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STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE CELL-WALL LIPOPOLYSACCHARIDE FROM *Salmonella senftenberg*

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ABSTRACT

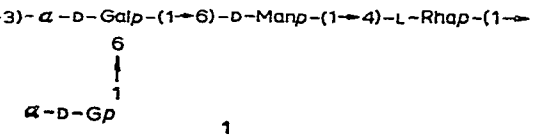
The structure of the O-specific side-chains of the cell-wall lipopolysaccharide (LPS) from *Salmonella senftenberg*, serogroup E_4, has been investigated. From the methylation analysis of the LPS and of a partially hydrolysed product, the linkages could be identified and the order of the sugar residues assigned. The terminal, non-reducing sugar residue in the side-chains was also identified, thereby defining the "biological" repeating units in the side-chains.

The anomeric nature of the different sugar residues was determined by polarimetric studies and by graded hydrolysis, followed by isolation and characterisation of a tetrasaccharide.

INTRODUCTION

Structural studies of the O-specific side-chains of different *Salmonella* LPS, using methylation analysis on the intact LPS and on chemically modified LPS, have been reported in previous publications[1-3]. The anomeric natures of some sugar residues were determined by polarimetric studies and for one LPS[4] from *S. muenster*, serogroup E_1, by isolation and characterisation of a trisaccharide.

The structure of the O-specific side-chains of the LPS from *Salmonella senftenberg* (1, 3, 19) belonging to group E_4 has been investigated by Staub and Girard[5], who used graded hydrolysis and thereafter isolated and characterised the oligosaccharides. As a result of these studies, the oligosaccharide repeating unit was assigned structure **1**. In the studies presented here, LPS isolated from the same bacteria has been investigated but different methods have been employed.



ISOLATION OF LPS

The bacteria were killed by γ-irradiation and disintegrated, and the cell-wall material was collected by centrifugation[1]. The LPS was isolated from the cell walls by extraction with phenol–water[1]. The presence of O-factors 1, 3, and 19 was demonstrated both for the bacteria and for the isolated LPS by slide agglutination and passive hemagglutination tests[1].

On acid hydrolysis, the LPS yielded L-rhamnose, D-mannose, D-galactose, D-glucose, and heptose in the relative proportions 19:19:29:24:9. The sugars were not isolated in the present study but were analysed, as their alditol acetates, by g.l.c.[6], and their identities confirmed by mass spectrometry[7] (m.s.). The sugars have been fully identified in previous investigations[5].

O-Acetyl groups are present[8] in the polysaccharide part of several *Salmonella* LPS. On treatment with methanolic hydrogen chloride, no methyl acetate was formed, as evident by g.l.c., demonstrating the absence of *O*-acetyl groups in the LPS from *S. senftenberg*.

METHYLATION ANALYSIS

The LPS was methylated in methyl sulphoxide by treatment with methylsulphinyl sodium and methyl iodide[9]. After hydrolysis, the methylated sugars were converted into alditol acetates and analysed by g.l.c.[10]–m.s.[11]. A typical chromatogram is given in Fig. 1, and the results are summarised in Table I.

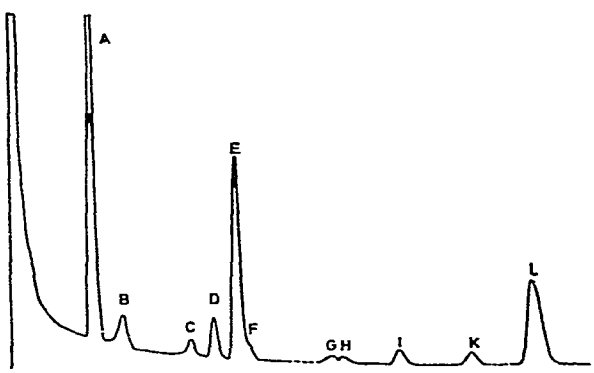


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated lipopolysaccharide.

Peak A (T = 1), from its m.s., was a mixture of products derived from 6-deoxy-2,3-di-*O*-methylhexoses and 2,3,4,6-tetra-*O*-methylhexoses. The components in this peak were resolved on an OV 225 SCOT column, demonstrating the presence of the alditol acetates derived from 2,3-di-*O*-methyl-L-rhamnose (T 0.91), 2,3,4,6-tetra-*O*-methyl-D-mannose (T 0.97), and 2,3,4,6-tetra-*O*-methyl-D-glucose (T 1.00).

On the basis of the m.s., all other peaks appeared to contain single components, which could be unambiguously identified from their T-values, m.s., and the knowledge of the parent sugars in the LPS.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED LIPOPOLYSACCHARIDE (*A*) AND OF PARTIALLY DEGRADED LIPOPOLYSACCHARIDE (*B*)

Sugars	*Peak*	T[a]	*Molar proportion* (%)	
			A	B
2,3-Di-*O*-methyl-L-rhamnose	A	1.00	18.7	18.7
2,3,4,6-Tetra-*O*-methyl-D-glucose			12.8	13.8
2,3,4,6-Tetra-*O*-methyl-D-mannose			1.9	2.0
2,3,4,6-Tetra-*O*-methyl-D-galactose	B	1.26	1.8	13.6
3,4,6-Tri-*O*-methyl-D-glucose	C	1.96	1.3	—
2,4,6-Tri-*O*-methyl-D-galactose	D	2.22	4.8	—
2,3,4-Tri-*O*-methyl-D-mannose	E	2.46	17.0	21.2
3,4,6-Tri-*O*-methyl-D-galactose	F	2.51	1.6	—
2,3,4-Tri-*O*-methyl-D-galactose	—	3.41	—	6.3
2,6-Di-*O*-methyl-D-galactose	G	3.68	0.8	—
2,6-Di-*O*-methyl-D-glucose	H	3.82	0.8	—
3,6-Di-*O*-methyl-D-glucose	I	4.36	1.7	—
2,4-Di-*O*-methyl-D-glucose	K	5.10	1.7	—
2,4-Di-*O*-methyl-D-galactose	L	6.35	12.8	3.1

[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.

Mild hydrolysis of the LPS with acid resulted in preferential cleavage of the L-rhamnosidic linkages. The poly- and oligo-meric material was recovered and subjected to methylation analysis. The results show an increase in 2,3,4,6-tetra-*O*-methyl-D-galactose, the appearance of a new component, 2,3,4 tri-*O*-methyl-D-galactose, and a decrease in 2,4-di-*O*-methyl-D-galactose, thereby demonstrating that L-rhamnose is linked to the 3-position of D-galactose in the original LPS.

ANOMERIC NATURE OF THE SUGAR RESIDUES

Lipid-free polysaccharide was prepared from the LPS by hydrolysis[12] with 1% aqueous acetic acid for 1 h at 100°. This material was treated with 0.25M sulphuric acid at 80°, and the change in optical rotation was followed (Fig. 2). The initial increase in rotation demonstrates that the L-rhamnosidic linkages, being the most readily hydrolysed, are α-linked.

A larger amount (100 mg) of lipid-free polysaccharide was hydrolysed under the same conditions for 5 h and the oligosaccharide mixture obtained was fractionated on a Sephadex G-25 (superfine) column (Fig. 3). The material in the tetrasaccharide region (9.0 mg) moved as a single spot on paper chromatography in two different systems. A hydrolysate of the tetrasaccharide contained D-glucose, D-galactose, D-mannose, and L-rhamnose in the relative proportions 1:0:1.0:1.0:1.0. The material was first reduced with borodeuteride, hydrolysed, reduced with borohydride, and acetylated. Only the L-rhamnitol contained deuterium, demonstrating that L-rhamnose constitutes the terminal reducing sugar in the tetrasaccharide. In the sugar analysis,

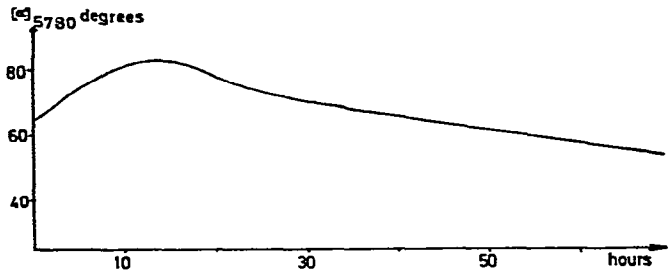


Fig. 2. Optical rotation *versus* time on acid hydrolysis of lipopolysaccharide.

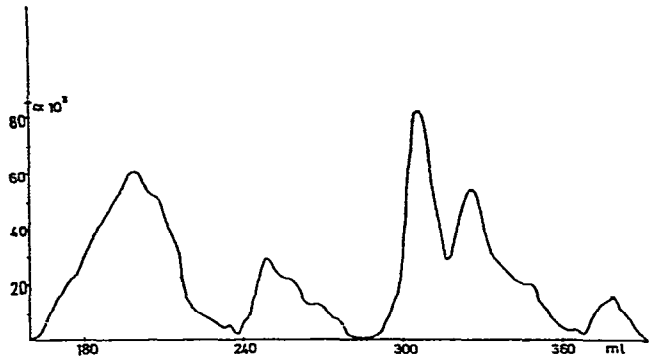
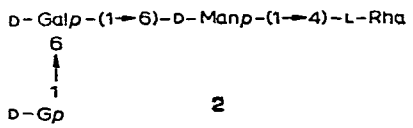


Fig. 3. Separation of oligosaccharides by gel filtration on Sephadex G-25 superfine.

D-arabinose was used as internal standard, and the total amount of tetrasaccharide was estimated as 8.0 mg. In accordance with the results of the methylation analysis, discussed in more detail below, these results demonstrate that the tetrasaccharide has structure **2**.



2

The 60 MHz n.m.r. spectrum of the fully trimethylsilylated tetrasaccharide alditol revealed, *inter alia*, three signals of similar intensities at τ 5.12 (J 1 Hz), 5.40 (J 3 Hz), and 5.58 (J 1 Hz), which were assigned to the three anomeric protons. The signal at τ 5.12 (J 3 Hz) is tentatively assigned to the anomeric proton of the D-galactose residue, and the signal at 5.58 (J 1 Hz) to the D-mannose residue, by comparison with the chemical shifts and coupling constants of the anomeric protons of the non-reducing sugar residues of fully trimethylsilylated melibiose (τ 5.10 J 3 Hz) and the trisaccharide α-D-Gal*p*-(1→6)-β-D-Man*p*-(1→4)-L-rhamnitol obtained from the LPS of *S. muenster*[4], respectively. The signal at τ 5.40 (J 3 Hz) should then originate from the anomeric proton of D-glucose. No relevant reference was available, but the shape of the peak is typical for an α-D-glucopyranose residue. More important, however, is that none of the signals showed a large coupling constant ($J \simeq 8$ Hz), typical for a β-linked D-glucose or D-galactose residue, demonstrating that both these

residues are α-linked in the tetrasaccharide. It is difficult, from the n.m.r., to determine the anomeric nature of the D-mannose residue.

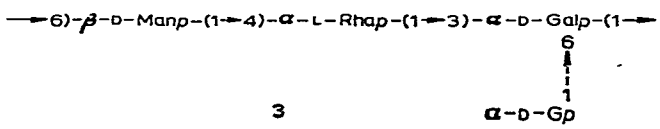
By using Hudson's rules of isorotation, the specific optical rotation of the all-α-D-linked tetrasaccharide and its alditol may be estimated as $[\alpha]_D + 132°$ (water) for both substances. If it is assumed that the D-mannose residue is β-linked, both values are reduced to +99°, in reasonable agreement with the observed values, $[\alpha]_D + 84°$ and +100°, and it is concluded that the D-mannose residue is β-linked.

DISCUSSION

With the exception of L-rhamnose, the pyranosidic nature of the sugar residues is evident from the methylation analysis. The rate of hydrolysis of the L-rhamnosidic linkages was, however, much lower than expected for L-rhamnofuranosides, and it is concluded that the L-rhamnose residues are also pyranosidic.

The sites of attachment between the sugar residues are demonstrated by the methylation analysis. The methylation analysis of the partially hydrolysed LPS showed that L-rhamnose is linked to D-galactose. Part of the D-galactose (25%) occurs as chain residues, and, accepting the concept of the repeating unit, must consequently be linked to D-mannose residues. This should, of course, also be true for the remaining 75%, which are substituted at C-6 by a terminal D-glucose residue.

The presence of 2,3,4,6-tetra-*O*-methyl-D-mannose demonstrates that the O-specific side-chains and the biological repeating unit are terminated by a D-mannose residue and that the average number of repeating units in the side chain is *ca.* 10. These results, together with the elucidation of the anomeric nature of the sugar residues, enable the structure of the biological repeating unit to be formulated (structure **3**). In this structure, the dotted line indicates those linkages that are only present in some repeating units. The conclusions of Staub and Girard[5] have thus been confirmed and elaborated.



The minor components in the methylation analysis are most probably derived from the core, and were found, in larger amount, on methylation analysis of LPS lacking the O-specific side-chains (R-mutants) or having a side-chain consisting of a single oligosaccharide residue only (SR mutants)[12].

Apart form the minor components originating from the basal core[12], a small proportion of 2,6-di-*O*-methyl-D-galactose (0.8%) was found. This indicates that, in some of the repeating units, D-galactose is substituted with a D-glucose residue in the 4-position. This structural feature could confer either of two sereological specificities to the bacteria *S. senftenberg*[14].

In serogroup A, B, and D_1, α-D-glucopyranose linked 1→4 to D-galactose residues confers $O12_2$ specificity to the bacteria, and in serogroup E_2 it confers O34

specificity. A weak cross-reaction between *S. senftenberg* bacteria and anti O34 factor serum was observed, whereas none was detected toward anti $O12_2$ factor serum. In serogroup A, B, and D_1, α-D-glucopyranose can be either (1→4) or (1→6)-linked to D-galactose in the repeating units, giving $O12_2$ and O1 specificity, respectively, to the bacteria. Both specificities may or may not be present in the bacteria, as demonstrated by immunological methods[15]. This phenomenon is called form variation and has not previously been observed in serogroup E_4.

The agreement between the sugar analysis and the methylation analysis is not very good, and a considerable part of the D-galactose (7%) and D-glucose (6%) is not accounted for in the latter analysis. The most likely explanation for this discrepancy seems to be that some of the basal core units in the LPS, which are composed of D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose residues[12], are not substituted with O-specific side-chains and are lost during dialysis of the methylated polysaccharide. Conclusions on the structural determination of the O-specific side-chains would be unaffected by this.

EXPERIMENTAL

The methods were the same as those used in the investigation of the *Salmonella muenster* LPS[4].

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