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Note

Structural characterization of the lipopolysaccharide O-antigen from atypical isolate of *Vibrio anguillarum* strain 1282

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ABSTRACT

Vibrio anguillarum is a Gram-negative bacterium associated with vibriosis in Atlantic cod (*Gadus morhua L.*). Although farmed cod in Norway is routinely vaccinated against the infection, outbreaks of *V. anguillarum*-associated vibriosis still occur. Here, we describe the structural characterization of the LPS O-chain polysaccharide (O-PS) from atypical isolates of *V. anguillarum* strain 1282 and show that it is distinct from that previously established for *V. anguillarum* serotype O2. The structure of the purified O-PS was shown by 1D/2D NMR (^{1}H, ^{13}C) spectroscopy and CE-MS studies to be a high-molecular mass linear polymer of tetrasaccharide repeating units, composed of 2-acetamido-3-(*N*-formyl-L-alanyl)amido-2,3-dideoxy-D-glucuronamide [GlcNAc3N(Fo-L-Ala)AN], 2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic acid (ManNAc3NAmA), 3-acetamido-3-dideoxy-D-quinovose (Qui3NAc), and 2,4-diacetamido-2,4-dideoxy-D-fucose (FucNAc4NAc).

OAc→[→4)-β-D-Glc*p*NAc3NAN-(1→4)-β-D-Man*p*NAc3NAmA-(1→4)-β-D-Qui*p*3NAc-(1→3)-α-D-Fuc*p*NAc4NAc-(1→]$_n$
Fo-L-Ala

NMR analysis of the partial hydrolysis-derived oligosaccharides confirmed the presence of an *O*-acetyl group at position O-4 of GlcNAc3N(Fo-L-Ala)AN and established that the above-mentioned structure represents the biological repeating unit of the O-PS. In addition, it was demonstrated that some of 2,3-diamino-2,3-dideoxy-glucuronamide in the O-PS was present in the form of 2,3-diamino-2,3-dideoxy-glucose.

Classical vibriosis associated with *Vibrio anguillarum* has been a major problem in cod farming. While over 23 serotypes have been described, only serotypes O1 and subtypes O2a and O2b have been associated with diseased cod.[1] Since juvenile cod is routinely vaccinated against vibriosis, it is thought that the occurring outbreaks of vibriosis are associated with other variants of the bacteria.[2] The immune system of Atlantic cod has been reported to differ from that of other bony fish species investigated so far and is characterized by weak antibody responses to *V. anguillarum* in vaccinated cod although they appear to be sufficient for protection. The specificity of the antibody responses has been shown to be associated with lipopolysaccharide (LPS).[3] Furthermore, cod immune sera produced against *V. anguillarum* serotype O2b were shown to distinguish between antigenic differences associated with LPS epitopes of each subtype.[4] We have previously determined the structure of the O-PS from *V. anguillarum* serotype O2.[5] In the present investigation we describe isolation and structural analysis of the LPS O-PS from atypical isolates of *V. anguillarum* strain 1282.

V. anguillarum strain 1282 was grown in a 30 L-fermentor in BHI supplemented with 2% NaCl, and LPS was extracted from enzyme-digested cells by the hot aqueous phenol method[6] and purified by ultracentrifugation. The O-PS was obtained by mild acid hydrolysis of the phenol layer LPS with 3% acetic acid, and purified by gel permeation chromatography on Bio-Gel P-2, followed by Bio-Gel P-10 column. The void volume fraction was applied to a column of Sephadex G-50 and afforded a high-molecular mass polymer (K_{av} 0.3). Aqueous layer LPS was found to consist mainly of α-glucan previously identified in LPS of *V. anguillarum*[5] and *V. ordalii*.[7]

Abbreviations: BHI, brain heart infusion; CE-MS, capillary electrophoresis-mass spectrometry; COSY, correlated spectroscopy; 1D/2D, one-/two-dimensional; Fo, formyl; dHex, deoxy hexose; Hex, hexose; HexNAc, 2-acetamido-2-deoxy-hexose; GLC, gas liquid chromatography; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; LPS, lipopolysaccharide; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; OAc, *O*-acetyl; O-PS, O-chain polysaccharide; TOCSY, total correlated spectroscopy.

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Table 1
^{1}H and ^{13}C NMR chemical shifts for the O-PS from *V. anguillarum* strain 1282

Residue	Nucleus	1	2	3	4	5	6	NOE/HMBC
A	^{1}H	4.64	3.73	4.25	3.95	4.04		**B**-4
	^{13}C	102.7	54.4	55.0	72.9	76.4		
B	^{1}H	4.83	4.52	4.00	3.77	3.79		**C**-4
	^{13}C	100.7	51.0	55.7	71.9	79.0		
B′	^{1}H	4.83	4.47	4.10	3.83	3.84		**C**-4
	^{13}C	101.1	52.0	52.9	72.7	78.8		
C	^{1}H	4.43	3.22	3.79	3.32	3.50	1.25	**D**-3
	^{13}C	104.7	72.4	56.2	83.5	73.1	17.6	
D	^{1}H	5.18	4.18	3.90	4.33	4.04	1.05	**A**-4
	^{13}C	97.8	48.7	76.6	53.9	67.5	16.7	
Ala	^{1}H		4.31	1.25				
	^{13}C	175.4	49.1	17.6				

Column NOE/HMBC contains interglycosidic correlations in NOESY and HMBC spectra from H-1 of the respective residue. Residue **B** contains Am at N-3, **B′** contains Ac at N-3; Amidine (Am): C-1 166.7, H-2/C-2 2.15/19.9 ppm. Residue **A** contains *N*-formyl-alanyl at N-3: C-1/H-1 164.3/7.96 ppm (D_2O, 45 °C).

Hydrolysis of the O-PS with 2 M TFA afforded 3-amino-3-dideoxy-D-quinovose as the only GLC–MS-detectable component. In addition, glycoses characteristic of the core component, D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose, were also identified.

The ^{1}H NMR and ^{13}C NMR spectra of the O-PS from *V. anguillarum* strain 1282 were fully assigned using 2D COSY, TOCSY, NOESY, and HMBC experiments (Table 1). The ^{1}H NMR spectrum of the O-PS showed resonances for four anomeric protons at δ 4.64, 4.83, 4.43, and 5.18 ppm. The ^{13}C NMR chemical shifts of the O-PS were fully assigned by HSQC (Fig. 1) and HMBC spectra, which showed correlations for four anomeric carbon resonances at 4.64 ppm (^{1}H)/102.7 ppm (^{13}C), δ 4.83 ppm (^{1}H)/100.7 ppm (^{13}C), 4.83 ppm (^{1}H)/101.1 ppm (^{13}C), and 4.43 ppm (^{1}H)/104.7 ppm (^{13}C), two CH_3 group at δ 1.25 ppm (^{1}H)/17.6 ppm (^{13}C) and at δ 1.05 ppm (^{1}H)/16.7 ppm (^{13}C), one *N*-alanyl group at δ 1.25 ppm (^{1}H)/17.6 ppm (^{13}C) (C-1 175.4 ppm), and one *N*-acetamidino group H-2/C-2 at δ 2.15 ppm (^{1}H)/19.9 ppm (^{13}C) (C-1 166.7) ppm. The presence of at least 10 signals of nitrogen-carrying carbons at 48–56 ppm suggested an unusual composition of the repeating unit. Based on the ^{1}H NMR and ^{13}C NMR chemical shift data, which were in agreement with literature values for their respective pyranosides,[8] four observed spin systems were attributed to 2-acetamido-3-amino-2,3-dideoxy-β-glucuronamide (residue **A**), 3-acetamidino-2-acetamido-2,3-dideoxy-β-mannuronic acid (residue **B**), 3-acetamido-3-dideoxy-β-quinovose (residue **C**), and 2,4-diacetamido-2,4-dideoxy-α-fucose (residue **D**). The sequence of monosaccharides and position of linkages in the repeating unit of the O-PS were established from the HMBC and NOESY spectra which showed interglycosidic correlations between C(H)-4**B** and H-1**A**, C(H)-4**C** and H-1**B**(**B′**), C(H)-3**D** and H-1**C,** C(H)-4**A** and H-1**D**, respectively. The combined evidence allowed the sequence of monosaccharides and position of linkages in the O-PS to be established as →4)-**A**-(1→4)-**B**-(1→4)-**C**-(1→3)-**D**-(1→.

Four main oligosaccharide-containing fractions were collected by HPLC and analyzed by NMR (Table 2). Main peak (**OS1**) represented reduced trisaccharide **A**–**B**–**C**, expected from the O-PS structure, with a missing *N*-formyl group at Ala residue. Position of Ala at A3 was confirmed by HMBC data.

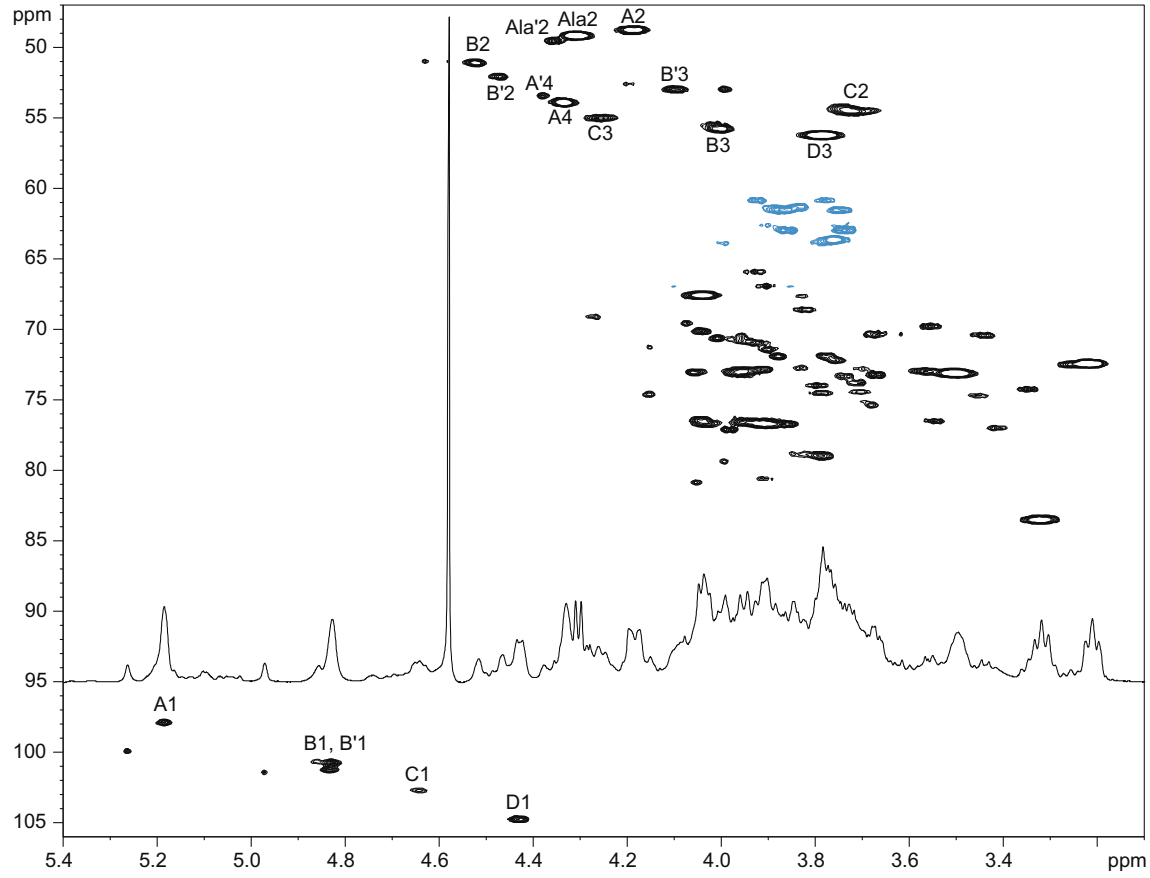
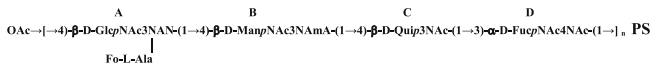


Figure 1. Fragment of the ^{1}H-^{13}C HSQC correlation spectrum of the O-PS from *V. anguillarum* strain 1282. Residues labeled with ' belong to the repeating units where amidine group on N-3 of the residue B is replaced by *N*-acetyl group (B = β-ManNAc3NAcA).

Table 2
^{1}H and ^{13}C NMR chemical shifts for reduced N-acetylated oligosaccharides of O-PS from *V. anguillarum* strain 1282

Compound residue	Nucleus	1	2	3	4	5	6	Amidine H-2/C-2
OS1 Residue **A′**	^{1}H	4.65	3.76	4.02	3.68	4.07		
	^{13}C	103.2	54.4	55.3	70.6	77.2	173.9	
OS1 Residue **B**	^{1}H	5.05	4.67	4.07	4.04	4.01		2.17
	^{13}C	101.2	51.0	55.9	76.0	77.2	173.2	20.0
OS1-3 Residue **C**	^{1}H	3.54	3.83	4.04	3.88	3.97	1.15	
	^{13}C	63.9	71.9	52.2	84.4	68.3	17.4	
OS1,2 Ala	^{1}H		4.21	1.32				
	^{13}C	178.2	51.3	18.1				
OS2 Residue **A′**	^{1}H	4.59	3.68	3.95	3.44	3.57	3.71/4.01	
	^{13}C	102.7	54.8	55.8	68.9	78.4	62.2	
OS2 Residue **B**	^{1}H	5.03	4.67	4.07	4.07	3.93		2.24
	^{13}C	101.1	51.0	55.9	75.3	78.0		20.0
OS3 Residue **A′**	^{1}H	4.67	3.89	4.21	5.00	4.15		
	^{13}C	102.9	53.7	53.2	71.2	74.9		
OS3 Residue **B**	^{1}H	5.05	4.67	4.07	4.04	3.96		2.20
	^{13}C	101.1	51.0	55.9	76.0	78.0		20.0
OS3 Ala	^{1}H		4.09	1.27				
	^{13}C		51.4	17.7				

Acetate signals C-1: 176.2–177.7 ppm; H-2/C-2: 1.98–2.09/22.8–23.5 ppm. Amidine: C-1 167.6 ppm in all products (D_2O, 25 °C).



β-D-GlcNAc3NAlaAN-(1→4)-β-D-ManNAc3NAmA-(1→4)-D-Qui3NAc-ol **OS1**
A B C

β-D-GlcNAc3NAla-(1→4)-β-D-ManNAc3NAmA-(1→4)-D-Qui3NAc-ol **OS2**
A B C

4-O-Ac-β-D-GlcNAc3NAlaAN-(1→4)-β-D-ManNAc3NAmA-(1→4)-D-Qui3NAc-ol **OS3**
A B C

Another important product, **OS3**, had the same structure, but contained an O-acetyl group at O-4 of the GlcNAc3NAlaA residue. This O-acetylated GlcNAc3NAlaA residue was present at the terminal non-reducing end of the polysaccharide chains. The *O*-acetyl group was also visible in the spectra of O-PS, but could not be assigned due to a poor quality. Thus, the structure of the repeating unit shown in Figure 3 represents the biological repeating unit. Oligosaccharide **OS2** unexpectedly contained 2-acetamido-3-alanylamido-2,3-dideoxy-glucose instead of 2-acetamido-3-alanylamido-2,3-dideoxy-glucuronic acid (residue **A**). Thus, some of 2,3-diamino-2,3-dideoxy-glucuronic acid in the O-PS is present in the form of 2,3-diamino-2,3-dideoxy-glucose.

The O-acetylation of 2-acetamido-3-(*N*-formyl-L-alanyl)amido-2,3-dideoxy-D-glucuronamide was also supported by results of CE-MS analysis of the O-PS carried out in a positive mode with the orifice voltage of 400 V that allowed fragmentation of the polysaccharide (Fig. 2). The CE-MS spectrum of the O-PS was consistent with the presence of three major species at *m/z* 800.7, *m/z* 987.8, and *m/z* 1029.8, corresponding to tri-, tetra-, and O-acetylated tetrasaccharide-containing species, respectively, with the molecular masses for constituent sugar residues being *m/z* 315.4 for GlcNAc3 N(FoAla)AN, *m/z* 258.4 for ManNAc3NAmA (Fig. 2). In order to confirm that the observed fragment ions generated through in-source collision-induced dissociation originated from the native O-PS, they were subjected to MS/MS analysis. Tandem MS analysis of a fragment ion at *m/z* 987.8 showed the presence of ions at *m/z* 572.3 consistent with the addition of GlcNAc3N(FoAla)AN to ManNAc3NAmA, while a fragment ion at *m/z* 614.3 indicated that GlcNAc3N(FoAla)AN residue was O-acetylated (Fig. 2B and C). Observed fragment ions at *m/z* 800.7 were consistent with a trisaccharide sequence (OAc)GlcNAc3N(FoAla)AN-ManNAc3NAmA-Qui3NAc. Fragment ions at *m/z* 1174.9 were consistent with the addition of Qui3NAc.

The L-configuration of alanine was determined by GLC–MS of its (*S*)-2-butyl ester *N*-acetyl derivative.[9] Absolute configurations of two monosaccharides, Qui3NAc and GlcN3NA, were determined as D using GLC of acetylated 2-butyl glycosides with chiral 2-butanol. For GlcN3NA a procedure including carboxyl reduction was used as described.[10] A computer-assisted structural analysis of regular polysaccharides on the basis of ^{13}C NMR data[11] was used to assign the absolute configuration of remaining monosaccharides in the repeating unit. Chemical shift of C-1 of β-D-Qui3NAc (104.7 ppm) indicated that FucNAc4NAc also had D-configuration. For DD pair, calculated value of this signal is 105.1 ppm, whereas for DL pair it should be expected at 100.7 ppm.

For ManN3NA, the negative β-effect (−1.2 ppm) for C-3 of ManN3NA in the 1,4-linked GlcN3NA-ManN3NA disaccharide showed the same (D) configuration of ManN3NA and GlcN3NA. Additionally, in ManN3NA-(1–4)-Qui3N disaccharide a large negative β-effect for C-3 of Qui3N (−2.2 ppm) is characteristic of the same (D) configuration of Qui3N and ManN3NA.[12] Therefore, it could be concluded that all monosaccharides in the O-PS of *V. anguillarum* strain LFI 1282 have D-configuration.

Based on the combined NMR, CE–MS, and structural evidence it could be concluded that the structure of the LPS O-PS from *V. anguillarum* strain 1282 as in Fig. 3 represents the biological repeating unit of the polysaccharide.

Preliminary CE–MS analysis of *V. anguillarum* isolates belonging to subtypes O2a and O2b performed on bacterial cells[13] indicated that structures of their O-antigens differ in the *N*-alanyl substitution, namely the O-PS of *V. anguillarum* serotype O2a isolate contains N-formylated L-alanine whereas the O-PS of *V. anguillarum* serotype O2b isolate contains N-acetylated L-alanine (Altman, unpublished). While *V. anguillarum* strain 1282 has been classified as atypical isolate on the basis of biochemical, serological, and genetic characteristics,[2] we have shown here that its O-antigen structure is distinct from that previously described for *V. anguillarum* serotype O2.[5] Both structures share the same structural element →4)-β-D-Glc*p*NAc3N(Fo-L-Ala)AN-(1→4)-β-D-Man*p*NAc3NAmA-(1→, probably responsible for the observed cross-reactivity with rabbit O2a antiserum[5] and consist of linear tetrasaccharide repeating units. However, as demonstrated in this report, the structure of O-PS from *V. anguillarum* strain 1282 is sufficiently different to allow for production of strain-specific antibodies in cod, warranting the incorporation of this atypical

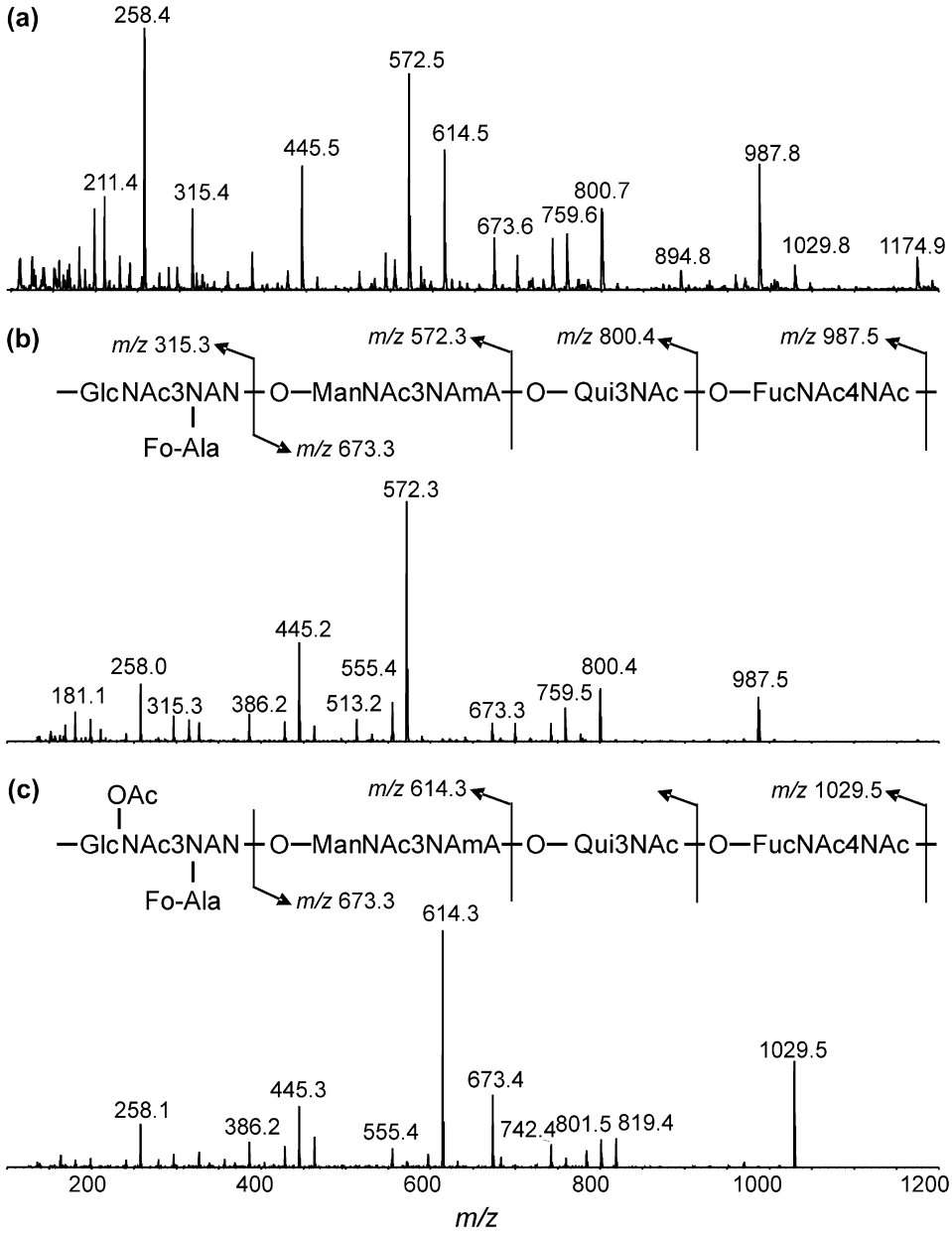


Figure 2. CE-MS and CE-MS/MS analysis (positive ion mode, orifice voltage 400 V) of the O-PS from *V. anguillarum* strain 1282: (A) extracted mass spectrum of the O-PS; (B) extracted MS/MS spectra of precursor ions at *m/z* 987.8; (C) extracted MS/MS spectra of precursor ions at *m/z* 1029.8.

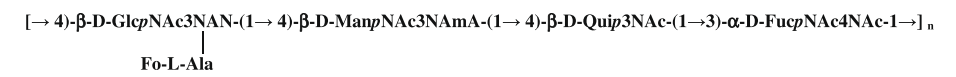


Figure 3. The proposed structure of the O-PS of *V. anguillarum* strain 1282.

variant into vaccine formulation currently based on serotypes O2a and O2b.[14]

1. Experimental

1.1. Bacterial culture

V. anguillarum strain 1282[4] was cultured in brain heart infusion (BHI) broth (Difco) containing 2% NaCl in a 30-L fermentor (new_MBR) at 15 °C for 22 h, dissolved oxygen was controlled at 20% and pH was maintained at 6.5 with 3 M NaOH. The cells were killed by addition of 500 g of phenol.

1.2. Isolation of LPS and preparation of the O-PS

Bacterial cells were washed with 10 mM phosphate-buffered saline, pH 7.4, digested enzymatically[13], and extracted by the method of Westphal et al.[6] Phenol and water layers were separated by low-speed centrifugation, collected separately, dialyzed against tap water until phenol-free and then lyophilized. The lyophilizates

were dissolved in 1% saline (w/v) and subjected to ultracentrifugation (105,000*g*, 4 °C, 16 h). The LPS pellets were re-dissolved in dist. water and lyophilized. Aqueous layer LPS was hydrolyzed with 1% acetic acid (100 °C, 2 h) and purified by gel permeation chromatography on a column of Bio-Gel P-10 (Bio-Rad) using 0.02 M pyridinium acetate (pH 5.4) as the eluant. The ^{1}H NMR spectrum of the void volume fraction indicated that it predominantly consisted of α-glucan.[5] This material was not further investigated. Phenol layer LPS (40 mg) was hydrolyzed with 3% AcOH (100 °C, 3 h). The reaction mixture was cooled down on ice and the insoluble lipid A was removed by low-speed centrifugation. The water-soluble part was lyophilized and purified by gel permeation chromatography on a column of Bio-Gel P-2 (Bio-Rad). The O-PS-containing fraction was further purified on a column of Bio-Gel P-10 (Bio-Rad) using 0.02 M pyridinium acetate (pH 5.4) as the eluant. The void volume fraction was collected and applied to a Sephadex G-50 column (Pharmacia Fine Chemicals, Uppsala, Sweden) irrigated with 0.02 M pyridinium acetate (pH 4.5) as the eluant. The gel-filtration properties of the eluted material are expressed in terms of their distribution coefficient K_{av}. $K_{av} = (V_e - V_t)/(V_t - V_o)$, where V_e is the elution volume of the specific material, V_o is the void volume of the system, and V_t is the total volume of the system.

1.3. Compositional analysis

Glycoses were determined by GLC as their alditol acetates. A sample of the O-PS was hydrolyzed with 2 M TFA at 100 °C overnight and the hydrolyzate was subjected to N-acetylation, followed by reduction ($NaBH_4$) and acetylation.[15] Absolute configurations of Qui3NAc and Ala were established by capillary GLC according to the method of Leontein et al.[9] and confirmed by comparison of the GLC retention time and MS with those of standards prepared with (*S*)- and (*R*)-2-butanol. For GlcN3NA a procedure including carboxyl reduction was used.[10] The absolute stereochemistry of remaining monosaccharides was determined on the basis of ^{13}C NMR data as described.[11]

1.4. Partial hydrolysis of the O-PS

The O-PS (20 mg) was dissolved in 10 M HCl (8 mL) and heated at 90 °C for 15 min. The reaction mixture was neutralized with 4 M NaOH and purified on a column of Bio-Gel P-10 (Bio-Rad) using 0.02 M pyridinium acetate (pH 5.4) as the eluant. The carbohydrate-containing fractions were collected, lyophilized, and subjected to re-N-acetylation. Briefly, the sample was dissolved in dry methanol (3 mL) and to it acetic anhydride (300 μL) and pyridine (60 μL) were added, and the reaction was allowed to proceed for 1 h at 22 °C. Following evaporation, the reaction products were purified on a column of Bio-Gel P-2 (Bio-Rad) using 0.02 M pyridinium acetate (pH 5.4) as the eluant. The oligosaccharide-containing fractions were reduced with $NaBH_4$ and further separated by HPLC on reverse phase column in 0.1% TFA with UV 220 nm detection. Four main peaks were collected and analyzed by NMR.

1.5. NMR spectroscopy

NMR spectra were performed using Varian INOVA 500 MHz and 600 MHz spectrometers employing standard software as described previously[16] at 25 °C or 45 °C using a 5 mm indirect detection probe with the ^{1}H coil nearest to the sample. The methyl resonance of acetone was used as an internal reference at δ 2.225 ppm for ^{1}H spectra and at 31.07 ppm for ^{13}C spectra.

1.6. CE-MS

All experiments were performed using a Prince CE system (Prince Technologies, The Netherlands) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Canada). A sheath solution (isopropanol–methanol, 2:1) was delivered at a flow rate of 1 μL/min. Separations were obtained on about 90 cm length bare fused-silica capillary using 15 mM ammonium acetate in deionized water, pH 9.0. The 5 kV of electrospray ionization voltage was used for positive ion mode and negative ion mode detections, respectively. Tandem mass spectra were obtained using enhance production ion scan mode (EPI) with a scan rate of 4000 Da/s. Nitrogen was used as curtain (at a value of 12) and collision gas (set to scale high). The separations were performed according to Li et al.[17]

Acknowledgments

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