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STRUCTURE OF THE LIPOPOLYSACCHARIDE ANTIGENIC O-CHAIN PRODUCED BY *Salmonella ohio* (O:6,7)

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ABSTRACT

Salmonella ohio, which belong to Group C_1 (0:6,7) of the Kauffmann–White classification system, produces a smooth lipopolysaccharide which by glycose analysis, methylation, deamination, and ^{1}H-n.m.r. studies was shown to have an O-polysaccharide chain composed of a repeating hexasaccharide unit having the structure {→2)-[α-D-Glc*p*-(1→3)]-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Man*p*-(1→}$_n$.

INTRODUCTION

The characterization and classification of *Salmonella* serotypes by the Kauffmann–White scheme[1] is based, in part, on the serological identification of the antigenic factors located in the O-chain polysaccharide portion of their lipopolysaccharide (LPS) components. The knowledge of the fine structures of the LPS O-chains is an essential element in the understanding of the molecular basis of the serospecific factors, and the application of the newer methods of polysaccharide analysis are providing structural information on lipopolysaccharides which is leading to advances in the elucidation of their immunobiological functions.

This paper describes the analysis of the LPS O-polysaccharide structure of *S. ohio* which belongs to group C_1 of the Kauffmann–White scheme, which is characterized by the O-antigenic factors 6, 7 and *14*, the latter factor being associated[2] with bacterial strains lysogenized by phage 14. The O-polysaccharide expressing factors 6 and 7 was found to be a polymer of a repeating hexasaccharide unit, and the structural analytical data provided background information for the identification of the epitopic features of the factors 6 and 7.

RESULTS AND DISCUSSION

Lipopolysaccharide production and hydrolysis. — Saline-washed and enzyme-digested[3] cells of *Salmonella ohio* were extracted by the hot phenol–water

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method[4]. The dialyzed and concentrated aqueous phase, on ultracentrifugation gave essentially pure LPS (1.0% yield, based on wet cells). Sodium dodecyl sulfate (SDS)–PAGE analysis of the LPS gave a typical S-type LPS banding ladder pattern in which the spacing was indicative of LPS composed of an O-chain with a large repeating oligosaccharide unit[5], and in addition showed bands corresponding to R-LPS, and S-R LPS[6,7] composed of O-chains having one, two, three, and four repeating units.

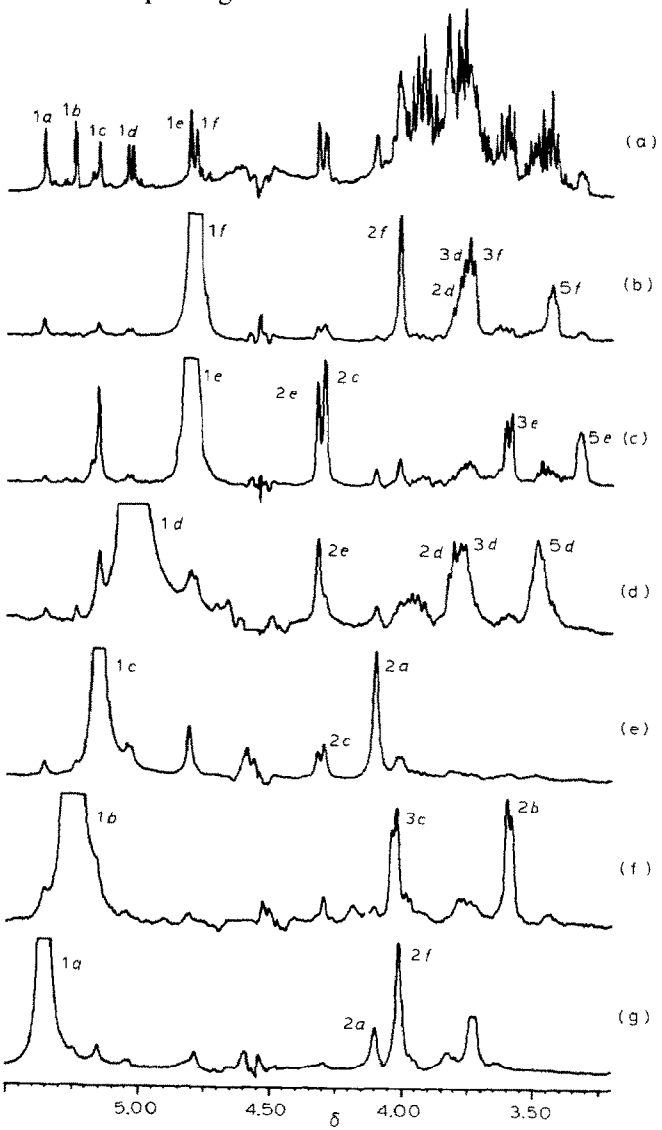


Fig. 1. (a) ^{1}H-N.m.r. spectrum of the O-chain of *S. ohio* LPS. N.O.e. difference spectra for the LPS O-chain of *S. ohio* obtained on saturation of the resonances: (b) H-1*f*, (c) H-1*e*, (d) H-1*d*, (e) H-1*c*, (f) H-1*b*, and (g) H-1*a*.

Partial hydrolysis of the LPS with hot, dilute acetic acid gave an insoluble lipid A (30%), and Sephadex G-50 gel permeation of the concentrated water-soluble products gave an O-chain polysaccharide fraction (14%) eluted at the void volume of the column, a pre-core fraction (K_{av} 0.43, 12%), a core fraction (K_{av} 0.70, 17%), and a fraction (K_{av} 1.00, 15%) containing 3-deoxy-D-*manno*-octulosonic acid and phosphate. These results were consistent with the observed SDS–PAGE analysis.

Polysaccharide O-chain. — The O-chain fraction had $[\alpha]_D^{20}$ +45° (*c* 1.0, water). *Anal.* Found: C, 38.10; H, 5.49; N, 1.13; and ash 0.0%. On quantitative analysis, the O-polysaccharide was found to be composed of D-mannose (60.3%), D-glucose (20.5%), and 2-acetamido-2-deoxy-D-glucose (19.2%). The configuration and identification of the glycoses were established by their isolation by paper chromatography, followed by g.l.c. of their acetylated (−)-2-butyl glycoside derivatives[8].

The ^{1}H-n.m.r. spectrum of the O-chain (500 MHz, 47°) (Fig. 1a) showed, among others, (a) a signal for a *N*-acetyl group at δ 2.04 (s, 3 H), (b) six signals in the anomeric region at δ 5.36 (unresolved, 1 H), 5.25 (d, 1 H, $J_{1,2}$ 3.8 Hz), 5.16 (unresolved, 1 H), 5.04 (d, 1 H, $J_{1,2}$ 7.8 Hz), 4.81 (unresolved, 1 H), and 4.79 (unresolved, 1 H). The methylated and hydrolyzed O-chain afforded, on hydrolysis, 2,3,4,6-tetra-*O*-methylglucose, 3,4,6-tri-*O*-methylmannose, 4,6-di-*O*-methylmannose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylamino)glucose (1.0:2.7:1.0:0.6) (Table I), identified by g.l.c.–m.s. analysis.

The aforementioned results indicated that the polysaccharide consists of a repeating hexasaccharide unit in which the D-glucopyranosyl residue is a non-reducing end group, three of the four D-mannopyranosyl residues are glycosidically substituted at O-2, the 2-acetamido-2-deoxy-D-glucopyranosyl residue is substituted at O-3, and the remaining D-mannopyranosyl residue forms a branch point with glycosyl substituents at O-2 and O-3.

N.m.r. analysis. — The structure of the O-chain could be resolved by n.m.r. experiments which established the glycose sequence, knowing only the chemical shifts of the proton signals corresponding to H-1, H-2, and H-3 of each glycose

TABLE I

METHYLATION ANALYSIS OF THE O-POLYSACCHARIDE FROM *S. ohio*

Partially methylated alditol acetates	T_{GM}[a]	*Percentage composition*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	18.8
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol	1.29	50.7
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methylmannitol	1.71	18.9
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol	3.69	11.6

[a] T_{GM} value relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

TABLE II

^{1}H-CHEMICAL SHIFTS[a] OF THE O-POLYSACCHARIDE FROM *S. ohio*

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a,b*
a	5.36	4.10	4.02	3.80	[b]	[b]
b	5.25	3.59	3.79	3.45	[b]	[b]
c	5.16	4.30	4.03	3.97	[b]	[b]
d	5.04	3.82	3.77	3.53	3.48	[b]
e	4.81	4.33	3.60	3.48	3.32	[b]
f	4.79	4.02	3.74	3.64	3.44	[b]

[a]δ values measured at 47° relative to the signal of internal acetone (δ 2.225). [b]Not determined.

residue. Each anomeric proton resonance was assigned the arbitrary designation H-1*a*, H-1*b*, H-1*c*, H-1*d*, H-1*e*, and H-1*f* in order of decreasing chemical shifts. By following the cross-peaks in a Cosy spectrum[9] of the O-chain, resonances for the glycose H-2 protons were easily detected, and the relay Cosy spectrum[10] similarly revealed H-3 and H-4 resonances. Table II lists the ^{1}H-chemical shifts for almost all the protons of the glycose residues in the O-chain. One-dimensional n.O.e. experiments[11] (Fig. 1b–g) led to the following linkage information: (a) *a* is linked α-(1→2) to *f*, (b) *b* is linked α-(1→3) to *c*, (c) *c* is linked α-(1→2) to *a*, (d) *d* is linked β-(1→2) to *e*, (e) *e* is linked β-(1→2) to *c*, and (f) *f* is linked β-(1→3) to *d*.

Residue *b* was identified from n.O.e., anomeric proton-coupling constant, and methylation evidence as an α-D-glucopyranosyl residue. Residue *d* from similar evidence was identified as the 2-acetamido-2-deoxy-β-D-glucopyranosyl unit. Of the four remaining D-mannopyranosyl units, residues *e* and *f* which are, from n.O.e. evidence, substituted through O-2 could be identified as β-D-mannopyranosyl residues since they showed intraring n.O.e.'s between H-1, H-3, and H-5, and the remaining residue *c*, which forms the branch point, and residue *a*,

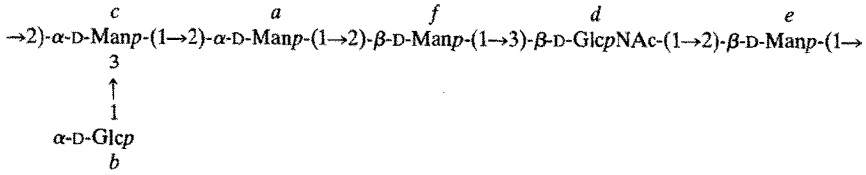
TABLE III

METHYLATION ANALYSIS OF THE OLIGOSACCHARIDE *A* FROM DEAMINATION OF THE O-POLYSACCHARIDE

Partially methylated acetates	*Anomer*	T_{GMA}	*Percentage composition*
3-*O*-Acetyl-2,5-anhydro-1,4,6-tri-*O*-methylmannitol		0.79	9.7
1-*O*-Acetyl-2,3,4,6-tetra-*O*-methylglucose	α	1.00	6.2
	β	1.03	9.4
1-*O*-Acetyl-2,3,4,6-tetra-*O*-methylmannose	α	1.15	15.5
	β		
1,2-Di-*O*-acetyl-3,4,6-tri-*O*-methylmannose	α	1.42	26.3
	β	1.48	13.2
1,2,3-Tri-*O*-acetyl-4,6-di-*O*-methylmannose	α	1.80	12.1
	β	1.88	7.6

[a]T_{GMA} value relative to that of 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α-D-glucose.

which is glycosidically substituted at O-2, could be identified as α-D-mannopyranosyl units. The accumulated evidence led to structure **1** for the repeating unit of the LPS O-chain. The proposed structure was confirmed by analysis of the oligosaccharide obtained after *N*-deacetylation and deamination of the O-chain.



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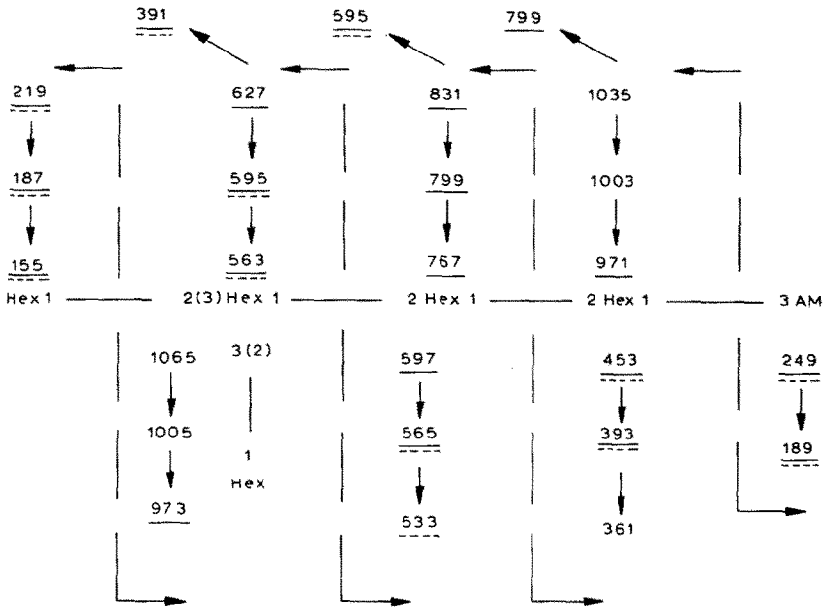
Deamination of the O-chain. — Nitrous acid deamination[12] of *N*-deacetylated O-chain (130 mg), followed by reduction ($NaBH_4$) and Bio-Gel P-2 gel filtration of the concentrated products gave an oligosaccharide *A* (2.0 mg, K_{av} 0.53) having $[\alpha]_D^{20}$ +52.0° (*c* 0.2, water), and composed of 2,5-anhydromannitol, D-mannose, and D-glucose (0.9:3.4:1.0) as determined by g.l.c. Methylation analysis of *A* (Table III) gave 2,3,4,6-tetra-*O*-methylglucose, 2,3,4,6-tetra-*O*-methylmannose, 3,4,6-tri-*O*-methylmannose, 4,6-di-*O*-methylmannose, and 2,5-anhydro-1,4,6-tri-*O*-methylmannitol, consistent with the proposed structure of the O-chain. Partial ^{1}H-n.m.r. (500 MHz, 47°) showed five anomeric protons at δ 5.39 (unresolved, 1 H), 5.27 (d, 1 H, $J_{1,2}$ 3.8 Hz), 5.18 (unresolved, 1 H), 4.78 (unresolved, 1 H), and 4.77 (unresolved, 1 H), and as expected, a proton signal corresponding to a



Scheme 1. Expected m.s. ion-fragmentation product of methylated reduced oligosaccharide *A* showing significant detected ions in c.i. mode (——) and in e.i. mode (----).

2-acetamido-2-deoxy-β-D-glucopyranosyl residue was absent. On direct-insertion m.s., methylated oligosaccharide *A* gave the fragmentation ions shown in Scheme 1 which are consistent with the patterns expected for the determined structure of the methylated and reduced deamination product of the O-chain polysaccharide.

The n.m.r. analysis and chemical evidence led to the unequivocal characterization of the LPS-O-polysaccharide as a polymer of a repeating hexasaccharide unit having structure **1**.

EXPERIMENTAL

Production of lipopolysaccharide. — Cultures of *S. ohio* (LCDC 79742, NRCC 4259) supplied by Dr. H. Lior, LCDC Health & Welfare Canada, Ottawa, were grown, in a fermenter (28 L, Microfirm, New Brunswick, Scientific), in a medium of 3.7% (w/v) brain heart infusion (Difco) at 37°, 200 r.p.m., and aeration at 25 L/min for 18 h. The collected cells (206 g) were washed with 2% (w/v) saline solution, digested with lysozyme, ribonuclease, and deoxyribonuclease[3], and subsequently extracted by the hot aqueous phenol method[4]. LPS was recovered from the dialyzed water layer by repeated ultracentrifugation at 105 000*g* (12 h at 4°) until judged pure by the carbocyanine dye assay[13].

Polysaccharide O-chain. — LPS (0.8 g) in 2% (v/v) acetic acid (100 mL) was heated for 2 h at 100°, and the precipitated lipid A removed by low-speed centrifugation. The lyophilized centrifugate was fractionated on a column of Sephadex G-50 with pyridinium acetate (0.05M, pH 4.7) as the eluent, and 10-mL fractions were monitored for neutral glycose, aminodeoxyglycose, 3-deoxyoctulosonate, and phosphate.

Nuclear magnetic resonance. — All ^{1}H-n.m.r. experiments were carried out with a Bruker AM-500 spectrometer at 47°. All samples were exchanged twice with 99.8% D_2O and the experiments performed on solutions in 0.5 mL of 99.99% D_2O. The internal reference was the methyl resonance of acetone set at δ 2.225. All n.m.r. experiments were acquired and processed with the standard software provided by Bruker (DISB87). The nuclear Overhauser enhancements (n.O.e.)[11] were measured by difference spectroscopy with 200-ms irradiation time. For the proton homonuclear shift correlation (Cosy)[9] and the two-step relay Cosy[19], 256 experiments of 1024 data points over a sweep width of 1200 Hz were acquired with 32 transients per experiment. For the relay Cosy, a delay of 32 ms was used for the two-step relay. The data were processed by use of unshifted sine-bell functions, zero-filling, a magnitude calculation, and symmetrization about the diagonal to give a final resolution of 1.2 Hz per point in both domains.

Analytical methods. — The quantitative methods used were: (a) the phenol–H_2SO_4 method for neutral glycose[14], (b) the modified Elson–Morgan method for aminodeoxyglycoses[15], (c) the periodate oxidation–thiobarbituric acid method for deoxyoctulosonate[16], and (d) the method of Chen *et al.*[17] for phosphate. Paper chromatography was done on Whatman No. 1 filter paper with: (a) 2:5:5 (v/v) (top

layer) pyridine–ethyl acetate–water, and (b) 4:1:5 (v/v) (top layer) butanol–ethanol–water as the mobile phases, and glycoses were detected with the periodate–alkaline $AgNO_3$ spray reagents[18].

Gel filtration was done on columns of Sephadex G-50 (2.5 × 80 cm), Sephadex G-15 (Pharmacia Fine Chemicals) (2.0 × 40 cm), and Bio-Gel P-2 (Bio-Rad Laboratories) (1.5 × 95 cm) at 20° with 0.05M pyridinium acetate buffer (pH 4.7) as the eluent. The gel-filtration properties of the eluted materials are expressed in terms of their distribution coefficient K_{av}. $K_{av} = (V_e - V_o)/(V_t - V_o)$, 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.

Glycoses were determined by g.l.c. of their derived alditol acetate[19] with *myo*-inositol as an internal standard. Oligo- and poly-saccharide samples (0.5–1.0 mg) were hydrolyzed with 10M HCl (1 mL) for 20 min at 85–90°. The hydrolyzates were evaporated to dryness, the residues taken into water (2 mL), and the solutions reduced with $NaBH_4$ (10 mg). The resulting alditols were acetylated prior to g.l.c. analysis.

The configuration of glycoses was established by capillary g.l.c. of their acetylated (−)-2-butyl glycosides according to the method of Leontein *et al.*[8].

G.l.c. was done with a Hewlett–Packard model 5830A gas chromatograph fitted with a hydrogen-flame detector. The following conditions were used with a capillary column (0.32 mm × 25 m), 007 series bonded-phase, fused silica OV-17 (Quadrex Corp.): (a) alditol acetates, start at 180°, 4°/min up to 240°; (b) methylated alditol acetates, start at 200°, 1°/min up to 240°; and (c) methylated glycose acetates, start at 150°, 4°/min up to 240°. Development was done with dry N_2 at 30–40 mL/min and retention times are quoted relative to D-glucitol hexaacetate (T_{GA}), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}), and 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose (T_{GMA}).

G.l.c.–m.s. was done with a Hewlett-Packard 5985B GLC-MS system employing the program conditions described above and an ionization potential of 70 eV. E.i.–m.s. and c.i.–m.s. of permethylated oligosaccharide was done by direct insertion into the source and the probe was heated ballistically from 40 to 350°.

Sodium dodecyl sulfate–PAGE. — LPS samples (1.0 μg) were analyzed in 14% poly(acrylamide) gels by electrophoresis in the presence of SDS. Bands were detected by use of the Ag-staining directions of Tsai and Frasch[20].

Methylation analysis. — Samples (1.0–2.0 mg) were methylated with sodium methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide according to the Hakomori procedure[21], and the products were purified by use of C18 Sep-Pak cartridges (Waters Associated). The methylated products were hydrolyzed with 10M HCl (1 mL) for 20–30 min at 85–90°. After evaporation, hydrolyzates were reduced with $NaBH_4$, followed by acetylation, or were directly peracetylated and examined by g.l.c.–m.s.

N-*Deacetylation and deamination of the O-polysaccharide*[12]. — O-Chain polysaccharide (30 mg) was dissolved in water (1.0 mL), and thiophenol (100 μL),

NaOH (400 mg) and dimethyl sulfoxide (5 mL) were added. After flushing with N_2, the sealed vial was heated for 16 h at 110°. The solution was poured into ice–water (15 mL), the base neutralized with 2M HCl, and the solution dialyzed against tap water. The dialyzate was concentrated to dryness, the residue dissolved in 33% acetic acid (15 mL), and 5% $NaNO_2$ solution (6 mL) was added. After 1 h at room temperature, the mixture was de-ionized with Rexyn 101 (H^+) ion-exchange resin, evaporated to dryness, and the residue reduced with aqueous $NaBH_4$. The reduced oligosaccharide product (*A*) was collected from the major fraction obtained on Bio-Gel P-2 column filtration.

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