ACTA CHEMICA SCANDINAVICA 25 (1971) 601–606

Fragmentation Analysis of the *Salmonella newport* Lipopolysaccharide

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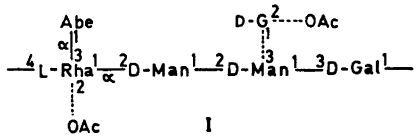
Fragmentation analysis of the lipopolysaccharide from *Salmonella newport* (6,8) has been employed to elucidate the structure of the oligosaccharide repeating unit of the O-specific side-chains. The anomeric nature of some of the glycosidic linkages has been investigated by NMR and optical rotation. From this and previous evidence, the detailed structure of the oligosaccharide repeating unit is proposed.

From the methylation analysis of the lipopolysaccharide (LPS) from *Salmonella newport* (6,8) and of chemically modified LPS, the structure of the biological repeating unit of the O-specific side-chains was formulated (see I). All sugar residues are pyranosidic. (The dotted lines indicate that only some of the repeating units contain these substituents.) The anomeric natures of the abequosidic and L-rhamnosidic linkages were determined by following the change in optical rotation on mild acid hydrolysis of the LPS.



I

The chemical studies did not reveal the mutual order of the "second", branched D-mannose residue and the D-galactose residue. The mutual order given in I was suggested by immunochemical evidence.[2]

In order to establish unequivocally the remaining structural features of the oligosaccharide repeating unit, the LPS has been subjected to graded acid hydrolysis, followed by isolation and characterisation of the oligosaccharides.

RESULTS

The LPS (150 mg) was treated with 0.1 % acetic acid at 100° for 1 h and worked up to give an essentially lipid-free polysaccharide (~100 mg).[8] This material was treated with 0.25 M sulphuric acid at 80° for 5 h, neutralised and the resulting mixture of oligosaccharides fractionated on a Sephadex G-25 column (Fig. 1). The fractionation was not complete and was therefore sup-

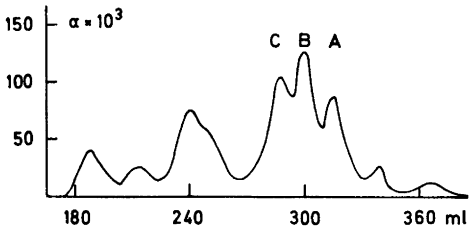
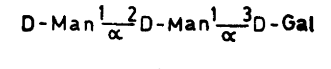


Fig. 1. Separation of the oligosaccharides obtained from the *S. newport* LPS on a Sephadex G-25 column.

plemented by paper chromatography. Three components were obtained pure, as shown by paper chromatography and subsequent analyses. One mixed fraction was also isolated and investigated as such.

The oligosaccharides were reduced to alditols with sodium borodeuteride, the alditols hydrolysed, the resulting mixture of sugars reduced with sodium borohydride and analysed, as the alditol acetates, by GLC.[3] The sugars containing deuterium and thus derived from the reducing terminal of the oligosaccharides were recognised by GLC-MS.[4] L-Arabinose was used as an internal standard and hence provided a basis for the calculation of specific optical rotations. Yields and properties of the oligosaccharides are given in Table 1.

The trisaccharide A_1 is a mannosylmannosylgalactose. The specific rotations of the trisaccharide and its alditol, $[\alpha]_D$ +102° and +77°, respectively, agree fairly well with the values, +90° and +61°, calculated using Hudson's rules of isorotation and assuming that both D-mannose residues are α-linked. If the original trisaccharide had contained one α- and one β-linkage values of +32° and +3°, respectively, would have been obtained. From this and previous[1] evidence, the structure A_1 given below is therefore proposed for the trisaccharide.

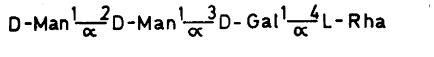


A_1

The tetrasaccharide B_1 contains one D-galactose and two D-mannose residues together with L-rhamnose which comprises the reducing terminal. The mutual order of the three hexoses should be the same as in A_1 and the tetrasaccharide may therefore be formulated as Man-Man-Gal-Rha. It has a surprisingly high paper chromatographic mobility and most probably contains an *O*-acetyl group linked to the L-rhamnose residue, a structural feature present in the oligosaccharide repeating unit.[1] The optical rotation of its

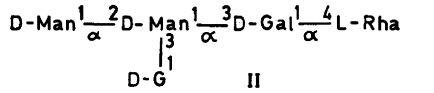
alditol, $[\alpha]_D$ +82°, lies between +112° and +51°, the values calculated for two α-D-mannosidic and one α-D-galactosidic or β-D-galactosidic linkage, respectively.

In the NMR spectrum of the fully trimethylsilylated alditol in carbon tetrachloride, the signals for the anomeric protons are well separated from other signals. A two-proton signal at τ 5.19, J 2 Hz, is assigned to the anomeric protons of the two D-mannose residues and a one-proton signal at τ 4.93, J 2 Hz, to the anomeric proton of the D-galactose residue. Even if these assignments were incorrect, all signals must represent anomeric protons and since none has the high coupling constant, J 7 – 8 Hz, observed for the anomeric proton in a β-D-galactopyranoside, it is concluded that the D-galactose residue is α-linked and that the tetrasaccharide has the structure B_1 given below



B_1

Fraction C_1 is a mixture of penta- and tetrasaccharides, and attempts to fractionate it further were not successful. Mass spectra from the alditol acetates revealed that only L-rhamnose is at the reducing end in these oligosaccharides. This is further supported by the methylation analysis on the reduced fraction C_1 (Table 2), where no penta-*O*-methyl-hexitol was detected. Since the methylation analysis afforded 4,6-di-*O*-methyl-D-mannose but no 2,4,6-tri-*O*-methyl-D-mannose, the terminal D-glucose residue is linked to the "second" D-mannose residue, in agreement with previous results, and the major component in fraction C_1 has structure II



II

A pure pentasaccharide, C_2, with a D-galactose residue as the reducing terminal, was also isolated. The results of the methylation and sugar analysis

Table 1.

Fraction	Yield mg	Paper chromatographic mobility [a] Solvent A	Solvent B	Sugar composition Rha:Man:Gal:Glu	$[\alpha]_D$ of oligosaccharide alditol
A_1	2.0	1.1	1.2	0:63:37:0	77°
B_1	2.0	–	1.8	25:50:25:0	82°
C_1	3.9	–	1.4 – 1.5	22:41:22:12	–
C_2	1.5	0.5	0.7	20:39:18:23	77°

[a] Relative to isomaltotriose.

Table 2. Methyl ethers from the hydrolysate of the methylated oligosaccharide alditol mixture C_1.

Sugars	T[a]	T^c	mol %[b]
1,2,3,5-Tetra-*O*-methyl-L-rhamnitol	–	–	3.0
2,3,4-Tri-*O*-methyl-L-rhamnose	0.46	–	6.1
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	0.97	13.1
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	1.00	10.7
3,4,6-Tri-*O*-methyl-D-mannose	1.95	–	15.8
2,4,6-Tri-*O*-methyl-D-galactose	2.28	–	14.8
4,6-Di-*O*-methyl-D-mannose	3.29	–	12.1

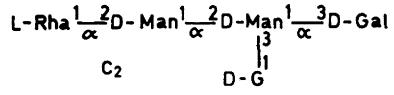
[a] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] As a considerable portion of 1,2,3,5-tetra-*O*-methyl-L-rhamnitol was lost during the methylation analysis, the percentages of the methylated sugars are given relative to the sum of the D-mannose derivatives, assumed to be the same as the percentage of D-mannose in the original oligosaccharide mixture.
[c] Retention times of the corresponding alditolacetates on the OV-225 S.C.O.T. column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Table 3. Methyl ethers from the hydrolysate of the methylated oligosaccharide alditol C_2.

Sugars	T^a	mol %[b]
1,2,4,5,6-Penta-*O*-methyl-D-galactitol	0.42	12.7
2,3,4-Tri-*O*-methyl-L-rhamnose	0.46	17.8
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	18.9
3,4,6-Tri-*O*-methyl-D-mannose	1.95	21.2
4,6-Di-*O*-methyl-D-mannose	3.29	17.8

[a] See Table 2.
[b] As part of 1,2,4,5,6-penta-*O*-methyl-D-galactitol was lost during the methylation analysis; the percentage of the methylated sugars are given relative to the sum of the D-mannose derivatives, assumed to be the same as the percentage of D-mannose in the original oligosaccharide.

of the pentasaccharide alditol (Table 3), in accordance with previous results, show that the pentasaccharide has the structure C_2 given below



The anomeric nature of the D-glucose residue was not established in these investigations. The optical rotation of the pentasaccharide alditol, is however, $[\alpha]_D$ +77°, and the corresponding calculated values, are for an α- or a β-glucosidic linkage +63° and +18°, respectively. The result therefore supports the assumption that the terminal D-glucose residue is α-linked, as previously suggested on immunochemical evidence.[5]

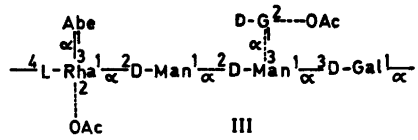
DISCUSSION

The oligosaccharide mixture obtained on hydrolysis of the *S. newport* LPS was more complicated than expected. Considerable losses therefore occurred during isolation of pure components and the yields reported are not representative of the amounts actually present in the hydrolysate.

Some of the *O*-acetyl groups in the O-specific side-chains were probably not removed during the hydrolysis and were present in the oligosaccharides (B_1, C_1), as indicated by their high paper chromatographic mobilities. This may have facilitated the isolation of pure B_1, although a simpler mixture would have been obtained had the *O*-acetyl groups been removed before the graded, acid hydrolysis.

The isolation of the pentasaccharide C_2 demonstrates that a considerable proportion of the D-galactosidic linkages has been hydrolysed. It was assumed that the α-L-rhamnosidic linkages would be the next most readily hydrolysed linkages in the O-specific side-chains (after the α-abequosidic), but the difference in rate of hydrolysis of these and the D-galactosidic seems to be smaller than expected. It is difficult to predict how the presence of an *O*-acetyl group in the 2-position of the L-rhamnose residue would influence the rate of hydrolysis of its glycosidic linkage.

The assignment of α-configuration to the L-rhamnose residue was based upon the change in optical rotation on mild acid hydrolysis. After an initial, rapid decrease, due to hydrolysis of α-abequosidic linkages, a weak maximum was observed and the rotation thereafter remained fairly constant. It thus appeared, that the increase in rotation, due to hydrolysis of the α-L-rhamnosidic linkages, was compensated, first by hydrolysis of remaining α-abequosidic linkages and later by hydrolysis of the hexosidic linkages, which resumably had the α-configuration. The fact that the α-D-galactosidic linkages are hydrolysed faster than expected does not affect this reasoning.



III

From the fragmentation analysis, a detailed structure III of the biological repeating unit of the O-specific side chains can now be proposed. The sequence for the D-mannose and D-galactose residues, suggested earlier, has been confirmed by the isolation of trisaccharide A_1. Other structural features have also been confirmed and the anomeric natures of the D-mannose, D-galactose, and D-glucose residues have been established.

EXPERIMENTAL

Gel chromatography. A Sephadex G-25 superfine column (97.5 × 2.5cm) was used at a flow rate of 7 ml/h. Three-ml fractions were collected.

Paper chromatography. Whatman No. 1 was used for analytical purposes. Solvent systems, A: ethyl acetate-acetic acid-water, 3:1:1 and B: butanol-pyridine-water, 6:4:3.

System A was run for 20 h and system B for 60 h. For preparative purposes, solvent system B and carefully washed Whatman 3 MM were used.

Methylation analysis. The oligosaccharide alditols were methylated with methylsulphinyl sodium and methyl iodide [7] and the methylated product isolated by distribution between water and chloroform. The fully methylated materials were treated as previously described.[1,6] The alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose and tetra-*O*-methyl-D-mannose were separated on an OV-225 S.C.O.T. column at 200°. The 1,2,3,5-tetra-*O*-methyl-L-rhamnitol derivative was resolved from the solvent peak on the ECNSS-M column at 50° and the *T*-value was not determined.

NMR. The tetrasaccharide alditol was converted into its trimethylsilyl derivative and the NMR recorded as described earlier.[6]

Acknowledgements. We are indebted to Mr. O. Larm for running the NMR spectra. The skilled technical assistence 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-656-04 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 Goljas minne.*

Added in proof. Recent results, using a specific oxidation method (Hoffman, J., Lindberg, B., and Svensson, S., to be published) indicate that the D-galactopyranose residues are β-linked. If this is correct, the NMR spectrum of C_2 has been misinterpreted, probably due to some impurity, which gives a signal in the same region as the anomeric protons. This question will be further studied.

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Received June 26, 1970.