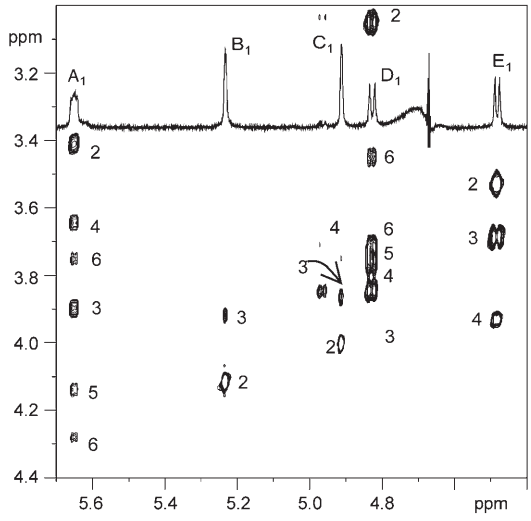


Figure 3. Anomeric region of the TOCSY spectrum of **1**, recorded at 600 MHz and 303 K.

ROESY spectrum by the spatial proximity of H-6 protons of GlcN I with the anomeric proton of GlcN II and supported by the scalar connectivity of this latter proton with the C-6 of the GlcN I residue, as deduced by the HMBC experiment. In agreement with the location of GlcN II in the lipid A backbone was the chemical shift of its C-6 indicating a mild glycosylation effect consistent with a substitution by a ketose residue. A $^{1}H,^{31}P$-HSQC experiment confirmed the phosphorylation at the anomeric centre of **A**, and at O-4 of **D**.

The two mannose residues **B** and **C** were α-configured according to the ^{13}C chemical shift values of their C-3 signals around 71 ppm (in the β-configuration, this signal occurs at around 74 ppm). Assignment of the proton and carbon chemical shifts for residue **B** was straightforward since the COSY spectrum showed clear cross-peak correlations for all of its protons. Additionally, the interpretation of the HSQC spectrum allowed the determination of the corresponding carbon chemical shifts, of which the signal of C-6 was displaced at low field (66.4 ppm) due to the glycosylation effect.

Proton resonance determination of the second mannose unit **C** was complicated by the absence of the H-4/H-5 cross peak in the COSY spectrum due to the similar resonances associated to these two protons. Therefore the position of H-5 was determined by its long range correlation with C-4 and confirmed by the correlation H-1/C-5 in the HMBC spectrum. The H-5/H-6b correlation was easily identified in the COSY spectrum, and the successive analysis of the HSQC spectrum suggested the terminal location of this residue, since all the carbon chemical shift values did not differ from those reported for the corresponding unsubstituted residue.[13]

The galactose unit **E** was β-configured on the basis of its anomeric carbon chemical shift at 104.6 ppm and its coupling constant ^{3}J(H-1,H-2) = 7.8 Hz. COSY analysis afforded the resonance identification of the first four protons, the absence of any coupling with H-5 prevented its initial assignment which was achieved exploiting its spatial proximity with the *syn–axial* anomeric proton of the β-anomer in the ROESY spectrum. Proton H-1 showed a medium strong NOE contact with H-5 in addition to the expected one with the already identified H-3 and two other ones later identified as the hydroxymethylene protons of Kdo **F**. Determination of the H-6 protons was possible by the heteronuclear long range correlation among H-6_a at 3.81 ppm and C-4, the corresponding H-6_a overlapped with H-6_a of **C**. Analysis of the carbon chemical shift of this residue suggested its terminal location in agreement with the methylation data.

As far as the two Kdo residues were concerned, their characteristic diastereotopic H-3_{ax} and H-3_{eq} proton signals in the high-field area of the proton spectrum were used as basis for spectroscopical assignments. The two methylene signals were present at δ 1.81/2.02 (H-3_{ax} of residues **F** and **G**, respectively) and δ 2.12/2.08 (H-3_{eq} of residues **F** and **G**, respectively). While exploring scalar connectivities for each Kdo residue, it was possible to assign the sequence only from H-3 through H-5. The H-5/H-6 cross peak was not present due to the small value of the coupling constant ^{3}J-(H-5,H-6). As for residue **F**, identification of proton H-6 was made possible by the presence of the two intra-residue NOE contacts H-4/H-6 (medium) and H-5/H-6 (strong). Scalar connectivities between the other exocyclic Kdo protons were also clearly present and carbon chemical shifts identified this unit as an O-8 glycosylated Kdo.

Determination of H-6 of **G** was more difficult due to the overlapping of H-5 of **G** and the H-6_b of **A**. In fact the ROESY spectrum showed a strong correlation between H-5 of **G** and H-6a of **A**. On the other hand, H-4 of **G** showed a medium NOE contact with a proton possessing the same chemical shift of H-6_a of **A**. These confirm the assignment of the H-6 of **G**, which overlaps with H-6_a of GlcN I. The resonance of proton H-7 was considered to overlap with that of H-6 due to the lack of any H-6/H-7 cross peak. Instead, a correlation with the hydroxymethylene proton H-8_a was identified in the COSY spectrum. The C-4 and C-5 carbon chemical shifts of **G** were clearly identified and were similar with those reported for a Kdo glycosylated at position 4 by a ketose and at position 5 by a *manno*-configured residue, a substitution pattern consistent with methylation analysis data. The C-6 and C-7 values were attributed on the basis of the literature data.[13]

The α-configuration of both Kdo residues was established on the basis of the chemical shift values of the H-3_{eq} protons.[14] In addition, the presence of the *inter*residual medium NOE between H-3_{eq} of **G** and H-6 of **F** indicated that **G** was substituted at position 4 by residue **F**,[15] defining residue **G** as the internal Kdo of the backbone and **F** as the external one.

The substitution pattern deduced for each monosaccharide from the spectroscopical data was in agreement with the methylation data, and with analysis of the dipolar couplings present in the ROESY spectrum together with the heteronuclear scalar long range couplings in the HMBC spectrum, allowed to ascertain the correct sequence among all the residues.

Residue **C** was linked to O-6 of **B**, since its H-1 showed an intense NOE contact with the diastereotopic proton H-6_a at 3.70 ppm of **B**, which was confirmed by the corresponding C–H long range correlation. Similar considerations showed that **B** was bound to O-5 of Kdo **G**, in turn bearing Kdo **F** at its O-4, as shown by the aforementioned NOE contact between its H-3_{eq} and H-6 of **F**. Galactose **E** was identified as substituent of O-8 of Kdo **F** as shown by the NOE contact between its H-1 and both the diastereotopic H-8 protons of this Kdo. This linkage was confirmed by the corresponding long range correlation in the HMBC spectrum. Kdo **G** did not show any diagnostic NOE correlation, still it was placed at O-6 of GlcN **D**, in agreement with both, literature data and the C-6 chemical shift of **D**.

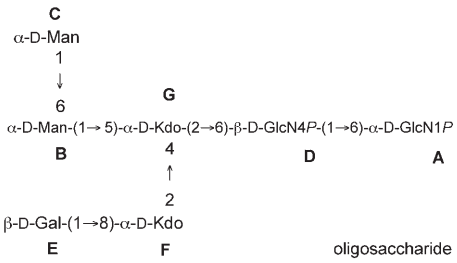
In summary, the above data identified the following chemical structure for oligosaccharide **1**.

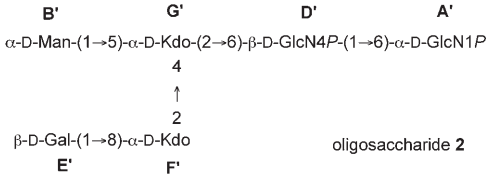
All residues were pyranoses. Oligosaccharide **1** represents the fully deacetylated backbone consistent with the *molecu-*

Table 1. 600 MHz ^{1}H and 150 MHz ^{13}C chemical shifts of oligosaccharide **1** isolated by HPAE chromatography, measured in D_2O at 303 K with acetone as internal standard.

	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
[6)-α-D-GlcN-1*P*]	5.67	3.41	3.91	3.66	4.16	3.75–4.29
A	91.7	55.6	70.6	70.6	72.7	70.7
[6)-α-D-Man-(1→]	5.25	4.12	3.93	3.84	4.16	3.70–4.08
B	101.9	71.2	71.6	67.2	72.6	66.4
[α-D-Man-(1→]	4.93	4.02	3.87	3.66	3.70	3.76–3.88
C	100.8	70.9	71.4	67.6	73.8	62.0
[6)-β-D-GlcN-4*P*-(1→]	4.84	3.06	3.86	3.76	3.73	3.46–3.71
D	100.8	56.5	73.2	75.0	75.9	63.4
[β-D-Gal-(1→]	4.50	3.54	3.70	3.94	3.73	3.75–3.81
E	104.6	71.9	73.5	69.6	75.7	62.0
	H-3$_{ax,eq}$/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8a,b/C-8
[8)-α-D-Kdo-(2→]	1.81/2.12	4.01	4.02	3.78	4.21	3.92–4.22
F	35.4	66.9	68.0	72.8	70.5	72.3
[4,5)-α-D-Kdo-(2→]	2.02/2.08	4.04	4.29	3.75	3.75	3.65/3.91
G	35.4	72.2	74.6	72.9*	70.5*	64.7

[*] Chemical shifts attributed on the basis of literature data.[13]





lar species at 2921.55 u in the ESI mass spectrum (Figure 1b).

The ^{1}H NMR spectrum of the second compound isolated by HPAEC (Figure 2b) showed the presence of one major molecular species together with another less abundant one that was not further investigated. The anomeric signals of the first (oligosaccharide **2**) were labelled **A′–G′** in analogy to oligosaccharide **1**. A comparison of the two proton spectra identified the main difference among the two oligosaccharides. Namely, **2** lacked the second mannose unit (**C** in **1**). In the spectrum of **2** four anomeric signals were present at 5.66 (**A′**), 5.25 (**B′**), 4.83 (**D′**) and 4.50 ppm (**E′**), together with the diastereotopic H-3 methylene signals of two Kdo residues (**F′** and **G′**) at high field. The in-depth 1D and 2D NMR analyses led to the complete assignment of all ^{1}H, ^{13}C NMR signals (Table 2), thus, residue **B′** was identified as a terminal mannose unit linked to position 5 of Kdo **G′**.

Taken together, the structure of **2** differed from that of **1** in the absence of the terminal mannose **C** unit.

Oligosaccharide **2** represented the molecular species consistent with the ion peak at 2759.61 u in the ESI mass spectrum (Figure 1b).

More straightforward was the structural elucidation of the third HPAEC fraction, that is, **3**; its ^{1}H NMR spectrum (Figure 2c) showed only three anomeric signals occurring at δ 5.65, 5.24 and 4.87 ppm, labelled as **A″, B″** and **D″**, respectively, in addition to the diastereotopic H-3 methylene signals of the two Kdo residues, **F″** and **G″**, at higher field. The NMR spectroscopical analysis led to the complete assignment of all ^{1}H, and ^{13}C signals (Table 3) and to the complete structural determination of **3** that differed from the previous one by lacking the terminal galactose unit.

This oligosaccharide could not be attributed to a pseudomolecular ion in the ESI-mass spectrum of the intact LOS mixture, probably due to its low abundance. Concluding, in-depth chemical and spectroscopical analyses of the carbohydrate backbone of the LOS fraction of the phytopathogenic bacterium *Agrobacterium tumefaciens* A1 identified three different oligosaccharides. Oligosaccharide **1** represented the most complex structure in which both, the external Kdo and the mannose **B** units bore an additional hexose, namely

Table 2. 600 MHz ^{1}H and 150 MHz ^{13}C chemical shifts of oligosaccharide **2** isolated by HPAE chromatography, measured in D_2O at 303 K with acetone as internal standard.

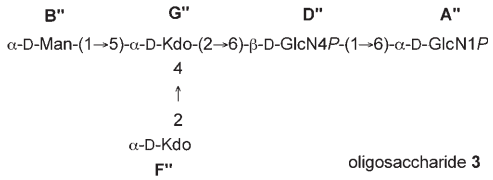
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
[6)-α-D-GlcN-1*P*]	5.66	3.41	3.92	3.66	4.18	3.78–4.31
A′	91.7	55.9	70.8	70.8	73.6	71.1
[α-D-Man-(1→]	5.25	4.15	3.97	3.84	4.07	3.89
B′	101.9	71.5	71.7	67.6	74.0	61.9
[6)-β-D-GlcN-4*P*-(1→]	4.83	3.05	3.85	3.72	3.72	3.49–3.73
D′	100.8	56.5	73.2	75.2	75.6	63.4
[β-D-Gal-(1→]	4.50	3.56	3.72	3.97	3.76	3.80
E′	104.5	72.3	74.0	70.0	76.4	62.2
	H-3$_{ax,eq}$/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8a,b/C-8
[8)-α-D-Kdo-(2→]	1.81/2.14	4.04	4.05	3.81	4.24	3.94–4.23
F′	36.3	67.1	68.3	73.2	71.0	72.7
[4,5)-α-D-Kdo-(2→]	2.03/2.09	4.07	4.30	3.78	3.78	3.38–3.71
G′	35.8	72.6	74.7	73.4*	70.8*	64.8

[*] Chemical shifts attributed on the basis of literature data.

Table 3. 500 MHz ^{1}H and 125 MHz ^{13}C chemical shifts of oligosaccharide **3** isolated by HPAE chromatography, measured in D_2O at 313 K with acetone as internal standard.

	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
[6)-α-D-GlcN-1*P*]	5.65	3.39	3.90	3.59	4.15	3.75–4.28
A″	91.7	56.0	71.1	71.1	73.7	70.9
[α-D-Man-(1→]	5.24	4.12	3.94	3.77	4.02	3.86
B″	101.8	71.5	71.7	67.8	74.0	62.0
[6)-β-D-GlcN-4*P*-(1→]	4.87	3.06	3.84	3.71	3.71	3.48–3.71
D″	101.0	57.0	73.9	75.5	75.5	63.8
	$H\text{-}3_{ax,eq}$/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-7/C-7	H-8a,b/C-8
[8)-α-D-Kdo-(2→]	1.80/2.14	4.02	4.00	3.72	4.01	3.76–3.94
F″	36.0	67.3	68.0	73.4	71.8	64.2
[4,5)-α-D-Kdo-(2→]	2.01/2.08	4.12	4.28	3.76	3.76	3.68–3.90
G″	35.8	71.7	74.4	73.5*	70.7*	64.9

[*] Chemical shifts attributed on the basis of literature data.

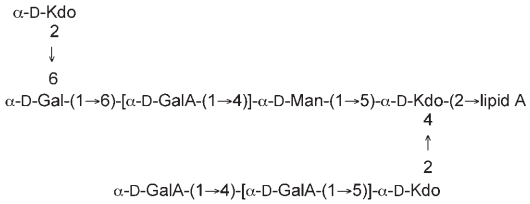


oligosaccharide **3**

a galactose and a mannose, respectively. Oligosaccharide **2** maintained the terminal galactose on the external Kdo, but lacked the mannose. It is noteworthy that all LOS species possessed low molecular mass, compared with LOS from other bacteria and that the few residues decorating the inner core moiety were usual hexoses. No heptoses were present. These features are important compared with data known about LPS of other phytopathogenic bacteria. Members of different genera[16] such as *Pseudomonas, Xanthomonas* and *Burkholderia* produce high-molecular mass LPS due to the presence of the O-specific polysaccharide. In addition, the monosaccharide composition showed a high content of deoxysugars such as rhamnose and fucose, or of acetamido residues, all of which are monosaccharides with less hydrophilic properties than their hexose counterparts. The same feature was confirmed when *A. tumefaciens* A1 LOS structure was compared with LPS structures from other members of this genus possessing a more pronounced phytopathogenic activity, such as *A. tumefaciens* strains B6,[6] C58[7] and F1.[8] These species synthesise high-molecular mass LPS, the hydrophobic pattern of which is maintained through a selection of deoxysugar derivatives. In this context, it appears that less hydrophilic sugar residues play an important role in pathogenesis, probably supporting an efficient adhesion process during the colonisation step of the bacterium on the plant cell wall; a process in which strain A1 is disadventaged with respect to other competitors due to the lack of these sugars on its external membrane surface.

Together with the potential role of these molecules in the pathogenic process of this bacterium, their structural analysis might be of importance in the chemotaxonomical classification Scheme of *Agrobacterium*. Recently, a debated issue centred on a possible amalgamation of the genus *Agrobacterium* in the genus *Rhizobium*, reflecting a proposal based on several phenotypic features shared by this two genera.[17]

The structural features of the inner core residues, which are rather conserved within members of the same species, have been generally considered as an additional tool inside the classification issue. The structures reported here represent the first core structure determined so far for *Agrobacterium tumefaciens.* Only one was reported for the closely related genus *Rhizobium*, that is, for the LPS of *R. etli* (reclassification of *R. leguminosarum* bv. *phaseoli* type I),[18] the structure of which is shown below.



Comparing the inner core structures from LPS of these two bacteria, it appears that they share the substitution pattern of a sugar residue attached to the internal Kdo unit, whereas the external one is also substituted but in a different fashion, having one galactose in *A. tumefaciens* and two *iso*-configured but acidic sugars in *R. etli*, located in different positions.

A different structural arrangement was found in the LPS of *A. larrymorey*,[19] another phytopathogenic species belonging to the *Agrobacterium* genus, for which a closer identity in the core structure was expected. Surprisingly the internal Kdo was substituted by a galactose and not by a mannose residue, whereas the external Kdo was simply terminal.

Rationalization of taxonomical relationship through the LOS inner core structure is an impossible task at this stage due to the low number of information available, but structural analysis might become an additional tool in the future, adding useful information to the field of taxonomical classification.

Experimental Section

Bacteria: *Agrobacterium tumefaciens* strain DSM 30150 (here referred as A1) was grown at 27 °C in liquid shaking culture (200 rpm) in Nutrient Broth (Fluka Nutrient Broth No 4 cod. 03856) for 18 h (early stationary phase).

The bacterial suspension was centrifuged (3500 × g, 5 min) and harvested cells were washed sequentially with 0.85 % aqueous NaCl, ethanol, acetone and diethyl ether.

Isolation and purification of the LOS: Dried cells were extracted with PCP[20] yielding the LOS (2% g_{LPS} per g_{cells}). The pellet was further extracted with hot phenol/water.[21]

Discontinuous SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis)[22] was performed with a 12% acrylamide separating gel on a miniprotean gel system (Bio-Rad). The samples were run at constant voltage (150 V) and stained with silver nitrate according to the procedure of Kittelberger.[23]

General and analytical methods: Determination of Kdo, neutral sugars including the determination of the absolute configuration of the hexoses, organic bound phosphate, and GLC-MS were all carried out as described elsewhere.[24] For methylation analysis of the Kdo region, LOS was first N-acetylated with acetic anhydride (15 μL) in 0.5 M NaOH (150 μL) for 10 min, then carboxy-methylated with methanolic HCl (0.1 M, 5 min) and subsequently with diazomethane in order to improve its solubility in DMSO. After methylation,[25] LOS was hydrolyzed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated as before, carboxyl-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analyzed by GLC-MS.[26]

Isolation of oligosaccharides 1, 2 and 3: LOS was dissolved in anhydrous hydrazine (25 mg mL^{-1}), stirred at 37 °C for 30 min, cooled, poured into ice-cold acetone (100 mL), and allowed to precipitate. The precipitate was then centrifuged (3000 × g, 30 min), washed twice with ice-cold acetone, dried, and then dissolved in water and lyophilized. The sample was de-N-acylated with 4 M KOH as described,[23] and desalted by gel-permeation chromatography [Sephadex G-10 (Pharmacia) 50 × 1.5 cm, in water, flow 0.5 mL min^{-1}]. The resulting oligosaccharide fraction eluted in the void volume and was further purified by HPAEC on a Carbopack PA-100 column (9 × 250 mm) eluted with a linear gradient of 30–37% of 1 M sodium acetate in 0.1 M NaOH at 2.0 mL min^{-1} over 140 min.

Electrospray ionisation (ESI) mass spectrometry: High resolution Fourier-transform mass spectrometer (FT-MS) equipped with a 7 Tesla actively shielded magnet and an Apollo ESI source (Apex II, Bruker Daltonics, Billerica, USA) was used for the analysis of native LOS. Mass spectra were acquired by using standard experimental sequences as provided by the manufacturer. Samples were dissolved at a concentration of ~10 ng μL^{-1} in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 2 μL min^{-1}. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 180 °C. The spectra, which showed several charge states for each component, were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses. Capillary skimmer dissociation (CSD) was induced by increasing the capillary exit voltage from −100 to −350 V.

NMR spectroscopy: 1D spectra were recorded with a Bruker DRX 600 spectrometer at 303 K, and 2D NMR spectra of **1** and **2** were recorded in 0.5 mL D_2O on the same instrument. 2D NMR spectra of oligosaccharide **3** were acquired at 313 K on a Varian Inova 500 of Consortium INCA (L488/92, Cluster 11). Both NMR spectrometers were equipped with a with a *z* gradients reverse probe and chemical shifts were referred relative to internal acetone [$\delta(^1H)$ 2.225, $\delta(^{13}C)$ 31.5]. ^{31}P NMR was measured for oligosaccharide **1** and were calibrated on 85% phosphoric acid which was used as external standard.

For the homonuclear experiment, solvent saturated DQF-COSY, TOCSY and ROESY spectra, 512 FIDs of 2048 complex data points were collected, with 48 scans per FID and using standard manufacturer software. The spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak and mixing times of 120 and 200 ms were used for TOCSY and ROESY, respectively. For the HSQC and HMBC (the last recorded only for oligosaccharide **1**) spectra, 256 FIDS of 2048 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Conversion of the Varian data and processing was performed with standard Bruker Xwinnmr 1.3 program, the spectra were assigned using the computer program Pronto.[27]

NOE's classification as strong, medium or weak, was evaluated directly on the ROESY spectrum counting the number of the contour levels in the 2D plot; *intra*residual NOEs cross peaks were analyzed first in order to related their size to the known distance (i.e., the medium NOE H-3/H-5 protons of residue **E**, is related to a distance of ca. 2.70 Å), on the basis of this preliminary examination, the contour levels of *inter*residual NOEs cross-peaks were counted and classified accordingly.

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