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Structure of the repeating unit of the O-specific polysaccharide of the lipopolysaccharide of *Yersinia kristensenii* strain 490 (O:12,25)

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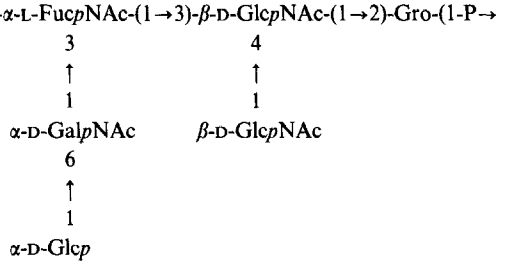
ABSTRACT

The O-specific polysaccharide isolated by mild acid degradation of the lipopolysaccharide of *Y. kristensenii* strain 490 (O:12,25) contained D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2,6-dideoxy-L-galactose, glycerol, and phosphate in the ratios 2:2:1:1:1:1. On the basis of ^{31}P- and ^{13}C-n.m.r. data, methylation analysis, dephosphorylation, solvolysis with anhydrous hydrogen fluoride, and Smith degradation, it was concluded that the repeating unit of the polysaccharide was a branched hexaosylglycerol phosphate with the following structure.



INTRODUCTION

A new species, *Y. kristensenii*, has been isolated from the *Yersinia enterocolitica* group on the basis of biochemical and DNA–DNA hybridisation data[1]. The structure **1** has been established[2] for the O-specific polysaccharide from the lipopolysaccharide (LPS) of *Y. kristensenii* strain 103 (O:12,26), which is built up of glycerol phosphate-containing hexasaccharide repeating units.

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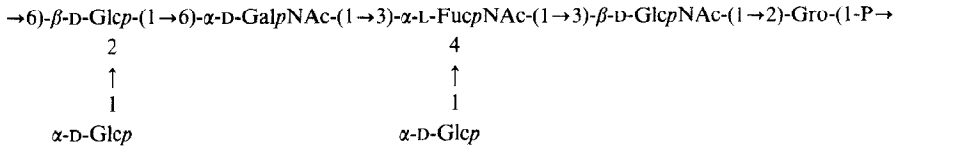


1

Now we report the structure of the O-specific polysaccharide of *Y. kristensenii* strain 490 (O:12,25) which is serologically related[3,4] to the strain 103 (O:12,26).

RESULTS AND DISCUSSION

LPS of *Y. kristensenii* strain 490 (O:12,25) was isolated[5] by phenol–water extraction. The crude preparation was separated by 3-fold ultracentrifugation at 105,000*g* to give, in precipitate, LPS-I (5.8% of the dry-cell weight), which was highly serologically active in the Ouchterlony test and in passive haemagglutination with the homologous O-antiserum. LPS-II (~ 1%) was isolated from the supernatant solution by gel-permeation chromatography on Sepharose 4B CL and further fractionated on Sephadex G-200 in the presence of sodium deoxycholate[6,7] to yield a high molecular weight fraction (LPS-III).

In sodium dodecyl sulphate-polyacrylamide gel electrophoresis[8] (SDS-PAGE), all preparations displayed progressive, ladder-like patterns of bands that proved the presence of molecules with increasing numbers of repeating units in the polysaccharide chain[9]. LPS-II and LPS-III each contained populations of molecules with long polysaccharide chains (a series of bands with E_f 0.1–0.3), LPS-I lacked these chains almost completely, and short polysaccharide chains ($E_f > 0.3$) were practically absent from LPS-III.

LPS-II was degraded by hydrolysis with aqueous 1% acetic acid at 100° for 1.5 h to give lipid A, which was removed by centrifugation at 18,000*g*, and a water-soluble portion that was subjected to gel-permeation chromatography on Sephadex G-50 to give a high molecular weight, serologically active, O-specific polysaccharide (PS-I, 30%), a core oligosaccharide fraction, and a low molecular weight fraction which contained mainly 3-deoxy-2-octulosonic acid.

General characterisation of lipid A and the oligosaccharide and monosaccharide fractions revealed close similarities to the corresponding products derived[2] from *Y. kristensenii* strain 103. The serological relationship of LPS of strains 103 and 490 and the identity of their monosaccharide components[4] suggest that the structures of their polysaccharide chains are similar. Paper electrophoresis indicated PS-I to be acidic and it was found to contain phosphate (2.6%). Acid hydrolysis of PS-I afforded glucose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, 2-amino-2,6-dideoxygalactose, and glycerol in the ratios 2:2:1:1:1, which were identified by using an amino acid analyser and g.l.c. of the alditol acetates prepared after deamination[10]. The monosaccharides were isolated from the hydrolysate by preparative p.c. The 2-amino-2,6-

dideoxygalactose was shown to be L and the other sugars to be D on the basis of the $[\alpha]_D$ values.

The ^{31}P-n.m.r. spectrum of PS-I contained only one signal; its position (−1.6 p.p.m.), which was not affected by change of pH in the range 3.5–9.8, proved that this signal belonged to a monophosphodiester group and excluded the presence of a glycosyl–phosphate linkage[11].

The presence of six signals for C-1 in the ^{13}C-n.m.r. spectrum (Table I) indicated PS-I to have a hexasaccharide repeating unit. There were also signals for four acetamido groups (CH_3 near 23 p.p.m. and CO near 175 p.p.m.) and one methyl group of a 6-deoxy sugar, which indicated the presence of four *N*-acetylated amino sugars, one of which was 2-acetamido-2,6-dideoxygalactose. There were two signals for carbon attached to nitrogen at 50.5 p.p.m. that were characteristic of C-2 of hexosamines having the *α-galacto* configuration, whereas the signals at 57.2 and 57.4 p.p.m. were characteristic of C-2 of those having the *β-gluco* configuration[12]. Hence, two of the 2-acetamido-2-deoxyglucose residues were *β* and the 2-acetamido-2-deoxygalactose and 2-acetamido-2,6-dideoxygalactose residues were *α*. The $J_{C\text{-}1,H\text{-}1}$ values showed[12,13] that each residue was pyranosidic with three *α* and three *β*. Thus, one glucose residue was *α* and the other was *β*.

The ^{13}C-n.m.r. spectrum of PS-I also contained a series of minor signals (10–15% of the intensity of the major signals). These signals were not due to the core oligosaccharide since the O-specific polysaccharide isolated from LPS-III, which included practically no low molecular weight components, had a ^{13}C-n.m.r. spectrum that was identical with that of PS-I. Therefore, the heterogeneity observed was associated, most probably, with structural features of the O-specific polysaccharide.

Methylation analysis[14] of PS-I revealed terminal glucose and 2-acetamido-2-deoxyglucose residues, 3,4-disubstituted 2-acetamido-2-deoxyglucose and 2-acetamido-2,6-dideoxygalactose redisues, and 6-substituted glucose and 2-acetamido-2-deoxygalactose residues.

Smith degradation of PS-I gave a phosphate-containing product, the ^{13}C-n.m.r. spectrum of which (Table I) was identical to that of the oligosaccharide **2**, obtained[2] by Smith degradation of the O-specific polysaccharide of *Y. kristenseniii* strain 103.

TABLE I

^{13}C-N.m.r. chemical shifts[a] (δ in p.p.m.)

Sugar unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Polysaccharide **6** (PS-I)						
→6)-*β*-D-Glc*p*-(1→	105.3	74.0	76.6	71.0	75.2	65.1
→3)-*α*-L-Fuc*p*NAc-(1→	99.8	50.5	75.5	80.4	69.0	17.1
4 ↑						
→6)-*α*-D-Gal*p*NAc-(1→	98.0	50.5	68.8	70.0	69.7	66.2
α-D-Glc*p*-(1→	99.8	72.9	74.5	71.0	72.9	62.2

(continued)

TABLE I (continued)

^{13}C-N.m.r. chemical shifts[a] (δ in p.p.m.)

Sugar unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
→3)-β-D-Glc*p*NAc-(1-	103.1	57.2	75.9	77.8	75.2	62.2
4						
↑						
β-D-Glc*p*NAc-(1→	101.8	57.4	74.7	72.7	76.5	62.2
→2)-Gro-(1→	66.2	81.6	63.6			
Oligosaccharide **5** (OS-I)						
β-D-Glc*p*(1→	104.8	73.7	76.6	70.5	77.2	62.1
→3)-α-L-Fuc*p*NAc-(1→	99.7	50.4	75.3	80.1	69.2	17.1
4						
↑						
→6)-α-D-Gal*p*NAc-(1→	97.9	50.9	68.6	70.0	69.3	66.3
α-D-Glc*p*-(1→	99.9	72.5	74.3	70.9	72.9	62.1
→3)-β-D-Glc*p*NAc-(1→	102.5	57.1	75.9	77.7	75.1	62.7
4						
↑						
β-D-Glc*p*NAc-(1→	101.7	57.2	74.6	72.5	76.5	61.9
→2)-Gro	61.5	82.6	63.5			
Oligosaccharide **8**						
β-D-Glc*p*-(1→	104.5	73.8	77.3	70.9	77.3	62.1
→4)-α-L-Fuc*p*NAc-(1→	99.5	50.9	70.1	80.9	69.1	17.1
→3)-β-D-Glc*p*NAc-(1→	102.9	56.5	75.8	77.5	75.1	61.9
4						
↑						
β-D-Glc*p*NAc-(1→	102.2	56.9	74.8	71.1	76.9	62.0
→2)-Gro	61.5	82.5	62.7			
Oligosaccharide **2**						
α-L-Fuc*p*NAc-(1→	99.1	50.7	69.0	72.5	68.1	16.5
→3)-β-D-Glc*p*NAc-(1→	102.7	57.0	79.9	69.8	77.1	62.0
→2)-Gro-(1→	66.0	81.2	62.0			
→1)-Gro	67.7	72.1	63.4			
Oligosaccharide **3**						
α-D-Gal*p*NAc-(1→	100.0	51.0	68.7	69.8	72.8	62.6
	100.3					
→3)-α-L-FucNAc	92.3	50.3	74.7	72.3	67.5	16.8
→3)-β-L-FucNAc	96.5	53.8	78.0	71.7	72.0	16.8
Oligosaccharide **4**[b]						
β-D-Glc*p*NAc-(1→	102.7	56.9	74.6	71.1	77.2	61.9
→4)-α-D-GlcN	90.3	55.3	69.7	80.1	72.1	61.6

[a] Assignments of the signals having differences in chemical shifts <0.5 p.p.m. could be interchanged. Additional signals: NAc at 23.3–23.4 (Me) and 175.4–176.0 (CO) p.p.m. [b] The data are for the major disaccharide in which the GlcN residue is α.

α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→2)-Gro-(1-P→1)-Gro

2

Solvolysis of PS-I with anhydrous hydrogen fluoride[15] at 0° yielded two disaccharides (**3** and **4**) that were isolated by gel-permeation chromatography on TSK 40. Analysis of the ^{13}C-n.m.r. spectra (Table I), taking into account the effects of glycosylation[16] and the results of sugar analysis, established the structures shown.

α-D-Gal*p*NAc-(1→3)-L-FucNAc

3

β-D-Glc*p*NAc-(1→4)-D-GlcN

4

Treatment of PS-I with aqueous 48% hydrofluoric acid gave an oligosaccharide product that was isolated by gel-permeation chromatography on TSK 40. The ^{13}C-n.m.r. spectrum (Table I), like that of PS-I, contained major and minor series of signals and, hence, this product was a mixture of two oligosaccharides which were isolated subsequently by reverse-phase h.p.l.c. Each oligosaccharide had the same sugar composition as PS-I and, thus, was a hexaosylglycerol.

Methylation analysis showed that the major oligosaccharide (OS-I) differed from PS-I in having a terminal glucose residue instead of the 6-substituted glucose residue. The ^{13}C-n.m.r. spectrum of OS-I (**5**, Table I) contained signals at 61–62 p.p.m. for two CH_2OH groups instead of the signals at 65.1 and 66.2 p.p.m. for two CH_2OP groups in the spectrum of PS-I. These data indicated that the repeating units in PS-I were connected by phosphodiester linkages between the hydroxymethyl groups of glycerol and one of the glucose residues.

The only signal for the *O*-substituted hydroxymethyl group in the ^{13}C-n.m.r spectrum of OS-I belonged to C-6 of the 2-acetamido-2-deoxygalactose residue. The relatively high-field position (66.3 p.p.m.) proved that this residue was substituted by an α-glycosyl residue (for a β-glycosyl residue, this signal would be expected at 68–69 p.p.m.)[2,16]. On the basis of this conclusion and the structures of **2**–**4**, structure **5** can be suggested for OS-I. Consequently, the repeating unit of PS-I can be represented by one of the alternative structures **6** and **7**.

β-D-Glc*p*-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→2)-Gro

3 4

↑ ↑

1 1

α-D-Gal*p*NAc β-D-Glc*p*NAc

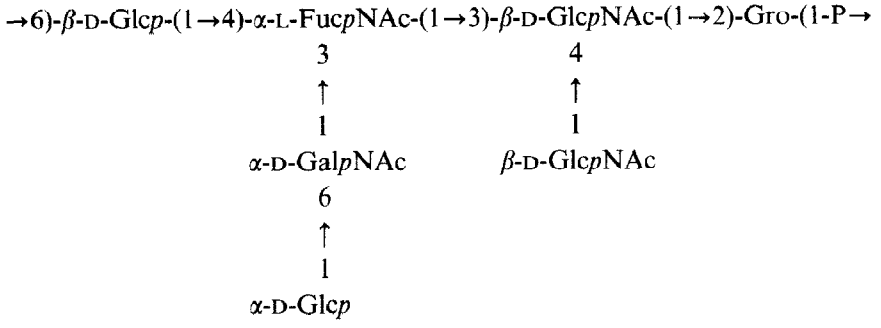
6

↑

1

α-D-Glc*p*

5 (OS-I)



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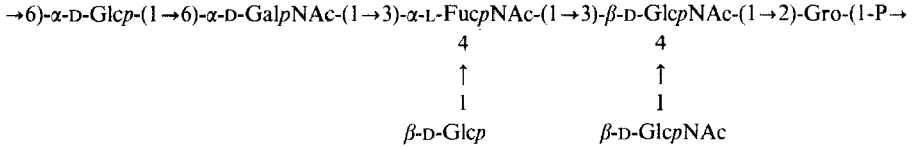

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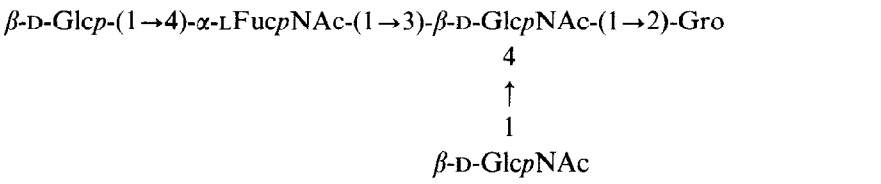
Smith degradation of PS-I with a limited amount of sodium periodate[2] (4 mol per 6 mol of oxidisable groups) gave PS-II, which contained glucose, 2-acetamido-2-deoxyglucose, 2-acetamido-2-deoxygalactose, 2-acetamido-2,6-dideoxygalactose, and glycerol in the ratios 1:2:0.7:1:1. Thus, only one terminal glucose residue had been oxidised fully, whereas the terminal 2-acetamido-2-deoxyglucose residue and the 6-substituted sugar residues were relatively stable.

When compared with the ^{13}C-n.m.r. spectrum of PS-I, that of PS-II lacked the signal at 99.7 p.p.m. (C-1 of an α sugar) and one of the signals at 66.2 p.p.m. (C-6 of a 6-substituted sugar). These data proved that the terminal α-glucose residue was originally 6-linked to 2-acetamido-2-deoxygalactose and, consequently, the repeating unit of PS-I had the structure **6**.

In order to confirm this structure, PS-II was dephosphorylated with aqueous 48% hydrofluoric acid, and two products were isolated from the resulting complex mixture of oligosaccharides by gel-permeation chromatography on TSK 40 followed by reverse-phase h.p.l.c. On the basis of sugar analysis and ^{13}C-n.m.r. data (Table I), it was found that the major oligosaccharide had structure **8**.



8

Sugar and methylation analysis indicated that the minor oligosaccharide differed from **8** by the presence of a terminal 2-acetamido-2-deoxygalactosyl group 3-linked to 2-acetamido-2,6-dideoxygalactose, which was in accord with the structure **5**.

Thus, it was concluded that the O-specific polysaccharide (PS-I) of *Y. kristensenii* strain 490 (O:12,25) had structure **6**.

This structure accorded with the tentative assignments of the ^{13}C-n.m.r. spectra of OS-I and PS-I (Table I), which were made by comparison with those of **2–4** and **8** and taking into account published data[16].

The origin of the minor signals in the ^{13}C-n.m.r. spectrum of PS-I remains obscure. The associated structure also included phosphodiester linkages, but the repeating unit was not a part of PS-I or PS-I with an additional sugar residue. The minor series of signals did not coincide with those[2] of the O-specific polysaccharide of *Y. kristensenii* strain 103. The serological cross-reactivity of LPS of strains 103 and 490 seems to be connected with the major polysaccharides.

EXPERIMENTAL

General methods. — ^{31}P-N.m.r. spectra were recorded with a Bruker Physics HX-250 instrument, using aqueous 85% phosphoric acid as an external standard (δ 0). ^{1}H-N.m.r. spectra were obtained with a Bruker WM-250 spectrometer on solutions in D_2O at 25°. ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 instrument on solutions in D_2O at 25° for oligosaccharides and 80° for the polysaccharide (external methanol, δ 50.15). Optical rotations were measured on a Jasco DIP 360 polarimeter at 25°.

G.l.c. was performed on a Hewlett–Packard 5890 instrument fitted with a capillary (25 m) Ultra 1 column at 150–290° (10°/min). G.l.c.–m.s. was carried out on an LKB 9000 instrument equipped with a column of 3% QF-1 on Gas-Chrom Q (100–120 mesh).

Gel-permeation chromatography was performed on a column (3.5 × 70 cm) of Sephadex G-50 in a pyridine acetate buffer (pH 5.5) or a column (80 × 1.6 cm) of TSK HW 40 (S) in water.

P.c. was carried out on Paper Filtrak FN-11, using 1-butanol–pyridine–water (6:4:3) or 1-propanol–NH_4OH (d 0.88)–water (6:3:1). Sugars were detected with alkaline silver nitrate, and amino sugars with ninhydrin.

Serological tests, growth of bacteria, and isolation of the lipopolysaccharide and O-specific polysaccharide were performed as described[2].

Acid hydrolysis was performed with 2M HCl at 100° for 3 h prior to analysis of neutral monosaccharides by g.l.c. of the alditol acetates. Amino sugars were analysed after hydrolysis with 4M HCl at 100° for 16 h, with the use of an amino acid analyser LG 5001 as described[17].

Methylation analysis was performed according to the Hakomori procedure[14] and the products were recovered by using a Sep-Pak C18 cartridge.

Dephoshorylation was performed by treatment with aqueous 48% HF for 4 days

at 0°, the solution was concentrated *in vacuo* at room temperature over solid NaOH, a solution of the residue in water was neutralised with aqueous ammonia, and the products were subjected to gel chromatography on TSK HW 40.

Solvolysis with anhydrous HF was carried out with stirring for 2 h at room temperature, the HF was evaporated over solid NaOH, and the residue was dissolved in water and subjected to gel chromatography as stated above.

Smith degradation. — Polysaccharide was treated with 0.1M sodium metaperiodate for 60 h at room temperature in the dark, an excess of ethylene glycol was added, the product was desalted using Sephadex G-50, then reduced with an excess of $NaBH_4$, desalted, and hydrolysed with aqueous 1% acetic acid (100°, 2 h), and the final product was isolated by gel-permeation chromatography on TSK HW 40.

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