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STRUCTURE OF THE MAJOR LIPOPOLYSACCHARIDE ANTIGENIC O-CHAIN PRODUCED BY *Salmonella carrau* (O:6, 14, 24)*†

JOSE L. DI FABIO, JEAN-ROBERT BRISSON, AND MALCOLM B. PERRY
Division of Biological Sciences, National Research Council, Ottawa, Ontario K1A 0RG (Canada)
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ABSTRACT

Salmonella carrau, which belongs to group H (O:6, 14, 24) of the Kauffmann–White classification system, produced a major (90%) smooth lipopolysaccharide which by SDS–PAGE, glycose analysis, methylation, periodate oxidation, deamination, and ^{1}H- and ^{13}C-n.m.r. studies was shown to have an O-polysaccharide moiety composed of a repeating pentasaccharide having the structure:

$$[\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow]_n$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-D-Glc}p \end{array}$$

INTRODUCTION

The Kauffmann–White classification of *Salmonella* serotypes[1] is based on serological reactions related to the structures of the O-polysaccharide moieties of the bacterial cell-wall lipopolysaccharides. A first step in the identification of the antigenic determinants is the elucidation of the chemical structures of the O-polysaccharides[2], which provides the initial information required to relate structures to serological specificities.

The aim of the present studies is to define the structural specificities related to the *Salmonella* O:1, 6, 14, 24, and 25 serological factors, and we now record the analysis of the LPS O-polysaccharide of *S. carrau* which expresses the antigenic factors O:6, 14, and 24. The O-polysaccharide was found to comprise a repeating pentasaccharide unit composed of D-mannose, D-glucose, and 2-acetamido-2-deoxy-D-glucose (3:1:1).

*Dedicated to Professor Bengt Lindberg.
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RESULTS AND DISCUSSION

Lipopolysaccharide production and hydrolysis. — Saline-washed and enzyme-digested[3] cells of *Salmonella carrau* were extracted by the hot phenol–water method[4], and the dialyzed and concentrated aqueous phase, on ultracentrifugation, gave essentially pure LPS (2.0% yield based on wet cells). SDS–PAGE of the LPS gave a typical S-type LPS banding ladder pattern in which the spacing was indicative of an LPS composed of repeating pentasaccharide units[5]. Partial hydrolysis of the LPS with hot dilute acetic acid gave an insoluble lipid A (8%), whereas gel filtration of the concentrated water-soluble products through Sephadex G-50 gave an O-chain polysaccharide fraction (39%) eluted at the void volume of the column, a core oligosaccharide (K_{av} 0.58, 8%), and a fraction (K_{av} 1.00, 29%) containing 3-deoxy-D-*manno*-octulosonic acid and phosphate.

Polysaccharide O-chain. — The O-chain fraction had $[\alpha]_D$ +109° (*c* 2.4, water) (*Anal.* Found: C, 37.29; H, 5.58; N, 1.37; ash. 1.4%) and was composed of D-mannose (60.3%), D-glucose (22.2%), and 2-acetamido-2-deoxy-D-glucose (17.5%). The configuration and identification of the glycoses were established by their isolation and characterization.

The ^{1}H-n.m.r. spectrum (500 MHz, 47°) of the O-chain (Fig. 1a) contained essentially five signals of equal intensity for anomeric protons at 5.345 (unresolved, 1 H), 5.253 (d, 1 H, $J_{1,2}$ 2.9 Hz), 5.157 (unresolved, 1 H), 4.896 (d, 1 H, $J_{1,2}$ 3.3 Hz), and 4.806 p.p.m. (unresolved, 1 H). Four minor (~10%) signals for anomeric protons were also present at 5.302 (unresolved), 5.040 (unresolved), 4.871 (d, $J_{1,2}$ 3.0 Hz), and 4.756 p.p.m. (unresolved), as was a major singlet at 2.03 p.p.m. (3 H) for NAc. The ^{13}C-n.m.r. spectrum (125 MHz, 27°) (Fig. 2a) showed five major signals for anomeric carbons at 102.7, 101.3, 100.6, 99.1, and 97.6 p.p.m., together with four minor signals at 103.4, 100.8, 100.3, and 97.9 p.p.m. Among other ^{13}C signals, one at 53.6 p.p.m. corresponded to C-2 of the 2-acetamido-2-deoxy-D-glucosyl residue and those at 22.9 ($NCOCH_3$) and 174.5 p.p.m. ($NCOCH_3$) to the NAc groups. These results indicated that the O-polysaccharide fraction might contain two distinct polymers, the major (90%) comprising pentasaccharide units, and the minor (10%) composed of tetrasaccharide units.

Methylation analysis of the O-chain fraction gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4,6-tri-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-mannose, 4,6-di-*O*-methyl-D-mannose, and 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose (1.0:1.4:0.9:1.1:0.4), identified by g.l.c.–m.s. of their alditol acetate derivatives (Table I). These results are consistent with the proposed pentasaccharide repeating-unit in which a D-glucopyranosyl group is a non-reducing end-group, two of the D-mannopyranosyl residues are 2- and 6-linked, the 2-acetamido-2-deoxy-D-glucopyranosyl residue is 3-linked, and the remaining D-mannopyranosyl residue forms a branch point with glycosyl substituents at positions 2 and 3.

Periodate oxidation of the O-chain. — Periodate oxidation of the O-chain fraction (105 mg) resulted, as expected, in the oxidation of two of the three D-

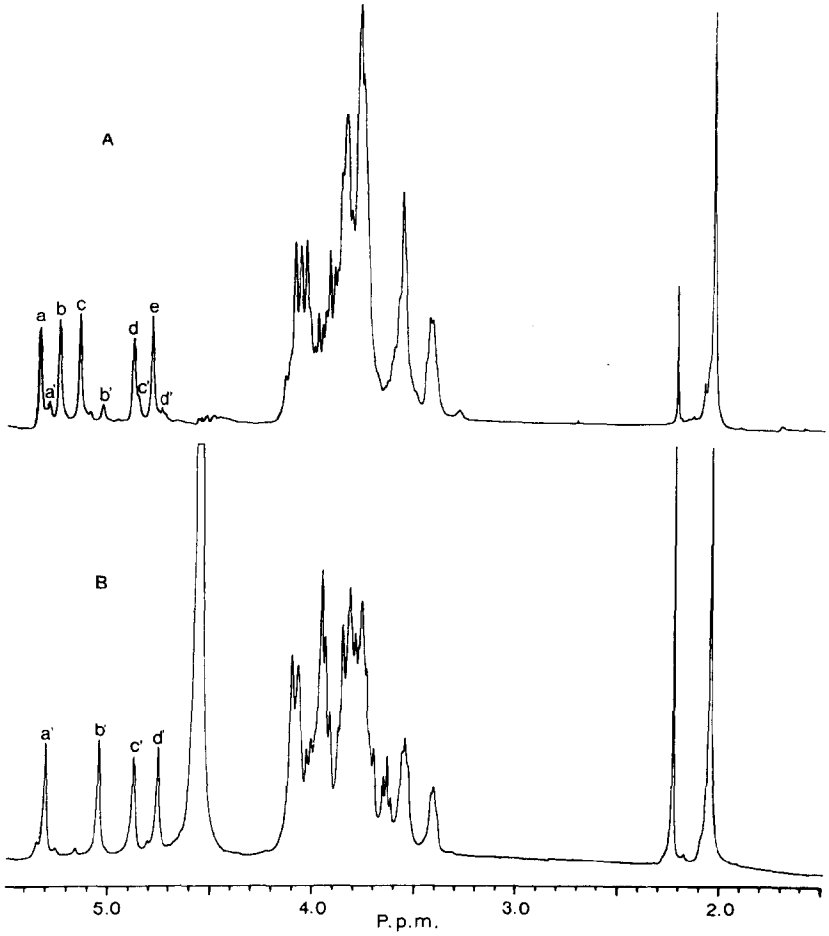


Fig. 1. Resolution-enhanced ^{1}H-n.m.r. spectra of (A) the LPS O-chain of *S. carrau* and (B) the LPS O-chain of *S. boecker*.

mannopyranosyl residues as well as the terminal D-glucopyranosyl units. Smith-type hydrolysis[6] of the reduced ($NaBH_4$) periodate-oxidized O-chain and gel filtration of the product through Bio-Gel P2 afforded a single oligosaccharide *A* (32 mg, K_{av} 0.71), $[\alpha]_D$ +41.5° (*c* 3.2, water), which was composed of glycerol, D-mannose, and 2-acetamido-2-deoxy-D-glucose (1:1:1). G.l.c.–m.s. of methylated *A* gave a major peak (*T* 12.3 min) for which the fragmentation ions were at *m/z*: 624, 612, 584, 552, 464, 438, 409, 394, 366, 348, 276, 246, 228, 219, 187, 157, 127, and 103. This pattern is consistent with the sequence shown in Fig. 3. Primary ions of the A series, characteristic of a terminal non-reducing hexose, were obtained at *m/z* 219 (aA_1) and 187 (aA_2), and, from the alditol end, fragments with *m/z* 103 (ald), 348 (bald), and 408 ($baldJ_1$) corresponding to a terminal glycerol residue substituted by a 2-acetamido-2-deoxy-D-glucopyranosyl derivative. The molecular ion ($M^+ + 1$)

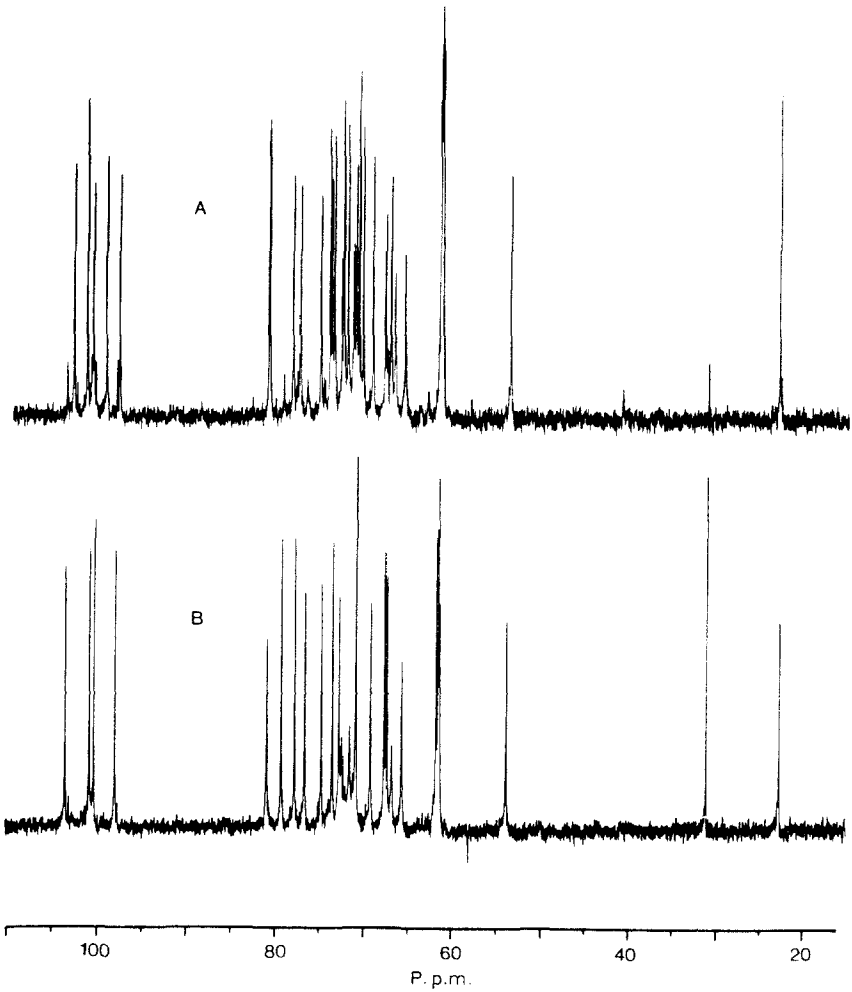


Fig. 2. ^{13}C-N.m.r. spectra of (A) the LPS O-chain of *S. carrau* and (B) the LPS O-chain of *S. boecker*.

at *m/z* 584 and fragments with *m/z* 612 (M + $C_2H_5^+$) and 624 (M + $C_3H_5^+$) confirmed the proposed structure. Methylation analysis of *A* (Table I) gave 2,3,4,6-tetra-*O*-methyl-D-mannose and 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose (1:1).

The configurations of the linkages in *A* were established from n.m.r. data. The ^{13}C-n.m.r. spectrum (50 MHz, 27°) contained signals for anomeric carbons at 101.4 ($J_{C,H}$ 160 Hz) and 98.0 p.p.m. ($J_{C,H}$ 173 Hz) (1:1), which the $J_{C,H}$ values indicated to be involved in β and α linkages, respectively. Consistent with this finding, the ^{1}H resonances (500 MHz, 57°) at 4.866 (d, 1 H, $J_{1,2}$ 3.5 Hz) and 2.047 p.p.m. (s, 3 H) can be assigned to H-1 and NAc of the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue, and the signal at 4.701 p.p.m., by inference, to H-1 of a β-D-mannopyranosyl residue.

TABLE I

METHYLATION ANALYSIS OF *S. carrau* LPS O-CHAIN FRACTION AND OF OLIGOSACCHARIDE *A*

Alditol acetate derivatives	T_{GM}[a]	*I*[b]	*II*[c]
		(percentage composition)	
3-*O*-Acetyl-2,5-anhydro-1,4,6-tri-*O*-methylmannitol	0.56		
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	1.00		60.5
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	19.8	
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol	1.29	30.4	
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol	1.41	17.7	
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methylmannitol	1.71	21.7	
1,3,6-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol	3.69	10.4	39.5

[a]Relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [b]Methylated O-chain fraction. [c]Methylated oligosaccharide *A*.

A ^{13}C DEPT[7] spectrum of *A*, considered in conjunction with the results of methylation analysis, showed that the glycerol moiety must be linked through O-1 since three hydroxymethyl signals at 63.3, 61.8, and 61.3 p.p.m. were due to C-6 of the D-mannose and 2-acetamido-2-deoxy-D-glucose residues and to the unsubstituted C-3 of the glycerol moiety. A fourth hydroxymethyl signal at 69.3 p.p.m. corresponded to the substituted C-1 of the glycerol moiety.

Thus, *A* was identified as β-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→1)-glycerol. Since the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue is 1-linked to glycerol, it can be inferred that it is 6-linked in the native polysaccharide to a D-mannopyranosyl residue, thus leading to a partial definition of the pentasaccharide repeating-unit in the O-chain structure as:

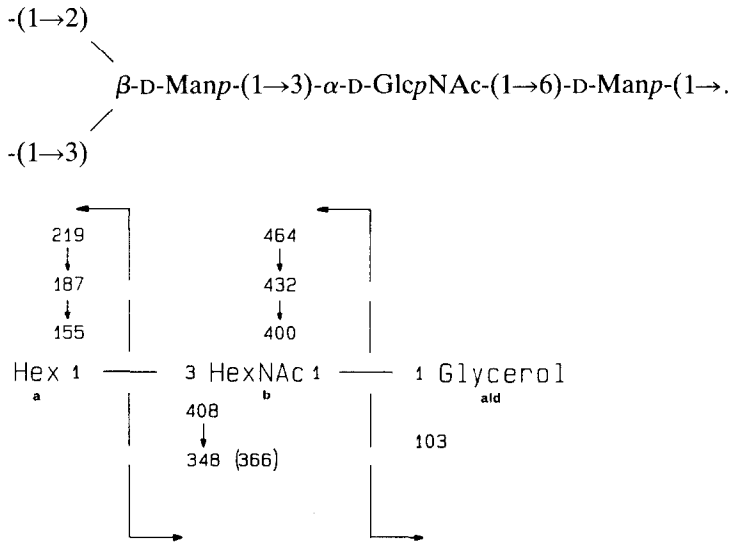


Fig. 3. Mass-spectral fragmentation of methylated oligosaccharide *A*.

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDE *B*

Derivative of sugar residue		T_{GMA}[a]	*Percentage composition*
3-*O*-Acetyl-2,5-anhydro-1,4,6-tri-*O*-methylmannitol		0.79	17.3
1-*O*-Acetyl-2,3,4,6-tetra-*O*-methylglucose	α	1.00	9.1
	β	1.03	11.2
1-*O*-Acetyl-2,3,4,6-tetra-*O*-methylmannose	α	1.15	22.0
	β	—	—
1,2-Di-*O*-acetyl-3,4,6-tri-*O*-methylmannose	α	1.42	15.3
	β	1.48	6.9
1,2,3-Tri-*O*-acetyl-4,6-di-*O*-methylmannose	α	1.80	11.2
	β	1.88	7.0

[a]Retention time relative to that of 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α-D-glucose.

Deamination of the O-*chain.* — Deamination[8] of *N*-deacetylated O-chain (100 mg) with nitrous acid followed by reduction ($NaBH_4$) and gel filtration of the concentrated products on Bio-Gel P2 gave an oligosaccharide *B* (18 mg, K_{av} 0.63), $[\alpha]_D$ +74° (*c* 1.8, water), composed of 2,5-anhydromannitol, D-mannose, and D-glucose (1.2:3.1:1.0) as determined by g.l.c. Methylation analysis of *B* (Table II) gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4,6-tetra-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, 4,6-di-*O*-methyl-D-mannose, and 2,5-anhydro-1,4,6-tri-*O*-methylmannitol consistent with the linkages deduced from the methylation analysis of the original O-chain, and with the characterization of oligosaccharide *A*.

G.l.c.–m.s. of methylated *B* gave a major peak (*T* 18.9 min) that had the following fragmentation pattern: *m/z* 861, 769, 657, 519, 437, 423, 407, 391, 355,

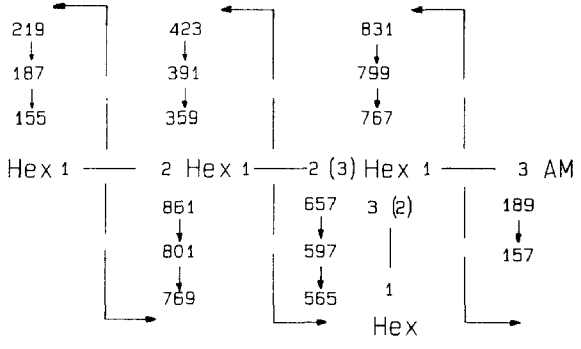


Fig. 4. Mass-spectral fragmentation of oligosaccharide *B*.

TABLE III

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

Residue[b]	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
a	5.345	4.075	4.040	3.785	3.885	n.d.[c]	n.d.
b	5.253	3.583	3.562	3.440	3.770	n.d.	n.d.
c	5.157	4.112	3.890	3.992	3.855	4.140	3.610
d	4.896	4.098	3.940	3.585	3.780	n.d.	n.d.
e	4.806	4.052	3.835	3.865	3.465	n.d.	n.d.

[a]Chemical shifts determined at 47° relative to internal acetone (2.225 p.p.m.). [b]See formula **1**. [c]Not determined.

281, 219, 189, 187, 157, 155, 88, and 45, which conformed to the structure given in Fig. 4, and allows two possible basic structures to be proposed for the O-chain:

$$[\rightarrow 2(3)\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-D-Glc}p\text{NAc-}(1\rightarrow 6)\text{-D-Man}p\text{-}(1\rightarrow]_n$$

3(2)
↑
1
D-Man*p*
2
↑
1
D-Glc*p*

or

$$[\rightarrow 2)\text{-D-Man}p\text{-}(1\rightarrow 2(3))\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-D-Glc}p\text{NAc-}(1\rightarrow 6)\text{-D-Man}p\text{-}(1\rightarrow]_n$$

3(2)
↑
1
D-Glc*p*

The exact structure of the O-chain was established on the basis of n.m.r. data. An almost complete assignment of the ^{1}H-n.m.r. spectrum of the O-chain was made by COSY[9] and relay COSY[10] 2-D experiments (Table III). Each anomeric proton resonance was assigned the arbitrary designation H-1a, H-1b, H-1c, H-1d, and H-1e in order of decreasing chemical shifts. By following the cross-peaks, the resonances for H-2 were detected easily, relay COSY spectra revealed the H-3 and H-4 resonances, and the H-5 signals were identified from the cross-peaks to the determined H-4 signals. Heteronuclear ^{1}H/^{13}C shift correlations[11]

TABLE IV

^{13}C-CHEMICAL SHIFTS OF THE O-POLYSACCHARIDE FROM *S. carrau*[a]

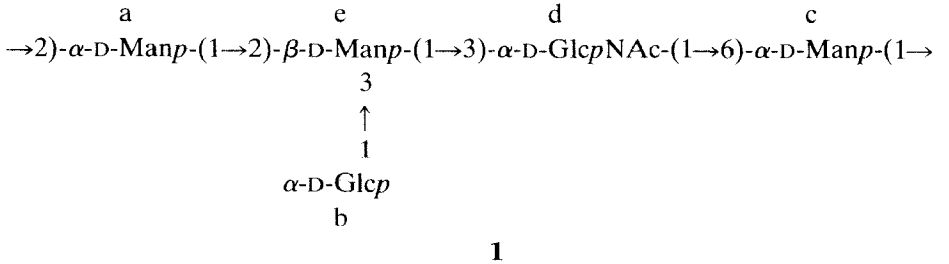
Residue[b]	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
a	99.1	78.1	71.1	67.2	72.5	n.d.[c]
b	101.3	72.0	74.0	70.3	72.5	n.d.
c	102.7	70.8	71.4	66.7	73.8	65.5
d	97.6	53.6	80.9	69.2	73.5	n.d.
e	100.6	75.1	80.8	67.7	77.4	n.d.

[a]Chemical shifts determined at 27° relative to internal acetone (31.07 p.p.m.) [b]See formula **1**. [c]Not determined.

established the ^{13}C assignments with reference to the determined proton n.m.r. assignments (Table IV). Correlation between the carbon signal corresponding to a substituted hydroxymethyl group (65.5 p.p.m.) with those of the protons at 4.14 and 3.61 p.p.m. gave the chemical shift of the resonances of H-6 and H-6′ which in turn was correlated by a COSY spectrum to H-5 of residue c, identifying c as the 6-linked α-D-mannopyranosyl residue. A correlation between the ^{13}C signal at 53.6 p.p.m. corresponding to C-2 of the 2-acetamido-2-deoxy-D-glucopyranose residue and the proton resonance with the chemical shift 4.098 p.p.m. (H-2d) identified d as the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue. Consideration of chemical shifts and coupling constants identified residue b as the α-D-glucopyranosyl unit.

Since substituted ^{13}C atoms experience significant deshielding, it can be concluded that residue a is 2-linked and that residue e is di-*O*-substituted at positions 2 and 3, by comparison of the ^{13}C chemical shift data with those of the methyl D-mannopyranosides[12].

N.O.e experiments[13] (Fig. 5) led to the following sequences: (*a*) α-D-Man*p* is 2-linked to e (*b*) α-D-Glc*p* is 3-linked to e, (*c*) α-D-Man*p* is 2-linked to a (*d*) α-D-Glc*p*NAc is 6-linked to c, and (*e*) β-D-Man*p* is 3-linked to d. Residue e exhibited n.O.e.'s between H-1e, H-3e, and H-5e as expected for the β-D-mannopyranosyl residue previously identified in oligosaccharide *B*. The combined chemical and physical evidence leads to the characterization of the LPS O-polysaccharide as containing a repeating pentasaccharide unit having the structure **1**.



1

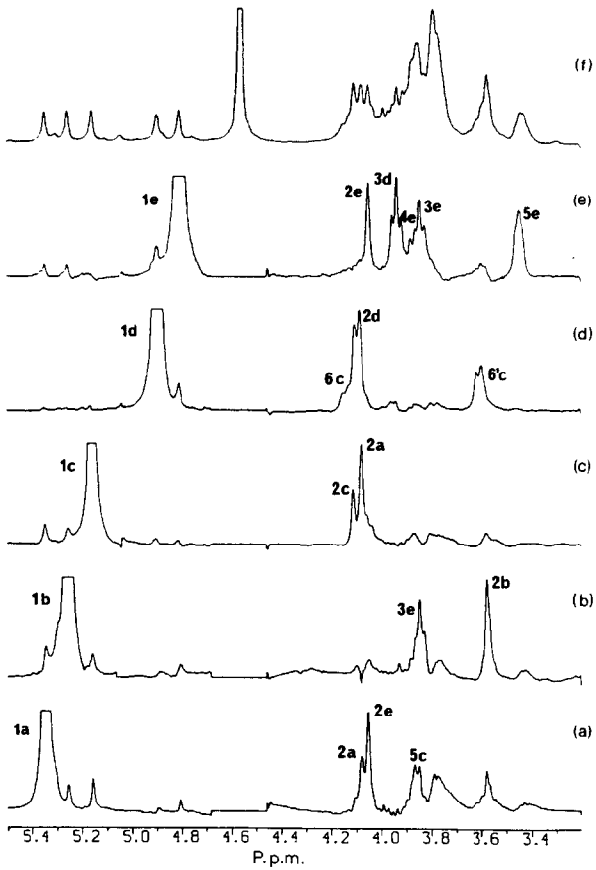


Fig. 5. N.O.e. difference spectra for the O-chain polysaccharide on saturation of the resonances (a) H-1a, (b) H-1b, (c) H-1c, (d) H-1d, (e) H-1e, and (f) ^{1}H-n.m.r. spectrum of the O-polysaccharide.

The minor (10%) O-polysaccharide present in the isolated O-chain fraction from *S. carrau* was shown to be identical with the major LPS O-chain produced by *S. boecker*[14] expressing the antigenic factors O:6 and 14, which was a polymer of a repeating tetrasaccharide unit, identical with the linear tetrasaccharide repeating-unit of the backbone of *S. carrau* LPS O-polysaccharide. From this early observation, it appears that the *Salmonella* O:24 antigenic factor can be related to an epitope involving the α-D-glucopyranosyl branch unit of the *S. carrau* major LPS O-polysaccharide.

EXPERIMENTAL

Production of lipopolysaccharide. — Cultures of *S. carrau* (LCDC S-886 NRCC 4226) supplied by Dr. H. Lior (LCDC Health & Welfare Canada, Ottawa) were grown in a fermenter (28 L, Microfirm, New Brunswick Scientific), using a

medium of 3.7% of 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% saline, digested with lysozyme, ribonuclease, and deoxyribonuclease[3], and then extracted with hot aqueous phenol[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[15].

Polysaccharide O-chain. — A solution of LPS (1.2 g) in aqueous 2% acetic acid (150 mL) was heated for 2 h at 100° and the precipitated lipid A was removed by low-speed centrifugation. The supernatant solution was lyophilized and the residue was eluted from a column of Sephadex G-50 using pyridinium acetate (0.05M, pH 4.7). Fractions (10 mL) were monitored for glycose, aminodeoxyglycose, 3-deoxyoctulosonate, and phosphate.

Analytical methods. — Quantitative methods used were (*a*) the phenol–sulfuric acid method for neutral glycoses[16], (*b*) the modified Elson–Morgan method for aminodeoxyglycoses[17], (*c*) the periodate oxidation–thiobarbituric acid method for deoxyoctulosonate[18], and (*d*) the method of Chen *et al.*[19] for phosphate. P.c. was done on Whatman No. 1 paper with (*a*) pyridine–ethyl acetate–water (2:5:5, upper layer) and (*b*) 1-butanol–ethanol–water (4:1:5, upper layer), and glycoses were detected with periodate–alkaline silver nitrate[20].

Gel filtration was done on columns of Sephadex G-50 (2.5 × 80 cm), Sephadex G-15 (Pharmacia) (2.0 × 40 cm), and Bio-Gel P-2 (Bio Rad Laboratories) (1.5 × 95 cm) at 20° using pyridinium acetate buffer (0.05M, pH 4.7). 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 acetates[21], using *myo*-inositol as the internal standard. Oligo- and poly-saccharide samples (0.5–1.0 mg) were hydrolyzed with 10M hydrochloric acid (1 mL) for 20 min at 85–90°. Each hydrolyzate was concentrated to dryness, and a solution of the residue in water (2 mL) was treated with sodium borohydride (10 mg). The resulting alditols were acetylated prior to g.l.c.

The configuration of glycoses was established[22] by capillary g.l.c. of their acetylated (−)-2-butyl glycosides.

G.l.c. was done with a Hewlett–Packard model 5830A gas chromatograph fitted with a flame-ionization detector. A capillary column (0.32 mm × 25 m), 007 series bonded phase, fused silica OV-17 (Quadrex Corp.) was used with the following temperature programmes: (*a*) alditol acetates, 180°→240° at 4°/min; (*b*) methylated alditol acetates, 200°→240° at 1°/min; and (*c*) methylated acetates, 150°→240° at 4°/min; (*d*) methylated oligosaccharides were separated with a capillary column (0.32 mm × 25 m), 007 series bonded phase, fused silica OV-1, 150°→350° at 10°/min. Nitrogen was the carrier gas at 30–40 mL/min and retention times are quoted relative to those of D-glucitol hexa-acetate (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 using a Hewlett–Packard 5985B instrument employing the above temperature programmes and an ionization potential of 70 eV.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). — LPS samples (1.0 μg) were analyzed in 14% polyacrylamide gels by electrophoresis in the presence of SDS. Bands were detected by silver-staining[23].

Methylation analysis. — Samples (1.0–2.0 mg) were methylated[24] using sodium methylsulfinylmethanide and methyl iodide in methyl sulfoxide, and the products were purified using C18 Sep-Pak cartridges (Waters Associates). The methylated products were hydrolyzed with 10M hydrochloric acid (1 mL) for 20–30 min at 85–90°, the hydrolyzates were concentrated, and the residues were reduced with sodium borohydride and acetylated, or directly acetylated, and examined by g.l.c.–m.s.

Periodate oxidation. — A solution of the O-chain polysaccharide (105 mg) in water (75 mL) containing sodium metaperiodate (750 mg) was stored for 5 days at 4°. Excess of periodate was reduced with ethylene glycol (1.5 mL) and the oxidized polymer was reduced with sodium borohydride (750 mg). After 16 h at room temperature, the excess of borohydride was decomposed with Rexyn 101 (H^+) resin, the solution was concentrated to dryness, and methanol was evaporated 3 times from the residue. Smith hydrolysis was done with aqueous 2% acetic acid (40 mL) for 2 h at 100° and the lyophilized product was reduced with aqueous sodium borohydride prior to its fractionation by gel filtration on Bio-Gel P2.

N-*Deacetylation and deamination of the O-polysaccharide*[8]. — To a solution of the O-chain (120 mg) in water (1.2 mL) were added thiophenol (120 μL), sodium hydroxide (500 mg), and methyl sulfoxide (5 mL). After flushing with N_2, the sealed vial was heated for 16 h at 110°. The solution was poured into ice–water (15 mL), neutralized with 2M hydrochloric acid, dialyzed against tap water, and concentrated to dryness. To a solution of the residue in aqueous 33% acetic acid (15 mL) was added aqueous 5% sodium nitrite (6 mL). After 1 h at room temperature, the mixture was deionized with Rexyn 101 (H^+) resin and concentrated to dryness, and the residue was reduced with aqueous sodium borohydride. The reduced oligosaccharide (*B*) was collected from the major fraction obtained by gel filtration on Bio-Gel P2.

N.m.r. spectroscopy. — ^1H- and ^{13}C-n.m.r. experiments were carried out using a Bruker AM-500 spectrometer at 47° and 27°, respectively. All samples were exchanged twice with 99.8% D_2O and run in 0.5 mL of 99.99% D_2O. The internal reference was the methyl resonance of acetone set at 2.225 p.p.m. for ^1H and 31.07 p.p.m. for ^{13}C. All n.m.r. experiments were acquired and processed using the standard software provided by Bruker (DISB87).

N.O.e.'s[13] were measured by difference spectroscopy with an irradiation time of 200 ms. For the proton homonuclear shift correlation (COSY)[9] and the two-step relay COSY[10], 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 using unshifted sine bell functions, zero-filling, a magnitude calculation, and symmetrization about the diagonal to give a final resolution of 1.2 Hz/point in both domains.

The DEPT experiments[7] were performed using a $3\pi/2$ proton pulse to distinguish between CH and CH_2 ^{13}C resonances. The heteronuclear ^{1}H–^{13}C shift correlation[11] consisted of 256 experiments of 400 transients and 2048 data points. The matrix was zero-filled to 512 × 2048 points and processed with Gaussian functions and a magnitude calculation to give a final resolution of 6.1 Hz/point for the ^{13}C domain and 4.7 Hz/point for the ^{1}H domain.

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