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Structural Studies on *Klebsiella* O Group 5 Lipopolysaccharides

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The structures of the O-specific side chains in two *Klebsiella* O group 5 lipopolysaccharides have been investigated using methylation analysis and Smith degradation as the principal methods. The results are consistent with the assumption that the side chains are composed of repeating units containing five α-D-mannopyranose residues, two of which are (1→3)-linked and three (1→2)-linked. The side chains are terminated by a 3-*O*-methyl-D-mannose residue.

In their studies on the sugar composition of lipopolysaccharides (LPS) from the different *Klebsiella* O groups, Nimmich and Korten [1] found that those of O group 5 contained mannose and a small amount of 3-*O*-methyl-mannose. The latter sugar is also a constituent of the *Escherichia coli* O:8 LPS,[2] *Streptomyces griseus*,[3] and of a polysaccharide found in the fungus *Coccidioides immitis*.[4]

In addition to mannose and 3-*O*-methyl-mannose the LPS contained galactose, glucose, *N*-acetylglucosamine, 3-deoxyoctulosonic acid (KDO), and heptose. In this communication structural studies on two O group 5 LPS, from the strains K57 and K75, are reported.

RESULTS AND DISCUSSION

The LPS were isolated as previously described.[1] Hydrolysis of the LPS yielded 3-*O*-methyl-mannose, mannose, galactose, glucose, and a heptose in the relative proportions 1.4:89.5:2.8:2.8:3.8 (K57) and 1.8:86.0:3.1:3.1:6.2 (K75), respectively, as determined by GLC-MS of the alditol acetates.[5,6] D-Arabinose was used as internal standard and the percentages of mannose were found to be 31 % and 32 %, respectively.

These analyses are in reasonably good agreement with those previously reported. Mannose was isolated from hydrolysates of the LPS and, from its

optical rotation, it was of the D-configuration. It seems reasonable to assume that 3-*O*-methyl-mannose has the same configuration. Neither LPS showed significant absorption around 1735 cm^{-1} in the IR, demonstrating the absence of *O*-acyl groups. The optical rotations of the LPS were $[\alpha]_{578}$ +29° (K57) and $[\alpha]_{578}$ +31° (K75). If it is assumed that the optical rotation is essentially due to the D-mannose residues these must be α-linked.

The two LPS were methylated by the Hakomori procedure.[7] In order to distinguish between ethers derived from D-mannose and from 3-*O*-methyl-D-mannose the methylation was performed with trideuteriomethyl iodide. The same method has been applied to an LPS containing L-rhamnose and 3-*O*-methyl-L-rhamnose.[8] The methylated polysaccharides were hydrolysed, transformed into a mixture of alditol acetates, and analysed by GLC – MS.[9] In order to facilitate interpretation of the mass spectra the sugars were reduced to alditols with sodium borodeuteride. The results of the two analyses are summarized in Table 1.

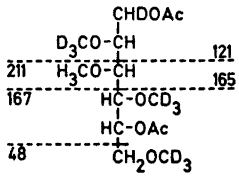
Table 1. Methyl ethers obtained in the methylation analyses of the *Klebsiella* K57 and K75 LPS.

Sugars [a]	T [b]	Mol %	
		K57	K75
2,3,4,6-G 2,3,4,6-Man	1.00	4.5	2.7
3,4,6-Man	1.95	54.6	58.4
2,4,6-Man	2.09	36.4	36.6
Others [c]	–	4.5	2.3

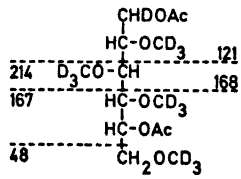
[a] 2,3,4,6-G = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.*
[b] Retention times of the corresponding alditol acetates on an ECNSS-M column, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[c] Most of these are probably non-sugar components.

As expected, the results for the two LPS are similar. The polysaccharide chains are essentially linear and composed of (1→2)- and (1→3)-linked D-mannopyranose residues in the proportion 3:2. In the gas chromatogram the peak at $T = 1$ could contain 2,3,4,6-tetra-*O*-methyl derivatives of both D-mannose and D-glucose. MS revealed that the material in the peak obtained from K75 LPS derived almost exclusively from 3-*O*-methyl-D-mannose as the fragments of *m/e* 165 and *m/e* 167 were of comparable intensities. The fragment of *m/e* 211 was strong but that of *m/e* 214 insignificant. The origins of the fragments from the 2,3,4,6-tetra-*O*-methyl derivatives of 3-*O*-methyl-D-mannose (I) and D-glucose (II) are illustrated below.

By similar arguments, the peak at $T = 1$ from K57 LPS contained comparable amounts of ethers deriving from 3-*O*-methyl-D-mannose and from D-glucose and/or D-mannose. The peak could not be resolved because of a minor contaminating non-sugar component.



I



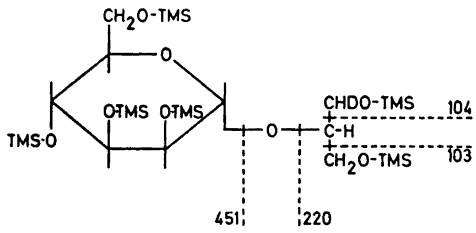
II

The O-specific side chains of bacterial LPS are generally composed of oligosaccharide repeating units. The smallest repeating unit for the *Klebsiella* O group 5 LPS, from the results of the methylation analysis, is a linear pentasaccharide, containing two (1→3)-linked and three (1→2)-linked α-D-mannopyranose residues. These could be combined in ten different ways. For five of these combinations, the two (1→3)-linked D-mannose residues are adjacent or occupy terminal positions. For the other five these residues are separated.

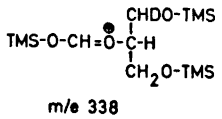
In order to distinguish between these alternatives, the K75 LPS was subjected to a Smith degradation,[10] *i.e.* periodate oxidation, borohydride reduction, mild acid hydrolysis, borohydride reduction, and characterisation of the products formed. Ideally, all acetalic linkages in the oxidized and reduced residues, but no glycosidic linkages, are broken during the hydrolysis. In our experience, however, it is difficult to achieve this result, and the results of the different reactions were therefore monitored by sugar and methylation analyses. Sugar analysis of the material obtained after periodate oxidation and borohydride reduction revealed that it contained mannose residues corresponding to about 90 % of the original, (1→3)-linked mannose residues. In a methylation analysis of this product the only tri-*O*-methyl-D-mannose obtained was the 2,4,6-isomer. These results demonstrate that the periodate oxidation had gone to completion and that no signification amounts of material had been lost.

A mild acid hydrolysis of this material, followed by reduction, should give either 2-*O*-glycerol-α-D-mannopyranoside or *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→2)-glycerol as the main component. After mild acid treatment the material was reduced with sodium borodeuteride. Part of the product was trimethylsilylated and investigated by GLC–MS using an XE-60 column.[11] The mixture contained only traces of the D-mannitol derivative, demonstrating that the hydrolysis had not been too drastic.

The main peak had $T_{man} = 3.55$ (retention time relative the TMS derivative of D-mannitol at 175°), and accounted for approximately 20 % of the calculated yield as determined by added internal standard (D-arabinose). It was identified as the TMS derivative of 2-*O*-glycerol-α-D-mannopyranoside (III). The MS, after correction for the deuterium labelling, was indistinguishable from that given by the TMS derivative of 2-*O*-glycerol-α-D-galactopyranoside ($T_{man} = 4.01$). Fragments were observed, *inter alia*, at *m/e* 73, 103, 104, 204, 217, 220, 338 (IV), 361, and 451. Fragment IV, by analogy with similar fragments formed from trimethylsilylated disaccharides,[12] should contain the aglycon, C-1 and the trimethylsilyloxy group from C-3.



III



IV

A second peak ($T_{man} = 15$), with not quite half the area of the main peak, was also obtained. As the TMS derivatives of disaccharide alditols (*e.g.* melibiitol, $T_{man} = 40$) and disaccharide glycerol glycosides (*e.g.* *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→2)-glycerol, $T_{man} = 52$) are so much slower on GLC, it could not be derived from a mannobiitol or a glycerol mannobioside. Its MS could not be interpreted. No peaks were observed in the region of the chromatogram where the TMS derivatives of a mannobiitol or a glycerol mannobioside should appear.

Part of the degraded material was subjected to methylation analysis. The proportion between 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-mannose was 3:1, indicating that 75 % of the oxidized reduced residues had been hydrolysed.

The assumed pentasaccharide repeating unit should consequently have the structure V, or a cyclic permutation of this.

→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1–

V

Assuming that all the side chains are terminated by 3-*O*-methyl-D-mannose the average number of repeating units, calculated from the percentages of 3-*O*-methyl-D-mannose and D-mannose in the sugar analyses, should be approximately 13 (K57) and 10 (K75), respectively. As the chains are terminated by 3-*O*-methyl-D-mannose, it seems reasonable to assume that the "biological" repeating unit is terminated by a 3-substituted D-mannose residue, as indicated in V.

EXPERIMENTAL

General methods. Concentrations were performed under reduced pressure at bath temperatures not exceeding 40°. Perkin-Elmer 900 or 990 instruments, equipped with flame ionisation detectors, were used for GLC. Glass columns (180 × 0.15 cm) containing 3 % ECNSS-M on Gas Chrom Q (100/120 mesh) were used for separation of alditol acetates (190°) and partially methylated alditol acetates (170°). OV-225 SCOT columns (50′ × 0.020″) were used for separation of alditol acetates and partially methylated alditol acetates in conjunction with MS. Glass columns (180 × 0.15 cm) containing 5 % XE-60 on Chromosorb W (80/100 mesh) were used at 175° or temperature-programmed from 125° to 190°, 4° min^{-1} (in quantitative estimations), for the separation of TMS-derivatives and also in conjunction with MS. GLC–MS was performed on a Perkin-Elmer 270 instrument under an ionisation potential of 70 eV, an ionisation current of

80 μA and ion-source temperature of 80° or, for the TMS derivatives, an LKB 9000 instrument under similar conditions except that the ion-source temperature was 220°. Optical rotations were recorded using a 10 cm micro-cell in a Perkin-Elmer 141 instrument, and for IR a Perkin-Elmer 257 instrument was used.

The LPS were isolated from *Klebsiella* O5:K57 (4425/51) and *Klebsiella* O5:K75 (645) as previously described.[1] The LPS showed $[\alpha]_{578}^{20}$ +29° (*c* 0.5, water) (K57) and $[\alpha]_{578}^{20}$ +30° (*c* 0.3, water) (K75). In the IR spectra (KBr) no significant absorptions around 1735 cm^{-1} (*O*-acyl-region) were observed.

Sugar and methylation analyses. For sugar analyses the LPS (2 mg) and D-arabinose (0.5 mg) were treated with 0.25 M sulphuric acid at 100° for 14 h. The solutions were neutralised with barium carbonate, the sugars were converted into alditol acetates as previously described [13] and the mixtures were analysed by GLC[5]–MS.[6] The *T*-value of the 3-*O*-methyl-D-mannose derivative was 8.8 on an ECNSS-M column. Larger samples (10 mg) of the two LPS were hydrolysed as above and the monosaccharides were isolated by gel filtration on a Sephadex G-15 column (25.0 × 1.4 cm). The sirupy samples showed $[\alpha]_{578}^{20}$ +11° (*c* 0.3, water) (K57) and $[\alpha]_{578}^{20}$ +10° (*c* 0.3, water) (K75). These values are corrected for the glucose and galactose content determined by GLC assuming the D-configuration of these sugars. For the methylation analyses, performed as previously described,[14] 5 mg samples of LPS were used. Sodium borodeuteride was used for the reduction of sugars to alditols. The methylation analysis of oligomeric material was performed in the same manner, except that the methylated products were recovered by partition between chloroform and water.

Smith degradation of the LPS. A mixture of the K75 LPS (30 mg) in water (20 ml) and 0.2 M sodium metaperiodate (5 ml) was kept in the dark at 4° for 120 h. Excess periodate was destroyed with ethylene glycol (1 ml), the solution was dialysed overnight and concentrated to 50 ml, sodium borohydride (250 mg) was added and the solution was kept at room temperature for 9 h. Excess borohydride was decomposed with 50 % acetic acid and the solution dialysed overnight. Part of the recovered material (3 %) was used for sugar analyses, as described above, with D-arabinose (0.5 mg) as internal standard. Another part (30 %) was lyophilized, dried and subjected to methylation analysis. After addition of 0.5 mg of D-arabinose (internal standard) the main part was treated with 0.25 M sulphuric acid at room temperature for 70 h (preliminary results had indicated that this would be a suitable time), neutralised with Dowex 3 (free base) ion exchange resin and reduced with sodium borodeuteride (50 mg) during 3 h at room temperature. The solution was treated with Dowex 50 (H^+) ion exchange resin and boric acid was removed by repeated distillations with methanol. The solution was divided into two equal parts which were lyophilized and dried *in vacuo* over phosphorus pentoxide. One part was subjected to methylation analysis as described above. The other part was dissolved in dry pyridine (1.5 ml) and treated with a mixture of trimethylchlorosilane (0.1 ml) and hexamethyldisilazane (0.2 ml). After 5 min at 40° the mixture was concentrated to dryness, dissolved in carbon tetrachloride and filtered. This solution was used for GLC–MS. The response for the TMS-derivatives of D-arabinitol (used as internal standard in the experiment) relative to a 2-*O*-glycerol-glycoside (2-*O*-glycerol-α-D-galactopyranoside) in the GLC-conditions used was checked in a model experiment and was found to be 1.5:1. Using the same response factor the main peak (T_{man} = 3.55) in the chromatogram accounted for 0.55 mg and the second peak (T_{man} = 15) for 0.2 mg (uncorrected for response factor) of the material.

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