Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

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

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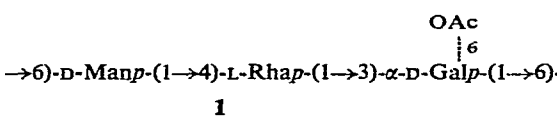
(Received June 8th, 1970; accepted for publication, June 29th, 1970)

ABSTRACT

The structure of the O-specific side-chains of the cell-wall lipopolysaccharide (LPS) of *Salmonella muenster* has been investigated by methylation analysis of undegraded LPS and of a material obtained from it on graded hydrolysis, and by partial hydrolysis studies, including the isolation and characterisation of a trisaccharide. As a result of these studies, a detailed structure of the biological repeating unit in these side chains is presented.

INTRODUCTION

The structure of the O-specific side-chains in the LPS from *S. anatum* (3,10) serogroup E_1, has been extensively studied by Robbins and co-workers[1,2]. These studies were essentially based on partial hydrolysis with acid, followed by the isolation and identification of oligosaccharides, and on periodate oxidation. They were later supplemented by immunochemical and biosynthetic studies[3]; structure **1** was thus proposed for the biological repeating unit of the O-specific side-chains.



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O-Factor 3 was correlated with the D-mannose residues linked to C-4 of L-rhamnose residues; O-factor 10 was correlated with the *O*-acetyl groups, tentatively located at C-6 in the D-galactose residues.

In our methylation studies of different *Salmonella* LPS[4–7], we have used g.l.c.–mass spectrometry (m.s.) as the main analytical tool. This method gives quantitative results, and is, in this respect, superior to other methods used in structural polysaccharide chemistry. It does not, however, give information on the anomeric nature of the sugar residues, which must be determined by other methods. In the present communication, the results are reported of an investigation of the LPS from *S. muenster* SH 1654 (3,10), using methylation analysis and partial hydrolysis studies.

METHODS AND RESULTS

Isolation of the LPS. — *S. muenster* SH 1654 was obtained from the collection of P. H. Mäkelä, State Serum Institute, Helsinki, Finland. The bacteria were killed by γ-irradiation and disintegrated, and the cell-wall material was collected and extracted by the phenol–water method[4], to yield the pure LPS. The presence of O-factor 3 was demonstrated, both in the bacteria and in the isolated LPS, by slide agglutination and passive hemagglutination inhibition[4], respectively. O-Factor 10 activity, however, could only be demonstrated for the intact bacteria.

A hydrolysate of the LPS yielded L-rhamnose, D-mannose, D-galactose, D-glucose, and a heptose in the relative proportions 28:28:32:3:8. The sugars were analysed by g.l.c. as their alditol acetates[8], the identifications being confirmed by m.s.[9]. These sugars have been fully characterized by earlier investigators[1].

On treatment of the LPS with methanolic hydrogen chloride, no methyl acetate was formed, demonstrating the absence of *O*-acetyl groups. This result was confirmed by subjecting the polysaccharide to acetalation, methylation, and acid hydrolysis[10], whereby the original sugars but no partially methylated sugars were detected.

Methylation analysis of the LPS. — The LPS was methylated, in methyl sulphoxide, by treatment[11] with methylsulphinyl sodium and methyl iodide. The fully methylated material was hydrolysed, and the methylated sugars were converted into their alditol acetates and analysed by g.l.c.[12]–m.s.[13]. A typical chromatogram, on an ECNSS-M column, is given in Fig. 1, and the results of the analysis are summarised in Table I. All components were identified by their retention times (*T* values) and by m.s. Most of the methylated sugars were also found and characterised earlier in the analysis of LPS from *S. strasbourg*[7]; the identification of these components will therefore not be discussed here.

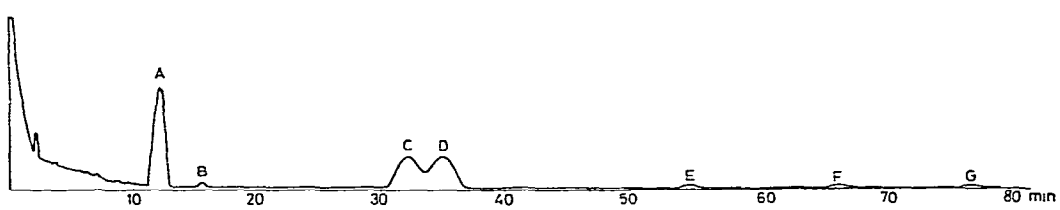


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated *S. muenster* LPS.

The m.s. of the material in peak *A* (*T* 1.0) demonstrated the presence of alditol acetates derived both from a 6-deoxy-2,3-di-*O*-methylhexose (L-rhamnose) and a 2,3,4,6-tetra-*O*-methylhexose. The latter could be either a D-glucose or a D-mannose derivative, as these are not separated on the ECNSS-M column. The three components can be separated on a silicon (OV-225) S.C.O.T.–column (*T* 0.91, 1.00, and 0.97, respectively), and on such a column the presence of the D-mannose and the D-glucose derivatives was demonstrated.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED LIPOPOLYSACCHARIDES

	Peak	T[a]	*Molar proportion, %*
2,3-Di-*O*-methyl-L-rhamnose	*A*	0.98	28.0
2,3,4,6-Tetra-*O*-methyl-D-mannose	*A*	1.00	3.3
2,3,4,6-Tetra-*O*-methyl-D-glucose	*A*	1.00	1.8
2,3,4,6-Tetra-*O*-methyl-D-galactose	*B*	1.25	2.1
2,4,6-Tri-*O*-methyl-D-galactose	*C*	2.28	27.4
2,3,4-Tri-*O*-methyl-D-mannose	*D*	2.48	26.0
3,4,6-Tri-*O*-methyl-D-galactose	*D*	2.50	1.6
2,6-Di-*O*-methyl-D-galactose	*E*	3.65	1.0
3,6-Di-*O*-methyl-D-glucose	*F*	4.35	1.6
2,4-Di-*O*-methyl-D-glucose	*G*	5.10	1.5

[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.

The component (26.0%) in peak *D* exhibited a m.s. of a 2,3,4-tri-*O*-methylhexose derivative. The *T* values for the corresponding D-glucose, D-mannose, and D-galactose derivatives are 2.49, 2.48, and 3.41, respectively. Since the total content of D-glucose residues in the LPS is only 3%, and since this amount is already accounted for in other peaks, the main component in this peak must be the D-mannose derivative.

The molar percentages of L-rhamnose, D-mannose, D-galactose, and D-glucose, calculated from the methylation analysis, are in good agreement with the results of the sugar analysis.

Methylation analysis of partially degraded LPS. — The LPS was subjected to a mild hydrolysis with acid, which was expected to cause preferential cleavage of the L-rhamnosidic linkages. The oligomeric and polymeric material was recovered and subjected to methylation analysis as above (Table II).

TABLE II

METHYL ETHERS FROM THE HYDROLYSATE OF PARTIALLY DEGRADED AND METHYLATED LIPOPOLYSACCHARIDES

	T[a]	*Molecular proportion, %*
2,3-Di-*O*-methyl-L-rhamnose	0.98	28.0
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	20.2
2,4,6-Tri-*O*-methyl-D-galactose	2.28	9.9
2,3,4-Tri-*O*-methyl-D-mannose	2.48	28.0

[a]See Table I.

The increase of 2,3,4,6-tetra-*O*-methyl-D-galactose, and the corresponding decrease in 2,4,6-tri-*O*-methyl-D-galactose, relative to the results from the methylation analysis for the original LPS, demonstrated that L-rhamnose is linked to D-galactose.

Acid hydrolysis studies. — A solution of the lipid-free polysaccharide[15] in 0.25M sulphuric acid was kept at 80°, and the change in optical rotation was followed polarimetrically (Fig. 2). Since L-rhamnosidic linkages are those most readily hydrolysed in this polysaccharide, the positive increase in optical rotation indicates that they are α-linkages.

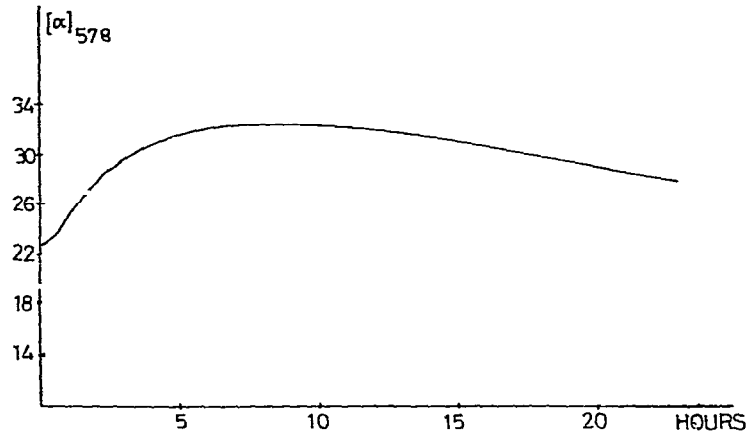


Fig. 2. Optical rotation *versus* time on acid hydrolysis of the *S. muenster* LPS.

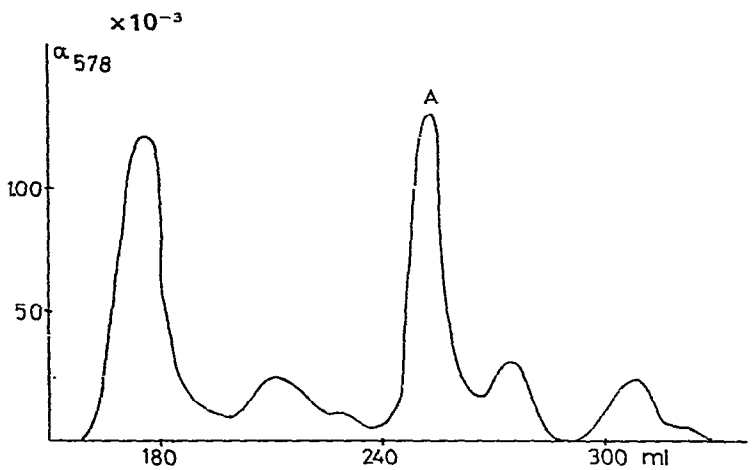


Fig. 3. Gel filtration on a Sephadex G-15 column of a 5-h hydrolysate of *S. muenster* LPS.

A larger amount of polysaccharide (100 mg) was hydrolysed as above, and the reaction was interrupted after 5 h. After neutralisation, the material was separated by gel filtration on a Sephadex G-15 column (Fig. 3). The component in the main peak *A* (15 mg) was homogenous on paper chromatography in two solvent systems and had the mobility expected for a trisaccharide.

The trisaccharide was converted into its alditol ($[\alpha]_D$ +50°) by borohydride

reduction (yield 13.5 mg). Part of this material was hydrolysed, and reduced by sodium borodeuteride, and the alditol mixture was analysed by g.l.c.–m.s. as their acetates. The relative proportions of L-rhamnitol, D-mannitol, and D-galactitol were 1.0:1.0:1.0. L-Arabinose, added as an internal standard, showed that these sugars account for essentially 100% of the amorphous preparation of trisaccharide alditol. Mass spectrometry showed that D-mannitol and D-galactitol, but not L-rhamnitol, contained deuterium. This shows that L-rhamnose is at the reducing end of the trisaccharide and agrees with the expectation that the trisaccharide was formed by hydrolysis of L-rhamnosidic linkages in the polysaccharide.

The n.m.r. spectrum (Fig. 4) of the fully trimethylsilylated trisaccharide alditol showed, *inter alia*, two equally large peaks at τ 5.52 and 5.17, corresponding to the two anomeric protons. All other signals appeared at higher fields. The coupling constants were small for each signal ($\approx$1 Hz.). Accurate values for the coupling constants could not be determined, even when a time-averaging computer was used to improve the signal to-noise ratio, but larger coupling constants, as typical for diaxial protons, would certainly have been observed.

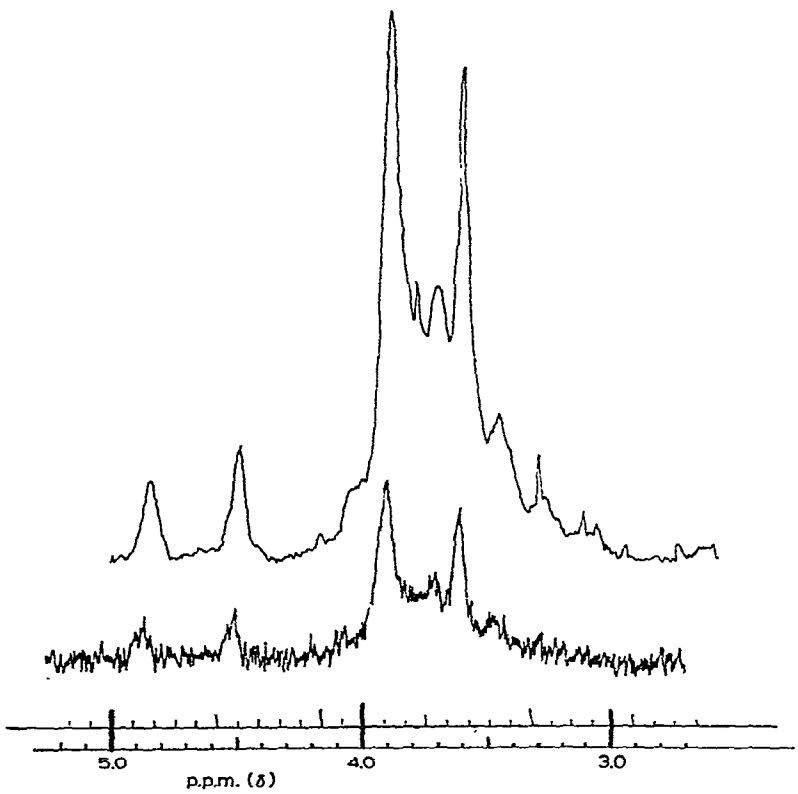
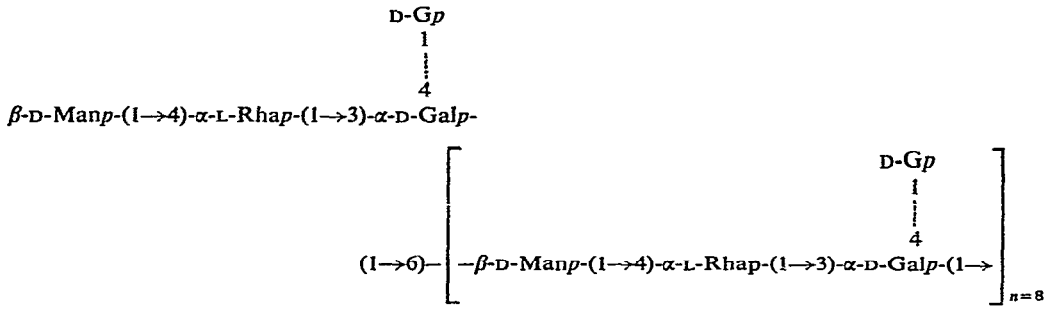


Fig. 4. N.m.r. spectrum of the fully trimethylsilyalted trisaccharide alditol of *S. muenster* LPS scanned one and twenty-five times, respectively.

DISCUSSION

From the present results, the structure **2** is proposed for the O-specific side-chains.



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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-rhamnose residue is also pyranosidic, as a furanosidic residue would have been more readily hydrolysed than was observed. From methylation analysis, the position to which the different sugar residues are substituted is obvious. The methylation analysis of the partially hydrolysed material shows that L-rhamnose is linked to D-galactose, and since there are only three sugar residues in the repeating unit their mutual order is readily evident.

The small proportion of 2,3,4,6-tetra-*O*-methyl-D-mannose observed in the methylation analysis of the LPS must derive from the terminal repeating unit. This finding indicates that D-mannose is the terminal residue in the repeating units which must therefore be 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 to be approximately 9.

The minor components, 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,6-di-*O*-methyl-D-galactose, found in the methylation analysis of the original LPS, indicate that a small part of the D-galactose residues in the side chains carry a terminal D-glucose residue in the 4-position. 2,6-Di-*O*-methyl-D-galactose does not originate from the basal core[14], but since it is sometimes difficult to estimate the structural significance of minor components, the presence of branched D-galactose residues should be regarded as tentative.

The other minor components obtained in the methylation analysis derive from the basal core[14] and have also been found in studies of other *Salmonella* LPS.

The investigation of the trisaccharide formed on partial, acid hydrolysis of the lipid-free polysaccharide, in conjunction with the results discussed above, demonstrate that it has the structure **3** and thus represents the "chemical" repeating unit of the O-specific side-chains.

D-Galp-(1→6)-D-Manp-(1→4)-L-Rhap

3

The anomeric natures of the D-galactopyranosidic and D-mannopyranosidic linkages were established from the optical rotation of the trisaccharide alditol and from the n.m.r. spectrum of its trimethylsilyl derivative. Neither of the signals at τ 5.52 and 5.17, assigned to the anomeric protons, showed the large coupling constant (6–8 Hz) expected for a β-D-galactopyranoside. In trimethylsilylated α-D-Gal*p*-(1→6)-D-G*p* (melibiose), the anomeric proton of D-galactose gives a signal at τ 5.10 and a coupling constant of 1 Hz. The corresponding value for β-D-Gal*p*-(1→6)-D-Gal*p* is τ 5.53 (*J* 6.5 Hz) (C. G. Hellerqvist *et al.* to be published.) It therefore seems reasonable to assume that the signal at τ 5.17 in the trisaccharide derivative is derived from the α-D-galactose residue, and the one at τ 5.52 from the D-mannose residue. This result gives no information on the anomeric nature of the D-mannopyranosidic residue, since both mannose anomers have approximately the same coupling constant.

The $[\alpha]_D$ value for an α-D-galactopyranosyl-α-D-mannopyranosyl-L-rhamnitol and an α-D-galactopyranosyl-β-D-mannopyranosyl-L-rhamnitol may be estimated as +115° and +55°, respectively. These values only give orders of magnitude, but the agreement between the latter value and that observed for the trisaccharide alditol, $[\alpha]_D$ +50°, strongly supports the assumption that the D-mannopyranose residue is β-linked. In one of their publications[2], Robbins and co-workers suggested that the D-mannose residue in the LPS of *S. anatum* (3,10) is α-linked, but experiments in support of this assumption have not been published.

The structure (**2**) of the "biological" repeating unit of the LPS from *S. muenster* (3,10) proposed by us and that proposed by Robbins and co-workers for the LPS of *S. anatum* (3,10) agree in most respects. Apart from a possible, but unlikely, difference in the anomeric nature of the mannosidic linkages, an important difference between the LPS of these two species is the presence of *O*-acetyl groups in *S. anatum* but not in *S. muenster*. Since both bacteria have O-factor 10, this discrepancy will be subjected to a more-detailed investigation.

EXPERIMENTAL

The methods used were essentially the same as in the investigation of the LPS from *Salmonella newport* and *S. kentucky*[6].

In the experiment on oligosaccharide isolation, the hydrolysate was neutralized with Dowex-3 (free base) ion-exchange resin. The material was concentrated to 2 ml and added to a column (97.5 × 2.5 cm) of Sephadex G-15 which was irrigated with distilled water (8 ml.h^{-1}). For paper chromatography, Whatman No. 1 paper was used with the solvent systems butyl alcohol–pyridine–water (6:4:3, 70 h) and ethyl acetate–acetic acid–water (3:1:1, 15 h).

The trimethylsilylation reaction was performed in dry pyridine (2 ml) with hexamethyldisilazane (0.4 ml) and chlorotrimethylsilane (0.2 ml) as silylation agents. After 5 min at 40°, the reaction mixture was concentrated to dryness, dissolved in carbon tetrachloride, and filtered into an n.m.r. tube. The n.m.r. spectra were recorded at 60 MHz on a Varian A-60 A spectrometer, fitted with a Varian C-1024 time-averaging computer device.

ACKNOWLEDGMENTS

We are indebted to Mr. O. Larm for running the n.m.r. spectra. The skilled technical assistance of Miss Gunnel Ljunggren and Mrs. Jana Cederstrand is acknowledged. This work was supported by grants from the Swedish Medical Research Council (to A.A.L. No. B 69-40 X-656-O 4 A and to B.L. No. B 69-13 X-2522-01) from the Swedish Natural Science Research Council, from Harald Jeanssons stiftelse, and from Sigurd and Elsa Goljes minne.

REFERENCES

1 P. W. ROBBINS AND T. UCHIDA, *Biochemistry*, 1 (1962) 323.
2 P. W. ROBBINS AND T. UCHIDA, *J. Biol. Chem.*, 1 (1965) 375.
3 P. W. ROBBINS, I. M. KELLER, A. WRIGHT, AND R. L. BERNSTEIN, *J. Biol. Chem.*, 240 (1965) 384.
4 C. G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohyd. Res.*, 8 (1968) 43.
5 C. G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Acta Chem. Scand.*, 23 (1969) 1588.
6 C. G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohyd. Res.*, 14 (1970) 17.
7 C. G. HELLERQVIST, B. LINDBERG, Å. PILOTTI, AND A. A. LINDBERG, *Acta Chem. Scand.*, 24 (1970) 1168.
8 J. S. SAWARDEKER, J. H. SLONEKER, AND A. R. JEANES, *Anal. Chem.*, 12 (1965) 1602.
9 O. S. CHIZHOV, L. S. GOLOVKINA, AND N. S. WULFSON, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1966) 1915.
10 A. N. DE BELDER AND B. NORRMAN, *Carbohyd. Res.*, 8 (1968) 1.
11 S. HAKOMORI, *J. Biochem.* (Tokyo), 55 (1964) 205.
12 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.
13 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohyd. Res.*, 5 (1967) 433.
14 C. G. HELLERQVIST AND A. A. LINDBERG, *Carbohyd. Res.*, 16 (1971) 39.
15 W. DRÖGE, O. LÜDERITZ, AND O. WESTPHAL, *Europ. J. Biochem.*, 4 (1968) 126.