

Structural Studies on the O-Specific Side-chains of the Cell-wall Lipopolysaccharide from *Salmonella Newington*

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The structure of the O-specific side-chains of the *Salmonella newington* I.S. 84 (3,15) lipopolysaccharide has been investigated by methylation analyses of the original lipopolysaccharide (LPS) and of the chemically modified LPS, and by identification of a trisaccharide, obtained on graded hydrolysis.

The structure of the O-specific side-chains of the LPS from *S. muenster* (3,10) serogroup E₁,¹ was recently investigated in this Laboratory. The results agreed in most respects with those obtained by Robbins and coworkers,² who studied the LPS from the related *S. anatum* (3,10), but some differences which may have immunological significance were revealed. Lysogenization of *S. anatum* (3,10) with bacteriophage ϵ ¹⁵ confers a new antigenic specificity to the bacteria.³ O-Antigen factor 15 appears with the concomitant disappearance of O-factor 10, and the bacteria are recognized as *S. newington* (3,15) of serogroup E₂. Structure I has been proposed for the O-specific side-chains of the LPS from *S. newington* (3,15) belonging to serogroup E₂.²

The present communication reports structural studies on the *S. newington* LPS, using the same methods described earlier for the *S. muenster* LPS.



I

ISOLATION OF THE LPS

The LPS from *S. newington* I.S. 84 was isolated from the bacteria as previously described.⁴ The presence of O-factors 3 and 15 were demonstrated both for the bacteria and for the LPS by slide agglutination and passive hem-

agglutination tests, respectively. The methods used were the same as previously described.⁴

The LPS on acid hydrolysis yielded L-rhamnose, D-mannose, D-galactose, D-glucose, and a heptose in the relative proportions 28:28:35:5:4. The values were derived by GLC of the alditol acetates,⁵ and the identities of the sugars were confirmed by MS.⁶ The sugars have been fully identified in a previous investigation.² The presence of *O*-acetyl groups in the LPS was demonstrated by treating it with methanolic hydrogen chloride and identifying the methyl acetate formed by GLC-MS.⁷

METHYLATION ANALYSES

The LPS was methylated by treatment with methylsulphonyl sodium and methyl iodide in methyl sulphoxide,⁸ and thereafter hydrolysed to a mixture of methylated sugars. These were analysed, as their alditol acetates, by GLC⁹-MS.¹⁰ A typical chromatogram is shown in Fig. 1. Peak A contained

Table 1. Methyl ethers from the hydrolysate of methylated lipopolysaccharide.

	Peak	<i>T</i> ^a	Mol%
2,3-Di- <i>O</i> -methyl-L-rhamnose	A	0.98	28.0
2,3,4,6-Tetra- <i>O</i> -methyl-D-mannose	A	1.0	1.7
2,3,4,6-Tetra- <i>O</i> -methyl-D-glucose	A	1.0	0.4
2,3,4,6-Tetra- <i>O</i> -methyl-D-galactose	B	1.25	1.4
2,4,6-Tri- <i>O</i> -methyl-D-galactose	C	2.28	27.4
2,3,4-Tri- <i>O</i> -methyl-D-mannose	D	2.48	26.4
3,4,6-Tri- <i>O</i> -methyl-D-galactose	D	2.50	1.2
2,6-Di- <i>O</i> -methyl-D-galactose	E	3.65	1.2
3,6-Di- <i>O</i> -methyl-D-glucose	F	4.35	0.9
2,4-Di- <i>O</i> -methyl-D-glucose	G	5.10	1.7

^a Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

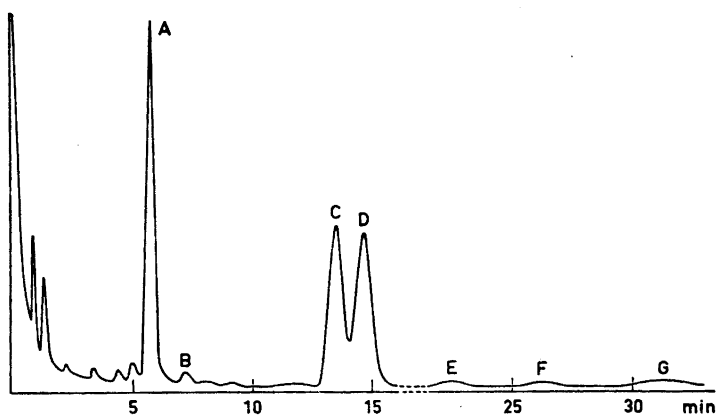


Fig. 1. GLC separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated *S. newington* LPS.

the alditol acetates derived from 2,3-di-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-mannose, and 2,3,4,6-tetra-*O*-methyl-D-glucose. These components were resolved on a silicon (OV-225) S.C.O.T. column (*T* 0.91, 0.97, and 1.00, respectively). The results of the methylation analysis are given in Table 1. The relative proportions of L-rhamnose, D-mannose, D-galactose, and D-glucose, as calculated from the methylation analyses, are 28:28:31:3, in reasonably good agreement with the sugar analysis.

The LPS was subjected to a mild acid hydrolysis, which should lead to preferential cleavage of the L-rhamnosidic linkages. The oligomeric and polymeric material was recovered and subjected to methylation analysis as above (Table 2). The increase of 2,3,4,6-tetra-*O*-methyl-D-galactose and the corre-

Table 2. Methyl ethers from the hydrolysate of partially degraded and methylated lipopolysaccharide.

	<i>T</i> ^a	Mol%
2,3-Di- <i>O</i> -methyl-L-rhamnose	0.98	28.0
2,3,4,6-Tetra- <i>O</i> -methyl-D-galactose	1.25	18.2
2,4,6-Tri- <i>O</i> -methyl-D-galactose	2.28	9.8
2,3,4-Tri- <i>O</i> -methyl-D-mannose	2.48	26.2

^a See Table 1.

sponding decrease in 2,4,6-tri-*O*-methyl-D-galactose, relative to the corresponding values from the methylation analysis of the original LPS, demonstrates that the L-rhamnose is linked to D-galactose in the 3-position.

The LPS was treated with methyl vinyl ether in order to protect all the free hydroxyl groups as acetals.¹¹ The modified polysaccharide was subjected to methylation analysis, whereby the original *O*-acetyl groups are replaced by *O*-methyl groups. Only one methylated sugar was obtained, and from its MS and *T*-value (5.10) it was identified as 6-*O*-methyl-D-galactose (3.5 %).

ACID HYDROLYSIS STUDIES

Lipid-free polysaccharide was prepared from the LPS by treatment with 0.1 % acetic acid for 1 h at 100°. A sample of this material was treated with 0.25 M sulphuric acid at 80°, and the change in optical rotation was followed polarimetrically (Fig. 2). The positive increase in optical rotation is ascribed

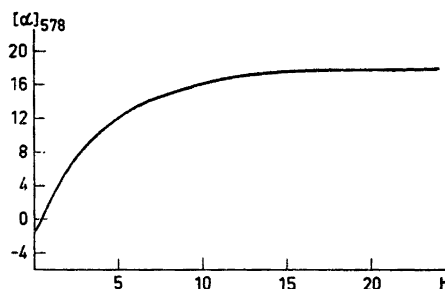


Fig. 2. Optical rotation versus time on acid hydrolysis of the *S. newington* LPS.

to the preferential hydrolysis of the α -linked L-rhamnosidic linkages in this polysaccharide.

A larger amount of polysaccharide (100 mg) was hydrolysed under the same conditions for 5 h, and the oligosaccharides obtained were fractionated on a Sephadex G-15 column. A major component (13.1 mg) was isolated, which was homogeneous on paper chromatography in two different solvent systems and had the mobility expected for a trisaccharide.

The trisaccharide was reduced with sodium borodeuteride to its alditol. Part of this material was hydrolysed, reduced by sodium borohydride, and the alditol mixture was analysed by GLC-MS. The relative proportions of L-rhamnose, D-mannose, and D-galactose were 1.0 : 1.0 : 1.0. Only the L-rhamnitol contained deuterium, showing that L-rhamnose occupied the reducing end of the trisaccharide. L-Arabinose was added as an internal standard, and the analysis showed that the three sugars accounted for 90 % of the amorphous trisaccharide alditol preparation, which thereby enabled the specific rotation, $[\alpha]_D - 27^\circ$, to be calculated.

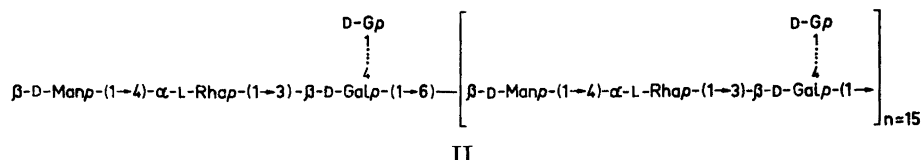
The $[\alpha]_D$ value for a β -D-galactopyranosyl- β -D-mannopyranosyl-L-rhamnitol, calculated according to Hudson's rules of isorotation, was -24° , in good agreement with the observed value. If we assume that one of the sugars is α -linked, the corresponding values are $+55^\circ$ and $+35^\circ$, and it is therefore concluded that they are both β -linked. These results, in conjunction with the results of the methylation analysis, therefore demonstrate that the trisaccharide has the structure



In the NMR spectrum of the trimethylsilyl derivative of the trisaccharide alditol, the signals from anomeric protons of the D-galactose and the D-mannose residues both appeared at $\tau = 5.5$ ppm, and hence individual coupling constants could not be determined. An isomeric trisaccharide alditol, α -D-Gal-(1 \rightarrow 6)- β -D-Man-(1 \rightarrow 4)-L-Rhamnitol ($[\alpha]_D + 50^\circ$) was obtained from the *S. muenster* LPS.¹ The NMR spectrum of the trimethylsilyl derivative revealed, *inter alia*, signals at $\tau = 5.52$ ppm and $\tau = 5.17$ ppm, which are assigned to the anomeric protons of the β -D-mannopyranosyl and the α -D-galactopyranose residue, respectively. The absence of signals at $\tau = 5.17$ ppm in the spectrum of the *S. newington* trisaccharide alditol therefore substantiates the conclusion that the D-galactopyranose residue is β -linked.

DISCUSSION

From the present results, the structure II is proposed for the O-specific side-chains of the *S. newington* LPS.



The pyranosidic nature of the D-mannose, D-galactose, and D-glucose residues is evident from the methylation analysis. It is assumed that the L-rhamnosidic linkages are also pyranosidic, as furanosidic residues would have been more readily hydrolysed than was observed. From the methylation analysis, the sites of attachment of the different sugar residues can be derived. The methylation analysis of the partially hydrolysed material shows that L-rhamnose is linked to D-galactose; since there are only three sugar residues in the repeating unit, their mutual order is readily deduced.

The small amount of 2,3,4,6-tetra-*O*-methyl-D-mannose observed in the methylation analysis of the LPS is derived from the terminal repeating unit. The biological repeating unit is thereby defined as Man-Rha-Gal. From the relative percentages of 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,4-tri-*O*-methyl-D-mannose, the average number of repeating units in a side-chain was estimated as 16.

Most of the other minor components in the methylation analysis are derived from the basal core¹³ and have also been found in studies on other *Salmonella* LPS.^{1,4,7,14}

2,3,4,6-Tetra-*O*-methyl-D-glucose (0.4 %) and 2,6-di-*O*-methyl-D-galactose (1.2 %) do not originate from the basal core, and the presence of these sugars may indicate that, in a small part of the repeating units, a terminal D-glucose residue is linked to D-galactose at the 4-position. This structural feature was also observed in the *S. muenster* LPS.¹ Such substitution may also occur in *Salmonella* serogroups A, B, and D LPS, where the D-glucose residues are α -linked and confer O-antigen 12₂ specificity to the bacteria. The same structural feature in serogroup E₃ gives the bacteria O 34 specificity.¹⁵ The *S. newington* LPS did not exert any O 12₂ specificity when tested in passive hemagglutination inhibition.

A small percentage of *O*-acetyl groups, linked to D-galactose at C-6, was found. The amount is so low, that there is a finite probability that it originates from the D-galactose residues in the basal core rather than from those in the *O*-specific side-chains. This, however, we consider less likely, as no acetyl substituents in this position were detected during previous structural studies of the basal core region.¹³ It is worthy of note that Robbins and coworkers³ found 6-*O*-acetyl-D-galactose residues in the *S. anatum* LPS (3,10), while in another serogroup E₁ LPS, from *S. muenster* (3,10), we were not able to find any.

The assignment of β -configuration to the D-mannopyranosidic residue is at variance with the results of Robbins and coworkers, who did not determine the configuration of the L-rhamnopyranosidic residue. In all other respects, there is good agreement between their results and those obtained by us.

EXPERIMENTAL

The experimental methods were the same as used in the similar study of the *S. muenster* LPS.¹

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