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Structure of the O-specific polysaccharide of the lipopolysaccharide from *Yersinia kristensenii* O:25.35

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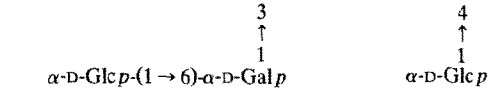
ABSTRACT

Mild hydrolysis of the lipopolysaccharide (LPS) from *Yersinia kristensenii* serovar O:25.35 with acid afforded the O-specific polysaccharide (PS) which contained D-glucose, D-galactose, 2-acetamido-2,6-dideoxy-L-galactose, 2-acetamido-2-deoxy-D-glucose, glycerol, and phosphate in the ratios 3:1:1:1:1. On the basis of ^{31}P and ^{13}C NMR spectroscopy, hydrolysis, methylation studies, Smith degradation, and dephosphorylation, the repeating unit of PS was shown to have the following structure.



INTRODUCTION

Comparative immunochemical studies[1] demonstrated that the LPSs from *Y. kristensenii* serovars O:12.25, O:12.26, and O:25.35 have similar sugar compositions and each gave a single precipitation band with homologous antisera on immunodiffusion in agar. Partial cross-reactivity and positive indirect hemagglutination were observed for the serovars O:12.25 and O:12.26, and for the serovars O:12.25 and O:25.35, thus indicating the correct serological typing and the presence of a common determinant in each pair.

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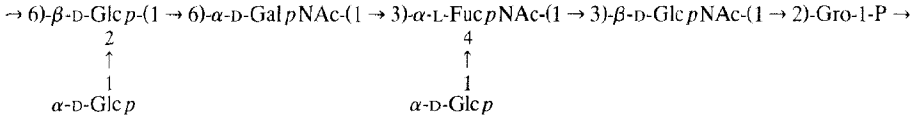
The structure of the repeating unit of the O-specific polysaccharide O:12.26 has been elucidated[2] as:



The structure of the O-specific polysaccharide of the LPS from serovar O:25.35 is now reported.

RESULTS AND DISCUSSION

The LPS was extracted from the dried micro-organism with hot aqueous phenol, and the nucleic acids were removed by precipitation with trichloroacetic acid. Centrifugation then gave LPS-1, and LPS-2 remained in the supernatant solution. LPS-1 and LPS-2 contained mainly the *R*- and *S*-forms, respectively.

Treatment of LPS-1 and LPS-2 with dilute acetic acid followed by molecular-sieve chromatography of each soluble fraction on Sephadex G-50 gave the O-specific polysaccharide (PS), in yields of 12 and 37%, respectively.

PS had $[\alpha]_{578}^{20}$ +30° (*c* 1.2, H_2O), gave a single precipitation band with the homologous antiserum on double immunodiffusion in agar, and contained 2.5% of phosphorus, 13.4% of 2-amino-2-deoxyglucose, and 13.5% of 2-amino-2,6-dideoxygalactose. Galactose, glucose, 2-amino-2-deoxyglucose, 2-amino-2,6-dideoxygalactose (in the ratios 1:3:1:1), and glycerol were identified in the polysaccharide hydrolysate by PC and by GLC of the alditol acetates.

The $[\alpha]_D$ values of the monosaccharides isolated from a hydrolysate of the LPS, using paper electrophoresis on a preparative scale followed by paper chromatography, indicated the glucose, galactose, and 2-amino-2-deoxyglucose to belong to the D series while the 2-amino-2,6-dideoxygalactose has the L configuration.

The ^{13}C NMR spectrum of PS contained signals for C-1 at 98.8, 99.3, 99.5, 101.2, 102.0, and 104.2 ppm (Fig. 1a) indicative of a hexasaccharide repeating unit. In addition, there were signals at 23.5 and 175.0 ppm for NAc and at 50.1, 57.0, and 16.9 ppm indicative of 2-acetamido-2,6-dideoxygalactose and 2-acetamido-2-deoxyglucose residues[3]. Analysis of the region for the C-2 resonances of the acetamidodeoxyhexoses demonstrated that the signal at 57.0 ppm could be assigned only to 2-acetamido-2-deoxy-β-D-glucopyranose and that at 50.7 ppm to 3-substituted 2-acetamido-2,6-dideoxy-α-D-galactopyranose[3]. The $J_{C\text{-}1,H\text{-}1}$ values (160 Hz for the signals at 102.0 and 104.2 ppm, and 170 Hz for the signals at 98.8, 99.3, 99.5, and 101.2 ppm) demonstrated[4] that each sugar residue was pyranoid, two were β, and four were α. *J*-Modulated spin-echo experiments showed that the ^{13}C signals at 61.9, 66.0, and 67.8 ppm could be assigned to hydroxymethyl groups, two of which were glycosylated[5]. The signal at 1.6 ppm in the ^{31}P NMR spectrum of PS indicated a disubstituted monophosphate residue in the sugar chain[6].

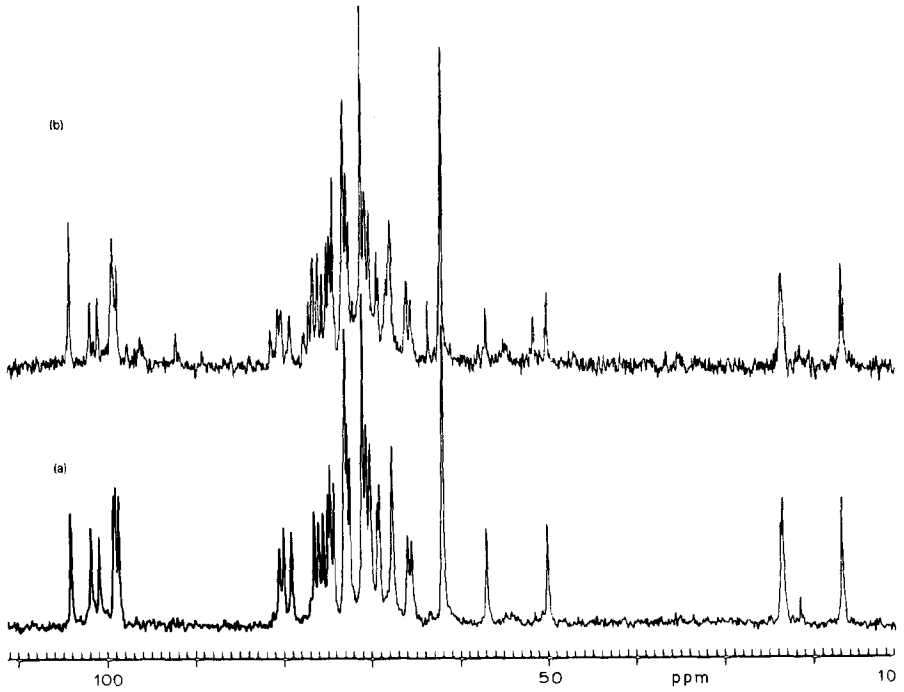


Fig. 1. ^{13}C NMR spectra of PS (a) and autohydrolysed PS (b).

Methylation of PS by the Hakomori method[7] followed by hydrolysis gave 2,3,4,6-tetra-*O*-methylglucopyranose, *O*-methylglycerol, 2,3,4-tri-*O*-methylglucopyranose, 2,3,4-tri-*O*-methylgalactopyranose, 2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)glucopyranose, and 2,6-dideoxy-2-(*N*-methylacetamido)galactopyranose in the ratios 2:1:1:1:1:1, identified by GLC–MS of the corresponding alditol acetates and methyl glycosides. These data indicated PS to be branched with 3,4-disubstituted 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2,6-dideoxy-L-galactose as the branch points, and to contain 6-linked D-glucose and D-galactose residues, and two terminal D-glucose residues.

Smith degradation[8] (periodate oxidation, borohydride reduction, and acid hydrolysis) of PS furnished 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2,6-dideoxy-L-galactose, and glycerol, thus demonstrating the resistance of these sugar residues to periodate oxidation. Mild acid hydrolysis of the polyalcohol yielded oligosaccharide OS-1, $[\alpha]_{578}^{20}$ −49° (*c* 1.14, H_2O), which contained the above sugar residues together with an equimolar amount of phosphate. The ^{31}P NMR spectra of OS-1 (Fig. 2) and PS contained identical signals.

Treatment of OS-1 with aqueous 40% hydrofluoric acid and gel-permeation chromatography of the products on TSK HW 40(F) afforded a dephosphorylated oligosaccharide (OS-2), $[\alpha]_{578}^{20}$ −59° (*c* 0.5, H_2O), and glycerol, identified by PC and GLC of the triacetate.

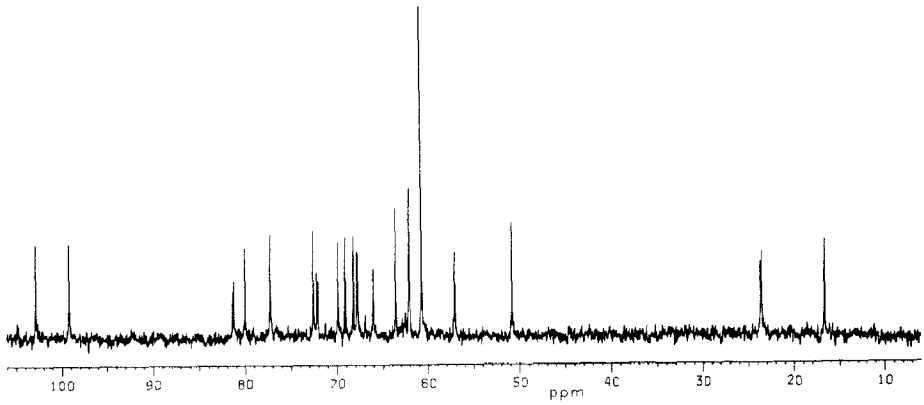


Fig. 2. ^{13}C NMR spectrum of the oligosaccharide OS-1.

The ^{13}C NMR spectrum of OS-2 contained C-1 signals at 102.4 and 98.9 ppm, as well as the characteristic signals at 57.0, 50.7, and 16.6 ppm, which indicated the presence of 2-acetamido-2,6-dideoxy-L-galactopyranose and 2-acetamido-2-deoxy-D-glycopyranose residues. Analysis based on glycosylation effects[9] and theoretical calculations indicated that OS-2 contained 2-acetamido-2,6-dideoxy-β-L-galactopyranose and 3-linked 2-acetamido-2-deoxy-β-D-glucopyranose.

The absolute configuration of 3-linked sugar residues may be elucidated using the glycosylation effect[9]. Thus, the β-effects of glycosylation for α-D-Hex*p*-(1 → 3)-α-D-Gal and α-D-Hex*p*-(1 → 3)-α-L-Gal are −1.5 and −0.7 ppm, respectively (at C-2 of Gal). We have observed the latter value, corresponding to the L configuration of the 2-acetamido-2,6-dideoxygalactose residue in OS-2. Assignment of all of the amino sugar signals (Table I) indicated that the glycerol residue is 2-linked in OS-2. On the basis of the ^{13}C NMR data, OS-2 is assigned the structure α-L-Fuc*p*NAc-(1 → 3)-β-D-Glc*p*NAc-(1 → 2)-Gro.

The signals in the ^{13}C NMR spectrum of OS-1 (Fig. 2, Table I) were assigned on the basis of the data for OS-2 and literature data concerning the values of α- and β-phosphorylation effects[10]. Comparison of the spectra of OS-1 and OS-2 showed differences in the signals for C-1 and C-2 of the glycerol residue. For OS-1, the signals of C-1 (d, 65.8 ppm, $J_{C\text{-}1,P}$ 4 Hz) and C-2 (d, 81.0 ppm, $J_{C\text{-}2,P}$ 8 Hz) indicated that a phosphate group is attached to position 1 of the glycerol residue. The signals at 67.5 ($J_{C\text{-}1,P}$ 5 Hz), 71.9 ($J_{C\text{-}1,P}$ 8 Hz), and 63.4 ppm in the spectrum of OS-1 indicated the presence of a second glycerol residue, and the structure L-Fuc*p*NAc-α-(1 → 3)-D-Glc*p*NAc-β-(1 → 2)-Gro-(1 P → 1)-Gro.

The results of methylation analysis and Smith degradation of PS indicated that the second glycerol residue in OS-1 originated from a 6-linked hexose residue of the backbone.

Dephosphorylation of PS with aqueous 40% hydrofluoric acid gave a complex mixture of oligosaccharides, from which a disaccharide (OS-3), $[\alpha]_{578}^{20}$ +58° (*c* 1.0, H_2O), that contained D-glucose and D-galactose was isolated by chromatography

TABLE I

^{13}C NMR data (chemical shifts in ppm) for PS and its fragments

Compound	Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
PS	→6)-β-Glc*p*-(1→	104.2	75.1	76.7	71.1	74.9	67.8
	→3,4)-α-Fuc*p*NAc-(1→	98.8	50.1	75.6	80.1	65.6	16.9
	→6)-α-Gal*p*-(1→	99.3 [a]	69.2	70.2	70.7	69.4	67.8
	α-Glc*p*-(1→	99.5	72.8	74.4	71.1	73.1	62.0
	→3,4)-β-Glc*p*NAc-(1→	102.0	57.0	79.3	74.9	76.2	62.0
	α-Glc*p*-(1→	101.2 [a]	73.1	74.4	71.1	73.1	62.0
	→2)-Gro1→	66.0	80.6	62.0			
OS-1	α-Fuc*p*NAc-(1→	98.9	50.6	69.0	72.4	68.0	16.5
	→3)-β-Glc*p*NAc-(1→	102.4	56.9	79.9	68.9	77.0	62.0
	→2)Gro1→	66.0	81.0	62.0			
	→1)-Gro	67.8	71.9	63.4			
OS-2	α-Fuc*p*NAc-(1→	98.9	50.7	69.1	72.5	68.2	16.6
	→3)-β-Glc*p*NAc-(1→	102.4	57.0	80.0	69.9	77.0	62.2
	→2)-Gro	61.6	82.0	61.5			
OS-3	α-Glc*p*-(1→	99.7	72.5	74.4	70.9	73.1	61.9
	→6)-β-Gal*p*-(1→	97.8	73.1	74.3	70.1	74.3	67.9

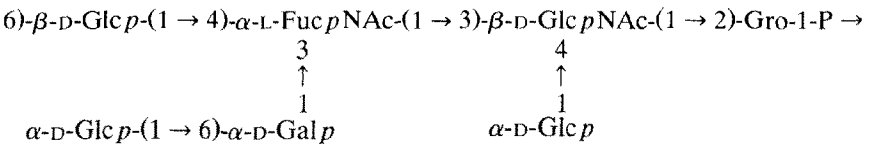
[a] Assignments may be reversed.

on TSK HW 40 (F) gel. The ^{13}C NMR spectrum of OS-3 contained signals for C-1 at 99.7 ppm for the non-reducing residue, and at 97.8 and 93.7 ppm for C-1α and C-1β of the reducing residue. Only single signals were observed for the other carbon atoms of the non-reducing residue and the chemical shifts indicated unequivocally α-D-glucopyranose. The ^{13}C signals of the β anomer of the reducing sugar residue showed chemical shifts (Table I) characteristic of 6-linked β-D-galactopyranose residue only[11,12]. Thus, OS-3 has the structure α-D-Glc*p*-(1→6)-α,β-D-Gal*p*.

In addition to OS-3, dephosphorylation of PS provided two oligosaccharide fractions (OS-4 and OS-5) which were separated by HPLC on Silasorb SPH C_{18}. The ^{13}C NMR spectra demonstrated OS-4 and OS-5 to be mixtures of disaccharides. The signals for C-1 at 104.2, 99.2, 99.5, and 99.0 ppm, for NAc at 23.5 and 175 ppm, for C-2 of 2-acetamido-2-deoxyhexoses at 50.5 and 51.2 ppm, and for 6-deoxy groups at 16.7 and 16.5 ppm indicated that OS-4 and OS-5 contained the disaccharide β-D-Glc*p*-(1→3 or 4)-α-L-Fuc*p*NAc. The signal at 104.2 ppm may be assigned only to a β-residue, whereas the signals at ~16 and 50–51.2 ppm are assigned to 2-acetamido-2,6-dideoxygalactopyranose. Thus, the repeating unit of PS possessed the partial structure: β-D-Glc*p*-[1→3(4)]-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→2)-Gro-(1-P→. Additional data concerning the structure of PS were provided by a comparison of its ^{13}C NMR spectrum (Fig. 1a) with that (Fig. 1b) of the degraded polysaccharide obtained by autohydrolysis. The spectrum of the latter polysaccharide contained additional signals at 16.6, 51.6, and 81.5 ppm

due to a FucNAc residue. In addition, the changes in the region 73–75 ppm are connected with the galactopyranose residue.

A diminution in the intensity of the signal at 99.3 ppm and the appearance of signals for C-1α and C-1β at 92.3 and 97.0 ppm indicated that the disaccharide fragment D-Glc*p*-(1 → 6)-Gal*p*-(1- was 3-linked to the 2-acetamido-2,6-dideoxy-L-galactopyranose residue. The signal for C-5 of the 2-acetamido-2-deoxy-β-D-glucopyranose residue, noted above at 77.0 ppm (Table I, OS-1), was not given by PS. This finding demonstrated that the α-D-glucopyranose residue was 4-linked to the 2-acetamido-2-deoxy-D-glucopyranose residue. On the basis of ^{13}C NMR data, the structure of the repeating unit of PS was assigned as:



A complete assignment of the signals in ^{13}C NMR spectrum of PS (Table I) was achieved by considering effects of glycosylation[9] and using literature data on the chemical shifts of the ^{13}C resonances of the constituents of the polysaccharide. The ^{13}C chemical shifts calculated for the structure suggested (S 1.4) coincided with the data in Table I and confirmed the structure.

EXPERIMENTAL

General methods.—Analytical and preparative PC were performed on Filtrak FN-15 and Whatman 3MM papers, using 6:4:3 1-butanol–pyridine–water. Paper electrophoresis was carried out in 0.025 M pyridine–acetate buffer (pH 4.5) at 28 V/cm on Whatman 3MM paper. Alkaline silver nitrate and ninhydrin were used for detecting sugars and amino sugars, respectively. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter at 20°C. Solutions were lyophilised or concentrated in vacuo. HPLC was performed on a column (0.4 × 25 cm) with Silasorb SPH C_{18} (LC) (7.5 μm) by elution with water. Gel-permeation chromatography was carried out on columns of Sephadex G-50 (3.0 × 75 cm), using aq 0.3% acetic acid, and Toyopearl TSK HW 40 (F) (1.5 × 80 cm), using water, with a refractive index detector. GLC was performed with a Pye Unicam 104 instrument equipped with glass columns (0.4 × 150 cm) packed with 3% QF-1 or OV-225 on Gas-chrom Q within the temperature ranges 175–225°C and 140–225°C, respectively, with Ar as the carrier gas. GLC–MS was performed on an LKB 9000S instrument, using the same columns. Amino acid analysis was performed with a Biotronic LC-2000 instrument on a column (0.22 × 6 cm) of DC 6A resin. Phosphorus contents were determined as described[13]. The ^{31}P (external aq 85% H_3PO_4) and ^{13}C NMR spectra (internal MeOH, 50.15 ppm) were obtained with a Bruker WM-250 spectrometer at 20 and 80°C, respectively.

Y. kristensenii serovar O:25.35 (strain 1647) was kindly provided by Professor H.H. Mollaret (Institut de Pasteur, Paris, France) and was grown in the standard nutrient medium.

Isolation of the LPS and the O-specific polysaccharide (PS).—The dried microorganisms (42 g) were extracted conventionally with aq 45% phenol. Nucleic acids were precipitated by the addition of aq 50% trichloroacetic acid. The precipitate was removed by centrifugation, and the supernatant solution was dialysed, concentrated, and ultracentrifuged at 105 000*g*. The resulting precipitate and supernatant solution were lyophilised to give LPS-1 (1.68 g) and LPS-2 (1.97 g), respectively. LPS-1 and LPS-2 (each 1 g) were hydrolysed with aq 1% acetic acid at 100°C for 2 h. Lipid A (420 and 100 mg, respectively) was removed by centrifugation and the supernatant solutions were lyophilised. The resulting polysaccharides (540 and 800 mg, respectively) were each subjected to chromatography on Sephadex G-50, to afford 3 fractions as follows: D-glucan (54 and 52 mg), PS (120 and 340 mg), and low molecular weight substances (320 and 340 mg), respectively.

Smith degradation of PS.—A solution of PS (70 mg) in 0.1 M sodium metaperiodate (7 mL) was kept in the dark for 72 h and the product was reduced conventionally with $NaBH_4$ for 4 h. The excess of borohydride was destroyed with acetic acid, the mixture was dialysed, and the solution was concentrated. The polyalcohol obtained was hydrolysed with aq 1% acetic acid for 2 h, the hydrolysate was concentrated, and the residue was reduced with $NaBH_4$ and chromatographed on TSK HW-40 (F) to yield oligosaccharide OS-1 (20 mg); $[\alpha]_{578}^{20}$ −49° (*c* 1.14, H_2O).

Dephosphorylation of OS-1.—A solution of OS-1 (16 mg) in aq 40% HF (1.5 mL) was kept for 48 h at 4°C. The excess of HF was removed over NaOH in vacuo and the residue (13.5 mg) was chromatographed on TSK HW 40 (F) to afford the dephosphorylated oligosaccharide OS-2 (4 mg); $[\alpha]_{578}^{20}$ −59° (*c* 0.5, H_2O), and glycerol (1 mg).

Dephosphorylation of PS.—A solution of PS (100 mg) in aq 40% HF (7 mL) was kept at room temperature for 96 h, then treated as described above. The product was chromatographed on TSK HW 40 (F) to furnish the disaccharide OS-3 (20 mg); $[\alpha]_{578}^{20}$ +58° (*c* 1.0, H_2O), and a mixture (50 mg) of oligosaccharides. OS-4 and OS-5 were isolated by HPLC from the mixture.

Hydrolysis.—Polysaccharides (5 mg) and oligosaccharides (2 mg) were each hydrolysed with 2 M HCl and 0.5 M trifluoroacetic acid (1 mL) at 100°C for 3 h and each hydrolysate was co-concentrated with MeOH. The products obtained were analysed by PC and by GLC of the alditol acetates. Each polysaccharide (3 mg) was hydrolysed with 4 M HCl at 100°C for 3 h, the hydrolysate was co-concentrated with MeOH, and the mixtures obtained were subjected to amino acid analysis.

PS (100 mg) was hydrolysed with 2 M HCl at 100°C for 3 h, the hydrolysate was co-concentrated with MeOH, and the mixture was subjected to paper electrophoresis on a preparative scale. The resulting fractions of sugars and amino

sugars were fractionated by PC to yield: D-glucose, $[\alpha]_{578}^{20}$ +92° (*c* 1.5, H_2O); D-galactose, $[\alpha]_{578}^{20}$ +75° (*c* 0.5, H_2O); 2-amino-2-deoxy-D-glucose hydrochloride, $[\alpha]_{578}^{20}$ +8° (*c* 0.6, H_2O); and 2-amino-2,6-dideoxy-L-galactose hydrochloride, $[\alpha]_{578}^{20}$ −82° (*c* 0.5, H_2O) lit. [14] $[\alpha]_D$ −95° (H_2O).

Methylation analysis.—PS (10 mg) was methylated by the Hakomori method[7]. A part (5 mg) of the product was methanolysed, and the resulting mixture of methyl glycosides was acetylated, then analysed by GLC–MS. The remainder of the product was formolysed and then hydrolysed. The products were converted into the alditol acetates and analysed by GLC–MS.

Autohydrolysis of PS.—A solution of PS (140 mg) in D_2O (2 mL) was kept at 70°C for 6 h in an NMR tube and then subjected to ^{13}C NMR spectroscopy.

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