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Structural Studies on the O-Specific Side-chains of the Cell-wall Lipopolysaccharide from *Salmonella paratyphi A* var. *durazzo*

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The structure of the O-specific side-chains of the cell-wall lipopolysaccharide (LPS) of *S. paratyphi A* var. *durazzo* has been investigated. Methylation analyses of the original LPS, of the material obtained on mild hydrolysis of the LPS with acid, and of a product obtained by acetalation of all of the free hydroxyl groups in the LPS, have provided the essential information in this study. The mixtures of sugars obtained were analysed, as alditol acetates, by GLC-mass spectrometry. A trisaccharide isolated after acid hydrolysis of the LPS was isolated and identified. As a result of these studies, a detailed structure of the repeating unit of these side-chains is presented.

Studies on the O-specific side-chains of the *Salmonella* serogroup B and D_1 lipopolysaccharides (LPS)[1] have demonstrated that they have closely similar structures. The most important difference is that whereas in the B-group the D-mannose unit in the oligosaccharide repeating unit is substituted at C-3 by an α-abequose (3,6-dideoxy-D-*xylo*-hexose) residue, in the D_1 group this position is occupied by an α-tyvelose (3,6-dideoxy-D-*arabino*-hexose) residue. The structure of the O-specific side-chains of *Salmonella* serogroup A LPS, which contain the 3,6-dideoxy-hexose, paratose, having the D-*ribo*-configuration, has not been determined. The O-factors 1_{12} and 12_2 are present in serogroup A as well as in serogroups B and D_1. The relationship between the groups is also reflected by the fact that lysogenization with the P 22 or ϕ 27 bacteriophages results in the presence of common factors.[2,3]

In the present communication, structural studies on the O-specific side-chains of *S. paratyphi A* var. *durazzo* I.S. 2 (2, 12_1, 12_3) are reported. The methods used in these studies are essentially the same as used in previous investigations of other *Salmonella* LPS.[4,6] Less extensive studies on the LPS from *S. paratyphi A* I. S. 256 ($2,12_2$) are briefly reported.

ISOLATION OF THE LIPOPOLYSACCHARIDE

Bacteria from *S. paratyphi A* variant *durazzo* ($2,12_1,12_3$) were killed by γ-irradiation, disintegrated, the cell-wall material collected, and the LPS extracted by the phenol-water method.[4] The presence of O-factors 2 and 12_3 was demonstrated both for the bacteria and for the isolated LPS by slide agglutination and hemagglutination tests, respectively.

Hydrolysis of the LPS yielded paratose, L-rhamnose, D-mannose, D-galactose, D-glucose, and heptose in the relative proportions 16:22:22:31:7:3. The sugars were not isolated in the present study, but were analysed by GLC, as their alditol acetates.[7] Their identity was confirmed by mass spectrometry (MS).[8] These sugars were fully characterized in previous studies.[9]

As part of the paratose was probably destroyed during the acid hydrolysis, it is conceivable that the O-specific side-chains of the LPS contain equimolar parts of 3,6-dideoxy-hexose, L-rhamnose, D-mannose, and D-galactose, as has been found for the serogroups B and D_1 LPS.[4,5]

The presence of *O*-acetyl groups in the LPS was indicated by an IR absorption at 1738 cm^{-1} and was confirmed by methanolysis of the LPS and identification of the methyl acetate formed by GLC–MS.

METHYLATION ANALYSIS

The LPS was methylated by treatment with methylsulphinyl sodium and methyl iodide in methyl sulphoxide,[10] hydrolysed, the mixture of methylated

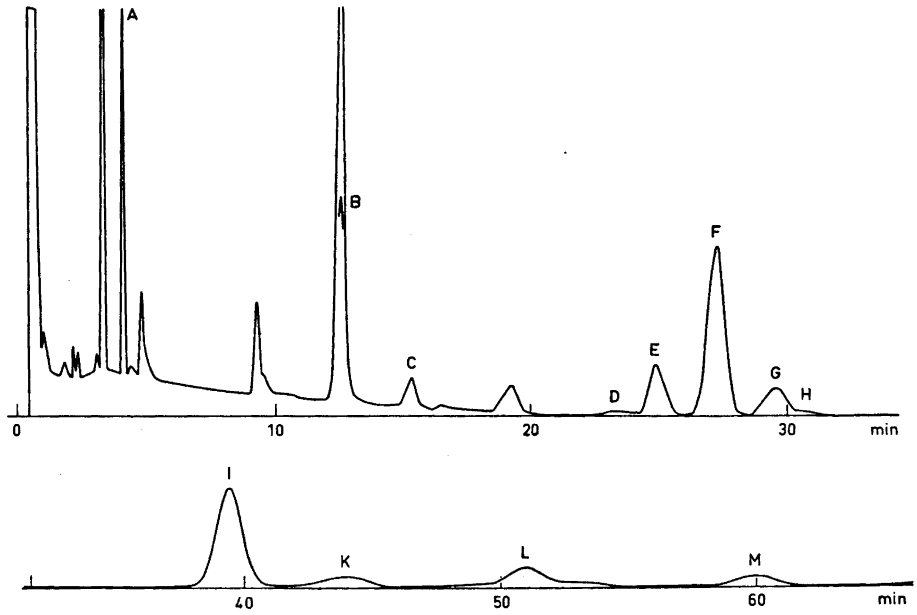

Fig. 1. GLC separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated lipopolysaccharide.

sugars converted into alditol acetates, and analysed by GLC-MS.[11,12] A typical chromatogram is shown in Fig. 1, and the results are summarised in Table 1.

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

Sugars	Peak	T^a	Mol%[b]
2,4-Di-*O*-methylparatose	A	0.34	6.9
2,3-Di-*O*-methyl-L-rhamnose	B	0.98	21.6
2,3,4,6-Tetra-*O*-methyl-D-glucose	B	1.00	1.5
2,3,4,6-Tetra-*O*-methyl-D-galactose	C	1.25	1.2
2,4,6-Tri-*O*-methyl-D-mannose	D	2.08	5.2
2,4,6-Tri-*O*-methyl-D-galactose	E	2.28	20.0
3,4,6-Tri-*O*-methyl-D-galactose	F	2.50	3.5
2,3,4-Tri-*O*-methyl-D-glucose	G	2.60	0.6
4,6-Di-*O*-methyl-D-mannose	H	3.30	16.9
2,6-Di-*O*-methyl-D-galactose	I	3.65	1.7
3,6-Di-*O*-methyl-D-glucose	K	4.30	3.5
2,4-Di-*O*-methyl-D-glucose	L	5.10	2.4

[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.

[b] As a considerable proportion of 2,4-di-*O*-methylparatose was lost during the methylation analysis, the mol % of the methylated sugars are given relative to that of 2,3-di-*O*-methyl-L-rhamnose, which, it is assumed, represents the mol % of L-rhamnose in the original lipopolysaccharide.

The two components in peak B, which were not resolved on the ECNSS-M column, were separated on a silicon (OV-225) S.C.O.T. column (*T* 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol 1.00, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol 0.97, and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol 0.91).

Most of the methylated derivatives were the same as found in the methylation analyses of groups B[4] and D[5] LPS, and the identification of these will not be discussed here.

The MS of the component in peak A corresponded to a 3,6-dideoxy-2,4-di-*O*-methyl-hexose derivative, presumably that of paratose, since this is the only 3,6-dideoxy-hexose in the LPS.

The component in peak K (*T* 4.30) was identified from its MS as a 3,6-di-*O*-methyl-hexose derivative, either from D-glucose (*T* 4.40) or D-galactose (*T* 4.35). 3,6-Di-*O*-methyl-D-glucose has previously been found in methylation studies on different R- and SR-mutants[13] and thus derives from the basal core, which is assumed to be the same in all *Salmonella* LPS. It seems most probable that the component in peak K is thus the D-glucose derivative. In previous studies on *Salmonella* LPS,[5,6,14,15] a minor peak with this *T*-value was assumed to contain the D-galactose derivative – a conclusion which is most probably erroneous.

From the methylation analysis, the molar proportions of paratose, L-rhamnose, D-mannose, D-galactose, and D-glucose are 7:22:22:26:8. In this analysis, it is assumed that the percentage of 2,3-di-*O*-methyl-L-rhamnose (the only L-rhamnose derivative) is the same as that of L-rhamnose in the sugar

analysis. A low value for paratose is expected, as part of the volatile 2,4-di-*O*-methyl-paratose and derivatives were evidently lost during concentrating. The values for D-mannose and D-glucose are in good agreement with those from the sugar analysis, that of D-galactose, however, is considerably lower. The probable explanation for this discrepancy is either that the D-galactose peak in the sugar analysis also contained some unknown material or that some D-galactose containing contaminant in the LPS was lost during the methylation analysis, probably during dialysis of the methylated LPS.

A sample of the LPS was subjected to a mild hydrolysis with acid which, as anticipated, caused preferential cleavage of all the paratosidic and part of the L-rhamnosidic linkages. The oligo and polymeric material was recovered and subjected to methylation analysis as above. From these results (Table 2) the mutual order of some sugar residues in the LPS may be deduced, as will be discussed below.

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

Sugars	T^a	Mol%[b]
2,3-Di-*O*-methyl-L-rhamnose	0.98	21.6
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	4.5
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	1.5
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	9.4
3,4,6-Tri-*O*-methyl-D-mannose	1.95	17.9
2,4,6-Tri-*O*-methyl-D-galactose	2.28	12.5

[a,b] See Table 1.

DETERMINATION OF THE POSITION OF THE *O*-ACETYL GROUPS

The LPS was treated with methyl vinyl ether,[16] to protect all free hydroxyl groups as acetals, and the modified LPS was subjected to methylation analysis. A mixture of sugars and methylated sugars was obtained, the methoxyl groups in the latter marking the positions of the original *O*-acetyl groups. In addition to the sugars, found in the sugar analysis, a new component (T 2.00) was observed, which from its T-value and MS was identified as a 3-*O*-methyl-L-rhamnose derivative. This compound accounted for about 20 % of the total L-rhamnose content.

ACID HYDROLYSIS

Lipid free polysaccharide was prepared by treating the LPS with 1 % acetic acid at 100° for 1 h.[17] A solution of the polysaccharide in 0.25 M sulphuric

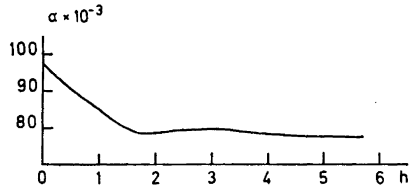

Fig. 2. Optical rotation *versus* time during acid hydrolysis of the polysaccharide.

acid was kept at 80°, and the change in optical rotation was followed polarimetrically (Fig. 2). The shape of the curve is similar to that obtained on acid hydrolysis of the *S. typhi* and *S. enteritidis* LPS[5] and, using the same arguments presented in the earlier work, indicates that the paratose residues and the L-rhamnose residues are α-linked.

Another part of the lipid free polysaccharide was hydrolysed by treatment with 0.25 M sulphuric acid at 100° for 5 h. The oligosaccharides obtained were fractionated on a Sephadex G 25 (superfine) column.

A pure component, eluted in the trisaccharide region was isolated. This, on reduction with sodium borodeuteride, acid hydrolysis, reduction with sodium borohydride and acetylation, yielded the acetates of L-rhamnitol, D-mannitol, and D-galactitol in equimolecular proportions. GLC-MS revealed that only the L-rhamnitol was labelled with deuterium. Consequently, the reducing terminal of the trisaccharide consists of an L-rhamnose residue. The analysis was performed using L-arabinose as internal standard, whereby the concentration of trisaccharide in an aliquot and hence its specific rotation, $[\alpha]_{578}^{20} + 115°$, could be determined. This is in good agreement with the calculated value, +112°, derived using Hudson's rules of isorotation, and assuming that both the D-galactose and the D-mannose residue are α-linked. The insertion of a β-linked residue would lead to considerably lower values, *e.g.* +55° for an α-D-galactose and a β-D-mannose residue. Even if the calculations are not very accurate, the results clearly indicate that both the D-mannose and the D-galactose residue are α-linked in the trisaccharide.

DISCUSSION

Both the sugar and the methylation analyses support the assumption that the O-antigenic side chains in the *S. paratyphi A* var. *durazzo* LPS are composed of tetrasaccharide repeating units, composed of paratose, D-mannose, L-rhamnose, and D-galactose. The methylation analysis revealed that, with the exception of L-rhamnose, these sugars were pyranosidic. An L-rhamnofuranosidic linkage should, however, be more readily hydrolysed than was observed, why this possibility was excluded.

The methylation analysis also demonstrates that the paratose residue is terminal, that the L-rhamnose and the D-galactose occur preponderantly as chain residues, linked at the 4- and 3-positions, respectively, and that the D-mannose is mainly present as a branched residue, linked at the 2- and 3-positions.

Since most of the D-mannose occurs as branched residues, linked through C-2 and C-3 in the original LPS, and as chain residues, linked through C-2 in the product resulting from the mild acid hydrolysis, it is concluded that the terminal paratose residues are linked to D-mannose at the 3-position. The fact that a considerable portion of terminal D-galactose was found in the hydrolysed product shows that many L-rhamnose residues, originally linked to D-galactose, have been hydrolysed. Only the sequence given in Fig. 3 is therefore possible, unless a less-ordered structure or a longer repeating unit is considered.

The presence of similar and small amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,6-di-*O*-methyl-D-galactose implies that some of the repeating

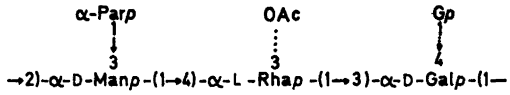

Fig. 3. Proposed structure for the repeating unit of the O-specific side-chains in the *Salmonella paratyphi A* var. *durazzo* lipopolysaccharide.

units carry a terminal D-glucose residue linked to D-galactose at the 4-position. This structural feature should confer O 12_2 specificity to the bacteria.[18] When LPS of *S. paratyphi A* var. *durazzo* was tested in concentrations up to 256 μg/ml, no inhibition of the passive hemagglutination system *S. typhimurium* 395 MS LPS/anti O 12_2 factor serum could be demonstrated. In fact, *S. paratyphi A* var. *durazzo* is the strain recommended for production of anti O 12_3 factor serum, since it lacks O 12_2 specificity.

The 2,4,6-tri-*O*-methyl-D-mannose found in the methylation analysis of original LPS is almost certainly derived from the 3-*O*-paratosyl-D-mannose residues of the terminal repeating unit of the *O*-specific side-chains. In the methylation analysis of degraded LPS, this ether corresponds to 2,3,4,6-tetra-*O*-methyl-D-mannose, which is of the same order of magnitude. The "biological" oligosaccharide repeating unit therefore starts with D-galactose; similar conclusions have also been reached for the groups B, C_2, C_3, D_1, D_2 and E LPS. The percentage of 2,4,6-tri-*O*-methyl-D-mannose, which is expected to be the same as those of the methyl ethers derived from the basal core [13] is, in fact, considerably higher. This may be due to contamination of the LPS with hapten. The average number of oligosaccharide repeating units in the side chains, 4, calculated from the methylation analysis, must therefore be regarded as a lower limit.

Part of the repeating units carry *O*-acetyl groups, linked to L-rhamnose at C-3.

The anomeric nature of the sugar residues, as presented in Fig. 3, is based on the hydrolysis studies and the characterisation of the trisaccharide α-D-galactopyranosyl-(1→2)-α-D-mannopyranosyl-(1→4)-L-rhamnose, obtained on partial hydrolysis.

As expected, the structure of the repeating unit resembles those of the serogroups B and D LPS. The main difference is of course that the α-linked paratose residue, associated with the presence of O-factor 2, replaces abequose or tyvelose at the 3-position of D-mannose. The distribution of the *O*-acetyl-groups, present in some of the repeating units, differs from that of the LPS from serogroups B and D_1.

Similar studies on the LPS extracted from *S. paratyphi A* I.S. 256 ($2,12_2$) showed the same basal structure for the repeating unit of the O-specific side-chains.

EXPERIMENTAL

The methods were essentially the same as those used in our previous investigation on *S. newport* and *S. kentucky*.[6]

Paper chromatography. Whatman 1 papers were used in the two systems: ethyl acetate-acetic acid-water, 3:1:1, 15 h; and butanol – pyridine – water, 6:4:3, 70 h.

Gel filtration. The column (98 × 2.5 cm) was packed with a Sephadex (G-25 super fine) gel and irrigated with distilled water (5 ml/h).

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