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Structure of the Polysaccharide O-Antigen of *Salmonella* Aberdeen (O : 11)

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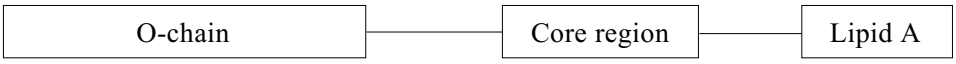
O-specific polysaccharide (OPS) of *Salmonella* Aberdeen was obtained from bacterial cell mass by water-phenol extraction procedure of lipopolysaccharide (LPS) followed by its mild acid hydrolysis and gel filtration of soluble carbohydrate material. Rhamnose, galactose, *N*-acetyl-glucosamine and mannose were detected and their linkages were established. Sugar configurations, D or L, were determined for (*S*)-(+)-2-butyl glycosides on an achiral capillary column. The structure of OPS was determined by analysis of spectra of ^{1}H and ^{13}C NMR and homonuclear and heteronuclear correlations spectra. Anomeric configurations were tentatively assigned by chromium trioxide oxidation and later proved by anomeric proton chemical shifts, H1-H2 coupling constants and proton coupled ^{13}C spectra. Sugar sequences were established from comparisons of specific carbon shifts with those from literature, two-dimensional nuclear Overhauser effect spectroscopy (NOESY) and heteronuclear multiple-bond correlation experiments (HMBC). The repeating unit of *S*. Aberdeen OPS has the structure:
→3)-β-D-Glc*p*NAc-(1→3)-[β-D-Man*p*-(1→4)-]α-D-Gal*p*-(1→4)-α-L-Rha*p*-(1→

Key words: *Salmonella* Aberdeen, O-antigen, repeating unit structure, NMR

The Kauffmann-White [1] classification system of the genus *Salmonella*, later modified by Popov and Le Minor [2], is based on cross reactions of specific antisera with O-somatic factors. These factors are borne by the O-specific polysaccharide chain of lipopolysaccharides (LPS) present in an external layer of outer membrane of the bacterial cell. LPS is a polymeric species with O-somatic chain linked to lipid A through core region. There is only one linkage in LPS, which is easily hydrolysed namely that between 3-deoxy-D-*manno*-octulosonic acid (Kdo) and lipid A. Diluted solution of acetic acid at 100°C splits it entirely.



It is a general believe that core region is rather a conservative part of LPS but not identical in all Gram-negative bacteria. In order to prepare the immune sera against O-specific antigens, rabbits are immunized with bacterial cells and interfering antifactors are removed from the sera by cross-absorption. In routine procedures *S.*

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Aberdeen is used for absorbing the anti-O:11 factor from the sera. As yet the chemical structure of the O-antigenic polysaccharide of *S.* Aberdeen has not been elucidated. In the Kauffmann-White system *S.* Aberdeen belongs to group F with the O:11 factor. The following sugar composition has been suggested for LPS O-chain and core unit: GlcN, Kdo, Hep, Gal, Glc, Man and Rha [3].

Several cross reactions of *S.* Aberdeen O-antigen with some bacteria and fungi have been established, namely with *Hanseniaspora osmophila* CBS 313, *Kloeckera javanica* CBS 282, *Kloeckera africana* CBS 277, *Candida tropicalis*, or *Escherichia coli* (O 75 and O 163) [4,5,6]. Cross-agglutination/absorption tests indicated that *C. tropicalis, E. coli* (O 75, O 163) and *S.* Aberdeen share the common antigenic factors. But no inhibition tests have been done in these studies [6].

As yet, no structure of representative antigen of *Salmonella* group F has been assigned. For this reason, we undertook a structural investigation of the *S.* Aberdeen O-somatic antigen using chemical and NMR techniques.

RESULTS AND DISCUSSION

Rhamnose, mannose, galactose and *N*-acetyl-glucosamine were identified in the hydrolysis product of O-polysaccharide in the respective molar ratios 1.08 : 1.01 : 1.00 : 0.80, but glucose and heptose, the sugars from core oligosaccharide, were found only at trace levels.

The sugar configurations, D or L, were established by comparison the chromatograms and the retention indice values of the acetyl derivatives of glycosides of (*S*)-(+)- and (±)-2-butanol on achiral liquid phase DB-23 with those of standard sugars. Good separations of the diastereoisomers were achieved in the temperature programme runs (Table 1). In this way, D configurations for mannose and galactose and L configuration for rhamnose were assigned. We have not succeeded in determination of *N*-acetyl-D-glucosamine configuration due to experimental problems. Nevertheless it is a general believe that this sugar has D configuration in bacterial products.

Table 1. GC retention indices[a] of diastereoisomeric glycosides of (*S*)-(+)- and (*R*)-(–)-2-butanol relatively to n-hydrocarbons (*e.g.* $C_{24} = 2400$).

	Furanoside				Pyranoside			
Sugar	α or β	α or β	α or β	α or β	α or β	α or β	α or β	α or β
	S-Agl[b]	*S*-Agl	*R*-Agl	*R*-Agl	*S*-Agl	*S*-Agl	*R*-Agl	*R*-Agl
L-rhamnose	2533	2579	2514	2540	2641	2702	2652	2703
D-mannose	3167	3174	3140	3171	3140	3269	3149	3256
D-galactose	3163	3192	3165	3220	3198	3370	3225	3387

[a] Retention indices (RI) in Kovats system were measured in temperature programme GC runs when even distances between hydrocarbon standards were obtained. These allowed us to calculate the RI by interpolation between two neighbour hydrocarbon standards assuming for them the values of RI as 100 folds of their numbers of carbon.

[b] *S*-Agl comes for aglycon of *S* configuration; L-sugar-*S*-Agl ≡ D-sugar-*R*-Agl in GC analysis.

Interresidual sugar linkages were deduced from the results of methylation analysis. The following products were identified as *O*-acetyl derivatives: 2,3-di-*O*-methyl-rhamnitol (0.7), 2,3,4,6-tetra-*O*-methyl-mannitol (1.0), 2,6-di-*O*-methyl-galactitol (1.1) and 2-(methylacetamide)-2-deoxy-4,6-di-*O*-methyl-glucitol (1.0), where the figures in parentheses correspond to GC peak intensities. Mannose is thus a terminal sugar, but rhamnose, galactose and *N*-acetyl glucosamine are substituted at carbon atoms 4, (4, 3) and 3, respectively.

The ^{1}H-NMR spectrum of *S.* Aberdeen O-antigen in part represents a set of easily assignable signals. Four anomeric resonances are present at 4.89, 5.06, 4.96 and 4.80 ppm, two of them being doublets with $^{3}J = 3.6$ Hz and $^{3}J = 8.4$ Hz. Two CH_3 signals appear at 1.32 and 2.06 ppm, representing the 6-deoxysugar and *N*-acetyl group, respectively. Some resonances in the ^{13}C NMR spectrum are also readily assignable. Those at 102.1, 100.8, 101.5 and 102.6 ppm are anomeric carbons, that of carbon carrying nitrogen is at 56.9 ppm, those of the two carbons from methyl groups are at 23.4 ppm (*N*-acetyl) and 18.1 ppm (deoxysugar), and that of the C=O group at 175.5 ppm. The results of sugar and methylation analysis and NMR spectroscopy, even without the full assignment of the resonances, show that the O-antigen of *S.* Aberdeen is composed of a repeating tetrasaccharide unit, in which all the sugars are pyranoid. All the ^{1}H and ^{13}C resonances of the oligosaccharide repeating unit were assigned using correlation techniques such as gCOSY, gDQCOSY, TOCSY and gHSQC. The anomeric ^{1}H and ^{13}C signals were used as entry windows for dividing the chemical shifts of proton and carbon resonances (Table 2) in the four sets of resonances. In turn, the resonance sets were assigned to the sugars in the following way. The previously assigned C-2 carbon atom of *N*-acetyl-glucosamine was used to identify the proton and carbon resonances of this sugar. Similarly, the proton resonance of mannose (H-2) was used in the same way to assign that sugar's signals, and the proton resonances of the CH_3 group in rhamnose identified the set of signals of this sugar. The final anomeric peaks at 5.06 ppm in the proton spectrum and at 100.8 ppm in the carbon spectrum were assigned to galactose.

Anomeric configuration of the specific sugar units was assigned from the chemical shift of the ^{1}H resonances, their coupling constants $J_{H1,H2}$, and heteronuclear coupling constants $J_{H1,C1}$. The anomeric configuration of two sugars, D-galactose and 2-deoxy-2-amino-D-glucose, can be easily assigned directly from the $J_{H1,H2}$ coupling constant as α (3.6 Hz) and β (8.4 Hz), respectively. The heteronuclear coupling constants $J_{H1,C1}$ of the other two sugars with the manno configuration, D-mannose and L-rhamnose, were found to be 165.7 Hz (β-D-mannose) and 171.6 Hz (α-L-rhamnose).

Sugar analysis of the chromium-trioxide-oxidised acetyl derivative of OPS indicated a significant decrease in mannose and the complete disappearance of *N*-acetyl-glucosamine, and confirmed the β configuration for both sugars in the O-polysaccharide.

Table 2. Chemical shifts of proton and ^{13}C resonances of the repeating unit of *Salmonella* Aberdeen OPS.

Sugar	^{1}H	δ	^{13}C	δ
→4)-α-L-Rha*p*-(1→	H-1	4.89	C-1	102.1
	H-2	3.81	C-2	72.1
	H-3	3.86	C-3	70.1
	H-4	3.51	C-4	81.7
	H-5	4.09	C-5	69.2
	H-6	1.32	C-6	18.1
→3,4)-α-D-Gal*p*-(1→	H-1	5.06	C-1	100.8
	H-2	3.99	C-2	69.4
	H-3	4.07	C-3	78.4
	H-4	4.39	C-4	77.2
	H-5	4.29	C-5	71.2
	H-6	3.71, 3.77	C-6	61.5
β-D-Man*p*-(1→	H-1	4.96	C-1	101.5
	H-2	4.04	C-2	71.6
	H-3	3.66	C-3	74.2
	H-4	3.56	C-4	68.3
	H-5	3.37	C-5	77.2
	H-6	3.73	C-6	62.2
→3)-β-D-Glc*p*NAc-(1→	H-1	4.80	C-1	102.6
	H-2	3.82	C-2	56.9
	H-3	3.64	C-3	82.8
	H-4	3.51	C-4	70.2
	H-5	3.51	C-5	77.0
	H-6	3.94, 3.78	C-6	62.4

Sugar sequences was established from comparisons of the specific carbon shifts with those from literature [13,14], heteronuclear multiple-bond correlation experiments (HMBC) and two-dimensional nuclear Overhauser effect spectroscopy (Table 3).

Table 3. Interglycosidic linkages for anomeric atoms deduced from NOESY and gHMBC spectra of *S.* Aberdeen O-antigen.

	Chemical shifts [ppm]			
Sugar residues	Anomeric proton atom	NOE to	gHMBC to	Connectivity to
→4)-α-L-Rha*p*-(1→	4.89	3.64	82.8	→3)-β-D-Glc*p*NAc-(1→
→3,4)-α-D-Gal*p*-(1→	5.06	3.51	–	→4)-α-L-Rha*p*-(1→
β-D-Man*p*-(1→	4.96	4.39	77.2	→4)-α-D-Gal*p*-(1→
→3)-β-D-Glc*p*NAc-(1→	4.80	4.07	78.4	→3)-α-D-Gal*p*-(1→

Thus the structure of the repeating unit of *S.* Aberdeen O-antigen is as below

$$\begin{array}{l} \qquad\qquad\qquad\quad \beta\text{-D-Man}p \\ \qquad\qquad\qquad\quad \downarrow{}^{1}_{4} \\ \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha-}(1\rightarrow \end{array}$$

The structure of tetrasaccharide unit is identical with that of *E. coli* O75 [15].

EXPERIMENTAL

Bacteria and their cultivation; isolation of the lipopolysaccharide. *Salmonella* Aberdeen O:11, KOS No 90 strain, was obtained from the National *Salmonella* Centre of Poland, KOS collection, Gdynia, Poland. The bacteria were cultivated on an enriched agar medium for 24 h at 37°C. The agar medium contained 0.4 g meat extract, 5.4 g casein hydrolysates, 1.7 g yeast extract, 3.5 g NaCl, 2.0 g glucose, and 20.0 g agar suspended in 1000 cm^3 distilled water. The isolated bacteria were washed from agar with 0.85% NaCl, killed with acetone and dried.

The lipopolysaccharide (LPS) was obtained by hot aqueous-phenol extraction of bacteria according to Westphal and Jann [7]. The dialysed solution was treated with ethanol (99.8%), first at pH 4.5 to obtain a 40% ethanol solution for the removal of nucleic acids, then at pH 7.0 to obtain an 80% ethanol solution for the precipitation of LPS. No ribose or deoxyribose was present in the preparations. The LPS was dissolved, dialysed against water, and freeze-dried.

Chemical methods, chromatography, mass spectrometry and NMR spectroscopy. The polysaccharide was prepared by mild acidic hydrolysis of LPS (1cm^3 1% acetic acid/10 mg LPS heated for 1 h in a boiling water bath). Following LPS degradation, the polysaccharide was isolated by gel filtration chromatography (GPC) on a Bio-Gel P-10 (100–200 mesh, BioRad Laboratories, Richmond, USA) column (100×0.9 cm) with water as eluent. The fractions were monitored with a differential refractometer detector (RIDK 101, Prague, Czech Republic). Two separate fractions containing the O-polysaccharide and the core oligosaccharide linked to a short repeating subunit were collected.

For sugar analysis, the polysaccharide was hydrolysed with 2 M trifluoroacetic acid at 120°C for 3 h. After reduction to alditol followed by acetylation, the monosaccharides were analysed by gas chromatography (GC) (CE 8000 TOP chromatograph). Acetyl derivatives of alditols and methylated alditols from methylation analysis were separated on bonded phase EC1 (30 m×0.25 mm) and DB-23 (60 m×0.30 mm) fused silica capillary columns. The standard temperature programmes were 4°C/min (EC1) and 2°C/min (DB-23) from 120°C to 260°C. Gas chromatography-mass spectrometry (GC-MS) was performed on a VG TRIO-3 instrument with an electron impact ionisation energy of 70 eV. The mass spectrometer was coupled directly to a HP 5890A gas chromatograph and controlled by a Digital Micro PDP11/53 computer.

The O-polysaccharide was methylated according to the method described by Hakomori using sodium methylsulfinylmethanide and iodomethane in DMSO [8]. The crude, methylated O-polysaccharide was dialysed (Spectrapor membrane tubing, MW cutoff 6000–8000) and freeze-dried. The permethylated polysaccharide was hydrolysed in aqueous 2 M trifluoroacetic acid at 120°C for 3 h. The products were reduced with $NaBH_4$ and acetylated. The partially methylated alditol acetates were analysed by GC and GC-MS and the spectra compared with a catalogue [9].

The sugar configurations, D or L, in the hydrolysed antigen mixtures of monosaccharides were established by capillary GC analysis of their acetylated (*S*)-(+)-2-butyl glycosides on a DB-23 column (60 m×0.3 mm) according to the method of Gerwig *et al.* [10] and Leontein *et al.* [11]. The GC peaks were identified by retention indices and coinjection of the acetyl derivatives of the reaction products of pure enantiomeric L-rhamnose, D-mannose and D-galactose with (±)-2-butanol and (*S*)-(+)-2-butanol (Aldrich). The identities of the GC peaks were confirmed by GC/MS analysis, which distinguished the five- and six-membered ring products.

Mannose and *N*-acetyl-glucosamine β-configurations were chemically assigned by oxidation of the peracetyl derivative of the polysaccharide with chromium trioxide in glacial acetic acid for 2 h with sonication at 50°C [12]. The reaction was terminated with 2-propanol. The oxidation product was diluted with water and extracted with chloroform. The chloroform solution was concentrated and the residue was deacetylated with methanol/sodium methanolate and sugar analysed.

All NMR spectra were recorded for the polysaccharide dissolved in deuterium oxide on a Varian Mercury 400 spectrometer. Proton spectra were recorded at 333 K for a solution of 4.6 mg/0.7 cm^3. Chemical shifts were expressed relative to internal acetone (2.225 ppm). Proton decoupled ^{13}C spectra, 100 MHz, were recorded at 333 K for solutions of 20 mg/0.7 cm^3 with acetone (31.41 ppm) as an internal standard.

Proton homonuclear-correlated two-dimensional NMR experiments (gCOSY, gDQCOSY, TOCSY, NOESY), the heteronuclear coupled and decoupled correlation spectra (gHSQC) and the heteronuclear multiple-bond correlation spectra (gHMBC) were obtained for 32 scans. The TOCSY spectrum was acquired with an MLEV-16 sequence with a mixing time of 110 ms. The NOESY spectrum was recorded with a mixing time of 300 ms.

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