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THE *Klebsiella* TYPE 38 CAPSULAR POLYSACCHARIDE: IDENTIFICATION OF 3-DEOXY-L-*glycero*-PENTULOSONIC ACID AND STRUCTURAL STUDIES

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ABSTRACT

D-Glucose and D-galactose (ratio 1:1) comprise ~80% of the capsular polysaccharide from *Klebsiella* type 38, and the acidic component has been identified as 3-deoxy-L-*glycero*-pentulosonic acid. The polysaccharide is composed of pentasaccharide repeating units, for which a structure is proposed on the basis of methylation analysis, and Smith-degradation and partial hydrolysis studies.

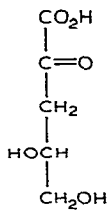
INTRODUCTION

The type-specific capsular polysaccharides from *Klebsiella* K1-K80, studied by Nimmich[1,2], are all acidic. Most of them contain uronic acid residues and some also contain pyruvic acid residues. The capsular polysaccharide from type 38 (K38)[1] was found to contain glucose, galactose, and an unknown acidic component that gave a positive reaction with the carbazole reagent. In the present communication, structural studies of this polysaccharide are reported.

RESULTS

The polysaccharide, which was isolated as previously described[1], yielded D-glucose and D-galactose in the proportion 1:1 on acid hydrolysis. These sugars accounted for ~80% of the polysaccharide material. On mild hydrolysis, the acidic component was liberated, and the residual polysaccharide was electrophoretically neutral. No other sugar was released during this treatment, thus demonstrating that the acidic component occupies a terminal position. With the periodate–thiobarbituric acid reagent[3], the acid gave a colour with an absorption maximum (550 nm) typical for 3-deoxyaldulosonic acids. The acid rapidly decomposed in acidic solution, yielding 2-furancarboxylic acid, which is a characteristic property of 3-deoxyaldulosonic acids[4,5]. The results demonstrate that the acidic component in the K38 polysaccharide is a 3-deoxypentulosonic acid.

The specific rotations of 3-deoxy-D- and -L-pentulosonic acid do not seem to have been determined, but the 2,4-dinitrophenylhydrazone of the γ-lactone of the L form is reported to have $[\alpha]_D$ −23° in *p*-dioxane[4]. This derivative, when prepared in crude form from the K38 acid, was levorotatory, indicating that the acid has the L configuration (**1**). The optical rotation increased rapidly with decreasing wavelength, becoming positive at 578 nm. Glycosides formed from the acid must be furanosidic (**2**) but the configuration of the anomeric linkage to the polysaccharide has not been settled.



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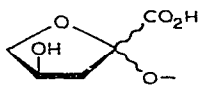

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The polysaccharide was methylated by the Hakomori procedure[6], the methylated product was hydrolysed, and the resulting partially methylated sugars were analysed as their alditol acetates by g.l.c.–m.s.[7] (Table I, column *A*). Two D-glucose and two D-galactose derivatives were obtained in approximately equal proportions. Methylation analysis of the neutral polysaccharide obtained on mild hydrolysis with acid also gave comparable amounts of four partially methylated sugars (Table I, column *B*). Three of these were the same in both analyses, but 6-*O*-methyl-D-galactose, in the former analysis, was replaced by 3,6-di-*O*-methyl-D-galactose in the latter analysis. The acidic sugar was thus attached to position 3 of the D-galactose residue.

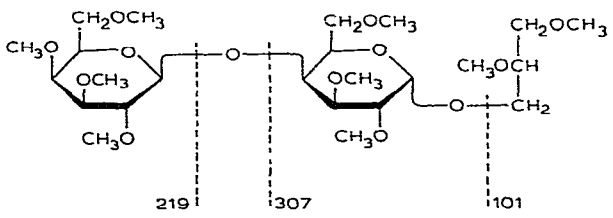
TABLE I

METHYL ETHERS OBTAINED FROM THE HYDROLYSATES OF THE FULLY METHYLATED, ACIDIC POLYSACCHARIDE (*A*) AND THE PARTIALLY HYDROLYSED, NEUTRAL POLYSACCHARIDE (*B*)

Sugar[a]	T[b]	*Mole %*	
		A	B
2,3,4,6-G	1.00	24.7	22.0
2,3,4,6-Gal	1.25	—	3.0
2,4,6-Gal	2.29	21.4	22.5
2,3,4-G	2.50	27.3	26.7
3,6-Gal	4.40	—	23.6
6-Gal	5.1	26.6	1.9

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

Part of the neutral polysaccharide was subjected to a Smith degradation[8], that is application in sequence of periodate oxidation, borohydride reduction, and mild hydrolysis with acid, during which the glycosidic linkages of non-oxidized residues should remain intact. Methylation analysis of the product gave a mixture of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose. The methylated, degraded product, on g.l.c.–m.s., gave a major peak with the mobility and m.s. expected for a fully methylated *O*-D-galactopyranosyl-(1→4)-*O*-D-galactopyranosyl-(1→1)-L-glycerol (**3**). Some typical, mass-spectral fragments are indicated in the formula; the anomeric nature of the sugar residues in **3** is discussed below.



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Partial hydrolysis of the neutral polysaccharide with acid, with fractionation of the product by paper chromatography, yielded the disaccharides listed in Table II.

TABLE II

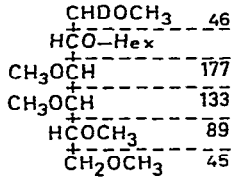
DISACCHARIDES OBTAINED ON PARTIAL HYDROLYSIS OF THE NEUTRAL POLYSACCHARIDE WITH ACID

Fraction	*Disaccharide*	*Amount (mg)*	$[\alpha]_{578}$ *(degrees)*	R_{MEL}[a]	T_{MEL}[b]	*Sugar analyses*[c] (%)	
						D-*G*	D-*Gal*
I	β-G-(1→3)-Gal	2.4			0.58		
			66	1.4		52	48*
	β-G-(1→2)-Gal	0.8			0.70		
II	β-Gal-(1→4)-Gal	0.6	67	1.2	0.94	—	100*
III	α-Gal-(1→6)-G	0.5	151	1.0	1.00	51*	49

[a]Mobilities on paper chromatography (Whatman No. 3; 1-butanol–pyridine–water, 6:4:3) relative to melibiose. [b]Retention times on g.l.c. of the corresponding permethylated alditols, relative to permethylated melibi-itol on an XE-60 column at 185°. [c]* indicates deuterium labelling at C-1 in the derived alditol. For fraction II, ~50% labelling of the galactitol was observed.

Each disaccharide was reduced with borodeuteride, the resulting disaccharide alditol was hydrolysed, the products were treated with borohydride, and the alditols were analysed, as the acetates, by g.l.c.–m.s. In this manner, the component sugars and the nature of the reducing sugar were determined. A portion of each disaccharide alditol was methylated and analysed by g.l.c.–m.s.[9]. The galactitol moiety could, according

to the methylation analysis, only be substituted in position 2, 3, or 4, and the presence or absence of the ions of *m/e* 134 and 90 distinguished between these alternatives, as indicated below for the partial structures **4**, **5**, and **6**.



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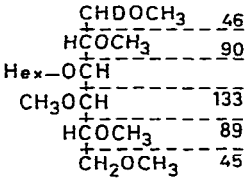

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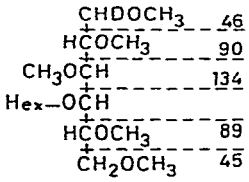

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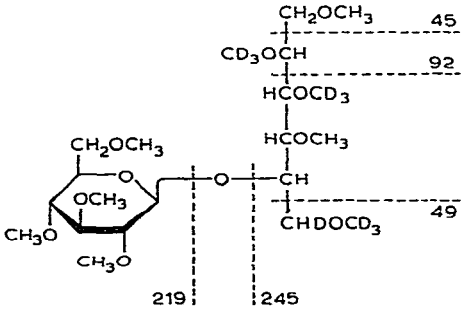
One of the components, a galactosylglucose derivative, had the same retention time and mass spectrum as fully methylated melibi-itol. The corresponding β-linked derivative had an almost identical mass spectrum but a different retention time. The disaccharide is thereby identified as melibiose, in agreement with its high optical rotation. Because of the small amounts available, the values for the optical rotations are not very accurate. The assignment of the β-D configuration to the other disaccharides is, however, supported by other evidence (see below).

Part of the methylated, neutral polysaccharide was subjected to mild hydrolysis with acid, the product was reduced with borodeuteride, methylated using trideuteriomethyl iodide, and analysed by g.l.c.–m.s. as in similar studies of a *Klebsiella* lipopolysaccharide[10]. The retention times and mass spectra of the two main components in the disaccharide region were consistent with the structures β-D-G-(1→2)-D-Gal and β-D-G-(1→3)-D-Gal in which the deuterium labellings are as indicated in **7** and **8**. The (1→3)-linked disaccharide predominated. The terminal D-glucose residue is thus linked to position 2 of the branching D-galactose residue, and the chain D-glucose residue is linked to position 3 of the chain D-galactose residue.



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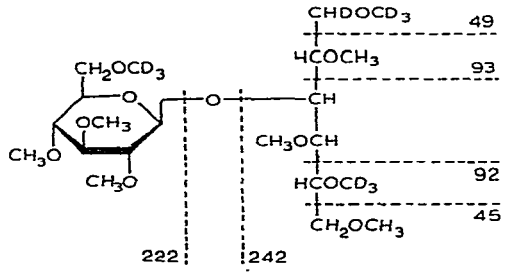

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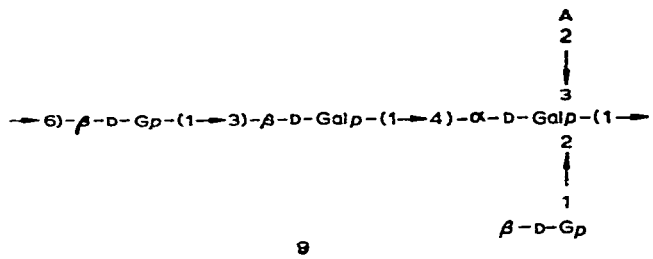
DISCUSSION

Bacterial capsular polysaccharides are generally composed of oligosaccharide repeating units. The present results indicate that the K38 polysaccharide is composed of pentasaccharide repeating units containing one 3-deoxy-L-*glycero*-pentulosonic acid residue (**2**), and two D-glucose and two D-galactose residues. The acidic sugar, which must exist as a furanoside, is terminal and linked to position 3 of D-galactose. However, the present results do not establish the anomeric configuration of the linkage.

The methylation analyses demonstrate that three of the hexose residues are pyranosidic. Also, the branching D-galactose residue is pyranosidic, as this residue is present in the melibiose obtained on graded hydrolysis (see below).

The Smith degradation establishes the sequence D-Gal-(1→4)-D-Gal-(1→6)-D-G- which thus, in conjunction with the methylation analyses, enables the sequence of sugar residues in the repeating unit to be formulated. It does not, however, distinguish between the two structures **9** and **10**; the anomeric nature of the sugar residues in **9** is discussed below.



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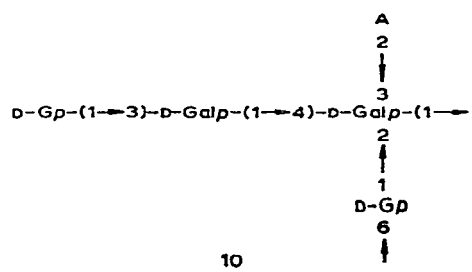
A = 3-Deoxy-L-*glycero*-pentulosonic acid (**2**)



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From the structure of the disaccharide derivatives (**7**, **8**) obtained on hydrolysis of the methylated polysaccharide, followed by reduction and trideuteriomethylation, it is evident that the sequence **9** is correct. Thus, the D-glucose residue in D-Glc*p*-(1→2)-D-Gal*p* is derived from a terminal D-glucose residue, and that in D-Glc*p*-(1→3)-D-Gal from a chain residue.

The isolation of melibiose on partial hydrolysis reveals that one of the D-galactose residues is α-linked. The optical rotation ($[\alpha]_{578}$ +45°) of the neutral

polysaccharide suggests that the other three hexose residues are β-D-linked. With this assumption, a value of +39° could be calculated, using Hudson's rules of isorotation. Further support for this assumption is provided by the optical rotations of the isolated disaccharides (Table II). Published $[\alpha]_D$ values for the disaccharides β-D-Gp-(1→3)-D-Gal, α-D-Galp-(1→4)-D-Gal, and β-D-Gal-(1→4)-D-Gal are +40°, +175°, and +68°, respectively[11].

The polysaccharide from *Klebsiella* K38 contains some unusual structural features. The 3-deoxy-L-*glycero*-pentulosonic acid has not been found previously as a component in polysaccharides. 3-Deoxy-D-*manno*-octulosonic acid (KDO) occurs in lipopolysaccharides from Gram-negative bacteria[12] and a 3-deoxy-*threo*-hexulosonic acid is a component in the extracellular polysaccharide from *Azotobacter vinelandii*[13]. The present and other 3-deoxyaldulosonic acids are formed when bacteria, such as *Pseudomonas saccharophila*, are grown on sugars[14,15].

Another unusual feature is the sugar residue that is doubly branched. This feature, although rare, has been observed before, *e.g.* in the plant gum leiocarpan[16].

EXPERIMENTAL

General methods were the same as previously described[10]. Isolation of the polysaccharide from *Klebsiella* K-type 38 (strain 8414) was performed as previously described[1]. The polysaccharide showed $[\alpha]_{578}$ +28° (*c* 0.5, water). For sugar analysis, the polysaccharide was hydrolysed with 0.25M sulphuric acid at 100° for 15 h. The hydrolysate was extracted with ether, and the sugars in the aqueous phase were analysed, as their alditol acetates, by g.l.c.–m.s.[17,18]. The analysis was performed in the presence of D-arabinose as internal standard. Glucose and galactose, in equal amounts, accounted for ~80% of the acidic polysaccharide. The sugars were isolated by paper chromatography and both showed positive optical rotations, demonstrating that they had the D configuration.

From the ether solution, 2-furancarboxylic acid, m.p. 133–134°, was isolated. It was indistinguishable (t.l.c., m.p., i.r., n.m.r.) from an authentic sample.

Characterization of the acidic component. — A solution of acidic polysaccharide (100 mg) and 2,4-dinitrophenylhydrazine (25 mg) in 90mM hydrochloric acid (35 ml) was kept at room temperature for 4 days. It was then kept at 80° for 1 h, cooled, and extracted with ether (4 × 35 ml). The ether solution was washed with hydrochloric acid (10 ml) and concentrated to dryness. The crude product, which contained unreacted 2,4-dinitrophenylhydrazine, showed $[\alpha]_{589}$ −5°, $[\alpha]_{578}$ +10°, and $[\alpha]_{546}$ +47° (*p*-dioxane).

Preparation of the neutral polysaccharide. — The polysaccharide (200 mg) was treated with 1% aqueous acetic acid (50 ml) at 100° and the product investigated by electrophoresis on glass fibre sheets in acetate buffer of pH 4. The polysaccharide, which was detected by spraying with sulphuric acid, gradually lost its electrophoretic mobility and became immobile after 5 h. It was recovered by precipitation with ethanol and showed $[\alpha]_{578}$ +45°.

Methylation analyses. — The acidic and the neutral polysaccharide and the oligosaccharides were methylated by the procedure previously described[10]. The results are given in Table I.

Smith degradation of the neutral polysaccharide. — The neutral polysaccharide (20 mg) was treated with 40mM sodium metaperiodate (25 ml) at 5° for 135 h. Ethylene glycol (1 ml) was added, the mixture was dialysed for 24 h, and sodium borohydride (30 mg) was added to the dialysed solution. After 10 h, the solution was again dialysed and then concentrated to 10 ml, and 0.5M sulphuric acid (10 ml) was added. After 8 h at room temperature, the solution was neutralized ($BaCO_3$), reduced with sodium borohydride (20 mg), deionized [Dowex-50 (H^+)], and concentrated, and boric acid was removed by repeated distillation of methanol from the residue. A solution of the product in water was lyophilized, and the residue was dried (P_2O_5) and methylated. Part of the methylated product was hydrolysed, and comparable amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose, as their alditol acetates, were detected in the hydrolysate by g.l.c.–m.s. Another part of the methylated product was investigated by g.l.c.–m.s. using a Dexil column. The main component, with T_{MEL} (retention time relative to permethylated melibi-itol) 3.03 at 230° and 2.70 at 250°, showed the following main peaks on m.s. (relative intensities in brackets): *m/e* 45(46), 71(26), 73(12), 75(21), 88(100), 89(12), 101(38), 103(13), 111(18), 127(12), 131(16), 155(12), 159(10), 163(18), 187(16), 219(7), 243(10), 275(10), and 307(2).

Partial hydrolysis of the neutral polysaccharide. — The neutral polysaccharide (80 mg) was hydrolysed with 0.25M sulphuric acid at 100° for 4 h. The hydrolysate was fractionated by paper chromatography, using ethyl acetate–acetic acid–water (3:1:1) followed by 1-butanol–pyridine–water (6:4:3). Three fractions were obtained (Table II), and the optical rotations of the components were determined. The components were then reduced with borodeuteride, part of each of the disaccharide alditols was hydrolysed, and the products were reduced with borohydride and analysed by g.l.c.–m.s. From the mass spectrum, the reducing moiety in the original disaccharide was identified. Another part was methylated and analysed by g.l.c.–m.s. The first fraction contained two components. G-(1→3)-Gal: *m/e* 43(27), 44(62), 45(83), 46(17), 59(21), 60(13), 67(13), 71(49), 75(41), 81(17), 88(94), 89(33), 90(19), 95(21), 101(100), 111(51), 115(16), 116(17), 127(16), 133(14), 155(14), 172(13), 177(6), 187(78), 219(14), 236(22). G-(1→2)-Gal: *m/e* 43(46), 45(69), 46(14), 59(17), 60(6), 67(14), 71(54), 75(37), 81(31), 88(100), 89(37), 90(6), 95(34), 111(34), 133(14),155(11), 177(11), 187(29), 219(11), 236(26). The second and the third fractions each contained one component. The second fraction was Gal-(1→4)-Gal: *m/e* 43(56), 45(67), 46(26), 71(41), 75(41), 88(100), 89(33), 90(17), 101(93), 102(26), 111(44), 115(25), 117(26), 134(15), 172(26), 187(44), 219(19), 236(37). The third fraction was Gal-(1→6)-G: *m/e* 45(55), 46(18), 71(40), 75(37), 88(100), 89(22), 90(27), 101(83), 102(19), 111(31), 115(31), 117(13), 127(12), 134(10), 146(19), 172(13), 178(8), 187(37), 219(12), 236(29). The methylated alditol from 6-*O*-β-D-galactopyranosyl-D-glucose was also prepared and investigated by g.l.c.–m.s. It showed T_{MEL} 1.36.

Partial hydrolysis of the methylated polysaccharide. — Methylated, neutral polysaccharide (15 mg) was treated with 90% aqueous formic acid (10 ml) at 80° for 75 min. The solution was concentrated to dryness, and a solution of the residue in water (2 ml) was reduced with sodium borodeuteride (20 mg) The deionized, dry product was methylated, using trideuteriomethyl iodide, and investigated by g.l.c.–m.s. Two main products in the disaccharide region had the same T_{MEL} values as G-(1→2)-Gal and G-(1→3)-Gal, respectively. Their fragmentations in the mass spectrometer were also the same; allowance was made for differences due to the detuerium labelling. It was evident from the m.s. that G-(1→3)-Gal contained a trideuteriomethyl group in the D-glucose moiety (*e.g.*, *m/e* 222 instead of 219) but that no such labelling was present in G-(1→2)-Gal.

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