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Note

Structure of the O-polysaccharide of *Providencia alcalifaciens* O2 containing ascarylose and *N*-(L-alanyl)-D-glucosamine

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ABSTRACT

The O-polysaccharide was obtained by degradation of the lipopolysaccharide of *Providencia alcalifaciens* O2 under mild acidic conditions followed by GPC. The polysaccharide was found to contain two unusual components: 3,6-dideoxy-L-*arabino*-hexose (ascarylose, Asc) and 2-(L-alanyl)amino-2-deoxy-D-glucose (GlcNAla). Ascarylose was partially split off during lipopolysaccharide degradation and could be eliminated completely by selective acid hydrolysis, which also partially cleaved the β-GalNAc-(1→6) linkage. The following structure of the branched pentasaccharide repeating unit was established by ^{1}H and ^{13}C NMR spectroscopy of the O-polysaccharide and O-deacetylated polysaccharide, as well as products of partial acid hydrolysis:

α-Asc*p*-(1→4)-α-D-Glc*p*A-(1→4)┐
→6)-β-D-Glc*p*NAla-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→
~60 % OAc—3)┘

Bacteria of the genus *Providencia* are widespread in nature and may cause urinary tract infections and enteric diseases in humans.[1] A combined O-antigen (O-polysaccharide)-based serotyping scheme for medically important *Providencia* species, *Providencia alcalifaciens*, *Providencia stuartii* and *Providencia rustigianii*, consists of 63 O-serogroups.[2] Aiming at elaboration of the chemical basis for this classification, O-polysaccharide structures have been established for 38 O-serogroups.[3–5] In this work, we elucidated the structure of the O-polysaccharide of the lipopolysaccharide (LPS) of *P. alcalifaciens* O2.

The LPS was isolated from bacterial cells by the phenol–water procedure[6] and degraded with aq 2% acetic acid. Fractionation of the carbohydrate portion by GPC on Sephadex G-50 Superfine resulted in an O-polysaccharide fraction (I) (PS-1) and two oligosaccharide fractions (IIa and IIb). Sugar analysis using GLC of the alditol acetates derived after full acid hydrolysis of PS-1 revealed ascarylose (3,6-dideoxy-L-*arabino*-hexose), glucose, GlcN, and GalN. GLC of the acetylated methyl glycosides showed the presence of glucuronic acid (GlcA). Further studies showed that the O-polysaccharide did not contain glucose, which was evidently a component of the LPS core. The L configuration of the 3,6-dideoxy-hexose and the D configuration of the other monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[7] and were in agreement with the glycosylation effects on ^{13}C NMR chemical shifts.[8] In addition to the monosaccharides, L-alanine was identified by GLC of the acetylated ester with (+)-2-octanol.

The ^{13}C NMR spectrum of PS-1 (Fig. 1, top) showed a structural heterogeneity, likely owing to non-stoichiometric O-acetylation (there was a signal for an *O*-acetyl group at δ 21.9). Mild alkaline treatment of PS-1 resulted in an O-deacetylated polysaccharide (PS-2), which was still irregular (Fig. 1, bottom) owing to partial loss of ascarylose during mild acid degradation of the LPS (see below). In addition, the sample was contaminated with a

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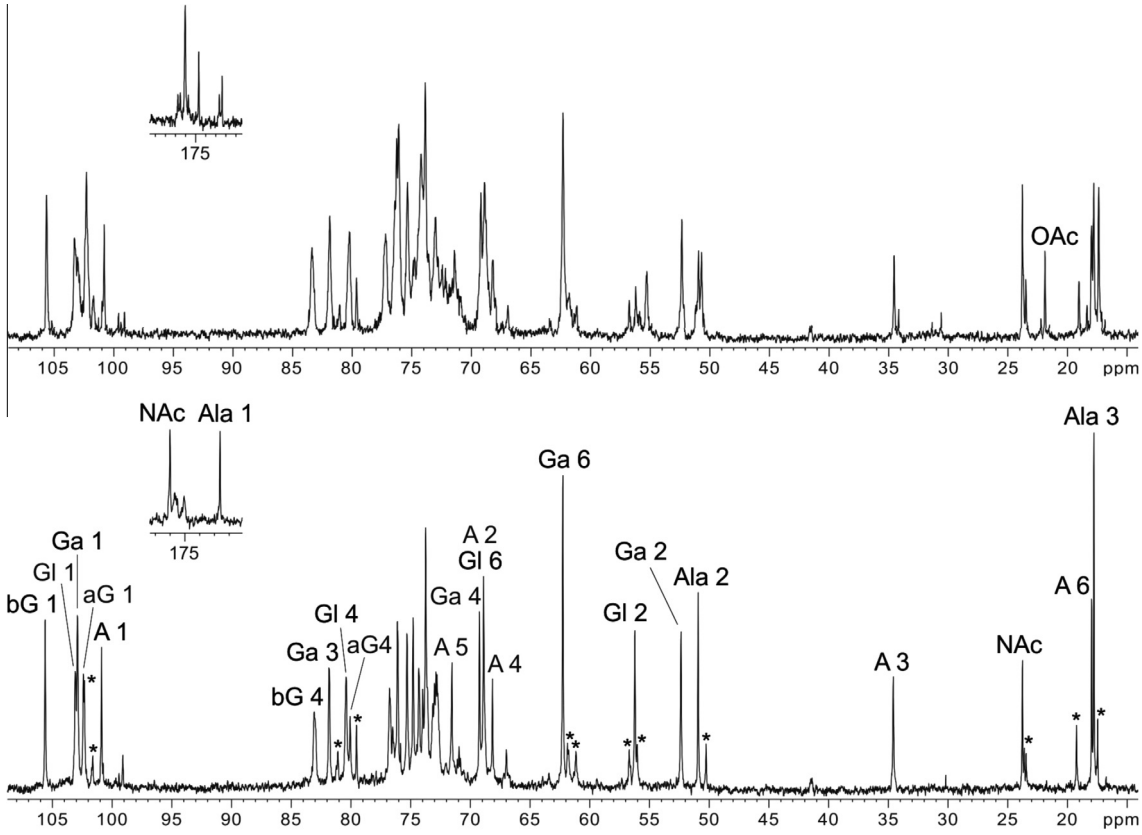

Figure 1. ^{13}C NMR spectra of the O-polysaccharide (PS-1) (top) and O-deacetylated polysaccharide (PS-2) (bottom) from *P. alcalifaciens* O2. Arabic numerals refer to carbons in alanine and sugar residues denoted by letters as follows: A, Asc; Gl, GlcN; Ga, GalN; aG, α-GlcA; bG, β-GlcA. Signals for the peptidoglycan-like polysaccharide[8] are marked with asterisk.

peptidoglycan-like polysaccharide reported earlier to be common for a number of *Providencia* serogroups.[9] In an attempt to obtain a regular Asc-lacking polymer, PS-2 was subjected to partial acid hydrolysis with 0.1 M CF_3CO_2H, and the products were fractionated by GPC on TSK HW-40 (S) to give fractions I-V.

The ^1H and ^{13}C NMR spectra of fractions I-V were assigned using 2D COSY, TOCSY, ROESY, ^1H, ^{13}C HSQC, HSQC-TOCSY, and HMBC experiments, and the NMR chemical shifts for fractions I, IV, and V are tabulated in Table 1. Fraction V was identified as free ascarylose.

Fraction IV was a tetrasaccharide (TS) composed of one linked residue each of α-Glc*p*A, β-Glc*p*A, and β-Glc*p*N, a GalN residue at the reducing end, as well as one alanyl and one *N*-acetyl group. The ^1H, ^{13}C HMBC spectrum of TS showed a cross-peak between C-1 (CO) of Ala and H-2 of GlcN at δ 172.7/3.81, thus demonstrating *N*-alanylglucosamine (GlcNAla); hence, GalN is N-acetylated. The HMBC spectrum also showed the following correlations between anomeric protons and linkage carbons and vice versa: α-GlcA H-1/GlcNAla C-4, GlcNAla H-1/β-GlcA C-4, β-GlcA H-1/α- and β-GalNAc C-3, α-GlcA C-1/GlcNAla H-4, GlcNAla C-1/β-GlcA H-4, and β-GlcA C-1/α- and β-GalNAc H-3. These data showed that TS is linear and has the structure shown in Chart 1, which was confirmed by low-field positions of the signals for linkage carbons (Table 1) and a ROESY experiment (data not shown).

The structure of TS was also corroborated by ESI MS; the negative ion mass spectrum showed the $[M-H]^-$ ion peak at *m*/*z* 804.2532 (calculated *m*/*z* 804.2528 for $C_{29}H_{46}N_3O_{23}^-$). Similar MS analysis showed that fraction III represented an octasaccharide consisting of two TS repeats ($[M-H]^-$: experimental *m*/*z* 1591.4994; calculated *m*/*z* 1591.5022 for $C_{58}H_{91}N_6O_{45}^-$). Fractions II and I were found to be mixtures of higher oligomers up to hexamer and octamer with dominant dodecasaccharide and eicosasaccharide, respectively (data not shown).

As compared with the ^{13}C NMR spectrum of TS, in the spectrum of the fraction I polysaccharide (PS-3) the signal for C-6 of GlcNAla shifted downfield from δ 61.8 to δ 69.0. In the ^1H, ^{13}C HMBC spectrum of PS-3, this signal showed a correlation with H-1 of β-GalNAc, thus indicating the Gal*p*NAc-(β1→6)-Glc*p*NAla linkage. These data suggested that the repeat of PS-3 corresponded to TS and had the structure shown in Chart 1.

Therefore, partial acid hydrolysis of PS-2 completely split off ascarylose, the terminal monosaccharide residue of a polysaccharide side chain, and selectively cleaved some GalNAc linkages in the main chain.

As compared with the NMR spectra of PS-3, the spectra of PS-2 showed additional signals for an ascarylose residue. A comparison with the ^1H and ^{13}C NMR data of the free monosaccharide (Table 1) indicated that ascarylose occurs as the α-pyranoside. The site of attachment of Asc was inferred by a downfield displacement of the signal for C-4 of α-GlcA from δ 73.1 in PS-3 to δ 80.1 in PS-2 and the presence of a Asc C-1/α-GlcA H-4 cross-peak at δ 100.9/3.62 in the ^1H,^{13}C HMBC spectrum of PS-2. Therefore, Asc is attached to α-GlcA at position 4, and PS-2 has the structure shown in Chart 1. In addition to the signals for PS-2, the NMR spectra of fraction I showed signals for PS-3, which was evidently due to partial loss of ascarylose during mild acid degradation of the LPS.

A comparison of the ^1H, ^{13}C HSQC spectra of PS-2 and PS-1 showed a partial displacement of the GlcNAla H-3/C-3 cross-peak from δ 3.74/74.3 in PS-2 to δ 5.19/76.2 in PS-1, which was evidently due to a deshielding effect of the *O*-acetyl group[10] and indicated O-acetylation of GlcNAla at position 3 (Chart 1). This conclusion was confirmed by upfield displacements (β-effects of

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and $^{3}J_{H,H}$ coupling constants (Hz)

Residue	C-1 *H-1* ($J_{1,2}$)	C-2 *H-2* ($J_{2,3ax}$)	C-3 *H-3* (*3equiv*, *3ax*) ($J_{2,3equiv}$; $J_{3equiv,4}$; $J_{3equiv,3ax}$)	C-4 *H-4* ($J_{3ax,4}$)	C-5 *H-5* ($J_{4,5}$)	C-6 *H-6* (*6a*,*6b*) ($J_{5,6}$)
O-Deacetylated polysaccharide (PS-2)						
→3)-β-D-Gal*p*NAc-(1→	102.9	52.4	81.8	69.2	76.1	62.3
	4.41	*4.05*	*3.80*	*4.09*	*3.63*	*3.72, 3.79*
→4)-β-D-Glc*p*A-(1→	105.6	73.7	75.3	83.1	76.8	175.0
	4.56	*3.34*	*3.62*	*3.65*	*3.82*	
→4,6)-β-D-Glc*p*NAla-(1→	103.1	56.2	74.3	80.4	74.8	68.9
	4.52	*3.76*	*3.74*	*3.63*	*3.78*	*3.75, 4.21*
→4)-α-D-Glc*p*A-(1→	102.4	73.1	73.0	80.1	73.6	175.7
	5.18	*3.67*	*3.73*	*3.62*	*4.12*	
α-Asc*p*-(1→	100.9	68.9	34.6	68.1	71.5	18.0
	4.57	*3.94*	*1.89, 2.02*	*3.58*	*3.99*	*1.21*
Asc-lacking polysaccharide (PS-3)						
→3)-β-D-Gal*p*NAc-(1→	103.0	52.3	82.0	69.2	76.1	62.3
	4.40	*4.06*	*3.79*	*4.10*	*3.65*	*3.73, 3.78*
→4)-β-D-Glc*p*A-(1→	105.6	73.8	75.3	83.4	77.5	176.1
	4.55	*3.33*	*3.60*	*3.61*	*3.72*	
→4,6)-β-D-Glc*p*NAla-(1→	103.2	56.2	74.9	80.1	74.3	69.0
	4.50	*3.75*	*3.77*	*3.63*	*3.72*	*3.74, 4.22*
α-D-Glc*p*A-(1→	102.3	73.0	74.2	73.1	74.7	177.4
	5.17	*3.64*	*3.68*	*3.50*	*3.99*	
Tetrasaccharide (TS)						
→3)-α-D-Gal*p*NAc	92.5	50.1	78.7	69.8	71.6	62.5
	5.20	*4.27*	*3.98*	*4.17*	*4.10*	*3.72*
→3)-β-D-Gal*p*NAc	96.4	53.6	81.7	69.1	76.2	62.3
	4.67	*3.98*	*3.80*	*4.11*	*3.68*	*3.74*
→4)-β-D-Glc*p*A-(1→	105.6, 105.4	73.6, 73.7	75.4	83.6	77.5	176.2
	4.56, *4.50*	*3.36*	*3.62*	*3.65,* *3.68*	*3.70*	
→4)-β-D-Glc*p*NAla-(1→	103.1	56.4	75.2	78.0	75.9	61.8
	4.54, *4.53*	*3.81*	*3.81*	*3.71*	*3.67*	*3.79, 3.93*
α-D-Glc*p*A-(1→	100.7	72.7	73.9	73.1	74.3	177.6
	5.42	3.60	3.68	3.49	3.97	
Ascarylose						
α-Asc*p*	93.93	69.31	34.11	68.35	70.93	18.10
	4.958	*3.918*	*2.049, 1.856*	*3.610*	*3.862*	*1.252*
	(1.4)	(3.2)	(4.3; 4.3, 13.6)	(11.1)	(9.1)	(6.3)
β-Asc*p*	95.84	69.19	38.03	68.08	77.18	18.39
	4.866	*3.934*	*2.201, 1.706*	*3.565*	*3.482*	*1.276*
	(1.1)	(3.6)	(4.3; 4.3; 13.9)	(11.6)	(9.3)	(6.2)
α-Asc*f*	103.30	76.44	32.66	83.13	69.31	19.39
	5.270	*4.205*	*2.389, 1.838*	*4.192*	*3.936*	*1.174*
	(<1)	(n.d.)	(6.4; 8.2; 14.0)	(8.2)	(5.5)	(6.6)
β-Asc*f*	101.01	72.27	31.36	81.96	70.19	19.03
	5.209	*4.273*	*2.299, 1.821*	*3.950*	*3.896*	*1.169*
	(4.4)	(9.9)	(7.4; 6.9; 12.3)	(8.9)	(n.d.)	(6.4)

^{1}H NMR chemical shifts are shown in italics.
n.d., not determined.
Additional chemical shifts for the *N*-alanyl group are δ_C 17.7–18.0 (C-3), 50.9 (C-2), and 172.6–172.7 (C-1), δ_H 1.52–1.53 (H-3) and 4.04–4.05 (H-2); for the *N*-acetyl group δ_C 23.7–23.8 (Me) and 176.0 (CO), δ_H 2.04–2.05 in the polysaccharides; δ_C 23.3 and 23.5 (both Me), 176.0 and 176.1 (both CO), δ_H 2.01 in TS; for the *O*-acetyl group δ_C 21.9 (Me) and 174.7 (CO), δ_H 2.08 in PS-1.

α-Asc*p*-(1→4)-α-D-Glc*p*A-(1→4)┐
→6)-β-D-Glc*p*NAla-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→ PS-1
OAc—3)┘

α-Asc*p*-(1→4)-α-D-Glc*p*A-(1→4)┐
→6)-β-D-Glc*p*NAla-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→ PS-2

α-D-Glc*p*A-(1→4)┐
→6)-β-D-Glc*p*NAla-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→ PS-3

α-D-Glc*p*A-(1→4)-β-D-Glc*p*NAla-(1→4)-β-D-Glc*p*A-(1→3)-D-GalNAc TS

Chart 1. Structures of the O-polysaccharide (PS-1), O-deacetylated polysaccharide (PS-2), Asc-lacking polysaccharide (PS-3), and tetrasaccharide (TS) from *P. alcalifaciens* O2. In PS-1 and PS-2, ascarylose is present in a non-stoichiometric amount owing to its partial cleavage during mild acid degradation of the LPS. The degree of O-acetylation in PS-1 is ~60%.

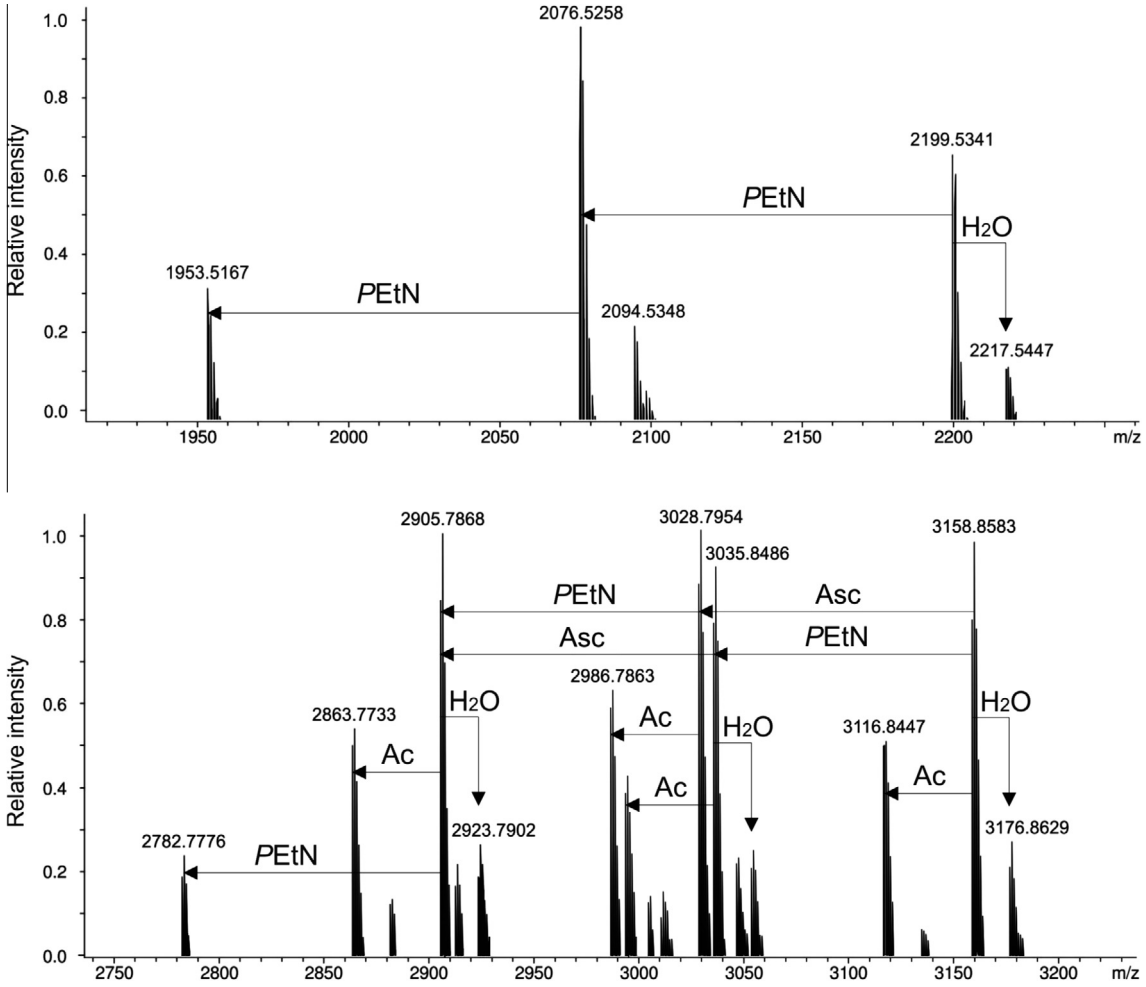

Figure 2. Parts of charge-deconvoluted negative ion ESI mass spectra of fractions IIa (bottom) and IIb (top) isolated from the degraded LPS. Shown m/z values refer to $[M-H]^-$ ions.

O-acetylation[9]) of the signals for the neighboring carbons C-2 and C-4 from δ 56.2 and 80.4 in PS-2 to δ 55.3 and 76.3 in PS-1, respectively. As judged by the ratio of integral intensities of the signals for OAc and NAc groups in the ^{1}H NMR spectra of PS-1, the degree of O-acetylation was ~60%.

Therefore, the O-polysaccharide of *P. alcalifaciens* O2 has the oligosaccharide repeat (O-unit) with the structure shown in Chart 1. This structure was confirmed by negative ion ESI MS analysis of fractions IIa and IIb isolated from the degraded LPS. The charge-deconvoluted spectrum of IIb (Fig. 2, top) showed the $[M-H]^-$ ion peak at *m*/*z* 2199.5341 for a $Hex_4GalA_1Hep_3Ara4N_1$-anhKdo$_1P_1PEtN_3$ core oligosaccharide (calculated *m*/*z* 2199.5347 for $C_{70}H_{123}N_4O_{66}P_4^-$), which has been reported earlier in the LPS of *P. alcalifaciens* O19 and O21.[11] In the spectrum of IIa (Fig. 2, bottom), the major $[M-H]^-$ ion peak at *m*/*z* 3158.8583 belonged evidently to the core bearing one O-unit, the O-unit having thus the molecular mass of 959.3242 Da (calculated *m*/*z* 3158.8578 for $C_{107}H_{180}N_7O_{92}P_4^-$; calculated molecular mass 959.3230 Da for $Asc_1GlcA_2GalNAc_1GlcNAla_1Ac_1$-$H_2O$). Both mass spectra also showed ion peaks for compounds lacking one and two *P*EtN groups (Δm ca. 123 Da) and that of Ia for compounds lacking Asc and/or O-acetyl group (Δm ca. 130 and ca. 42 Da, respectively).

A peculiar feature of the O-polysaccharide of *P. alcalifaciens* O2 is the presence of ascarylose and *N*-(L-alanyl)-D-glucosamine. Ascarylose has been known as a component of a few other bacterial polysaccharides, including *Yersinia pseudotuberculosis* 5a[12,13] and *Vibrio cholerae* O:3.[14] To our knowledge, *N*-(L-alanyl)-D-glucosamine has been hitherto reported only once as a component of the O-polysaccharide of *Proteus penneri* 25 (O69)[15], a representative of a genus closely related to *Providencia*.

1. Experimental

1.1. Bacterial strain and isolation of the lipopolysaccharide

Providencia alcalifaciens O2:H2 strain 67002 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.

LPS was isolated in a yield of ~6.2% of dry bacterial mass by phenol–water extraction[6] followed by dialysis of the extract without layer separation and freed from insoluble contaminations by centrifugation. The resultant solution was treated with cold (4 °C) aq 50% CCl_3CO_2H; after centrifugation the supernatant was dialyzed against distilled water and freeze-dried.

1.2. Preparation of the O-polysaccharide

A portion of the LPS (445 mg) was heated with 2% HOAc (9 mL) for 2.5 h at 100 °C, a lipid precipitate was removed by centrifugation at 13,000*g* for 20 min. The carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-

50 Superfine in 0.05 M pyridinium acetate buffer pH 4.5 to give one polysaccharide (PS-1) and two oligosaccharide fractions in yields 20.6, 31.0, and 108.9 mg, respectively.

1.3. O-Deacetylation and partial acid hydrolysis

PS-1 was subjected to O-deacetylation with 12% aqueous ammonia (0.5 mL, 37 °C, 16 h). Ammonia was removed with a stream of nitrogen at 20 °C, and the residual solution was lyophilized to give O-deacetylated polysaccharide (PS-2).

PS-2 (18.4 mg) was hydrolyzed with 0.1 M CF_3CO_2H (1 mL) at 100 °C for 2 h. Following acid evaporation the residue was fractionated by GPC on a column (80 × 1.6 cm) of TSK HW-40 (S) in 1% HOAc to give fractions I (PS-3), II, III, IV (TS), and V (Asc) in yields 3.3, 4.1, 3.6, 2.0, and 1.0 mg, respectively

1.4. Composition analysis

A PS-1 sample was subjected to hydrolysis with 0.5 M CF_3CO_2H (100 °C, 30 min) or 2 M CF_3CO_2H (120 °C, 2 h) followed by reduction with a solution of $NaBH_4$ (0.4 mL, 10 mg mL^{-1}) in 1 M NH_4OH (20 °C, 2 h), or to methanolysis (1 mL MeOH, 0.1 mL AcCl, 85 °C, 16 h). The products were acetylated with a 1:1 Ac_2O-pyridine mixture (100 °C, 1 h) and analyzed by GLC using a Maestro (Agilent) 7820 GC (Interlab, Russia) equipped with a HP-5 ms column and a temperature gradient of 7 °C min^{-1} from 160 to 290 °C. For determination of the absolute configurations of the monosaccharides, a PS-1 sample was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h) and N-acetylated (400 μL saturated $NaHCO_3$ solution, 60 μL Ac_2O, 0 °C, 1 h) or (for GlcA) subjected to methanolysis as above. The products were heated with (*S*)-2 octanol (100 μL) in the presence of CF_3CO_2H (15 μL) at 120 °C for 16 h, acetylated, and analyzed by GLC as above. Tyvelose (3,6-dideoxy-D-*arabino*-hexose) derived from the O-polysaccharide of *Yersinia pseudotuberculosis* O4b[16] was used for preparation of the authentic samples for identification of ascarylose by GLC of the acetylated alditols and (*S*)-2-octanol.

1.5. NMR spectroscopy

Samples were freeze-dried twice from a 99.9% D_2O soln and dissolved in 99.95% D_2O. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer (Germany) at 20 or 30 °C. Internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0; δ_C -1.6) was used as a reference for calibration. 2D NMR spectra were obtained using standard Bruker software, and Bruker TopSpin 2.1 program was used to acquire and process the NMR data. A mixing time of 150 and 200 ms was used in TOCSY and ROESY experiments, respectively. A 60-ms delay was used for evolution of long-range couplings to optimize the 2D ^1H,^{13}C HMBC experiment for coupling constant $J_{H,C}$ 8 Hz.

1.6. Mass spectrometry

High-resolution ESI MS was performed in the negative ion mode using a maXis instrument (Bruker Daltonics). Oligosaccharide samples (ca. 50 ng μL^{-1}) were dissolved in a 1:1 (v/v) water–acetonitrile mixture and injected with a syringe at a flow rate of 3 μL min^{-1}. Capillary entrance voltage was set at 3 kV and shield voltage at −500 V. Nitrogen was used as the drying gas, and the interface temperature was set at 180 °C. Internal calibration was done with ESI Calibrant Solution (Agilent).

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