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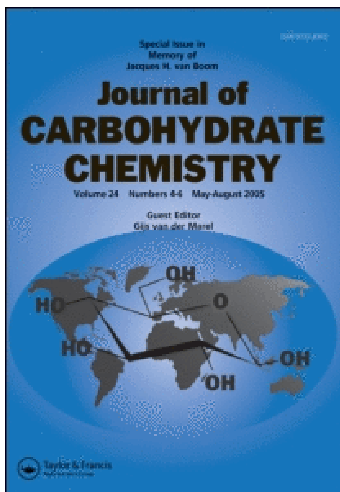

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The Full Structure of the Carbohydrate Chain of the Lipopolysaccharide of *Providencia alcalifaciens* O19

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The Full Structure of the Carbohydrate Chain of the Lipopolysaccharide of *Providencia alcalifaciens* O19

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An oligosaccharide isolated from the lipopolysaccharide of *Providencia alcalifaciens* O19 was found to consist of a single O-antigen repeating unit linked to the core. The full oligosaccharide structure was elucidated by 2D ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. It was shown that the inner core region has the same structure as in other *Providencia* strains studied, whereas the outer core is structurally diverse between *Providencia* strains. A pyruvic acid acetal was found in the isolated oligosaccharide and in the long-chain O-antigen, whose structure has been established earlier. The biological O-unit structure was elucidated in the short-chain lipopolysaccharide.

Keywords *Providencia alcalifaciens*, O-polysaccharide, O-unit, Core oligosaccharide, Lipopolysaccharide structure

INTRODUCTION

Bacteria of the genus *Providencia* are opportunistic human pathogens that cause intestinal and urinary tract infections. The O-antigen-based serological

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classification scheme of *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii* includes 63 O-serogroups. The O-antigen represents a polysaccharide chain of the lipopolysaccharide (LPS), which is built up of oligosaccharide repeating units (O-units). The O-antigens are structurally diverse even within one species, whereas the other LPS domains, lipid A and a core oligosaccharide, are more conserved. Together with the S-type LPSs having a long-chain O-antigen, many bacteria produce SR- and R-type LPSs, which have a single O-unit linked to the core or no O-antigen at all, respectively.

Chemical structures of the O-antigens have been established for about half *Providencia* O-serogroups (ref. [1] and refs. cited therein). In most of them, the O-antigens are acidic due to the presence of uronic acids, ulosonic acids, amides of uronic acids with amino acids, ether-linked lactic and 2,4-dihydroxypentanoic acids, or phosphate. Recently, also the core structures have been elucidated in five *Providencia* O-serogroups.[1−3]

Now we report on the full structure of the carbohydrate chain of the SR type LPS of *P. alcalifaciens* O19, including the O-unit and core structures. In addition, we revised the structure of the long-chain O-antigen of this bacterium and confirmed that it contains a pyruvic acid acetal, which was overlooked in our previous studies.[4]

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MATERIALS AND METHODS

Bacterial Strains, Growth, and Isolation of the LPS

P. alcalifaciens O19:H2 strain 691 obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized.

The LPS was isolated from dried bacterial cells by the phenol-water extraction,[5] the extract was dialyzed against tap water without layer separation and freed from insoluble contaminations by centrifugation (5000 rpm, 40 min), the crude LPS solution was treated with cold aq 50% CCl_3CO_2H, the precipitate was removed by centrifugation, and the supernatant was dialyzed against distilled water and freeze-dried.

Degradations of the LPS

An LPS sample (100 mg) was hydrolyzed with aqueous 2% HOAc at 100°C for 2 h; a lipid precipitate was removed by centrifugation at 13,000 × *g* for 20 min. The carbohydrate portion was fractionated by gel-permeation chromatography on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Amersham Biosciences) in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a differential

refractometer (Knauer) to give an O-polysaccharide, two oligosaccharide fractions (A and B), and low-molecular-mass compounds.

For MS studies, the oligosaccharides were used without further purification. For NMR spectroscopic studies, fraction A oligosaccharide was purified by anion-exchange chromatography on a 5-mL HiTrap Q column (Amersham Biosciences) in a gradient of $0 \rightarrow 1$ M NaCl over 1 h at a flow rate 3 mL min^{-1} using pulse amperometric detector (Dionex) for monitoring followed by desalting by gel filtration on a column (50×1.6 cm) of Sephadex G-15 (Amersham Biosciences).

For O-deacylation, an LPS sample (60 mg) was heated with aq 0.16 M NaOH (1.5 ml) at 100°C for 1 h, the reaction mixture was cooled and neutralized with 2 M HCl, the precipitate was removed by centrifugation, and the supernatant was desalted by gel filtration on a column (60×2.5 cm) of Sephadex G-50 (S) to yield the O-deacylated LPS (19 mg).

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NMR Spectroscopy

Samples were freeze-dried twice from a ^2H_2O solution and dissolved in 99.96% ^2H_2O with internal acetone (δ_H 2.225, δ_C 31.45) as reference. ^1H, ^{13}C, and ^{31}P NMR spectra of the oligosaccharides were recorded at 25°C on a Varian UNITY/Inova 500 spectrometer using standard pulse sequences COSY, TOCSY (mixing time 120 ms); NOESY (mixing time 300 ms); ^1H, ^{13}C HSQC, and HSQC-TOCSY (mixing time 80 ms); ^1H, ^{31}P HMQC (^1H-^{31}P coupling constant value was set to 11 Hz), and HMQC-TOCSY (mixing time 80 ms). Spectra were assigned with the help of the computer program PRONTO.[6] ^1H and ^{13}C NMR spectra of the O-deacylated LPS were recorded at 60°C on a Bruker DRX-500 spectrometer.

Mass Spectrometry

Ion-cyclotron resonance Fourier transform ESI MS of the oligosaccharides was performed in the negative ion mode using an APEX II Instrument (Bruker Daltonics) equipped with a 7 Tesla magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Mass accuracy has been checked through the external calibration. Samples (~10 ng μL^{-1}) were dissolved 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}. The capillary entrance and exit voltage was set to 3.8 kV and −100 V, respectively, and drying gas temperature was set to 150°C. The spectra, which showed several charge states for each component, were charge deconvoluted using Bruker XMASS 6.0.0 software, and mass numbers given refer to the monoisotopic molecular masses.

RESULTS AND DISCUSSION

The LPS was isolated by the phenol-water procedure from *P. alcalifaciens* O19 biomass and hydrolyzed under mild acidic conditions to cleave the acid-labile linkages of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, including the linkage between the core and lipid A. The products were fractionated by gel-permeation chromatography on Sephadex G-50 to yield a polysaccharide and two oligosaccharide fractions, A and B.

The O19 polysaccharide (long-chain O-antigen) structure has already been elucidated.[4] Both oligosaccharide fractions have been studied earlier by high-resolution ESI MS[1,2] and found to be heterogeneous owing to the occurrence at the reducing end of a Kdo residue in multiple forms, including anhydro forms, and the presence of multiple phosphoetanolamine (*P*EtN) groups in non-stoichiometric quantities. The highest molecular mass compound in fraction B corresponded to a $Hex_4GalA_1Hep_3Kdo_1Ara4N_1P_1PEtN_3$ core oligosaccharide (experimental and calculated molecular masses 2200.55 and 2200.53 u, respectively).[2] The highest molecular mass oligosaccharide in fraction A (Fig. 1) included the same core and an additional $HexNAc_2dHexNAc_1Hex_2Ac_1$ fragment (3177.91 and 3177.90 u, respectively),[1] which fully corresponded to the $GlcNAc_2Fuc3NAc_1Gal_2Ac_1$ O-unit.[4] Hence, fractions A and B have been suggested to derive from the SR- and R-type LPS, respectively.

Fraction A oligosaccharide was studied by two-dimensional ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, using ^{1}H,^{1}H COSY, TOCSY, NOESY (Fig. 2), ^{1}H,^{13}C HSQC-DEPT, HSQC-TOCSY, ^{1}H, ^{31}P HMQC, and HMQC-TOCSY experiments, essentially as described.[2,7] The ^{1}H and ^{13}C NMR chemical shift patterns as well as the phosphorylation pattern of the inner core region (residues C, E-G, K, Z) were essentially the same as in all LPS-derived oligosaccharides from various *Providencia* species studied earlier.[1−3] This finding confirmed that the inner core structure is conserved within the genus *Providencia*. A peculiar feature of this LPS region is charged substituents on three L-*glycero*-D-*manno*-heptose (Hep) residues, namely *PP*EtN, *P*EtN, and GalA (Fig. 3).

The assignment of the ^{1}H and ^{13}C NMR signals shown in Table 1 was performed for the outer core and O-unit moieties. The sugar spin systems were identified by H-1 to H-6 correlations for the *gluco*-configurated monosaccharides (except for Glc **H**) and H-1 to H-4 correlations for the *galacto*-configurated monosaccharides and Glc **H** in the TOCSY spectrum (Fig. 2, grey). The assignment for Fuc3NAc was completed by H6,H5 and H6,H4 correlations and for all Gal residues by H4/H5 correlations in the NOESY spectrum. The spin systems for the amino sugars were distinguished by correlations between protons at the nitrogen-bearing carbons and the corresponding carbons (H-2,C-2 for GlcNAc and H-3,C-3 for Fuc3NAc). Signals for two residues were split significantly to two series owing to nonstoichiometric acetalation with pyruvic acid (GlcNAc **X**/**X'**) or nonstoichiometric phosphorylation with *P*EtN (Gal **N**/**N'**) (see below).

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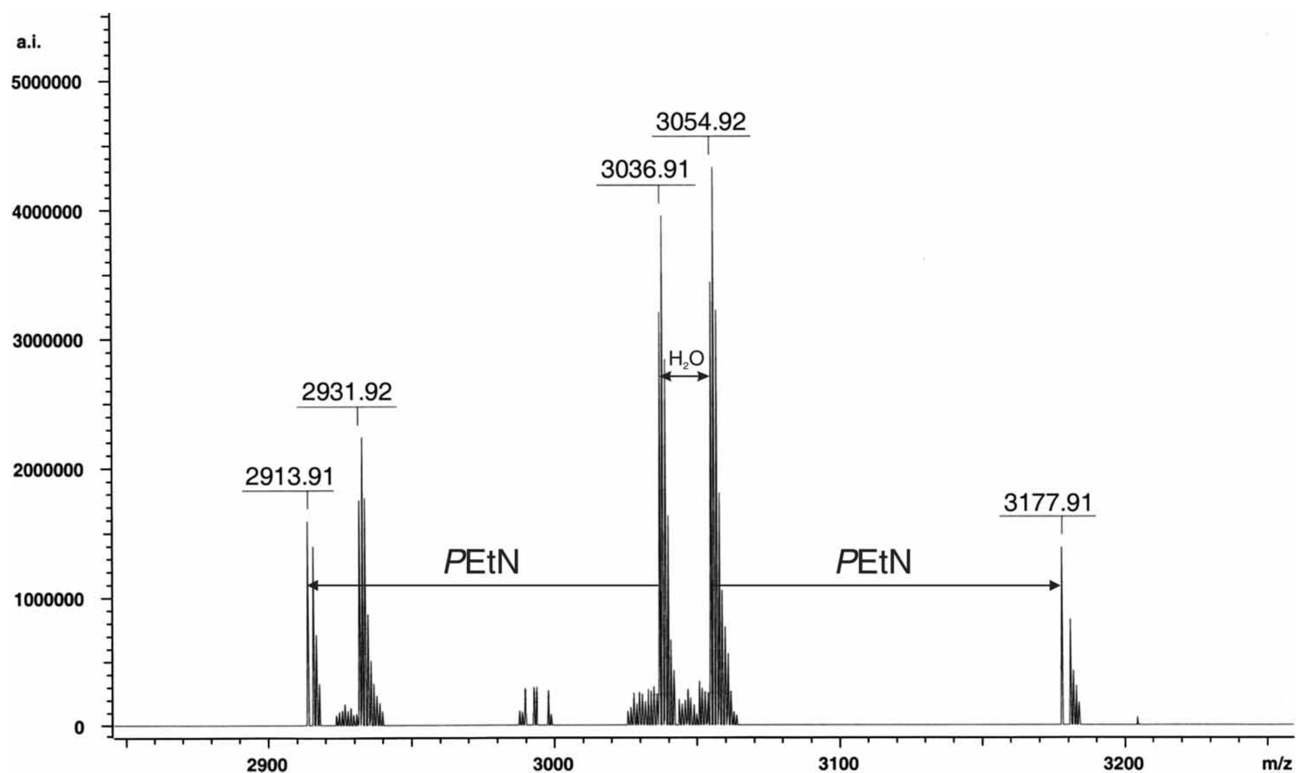

Figure 1: Charge-deconvoluted negative ion mode ESI ICR FT mass spectrum of fraction A oligosaccharide **1** from *P. alcalifaciens* O19. A peak at 3177.91 u corresponds to a $Hex_4GalA_1Hep_3Kdo_1Ara4N_1P_1PEtN_3$ core oligosaccharide bearing one $HexNAc_2dHexNAc_1Hex_2Ac_1$ O-unit.

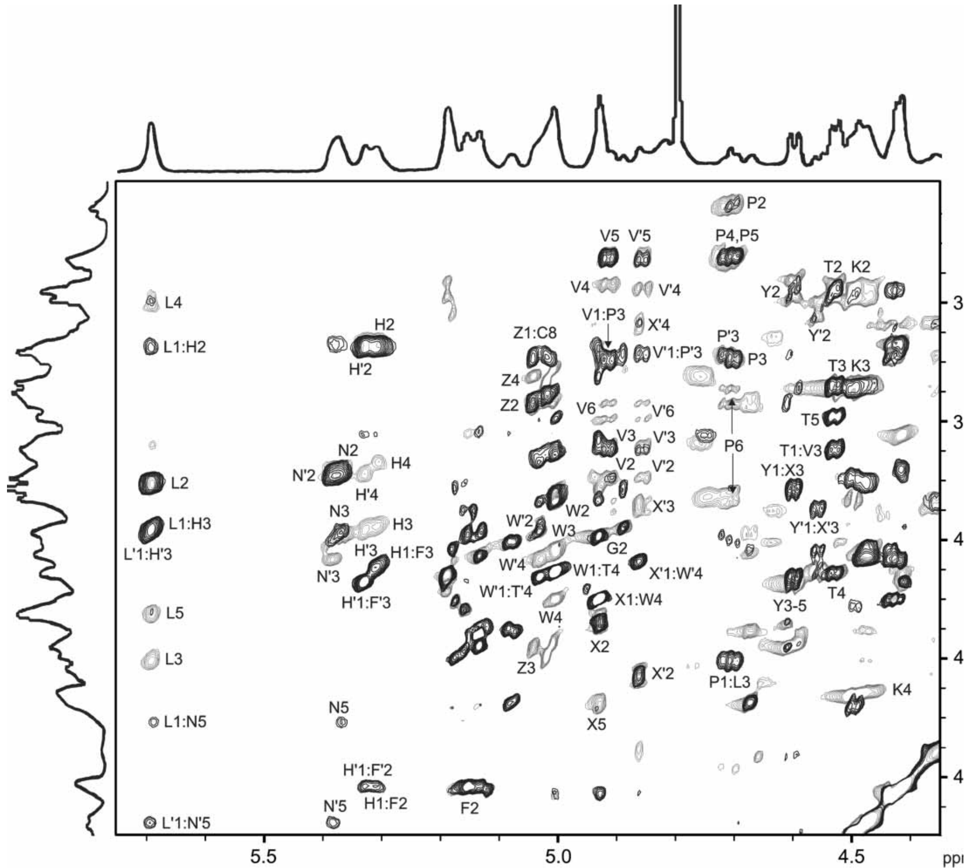

Figure 2: Parts of 2D TOSCY (grey) and NOESY (black) spectra of fraction A oligosaccharide **1** from *P. alcalifaciens* O19. For monosaccharide abbreviations see Table 1. Annotated are interresidue cross-peaks and intraresidue cross-peaks for anomeric protons.

Splitting of some proton signals for neighboring sugar residues was observed too but was insignificant (Table 1).

A comparison of the ^{1}H and ^{13}C NMR chemical shifts of the minor series for nonacetalated fraction A oligosaccharide with published data of the O19 polysaccharide[2] enabled recognition of the O-unit components: Fuc3NAc **Y**, GlcNAc **X**, Gal **W**, Gal **T**, and GlcNAc **V**, which have the same anomeric configurations in both compounds. The glycosylation pattern and the monosaccharide sequence in the O-unit moiety of the oligosaccharide were determined by the NOESY experiment, which revealed the following correlations between the anomeric protons and protons at the linkage carbons: Fuc3NAc **Y** H-1,GlcNAc **X** H-3; GlcNAc **X** H-1,Gal **W** H-4; Gal **W** H-1,Gal **T** H-4; and Gal **T** H-1,GlcNAc **V** H-3 (Fig. 2, black). Similar correlations were observed in the major series for the acetalated fraction A oligosaccharide (Fig. 2, black).

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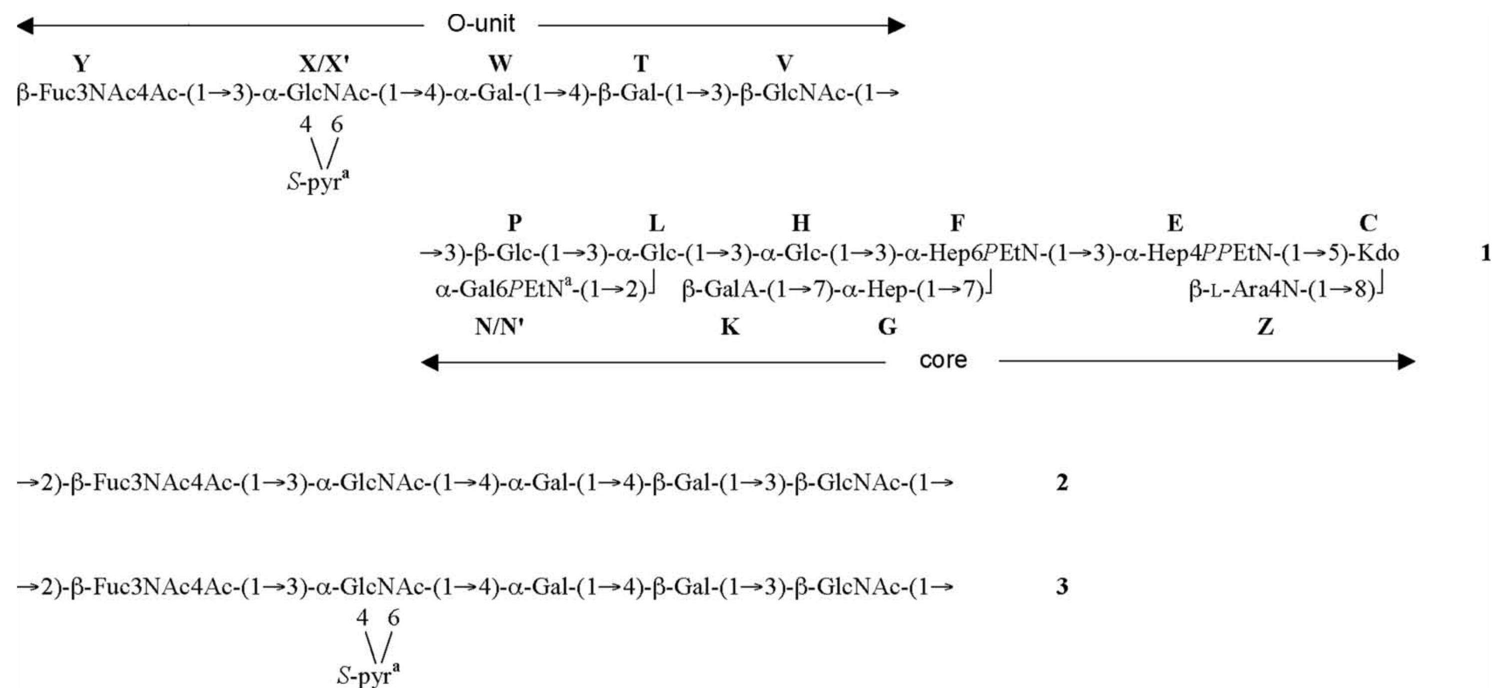

Figure 3: Structure of fraction A oligosaccharide (**1**), structure of the O-polysaccharide reported earlier (**2**),[4] and revised structure of the O-polysaccharide (**3**) from *P. alcalifaciens* O19. Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Hep, L-*glycero*-D-*manno*-heptose; EtN, ethanolamine; *S*-pyr, pyruvic acid (*S*)-acetal. [a]Substitution with pyr and *P*EtN is nonstoichiometric.

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Table 1: NMR chemical shifts (δ) of the oligosaccharide **1**.

Residue	Nucleus	1	2	3	4	5	6 (6a)	6b
β-Fuc3NAc4Ac **Y**/**Y**′	^{1}H	4.60/4.56	3.57/3.65	4.07	5.18	4.03	1.17	
	^{13}C	104.7	68.9	54.2	72.9	71.6	16.3	
α-GlcNAc **X**	^{1}H	4.92	4.13	3.91	3.68	4.27	3.78	3.87
	^{13}C	99.6	53.8	81.4	71.4	68.7	61.3	
α-GlcNAc4,6pyr **X**′	^{1}H	4.86	4.22	3.95	3.64	4.35	3.68	3.92
	^{13}C	100.1	54.5	77.4	73.1	63.8	65.2	
α-Gal **W**/**W**′	^{1}H	5.01/5.04	3.93/3.98	4.02/4.04	4.10/4.04	4.42	3.63	3.68
	^{13}C	101.4	69.8	70.2	78.3	72.4	60.4	
β-Gal **T**/**T**′	^{1}H	4.52	3.58	3.73	4.06/4.07	3.78	3.78	3.85
	^{13}C	104.9	71.6	73.5	78.7	76.4	61.3	
β-GlcNAc **V**/**V**′	^{1}H	4.91/4.85	3.89	3.85	3.57	3.51	3.78	3.94
	^{13}C	101.2	55.9	83.6	68.8	76.5	61.3	
β-Glc **P**/**P**′	^{1}H	4.70/4.72	3.43/3.44	3.69/3.68	3.53	3.52	3.75	3.93
	^{13}C	103.6	74.0	85.7	69.1	76.5	61.7	
α-Glc **L**/**L**′	^{1}H	5.69	3.91	4.20	3.59	4.12	3.83	
	^{13}C	96.4	73.6	78.7	69.1	72.6	61.3	
α-Glc **H**/**H**′	^{1}H	5.31/5.33	3.67	3.98/3.99	3.87/3.89			
	^{13}C	101.4	71.5	79.9	69.3			
α-Gal **N**	^{1}H	5.37	3.88	3.98	3.98	4.30	3.72	3.76
	^{13}C	95.1	69.2	70.4	70.4	71.9	62.3	
α-Gal6*P*EtN **N**′	^{1}H	5.38	3.88	4.02	4.02	4.47	4.01	4.01
	^{13}C	95.1	69.2	70.3	69.6	70.8	65.9	

All monosaccharides are in the pyranose form and have the D configuration. Nonprimed and primed letters designate sugar residues in the acetalated and nonacetalated oligosaccharide, respectively. EtN, ethanolamine; pyr, pyruvic acid acetal. Additional signals for EtN are at δ_H 3.36 (CH_2N) and 4.16–4.22 (CH_2O), δ_C 41.7 (CH_2N) and 63.4–63.9 (CH_2O); *S*-pyr at δ_H 1.49, δ_C 103.3 (OCO) and 25.6 (Me); NAc at δ_H 1.95–2.06, δ_C 23.1–24.0 (Me); OAc at δ_H 2.21, δ_C 21.5 (Me).

That, as in the O19 polysaccharide, the Fuc3NAc **Y** residue is 4-O-acetylated in the oligosaccharide followed from the presence of NMR signals for an O-acetyl group at δ_H 2.21 and δ_C 21.1 (Me) and a low-field position of the H-4 signal at δ 5.18 (compare δ 5.05 in the polysaccharide[2]). The terminal nonreducing position of Fuc3NAc **Y** was confirmed by the C-2 chemical shift of δ 68.9 (compare the value δ 75.8 for C-2 of the 2-substituted Fuc3NAc residue in the O19 polysaccharide[2]). Therefore, GlcNAc **V** is the first monosaccharide of the O-unit, whose linkage connects the O-unit to the core.

In the NMR spectra of fraction A oligosaccharide, there were signals for a pyruvic acid acetal at δ_H 1.49 and δ_C 25.6, 103.3, and 176.2. As compared with non-acetalated GlcNAc **X**, the signals for C-4 and C-6 of acetalated GlcNAc **X**′ were shifted downfield from δ 71.4 and 61.3 to δ 73.1 and 65.2, respectively, and the C-5 signal was shifted upfield from δ 68.7 to δ 63.8. These data proved unambiguously the location of the acetal group at positions 4 and 6 of the GlcNAc residue. A comparison with published data[8] showed the *S* configuration at the pyruvic acid acetal carbon. The integral intensity ratios of the signals for GlcNAc **X** and GlcNAc **X'** and for methyl groups of pyr and NAc showed that pyruvic acid is present on 35–40% GlcNAc residue. However, the initial content of pyruvic acid in the LPS may be higher as partial deacetalation could occur in the course of mild acid degradation of the LPS.

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The remaining four hexose residues (Glc **H**, **L**, and **P** and Gal **N**) were assigned as components of the outer core region. Based on $^3J_{H,H}$ coupling constant values determined from the two-dimensional spectra, these residues are α-linked, except for Glc **P** that is β-linked. The NOESY spectrum (Fig. 2, black) showed the following interresidue correlations: GlcNAc **V** H-1,Glc **P** H-3; Glc **P** H-1,Glc **L** H-3; Glc **L** H-1,Glc **H** H-3; and Glc **H** H-1,Hep **F** H-3. A Gal **N**/**N**′ H-1,Glc **L**/**L**′ H-2 cross-peaks were weak and partially coincided with intraresidue Gal **N**/**N**′ H-1/H-2 cross-peaks but the **N**/**N**′-(1 → 2)-**L**/**L**′ linkage was confirmed by strong Gal **N**/**N**′ H-1,Glc **L**/**L**′ H-1 and Glc **L**/**L**′ H-1,Gal **N**/**N**′ H-5 cross-peaks. These data defined the structure of the outer core and indicated that the O-unit is attached at position 3 of Glc **P**. The C-6 signal of Gal **N'** was observed at δ 65.9 i.e. was shifted downfield as compared with its position at δ 62.3 in Gal **N**′, thus suggesting nonstoichiometric phosphorylation of the Gal residue at position 6. This conclusion was confirmed by the ^{1}H,^{31}P HMQC and HMQC-TOCSY experiments, which showed correlations of the phosphate group with CH_2O groups of both EtN and Gal **N**. Integral intensities of the signals for Gal **N** and Gal **N'** were almost equal and, hence, the degree of phosphorylation is ~50%.

The data obtained demonstrate that fraction A oligosaccharide derived from the SR-type LPS of *P. alcalifaciens* O19 has the structure **1** shown in Figure 3.

Earlier, the structure **2** (Fig. 3) has been established for the O-polysaccharide of *P. alcalifaciens* O19, which was derived from the S-type LPS by mild acid hydrolysis.[4] The O-unit in the O19 polysaccharide corresponds to that in oligosaccharide **1**, except for the lack of pyruvic acid acetal from the former. The acetal group is known to be highly acid-labile and could be lost upon stronger conditions of mild acid hydrolysis of the LPS used for the O19 polysaccharide[4] (NaOAc buffer pH 4.2 at 100°C for 9 h followed by aq 2% HOAc at 100°C for 1 h vs. aq 2% HOAc at 100°C for 1 h in this work). In order to confirm the presence of the pyruvic acid acetal in the O19 polysaccharide too, the LPS was subjected to alkaline hydrolysis, which does not affect the acetal group.

In addition to the expected sugar and O-deacylated lipid A signals, the ^{1}H and ^{13}C NMR spectra of the O-deacylated LPS showed a signal for the methyl group of pyruvic acid at δ_H 1.43 and δ_H 26.1, which are similar to those in oligosaccharide **1**. The ^{1}H NMR signal had the integral intensity 0.64 compared with the intensity of the *N*-acetyl group signal. Therefore, the pyruvic acid acetal is present in about two thirds of the O-units in the O19 polysaccharide. Based on these data and the structure of the O-unit in oligosaccharide **1**, the O19 polysaccharide has the full structure **3** shown in Figure 3.

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The inner core of the LPS of *P. alcalifaciens* O19 has the same structure as that of other *Providencia* strains from various species established earlier,[1–3] whereas the outer core is different. *P. rustigianii* O14[1] and O34[3] have the same outer core with alternating lateral Glc and GlcNAc residues, a characteristic feature that is observed in none of *P. alcalifaciens* and *P. stuartii* O-serogroups.[1,2] The outer core of *P. alcalifaciens* O19 differs also in the carbohydrate structure and the presence of *P*EtN on a lateral Gal residue (Fig. 3). In *P. alcalifaciens* O21, O29, and O32, the outer core also consists of four hexose residues and *P*EtN,[1,2] and, hence, belongs to the same type as that of *P. alcalifaciens* O19. In five more *P. alcalifaciens* O-serogroups, the outer core contains *P*EtN too but has only three (O6, O16, O35 and O39) or two (O36) hexose residues. The core structure of the *P. alcalifaciens* O35 LPS has been elucidated[2] and the outer core region found to be significantly different from that of *P. alcalifaciens* O19. Therefore, the LPS outer core is structurally diverse both between *Providencia* species and within one species.

Elucidation of the structure of oligosaccharide **1** defines also the site of attachment of the O-antigen to the core and the biological O-unit structure in the SR-type LPS of *P. alcalifaciens* O19. With these data, the full structure of the carbohydrate chain can be inferred also in the S-type LPS with the long-chain O-antigen. Earlier, the biological O-unit structures have been established in *P. rustigianii* O14[1] and *P. rustigianii* O34,[3] and D-GlcNAc has been found to be the first monosaccharide of the O-unit, whose transfer to

the lipid carrier initiates the O-antigen biosynthesis in the Wzy-dependent pathway.[9] When present, an *N*-acetyl-D-hexosamine (GlcNAc or GalNAc) was suggested to be the first sugar in the O-units of all other *Providencia* O-antigens but in some of them, as in *P. alcalifaciens* O19, the O-unit contains more than one *N*-acetyl-D-hexosamine residue. In this case, the unambiguous choice, and thus the biological O-unit structure elucidation, is enabled only experimentally.

As in most *Providencia* O-serogroups, in *P. alcalifaciens* O19 the nonreducing end of the O-unit, and, hence, of the whole long-chain O-antigen, is occupied by the least common component, namely, Fuc3NAc4c. One can speculate that in this way the bacteria achieved the most O-antigen diversity that may offer the various clones selective advantage in the specific niche.

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