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Structural Studies on the *Klebsiella* O Group 7 Lipopolysaccharide

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The structure of the O-specific side chains in the *Klebsiella* O group 7 lipopolysaccharide has been investigated, using methylation analysis, partial hydrolysis and Smith degradation studies. In the modification of the Smith degradation used here, the polyalcohol obtained after periodate oxidation-borohydride reduction was methylated before and ethylated after the mild acid hydrolysis. The product obtained was then hydrolysed and analysed, as alditol acetates, by GLC-MS. From these studies, it is concluded that the side chains are composed of tetrasaccharide repeating units, and a structure for these units is proposed.

The twelve different *Klebsiella* O group lipopolysaccharides (LPS) studied by Nimmich and Korten [1] all contain glucose, galactose, a heptose, *N*-acetyl-glucosamine and a 3-deoxyoctulosonic acid (KDO), which most probably are components of the basal core. In two of them, O7 and O10, the O-specific side chains contained rhamnose and ribose. The O group 10 LPS also contained a low percentage of 3-*O*-methyl-L-rhamnose, not observed in the O7 LPS. We recently reported structural studies on the O10 LPS [2] and now report similar studies on the O7 LPS.

The LPS was isolated as previously described [1] and showed $[\alpha]_{578}$ $-26°$. Analysis of a hydrolysate by GLC [3]-MS [4] showed that the LPS contained rhamnose, ribose, galactose, glucose, and a heptose in the relative proportions 71:20:2:4:3. D-Arabinose was used as internal standard and the analysis showed that the LPS contained 45 % of "anhydro-sugar" residues.

The main components of the hydrolysate, rhamnose and ribose, were isolated and proved to have the L- and D-configurations, respectively. The LPS showed only a weak absorption around 1735 cm^{-1} in the IR, indicating the absence of *O*-acetyl or other *O*-acyl groups in the O-specific side chains. This was confirmed by subjecting the LPS to an *O*-acyl determination by the

method devised by de Belder and Norrman [5] whereby no methylated sugars were produced.

The LPS was methylated by the method of Hakomori,[6] hydrolysed and the mixture of methylated sugars analysed, as the alditol acetates, by GLC-MS.[7] The results are given in Table 1, column A. The O-specific side chains in LPS from Gram-negative bacteria are generally composed of oligosaccharide repeating units. There are, however, no simple stoichiometric proportions be-

Table 1. Methylation analyses of the original and modified *Klebsiella* O group 7 LPS.

Methylated sugar	T[b]	Mol % [c]			
		A	B	C	D
2-Et-3,5-Rib [a,d,e]	0.38	–	–	–	20
2,3,4-Rha [a,d]	0.46	–	18	–	–
3-Et-2,4-Rha [d,e]	0.44	–	–	–	4
3,5-Rib	0.77	19	12	26	9
3,4-Rha	0.92	22	24	< 1	< 1
2,4-Rha [e]	0.98	50	33	68	58
2,3,4,6-G [e]	1.00	2	5	2	< 1
Others [f]	–	8	9	4	9

[a] 2-Et-3,5-Rib = 2-*O*-ethyl-3,5-di-*O*-methyl-D-ribose, 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.*

[b] Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column.

[c] A, original lipopolysaccharide; B, lipopolysaccharide, partially degraded with acid; C, periodate oxidized and reduced lipopolysaccharide; D, Smith degraded lipopolysaccharide (see text).

[d] Part of these volatile derivatives was probably lost during working up.

[e] These compounds were separated on an OV-225 SCOT-column.

[f] Some of these are probably non-sugar components.

tween either L-rhamnose (71 %) and D-ribose (20 %) in the sugar analysis or between 3,5-di-*O*-methyl-D-ribose (19 %), 3,4-di-*O*-methyl-L-rhamnose (22 %), and 2,4-di-*O*-methyl-L-rhamnose (50 %) in the methylation analysis. D-Ribose is more labile than L-rhamnose during acid hydrolysis but this was compensated for in the sugar analysis. The most reasonable assumption seems to be that the O-specific side chains are composed of linear tetrasaccharide repeating units, containing one D-ribofuranose and three L-rhamnopyranose residues. Although a nonasaccharide repeating unit would give better agreement with the experimental results, repeating units of this size have not been demonstrated in LPS. The D-ribofuranose residue and one of the L-rhamnopyranose residues should be linked at the 2-positions, the two other L-rhamnopyranose residues should be linked at the 3-position. Assuming that the main contribution to the optical rotation of the LPS is due to the O-specific side chains, all or, less probably, three of the four sugar residues should give negative contributions to the optical rotation (*cf.* β-D-ribofuranosides and α-L-rhamnopyranosides).

The D-ribofuranosidic linkages in the LPS were cleaved by mild acid hydrolysis and the product was then reduced with sodium borohydride and

subjected to methylation analysis. The results are given in Table 1, column B. The 1,3,4,5-tetra-*O*-methyl-D-ribitol, from the D-ribosidic end-groups, is volatile and was not accounted for. Although the stoichiometry in the analysis is not very good, the formation of 2,3,4-tri-*O*-methyl-L-rhamnose and the decrease in the 2,4-di-*O*-methyl-L-rhamnose demonstrate unambiguously that the D-ribofuranose residues are linked to the 3-position of L-rhamnose in the original polysaccharide.

In order to determine the complete sequence, the LPS was subjected to a Smith degradation.[8] The procedure was somewhat modified in that the polyalcohol obtained after periodate oxidation-borohydride reduction was methylated before and ethylated after the mild acid hydrolysis. Part of the methylated polyalcohol was hydrolysed and the methylated sugars analysed, as the alditol acetates, by GLC-MS. The results given in Table 1, column C, demonstrate that all the 2-linked L-rhamnose residues have been oxidised. A sugar analysis using an internal standard showed that the other sugar residues remained essentially intact. The analysis of the hydrolysed and ethylated product is given in Table 1, column D. The presence of 3,5-di-*O*-methyl-D-ribose and 3-*O*-ethyl-2,4-di-*O*-methyl-L-rhamnose reveals that not all the acyclic acetals on the modified L-rhamnose residues have been hydrolysed and that some of the acid-labile D-ribofuranosidic linkages have been hydrolysed. A comparable amount of 3-*O*-ethyl-2,4-di-*O*-methyl-L-rhamnose was also formed when a sample of fully methylated original LPS was hydrolysed under the same conditions as in the Smith degradation experiment and subjected to ethylation analysis. The high percentage of 2-*O*-ethyl-3,5-di-*O*-methyl-D-ribose, however, demonstrates that the 2-linked L-rhamnose is linked to the 2-position of the D-ribofuranose residue in the original LPS.

As a result of these studies, structure I is proposed for the tetrasaccharide repeating unit in the O-specific side chains of the *Klebsiella* O group 7 LPS. It cannot be decided whether the "biological" repeating unit has this structure or a cyclic permutation of this, as no methylated sugar deriving from the non-reducing terminal was observed in the methylation analysis. This negative evidence would suggest that the O-specific side chains are long. We cannot exclude the possibility that the anomeric nature of one of the four sugar residues in this structure should be reversed.

$$\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \quad \text{I}$$

The modified procedure for the Smith degradation described above has some advantages. Acetal migration during the hydrolysis of the polyalcohol, which may complicate the results, is eliminated. It is possible to perform a degradation with only small amounts of material. With the methylation-ethylation technique, structural information may be obtained which is lost in a conventional Smith degradation.

EXPERIMENTAL

General methods were the same as in a previous investigation.[9] The LPS was isolated from strain *Klebsiella* O7:K67 (264[2]) as previously described,[1] and showed $[\alpha]_{578}^{20}$ −26°

(*c* 0.3, water). In the IR spectra (KBr) no significant absorption around 1735 cm^{-1} was observed. Preparation of acetalated LPS by reaction with methyl vinyl ether and methylation analysis of the product were performed as previously described.[5,9] The content of phosphorus in the LPS was 0.9 %.

Sugar analysis. The LPS (3 mg) and D-arabinose were treated with 0.12 M sulphuric acid at 100° for 14 h. In separate experiments it was demonstrated that complete hydrolysis was obtained under these conditions but that 7 % of the D-ribose was decomposed. The sugars in the hydrolysate were analysed, as alditol acetates, as previously described.[3,4,9] L-Rhamnose, $[\alpha]_{578}^{20}$ + 7° (*c* 0.3, water) and D-ribose, $[\alpha]_{578}^{20}$ − 19° (*c* 0.1, water), were isolated from a hydrolysate of the LPS (20 mg) by paper chromatography.

Methylation analyses of original and partially degraded LPS were performed as previously described.[6,10,11] Ethylation was performed by procedures similar to those used for methylation. The location of ethyl groups in partially etherified alditol acetates by MS was unambiguous and will not be discussed.

Analysis of partially hydrolysed LPS. The LPS (8 mg) in 0.01 M sulphuric acid (4 ml) was kept at 100° for 45 min. After neutralization with Dowex 3 (free base), sodium borohydride (30 mg) was added and the solution was kept at room temperature for 4 h. The solution was then treated with Dowex 50 (H^+), concentrated and boric acid removed by repeated distillations with methanol. The product was dissolved in water, lyophilized and subjected to methylation analysis (column B in the Table).

Smith degradation of the LPS. The LPS (28 mg) was dissolved in 0.1 M acetate buffer of pH 3.9, 0.2 M sodium metaperiodate (5 ml) was added and the solution was kept in the dark at 4° for 120 h. Excess periodate was destroyed with ethylene glycol (1 ml), the solution was dialysed against tap water overnight and concentrated to 50 ml. Sodium borohydride (300 mg) was added and the solution kept at room temperature for 8 h. Excess borohydride was decomposed by addition of 50 % aqueous acetic acid and the solution was dialysed overnight. Part of this material (6 %) was used for sugar analysis, with D-arabinose (0.5 mg) added as internal standard. The main part was lyophilized and methylated. Part of this product (30 %) was hydrolysed and analysed as alditol acetates (column C in Table 1). The other part was treated with 50 % aqueous acetic acid for 1 h at 100°, concentrated to dryness, dissolved in dioxane-ethanol (3:1, 10 ml), reduced with sodium borohydride (50 mg) at room temperature overnight, worked up using Dowex 50 (H^+) followed by distillations with methanol and ethylated. The analysis of this material is given in Table 1, column D.

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