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Structure of the polysaccharide O-antigen of *Salmonella riogrande* O:40 (group R) related to blood group A activity

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ABSTRACT

The structure of the *Salmonella* O:40 (Group R) antigen was determined from an analysis of the antigenic O-polysaccharide component of the lipopolysaccharide produced by *Salmonella riogrande* O:40. Using ^{1}H- and ^{13}C-NMR spectroscopy, methylation analysis, and periodate degradation methods, the *O*-polysaccharide was found to be a high molecular weight branched polymer of repeating pentasaccharide units having the structure:

$$\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \beta\text{-D-Glc}p\text{NAc} \end{array}$$

The reported human blood group A activity was concluded to reside in an epitope of a terminal trisaccharide portion of the O-chain involving α-D-Gal*p*NAc and β-D-Glc*p*NAc residues linked $(1\rightarrow 3)$ and $(1\rightarrow 2)$, respectively, to β-D-Man*p* branched residues in which the α-D-Gal*p*NAc residue would appear to be the critical antigenic factor recognized by polyclonal blood group A antisera.

INTRODUCTION

Common carbohydrate antigenic factors between human blood group substances and bacterial immunogens are probably responsible for the origin of many heterophile antibodies [1–3]. In the case of *Salmonella* species, the common antigenicities between *Salmonella* group U and human blood group B [4–6], and between group R and blood group A [6–8], resides in the lipopolysaccharide (LPS) antigenic *O*-polysaccharide components of the respective O:43 (group U) and O:40 (group R) serotypes as defined in the Kauffmann–White classification scheme [8].

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The structural difference which determines the blood group B or A specificities lies in the replacement of the terminal α-D-Gal*p* residues in the blood group B antigen, by α-Gal*p*NAc residues in the case of the blood group A antigenic end-groups. A recent study of the *Salmonella* O:43 antigen [10] demonstrated that its blood group B activity could be related to a terminal, non-reducing trisaccharide composed of α-D-Gal*p* and α-L-Fuc*p* residues linked (1 → 3) and (1 → 2), respectively, to a β-D-Gal*p* residue, identical in structure with the non-reducing terminal end of the blood group B antigen.

The present study records the analysis of the LPS O-chains of the *S. riogrande* O:40 reported to have blood group A antigenic activity [6,8]. The O:40 LPS O-chain was found to be polymers of a branched repeating pentasaccharide units composed of D-GalNAc, D-GlcNAc, D-Man, and D-Glc (2:1:1:1) residues and concludes that its blood group A activity is essentially determined by the α-D-Gal*p*NAc residue contained in the non-reducing end of the trisaccharide structure, shown below, that is a part of the pentasaccharide repeating unit.

α-D-Gal*p*Nac-(1 → 3)-β-D-Man*p*-(1 → —
2
↑
1
β-D-Glc*p*NAc

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) produced by *S. riogrande* O:40 was isolated in 5% yield by ultracentrifugation of the dialyzed concentrated aqueous phase of cells extracted by the phenol–water method [11]. Hydrolysis of the LPS with hot dilute acetic acid afforded an insoluble lipid A (20%), and Sephadex G-50 gel filtration chromatography of the water soluble product gave *O*-polysaccharide (K_{av} 0.04 to 0.11, 75%), a core oligosaccharide (K_{av} 0.53, 17%) and a fraction (K_{av} 0.98, 8%) containing 3-deoxy-D-*manno*-octulosonic acid and phosphate.

The *O*-polysaccharide had $[\alpha]_D + 107°$ (*c* 1.4, H_2O) which on hydrolysis was shown to be composed of D-Man, D-Glc, D-GlcNAc, and D-GalNAc in the relative proportions 1:1:1:2, and minor amounts (~0.2%) of aldoheptose probably arising from attached core residues. The identities of the sugars and their D configurations were established by capillary GLC of derived alditol acetates [12] and their trimethylsilylated 2-(−)-butyl glycosides [13].

The ^{1}H-NMR spectrum of the *O*-polysaccharide showed, inter alia, signals for *N*-acetyl groups at 2.03 (3 H) and 2.08 ppm (6 H), and five anomeric protons (Table I) whose chemical shifts and $J_{1,2}$ coupling constants indicated that, consistent with the composition data, the O-chain was composed of a repeating pentasaccharide unit and, that three of the glycoses had the β anomeric configuration and two the α configuration, that they had pyranosyl ring forms, and that the aminodeoxyglycoses were *N*-acetylated.

TABLE I

^{1}H-NMR data [a] for the anomeric protons of the LPS O-chain of *S. riogrande* O:40 and its degradation products

Derivative	Glycopyranosyl residue				
	→4)-α-D-Gal*p*NAc-(1→	→3)-α-D-Gal*p*NAc-(1→	→4)-β-D-Glc*p*-(1→	→2,3)-β-D-Man*p*-(1→	β-D-Glc*p*NAc-(1→)
O-chain	5.22 (3.2)	4.93 (3.8)	4.76 (8.2) [b]	4.73 (1.0)	4.63 (7.5) [b]
Oligosaccharide A	5.21 (3.9)	4.96 (3.7)	–	4.77 (0.8)	–
Oligosaccharide B	5.17 (3.9)	–	–	4.82 (0.8)	–
Glycoside C	–	–	–	4.81 (0.8)	–

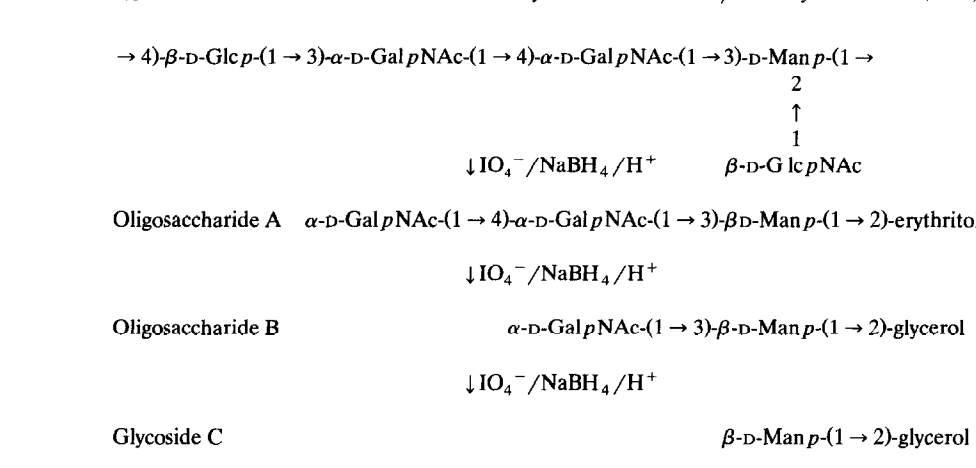
[a] Observed chemical shifts (ppm) measured at 320 K, using acetone as an internal reference (2.225 ppm), and coupling constants measured in Hz are given in parentheses. [b] Assignments may be reversed.

TABLE II

^{13}C-NMR data [a] for the anomeric carbon atoms of the LPS O-chain of *S. riogrande* O:40 and its degradation products

Derivative	Glycopyranosyl residue				
	→4)-α-D-Gal*p*NAc-(1→	→3)-α-D-Gal*p*NAc-(1→	→4)-β-D-Glc*p*-(1→	→2,3)-β-D-Man*p*-(1→	β-D-Glc*p*NAc-(1→
O-chain	100.1 (176)	99.2 (172)	101.8 (164) [b]	102.1 (159)	104.6 (162) [b]
Oligosaccharide A	100.5 (176)	99.2 (172)	–	100.7 (159)	–
Oligosaccharide B	100.2 (177)	–	–	100.7 (159)	–
Glycoside C	–	–	–	100.4 (159)	–

[a] Observed chemical shifts ppm measured at 320 K, using acetone as an internal reference (31.07 ppm), and coupling constants measured in Hz are given in parentheses. [b] Assignments may be reversed.



Scheme 1. Smith-type periodate degradation of the LPS O-chain of *S. riogrande* O:40.

Further ^{1}H- and ^{13}C-NMR data obtained from the spectra of the oligosaccharides, derived in Smith-type periodate oxidation–degradation [14] (Scheme 1) of the O-chain, allowed the assignments of anomeric proton signals recorded in Table I to be made.

The ^{13}C-NMR spectrum of the *O*-polysaccharide was consistent with the proposed pentasaccharide repeating unit in the O-chain. The spectrum showed inter alia, acetamido signals at 175.1, 175.2, and 175.5 ppm ($NHCOCH_3$) and at 22.88 (2 C) and 23.26 ppm (1 C) ($NHCOCH_3$), as well as C-2 signals at 49.7 (2 C) and 50.9 ppm (1 C) from the three acetamidodeoxyglycose residues. The spectrum also showed five anomeric carbon signals whose chemical shifts (Table I) were assigned from a heteronuclear ^{13}C–^{1}H shift correlation measured in the ^{1}H-detection mode via multiple quantum coherence [$^{1}H(^{13}C)$HMQC] [15]. The previously assigned anomeric configurations determined from the ^{1}H-NMR data were confirmed by the measured $J_{C,H}$ coupling constants [16,17].

Methylation analysis of the O-chain (Table III) showed that it contained a D-Glc*p*NAc non-reducing end-group, a D-Man*p* branch point substituted at O-2 and O-3, a D-Glc*p* residue substituted at O-4, a D-Gal*p*NAc residue substituted at O-4, and a second D-Gal*p*NAc residue substituted at O-3.

As expected from the above substitution data, Smith-type degradation, involving periodate oxidation, reduction ($NaBH_4$), and mild acid hydrolysis [14], resulted in oxidation of the 4-*O*-substituted D-Glc*p* residue, and the D-Glc*p*NAc end-groups. Bio-Gel P2 gel-filtration chromatography of the product gave a major fraction (K_{av} 0.40), which was pure by paper chromatography (R_{Gal} 0.22), had $[\alpha]_D$ + 159° (*c* 0.9, H_2O) and on hydrolysis and GLC analysis was shown to be an oligosaccharide (A) (Scheme 1) composed of D-GalNAc, D-Man, and erythritol in the molar ratios 2:1:1. Methylation analysis of A (Table III) showed it to have a non-reducing glycose end group of D-Gal*p*NAc, an internal 3-*O*-substituted D-Man*p* residue, and a 4-*O*-substituted D-Gal*p*NAc residue together with an erythritol end-group which must originate from the 4-*O*-substituted D-Glc*p* residues. The above evi-

TABLE III

Methylation data for the LPS O-chain of *Salmonella riogrande* and its degradation products

Derivative	T_{GM} [a]	Relative molar ratios		
		O-chain	Oligo A	Oligo B
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol-1-*d*	1.40	1.00		
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol-1-*d*	1.42		1.00	1.00
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methyl-D-mannitol-1-*d*	1.74	0.96		
1,5-Di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol-1-*d*	2.65	0.89		
1,5-Di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-galactitol-1-*d*	3.03		0.91	0.92
1,4,5-Tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-galactitol-1-*d*	3.55	0.87	0.90	
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamido-D-galactitol-1-*d*	3.59	0.90		

[a] Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM} = 1.00). Fused-silica OV-17 coated capillary column (0.3 mm × 25 m); temperature program 200° for 2 min to 240° at 2°/min.

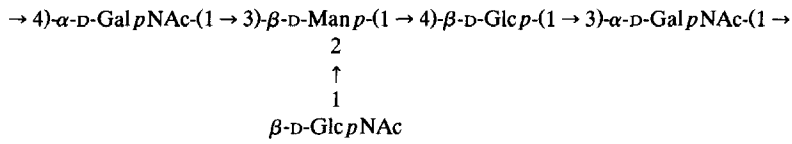
dence indicates that the non-reducing side groups in the O-chain are single D-Glc*p*NAc units linked at the O-2 position of the 2,3-di-*O*-substituted D-Man*p* branch points and this D-Man*p* unit is (1 → 3) linked in the linear backbone of the O-chain.

The characterisation of the trisaccharide nature of oligosaccharide A provides evidence that the backbone of the O-chain is a linear tetrasaccharide sequence in which only the 4-*O*-linked D-Glc*p* residues are oxidized by periodate. The above initial evidence does not permit the order of the 4-*O*-linked D-Gal*p*NAc and 3-*O*-linked D-Man*p* residues to be defined.

Smith-type degradation of A (Scheme 1), followed by Bio-Gel P2 chromatographic separation of the product, gave an oligosaccharide B that was pure on paper chromatography (K_{av} 0.51, and R_{Gal} 0.60) which had $[\alpha]_D$ + 105° (*c* 0.8 H_2O) and was composed of D-GalNAc, D-Man, and glycerol (1 : 1 : 1). Methylation analysis of B (Table III) showed it to have a non-reducing glycose D-Gal*p*NAc end group and a 3-*O*-substituted internal D-Man*p* residue. Considered in conjunction with ^{1}H- and ^{13}C-NMR analysis (Tables I and II) B can be identified as α-D-Gal*p*NAc-(1 → 3)-β-D-Man*p*-(1 → 2)-glycerol, thus establishing the linkage sequence of these two glycoses in the original O-chain. The fact that the Smith-type degradation of A afforded oligosaccharide B which still contained the D-Man residue leads to an unambigious linkage sequence of A, which, from subsequent ^{1}H- and ^{13}C-NMR analysis (Tables I and II), is identified as having the structure α-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*NAc-(1 → 3)-β-D-Man*p*-(1 → 2)-erythritol, thus establishing the complete linkage sequence in the original O-chain.

A Smith-type periodate degradation of B (Scheme 1) afforded after BioGel P2 chromatography, the glycoside C, β-D-Man*p*-(1 → 2)-glycerol (K_{av} 0.71), which had $[\alpha]_D$ − 34° (*c* 0.5, H_2O) and R_{Gal} 0.94 (PC), and gave anomeric NMR data consistent with this structure (Tables I and II).

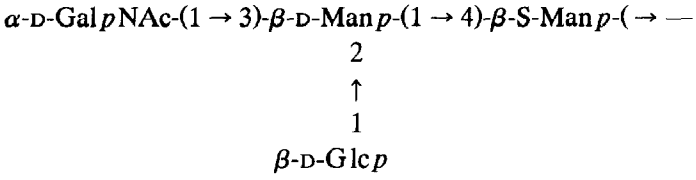
From the analytical data the polysaccharide O-chain can be unambiguously defined as a polymer of repeating branched pentasaccharide units having the structure:



The β-D configuration of the D-Glc*p*NAc side chain substituents and the (1 → 4) linked D-Glc*p* residues were assigned from their O-chain ^{1}H- and ^{13}C-NMR spectral anomeric signal chemical shifts and coupling constants.

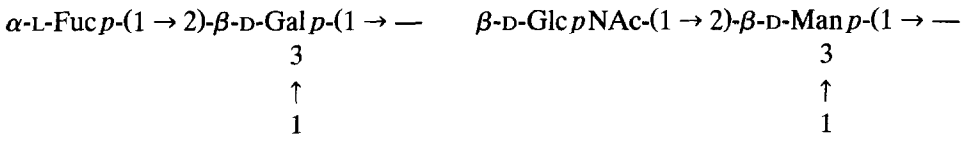
From a consideration of the structures of the *Salmonella* O:40 LPS antigenic O-chain and blood group A substance, the serological cross reactions between polyclonal *Salmonella* O:40 and blood group A antisera and blood group A erythrocytes and *Salmonella* O:40 bacterial cells, appear to be mainly determined by an epitope involving non-reducing α-D-Gal*p*NAc end-groups.

It is interesting to note that the blood group A activity of the LPS O-chain of *Escherichia coli* O:6 [18] can be similarly related to an epitope in part of its repeating unit as shown below:



This suggestion is supported by the fact that methyl 2-acetamido-2-deoxy-α-D-galactopyranoside was reported to be a marked inhibitor of the above blood group A cross reactions [8].

In contrast to the *Salmonella* O:43 antigen in which the blood group B activity resides in a non-reducing end-group trisaccharide unit having exactly the same structure as the blood group B antigen [10], the *Salmonella* O:40 corresponding serological reactivity resides in a trisaccharide end-group having apparent limited similarities as seen by a comparison of the two structures shown below:



Blood group A terminus — *Salmonella* O:40 O-chain terminus

A murine monoclonal antibody to the antigenic determinant of blood group B, prepared by immunization with a protein conjugate of a synthetic group-B trisac-

charide immunodeterminant, and specific selection of hybridomas using the same trisaccharide [19], showed the same activity to blood group B substance and to LPS O:43 O-chain, in passive haemagglutination and ELISA assay systems.

A similar monoclonal antibody (Synaff. Anti-A, Chembiomed, Alberta, Canada) prepared to the synthetic blood group A terminal trisaccharide, which was specific for the A antigen and negative for the B antigen, failed to show positive reaction with the LPS O:40 O-chain in passive haemagglutination and ELISA assay systems.

EXPERIMENTAL

Production of lipopolysaccharide and O-polysaccharide.—S. riogrande O:40 (NRCC 4410; Institut Pasteur 231 K) was grown in 3.7% brain–heart infusion (Difco) at 37°, 200 rpm, and aeration at 25 L/min for 18 h using a fermenter (28-L Microferm). Cells were killed by the addition of phenol to 0.75% final concentration and harvested using a Sharples centrifuge (yield 14 g wet wt/L). LPS was isolated from the cells by the enzyme digest phenol–water extraction method [11], followed by ultracentrifugation of the concentrated dialyzed aqueous extraction phase (yield 3.9% LPS based on dry wt cells). Solutions of the LPS (1.5%) in 2% acetic acid were hydrolyzed for 2 h at 100°, and, following removal of precipitated lipid A, the lyophilized water-soluble products were fractionated by Sephadex G-50 gel-filtration chromatography to yield O-polysaccharide as a fraction eluting at the void volume of the system.

Analytical methods.—Quantitative analyses of glycoses were made as previously described [20], and GLC–MS was performed as described in ref. 21. Paper chromatography was done using pyridine–EtOAc–water (2:5:5 v/v, top layer) as the mobile phase, and mobilities are quoted relative to D-galactose (R_{Gal}). Methylation analyses and Smith-type periodate oxidations were also made under described conditions [21].

NMR spectroscopy.—Measurements were made at 320 K on solutions in D_2O with a Bruker AMX-500 spectrometer, using standard Bruker software. Heteronuclear ^{13}C–^{1}H chemical shift correlations were measured in the ^{1}H-detection mode via multiple quantum coherence [$^{1}H(^{13}C)$] (ref. 15) with a Bruker 5-mm inverse broad-band probe using reverse electronics as previously described [22].

General methods.—Concentrations were made under reduced pressure below 40°. Optical rotations were determined at 20° in 10-cm microtubes using a Perkin–Elmer 243 polarimeter.

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