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Structural studies of the *Shigella boydii* type 5 O-antigen polysaccharide

M. John Albert [a], Tord Holme [b], Bengt Lindberg [c,*], Johan Lindberg [c], M. Mosihuzzaman [d], Firdausi Qadri [a], M. Mahbubur Rahman [b,c]

[a] *Department of Laboratory Research, International Centre for Diarrhoeal Research, Bangladesh, (ICDDR, B), Dhaka-1000, Bangladesh*

[b] *Department of Bacteriology, Karolinska Institute, S-10401 Stockholm, Sweden*

[c] *Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-10691 Stockholm, Sweden*

[d] *Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh*

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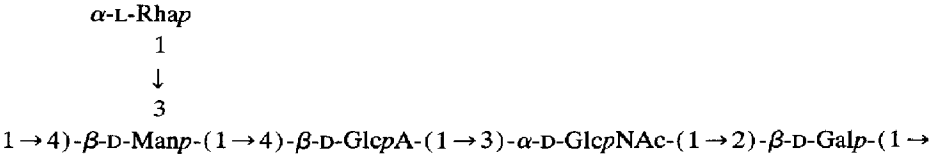
Abstract

The structure of the *Shigella boydii* type 5 O-antigen polysaccharide has been investigated by sugar and methylation analyses, and specific degradations. It is proposed that it is composed of hexasaccharide repeating units with the following structure. The repeating unit also contains an *O*-acetyl group, linked to one of the primary positions.



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1. Introduction

During studies of *Aeromonas* species isolated from humans with diarrhoeal illness, it was observed that strains of *Aeromonas caviae*, isolated from 16 patients, cross-reacted with antisera to *Shigella boydii* type 5 [1]. The structures of 9 of the 15 O-antigen types of *S.*

* Corresponding author.

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Table 1
Sugar analyses of PS and degradation products

Sugar	Detector response (%)			
	PS	PS-1	OS-1	OS-2
L-Rha	12			
D-Man	39	54		70
D-GlcA	13	15	34	
D-Gal	19	22	40	
D-GlcNAc	15	10	26	30
Hep	1.4			

boydii have been determined, all of them by Kochetkov and/or his co-workers (see Ref. [2] and previous papers). The structure of the O-antigenic polysaccharide from type 5, however, has not been reported. We now report structural studies of this polysaccharide.

2. Results and discussion

The lipopolysaccharide (LPS) from *S. boydii* type 5 was isolated by extraction with phenol–water [3]. On polyacrylamide gel electrophoresis it gave multiple bands, demonstrating that the O-antigen polysaccharide contained on average 10–15 repeating units. The LPS was treated with acid under mild conditions, and the polysaccharide (PS) isolated by chromatography on a Sephadex G-50 column. Sugar analyses of the PS, by GLC of the alditol acetates, gave L-Rha, D-Man, D-Gal, D-GlcNAc, and heptose (Table 1). The low percentage of heptose is in agreement with the presence of long O-antigenic side-chains. D-Glucitol was also obtained in the sugar analysis. D-Glc is, however, not a component of the O-antigen polysaccharide but is derived from D-GlcA, which is released, lactonised, and reduced to D-glucitol during this analysis. When the reduction was performed using sodium borodeuteride, D-(1-^{2}H,6,6-^{2}H$_{2}$)glucitol was obtained. The absolute configurations of the sugars were determined as described by Gerwig et al. [4,5]. No glucose derivative was obtained in the methylation analysis (Table 2), unless the methylated product was carboxyl-reduced before hydrolysis. It could not be decided from the methylation analysis whether D-GlcA and one of the D-Man are pyranoid and substituted on O-4, or furanoid and substituted on O-5. Since all signals for hydroxymethyl groups in the ^{13}C NMR spectrum of the *O*-deacetylated PS appear at 62–60 ppm, it is concluded that this D-Man is pyranoid. That D-GlcA is pyranoid is evident from the structure of oligosaccharide OS-1, as discussed below. These results therefore indicate that the O-antigen polysaccharide is composed of hexasaccharide repeating units, containing terminal L-Rha, D-Man linked through O-3, D-Man linked through O-3 and O-4, D-Gal linked through O-2, D-GlcNAc linked through O-3, and D-GlcA linked through O-4. The ^{1}H and ^{13}C NMR spectra revealed that the polysaccharide contains an *O*-acetyl group per repeating unit. The shift of a signal at δ 63.2 to 60.7 on *O*-deacetylation indicates that the *O*-acetyl group is linked to a primary position.

Table 2
Methylation analyses of PS, carboxyl-reduced PS, and degradation products

Sugar [a]	T [b]	Detector response (%)					
		PS	C [c]	U [d]	PS-1	OS-1	OS-2
1,3,4,5,6-Gal	0.45					28	
2,3,4-Rha	0.63	15	15	12			
2,3,4,6-Man	1.13						35
2,3,6-Man	1.32				28		45
2,4,6-Man	1.36	22	19	16	32		
3,4,6-Gal	1.39	15	11	23	26		
2,6-Man	1.60	29	22	24			
2,3-Glc	1.81		14				
2,3,4,6-GlcNAc	2.53			25 [e]			
2,4,6-GlcNAc	2.85	19	18		13	72	20

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, etc. [b] Retention time on an HP-5 column, see Experimental. [c] Carboxyl-reduced PS. [d] Uronic acid-degraded PS. [e] OCD_3 in 3-position.

The ^1H and ^{13}C NMR spectra of the *O*-deacetylated PS (Figs. 1 and 2) confirm the presence of hexasaccharide repeating units. 2D correlation spectra, namely, H,H-COSY, C,H-COSY, and H,H-HOHAHA, demonstrate that L-Rha and D-GlcNAc are α-linked (Table 3). Because of overlapping signals, some signals for the other sugar residues could not be assigned. From the $J_{C\text{-}1,H\text{-}1}$ values [6], 177.1, 170.0, 162.0, 162.0, 161.1, and 160.0

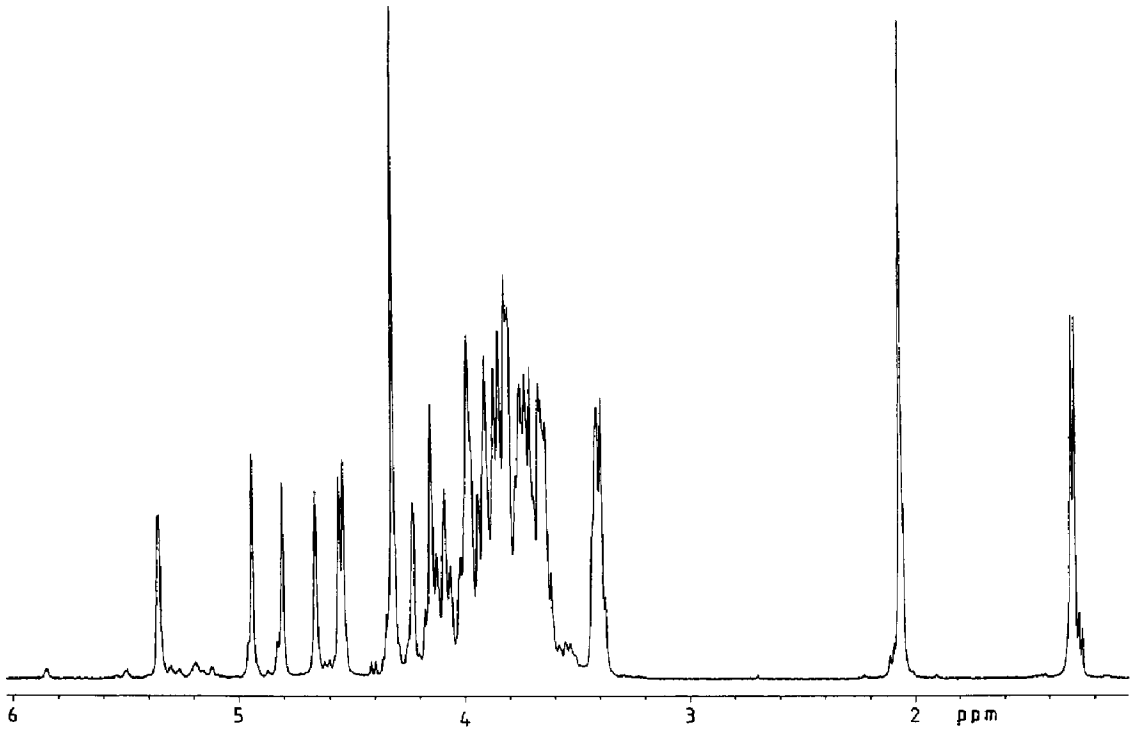

Fig. 1. ^1H NMR spectrum of the *O*-deacetylated *Shigella boydii* type 5 O-antigen polysaccharide.

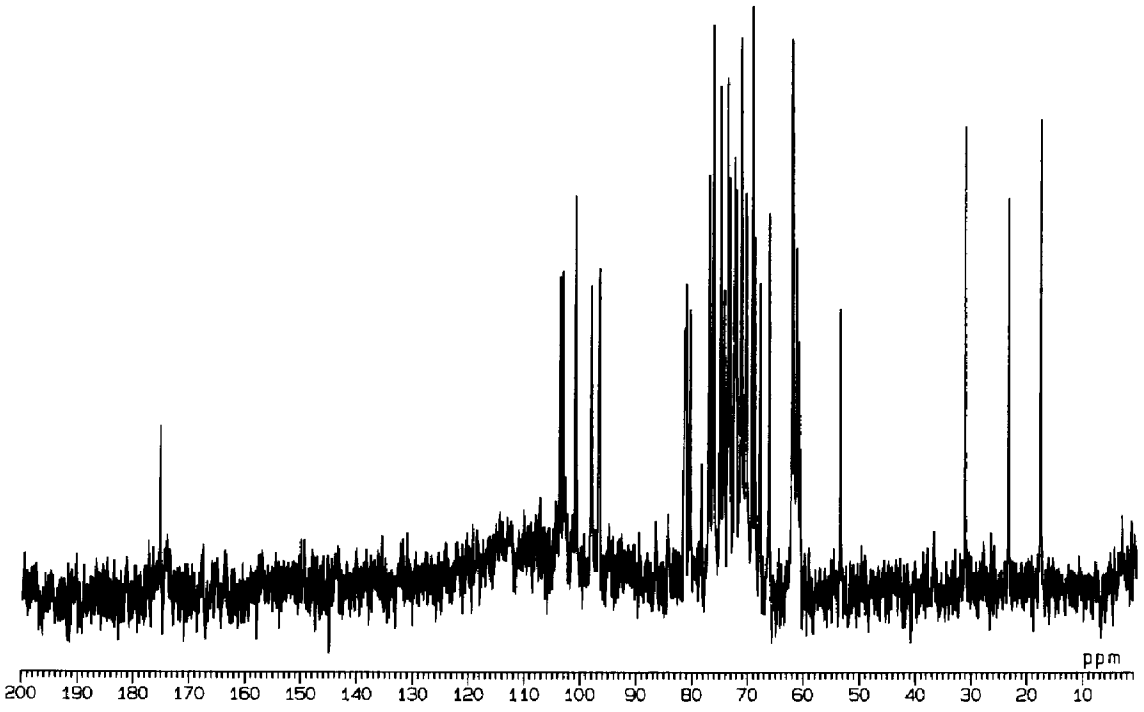

Fig. 2. ^{13}C NMR spectrum of the *O*-deacetylated *Shigella boydii* type 5 O-antigen polysaccharide.

Hz, respectively, there are two α-linked sugar residues, and D-Gal, D-GlcA, and the two D-Man residues are consequently β-linked. The signals for the anomeric protons of the two β-D-Man*p* residues appeared at δ 4.81 and 4.66, and those of β-D-Glc*p*A and β-D-Gal*p* overlapped at δ 4.55.

The PS was treated with 0.5 M trifluoroacetic acid at 80°C for 10 h, conditions during which most of the rhamnosyl and some of the other glycosyl linkages should be cleaved. The product was reduced with sodium borodeuteride, and fractionated on a Bio-Gel P-2 column. An oligosaccharide fraction (OS-1) and a fraction of higher molecular weight (PS-1) were obtained. The former was further purified by HPLC on a C18 column, and proved to be a trisaccharide-alditol, composed of D-GlcA, D-GlcNAc, and D-(1-^{2}H)galactitol. The ^{1}H NMR spectrum contained, *inter alia*, signals for the anomeric protons of α-D-Glc*p*NAc at δ 5.15 (1 H), $J_{1,2}$ 3.7 Hz, and β-D-Glc*p*A at δ 4.50 (1 H), $J_{1,2}$ 7.7 Hz,

Table 3
^{1}H and some ^{13}C chemical shifts for the 2-acetamido-2-deoxy-D-glucopyranosyl residue and the L-rhamnopyranosyl group in the *O*-deacetylated *Shigella boydii* type 5 O-antigen polysaccharide

Sugar residue	Chemical shift (δ)					
	H-1/C-1	H-2/C-2	H-3	H-4	H-5	H-6
→3)-α-D-Glc*p*NAc-(1→	5.35 96.6 (177 Hz)	4.10 53.4	3.96	3.63	4.07	~3.83
α-L-Rha*p*-(1→	4.94 96.5 (170 Hz)	3.99	3.40	4.33	4.33	1.30

and for *N*-acetyl at δ 2.04 (3 H). Methylation analysis (Table 2) showed that the D-Glc*p*NAc was 3-substituted and the galactitol 2-substituted, demonstrating that the trisaccharide-alditol has structure **1**.

β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-D-(1-^{2}H)Galactitol

1

Methylation analysis of PS-1 (Table 2) gave, *inter alia*, 2,3,6-tri-*O*-methyl-D-mannose, but no 2,6-di-*O*-methyl-D-mannose or 2,3,4-tri-*O*-methyl-L-rhamnose, thus indicating the partial structure **2**.

→4)-β-D-Man*p*-(1→
3
↑
1
α-L-Rha*p*

2

The (1→3) linkage between β-D-Glc*p*A and α-D-Glc*p*NAc was confirmed by treatment of the methylated PS with sodium methylsulfinylmethanide in dimethyl sulfoxide, methylation with trideuteriomethyl iodide, hydrolysis, and analysis of the methylated sugars as the alditol acetates by GLC–MS. During this treatment, the sugar residues linked to the uronic acid residue are released as a non-reducing and a reducing residue, respectively [7], and the latter is to some extent further degraded.The formation of 2-deoxy-3,4,6-tri-*O*-methyl-2-methylacetamido-D-glucose, with a trideuteriomethyl group on O-3 (Table 2), demonstrates that the uronic acid is linked to this position in the PS.

Smith degradation of the PS, that is periodate oxidation, borohydride reduction, and hydrolysis under mild conditions [8], gave a main product (OS-2), eluted in the oligosaccharide region on a Bio-Gel P-2 column. The ^{1}H NMR spectrum of this product indicated that it was not a pure oligosaccharide. In addition to the signals for the anomeric protons of two β-D-Man residues, at δ 4.84 and 4.68, respectively, a signal at δ 4.52, $J_{1,2}$ 8.0 Hz, and a signal for *N*-acetyl at δ 2.04, it contained, *inter alia*, a complex signal at δ 5.03–5.11, in part assigned to the α-D-GlcNAc residue. The reason for the complexity is probably that the oxidation was incomplete and, more importantly, that the hydrolysis of the acetal linkages in modified residues, in particular those from β-D-Glc*p*A [7], was also incomplete. Methylation analysis (Table 2), however, gave 2,3,4,6-tetra-*O*-Me-Man and 2,3,6-tri-*O*-Me-Man, thus demonstrating the relative order of the two mannose residues as in partial structure **3**.

→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→
3
↑
3

From the combined evidence it is proposed that the O-antigen polysaccharide from *Shigella boydii* type 5 is composed of hexasaccharide repeating units with the structure **4**. The repeating unit further contains an *O*-acetyl group, linked to one of the primary positions.

α-L-Rha*p*
1
↓
3
→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Gal*p*-(1→
4

3. Experimental

General methods.—Concentrations were performed under diminished pressure or by flushing with air. For GLC, a Hewlett–Packard 5830 A instrument, a flame-ionisation detector, and a DB-225 fused-silica capillary column were used. GLC–MS was performed on a Hewlett–Packard 5970 instrument.

NMR spectra of solutions in D_2O were recorded at 70°C (^{13}C) and 85°C (^1H) using a Jeol GSX-270 instrument. Chemical shifts are reported in ppm, using acetone (δ 31.00) for ^{13}C and sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00) for ^1H as internal references. The 2D experiments (H,H-COSY, C,H-COSY, and H,H-HOHAHA) were performed using standard pulse sequences available in the Jeol software.

Fractionations on Sephadex G-50 or Bio-Gel P-2, using aq 1% pyridinium acetate buffer (pH 6.5) as irrigant, were monitored with a differential refractometer. Oligosaccharides were purified by reversed-phase chromatography, performed on a Shimadzu LC6A HPLC system. The C18 (μ Bondapac) column used was irrigated with 7:93 MeCN–water.

Sugar and methylation analyses were performed as previously described [9,10].

SDS-PAGE was carried out with a Bio-Rad Mini Protein Electrophoresis Equipment and silver staining [11].

Isolation of the PS.—A culture of *Shigella boydii* serotype 5 was grown in trypticase soy broth supplemented with 0.5% yeast extract. A 35-L fermentor containing 20 L of medium was inoculated with 5 L of late logarithmic phase culture. The mixture was stirred, aerated (8 L/min), and kept at pH 7.2. The bacteria were killed in the late logarithmic or early stationary phase by addition of formaldehyde, and after 18 h at 4°C the cells (43 g) were harvested by centrifugation and freeze-drying.

LPS (2.2 g) was obtained from the cells (43 g) by extraction with phenol–water, followed by ultracentrifugation [3]. The LPS (200 mg) in aq 1% AcOH (20 mL) was kept for 90 min at 100°C, cooled, and centrifuged. The supernatant solution was extracted with diethyl ether and freeze-dried, and the product fractionated on a column (90 × 2.6 cm) of Sephadex G-50. The PS (80 mg) was eluted shortly after the void volume and recovered by freeze-drying. Part of the PS was *O*-deacetylated by treatment with aq 0.1 M NaOH at room temperature for 18 h.

Hydrolysis of the PS under mild conditions.—A solution of *O*-deacetylated PS (10 mg) in 0.5 M CF_3CO_2H (1 mL) was kept at 80°C for 10 h, then concentrated to dryness. The residue was dissolved in water (0.5 mL), $NaBD_4$ (10 mg) was added, and the solution kept at room temperature for 2 h. After conventional work-up, the product was fractionated on a column (90 × 1.6 cm) of Bio-Gel P-2. Two fractions, PS-1 (2 mg) and OS-1 (1 mg)

were collected and the latter was further purified on a reversed-phase C18 column.

Uronic acid degradation of the PS.—To a solution of the methylated PS (2 mg) in dimethyl sulfoxide (1 mL) was added *p*-toluenesulfonic acid (a trace) and 2,2-dimethoxypropane (0.1 mL) in order to eliminate any water present. Sodium methylsulfinylmethanide in dimethyl sulfoxide (2 M, 1 mL) was added, and the mixture was agitated in an ultrasonic bath for 30 min, then kept standing for 18 h. Trideuteriomethyl iodide (0.5 mL) was added with external cooling, and the mixture was agitated in the ultrasonic bath for 30 min. The excess of MeI was removed by flushing with N_2, and the solution was diluted with water and transferred to a Sep-Pak C18 cartridge. This was washed with water and the product eluted with MeCN.

Smith degradation of the PS.— A solution of *O*-deacetylated PS (5 mg) and sodium metaperiodate (17.5 mg) in 0.1 M acetate buffer (pH 3.9, 2 mL) was kept in the dark at 5°C for 48 h. Excess of periodate was reduced with ethylene glycol, the solution concentrated to dryness, and the polymer isolated by chromatography on a Bio-Gel P-2 column. This product was dissolved in water (1 mL), treated with $NaBH_4$ (10 mg), and recovered by chromatography on the Bio-Gel P-2 column. A solution of the polyalcohol in 0.5 M CF_3CO_2H was kept at room temperature for 154 h, diluted with water (3 mL), and freeze-dried. The product was reduced with $NaBH_4$ and an oligosaccharide fraction (OS-2, 2 mg) isolated by chromatography on the Bio-Gel P-2 column.

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