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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 52

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ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* Type 52 has been investigated Methylation analysis, characterization by gas–liquid chromatography–mass spectrometry of oligosaccharide derivatives obtained on partial hydrolysis of the methylated polysaccharide with acid, and specific degradation of the methylated polysaccharide by successive treatments with base and acid followed by characterization of the product, were the principal methods used The polysaccharide is composed of hexasaccharide repeating-units containing D-glucuronic acid, D-galactose, and L-rhamnose, in the ratios 1 3 2 A structure for these units, disregarding the anomeric natures of the sugar residues, is proposed

INTRODUCTION

We have previously reported structural studies of capsular polysaccharides from *Klebsiella* Type 9[1] and Type 47[2] Both these polysaccharides are composed of D-glucuronic acid, D-galactose, and L-rhamnose residues The Type 52 polysaccharide contains the same sugars[3], and structural studies of this polysaccharide are now reported

RESULTS AND DISCUSSION

The polysaccharide, isolated as previously described[3], had $[\alpha]_D$ +42° It did not show any significant ir absorption in the 1735 cm^{-1} region, demonstrating the absence of *O*-acetyl or other *O*-acyl groups Glucose, galactose, and rhamnose, in the relative proportions 1.0 3 0 2 0, were obtained from a hydrolysate of the polysaccharide *via* reduction of the trimethylsilyl ethers[1,4] with lithium aluminium hydride Similar proportions (1 0 3 1·2 2) were obtained on analysis of the carboxyl-

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reduced polysaccharide prepared by the method of Taylor and Conrad[5] Glucose derived from the glucuronic acid, as this sugar was not obtained on hydrolysis of the original polysaccharide Rhamnose and galactose were isolated from a hydrolysate and proved to belong to the L- and D series, respectively. It seems reasonable to assume that the glucuronic acid belongs to the D series

The polysaccharide was methylated by the Hakomori procedure[1,6], and part of the product was reduced with lithium aluminium deuteride[1] The two samples were hydrolysed and the sugars in the hydrolysates were analysed, as their alditol acetates, by g l c–m s [7]. The carboxyl-reduced material was also subjected to methylation analysis The results of the three analyses (Table I, columns *A*, *B*, and *C*) are consistent. The L-rhamnose and D-galactose derivatives are the same in the three analyses. The uronic acid residue appears as 2,3-di-*O*-methyl-D-glucose, dideuterated at C-6, in the analysis of the methylated, reduced sample, and as 2,3,6-tri-*O*-methyl-D-glucose in the analysis of the reduced, methylated sample

TABLE I

METHYLATION ANALYSES OF ORIGINAL AND MODIFIED *Klebsiella* TYPE 52 POLYSACCHARIDES

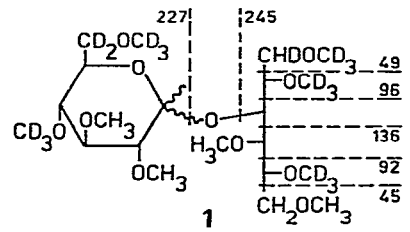
Methylated sugar[a]	T[b]	*Mole %*[c]				
		A	*B*	*C*	*D*	*E*
1,2,4,5,6-Gal	0 42	—	—	—	—	8[d,e]
3,4-Rha	0 92	17	16	17	10[f]	10
2,3-Rha	0 98	23	19	16	23	23
2,3,4,6-Gal	1 25	22	16	15	18	26
2,4,6-Gal	2 28	26	18	17	21[g]	9
3,4,6-Gal	2 50	—	—	—	—	19[h]
2,3,6-G	2 50	—	—	16	—	—
4,6-Gal	3 65	13	17	19	27	5
2,3-G	5 39	—	14[i]	—	—	—

[a]1,2,4,5,6-Gal = 1,2,4,5,6-penta-*O*-methyl-D-galactitol, 3,4-Rha = 3,4-di-*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 at 170° [c]Polysaccharide *A*, original, *B*, carboxyl-reduced after methylation, *C*, carboxyl-reduced before methylation, *D*, degraded polysaccharide (see Text), *E*, degraded, trideuteriomethylated polysaccharide (see Text) [d]Monodeuterated at C-1, and trideuteriomethylated at C-1 and C-5 [e]Part of this volatile ether and derivatives was probably lost during work-up [f]About 10% monodeuteration at C-1 [g]About 45% monodeuteration at C-1 [h]Trideuteriomethylated at position 3 [i]Dideuterated at C-6

The results of the sugar and methylation analyses suggest that the polysaccharide contains a hexasaccharide repeating-unit composed of two L-rhamnose residues, three D-galactose residues, and one D-glucuronic acid residue The results also give information on the positions through which the sugar residues are linked

The methylated polysaccharide was treated with 90% formic acid at 100° for 45 min, and the product was reduced with lithium aluminium deuteride, remethylated using trideuteriomethyl iodide, and analysed by g l.c–m s.[1,2,8–12]. The single

component (**1**), eluted in the disaccharide region, was shown by m s to be derived from an aldobiouronic acid Thus, the ion of the A_1-type from a fully methylated hexopyranoside should have *m/e* 219[8–12]. The observed shift to *m/e* 227 demonstrates the presence of eight deuterium atoms, two at C-6 and six in the trideuteriomethyl groups, known to be located at C-4 and C-6 The m s. further demonstrates, as indicated in the structure **1** below, that the galactitol part is substituted at C-3, and derives from the branching D-galactose residue as it contains a trideuteriomethyl group at C-2.



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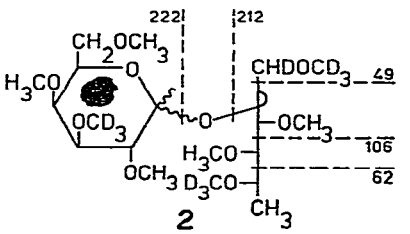

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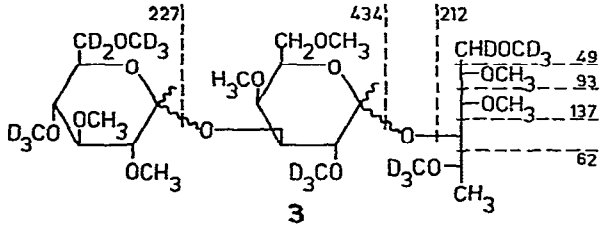
This experiment was repeated, using milder conditions of hydrolysis In addition to **1**, another disaccharide derivative (**2**) and a trisaccharide derivative (**3**) were obtained in the product The disaccharide derivative **2** derived from D-Gal*p*-(1→2)-L-Rha*p*, and the presence of one trideuteriomethyl group in the D-galactose residue demonstrated that it had been a chain residue in the original polysaccharide The trisaccharide derivative **3** derived from D-GA*p*-(1→3)-D-Gal*p*-(1→4)-L-Rha*p* That the D-galactose had been a branching residue in the original polysaccharide was corroborated by the A_1-type fragment of *m/e* 434, obtained by fission of the galactosidic linkage The assignment of substitution in the L-rhamnitol parts of **2** and **3** from analyses of the m s was difficult, as some pertinent peaks were weak and appeared in regions containing stronger peaks from other parts of the molecules It was, however, supported by the degradation experiment discussed below.

The course of the partial hydrolysis of the second experiment above was followed by complete hydrolyses of the trideuteriomethylated products and analyses of the mixtures, as the alditol acetates, by g l c –m s [7]. Three sugars were trideuteriomethylated to a considerable extent. 2,3,4,6-tetra-*O*-methyl-D-glucose (27%) to 100%



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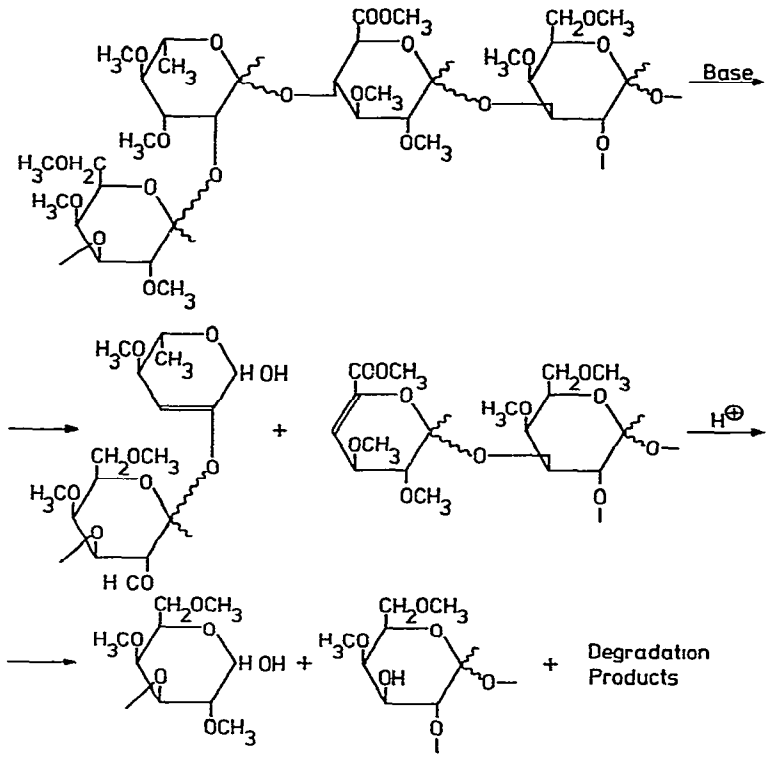
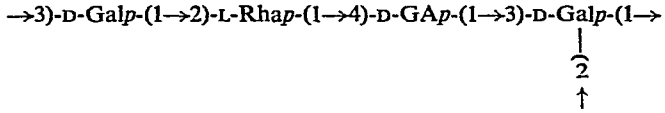
at C-4 and C-6, 2,3,4,6-tetra-*O*-methyl-D-galactose (35%) to ~80% at C-3, and 2,4,6-tri-*O*-methyl-D-galactose (12%) to ~70% at C-2 This demonstrates that three types of glycosidic linkages were more readily hydrolysed than the others. Two of these are the L-rhamnosidic linkages, as indicated by the decrease in 3,4- and 2,3-di-*O*-methyl-L-rhamnose (to 11 and 6%, respectively) The third linkage should be that of the terminal D-galactose residue, as most of the 2,3,4,6-tetra-*O*-methyl-D-galactose is labelled and thus derives from other D-galactose derivatives In addition to these ethers, 2,3,6-tri-*O*-methyl-D-glucose (8%, 100% trideuteriomethylated at C-6) and small proportions of 4,6-di-*O*-methyl-D-galactose, 1,2,4,5,6-penta-*O*-methyl-D-galactitol (100% trideuteriomethylated at positions 1, 2, and 5), and 2,3,4-tri-*O*-methyl-L-rhamnoses (trideuteriomethylated at positions 2 or 4) were observed The tetra-*O*-methyl-L-rhamnitols and the hexa-*O*-methylgalactitols formed from the reducing sugars liberated during the graded hydrolysis are considerably more volatile than the other derivatives and are not accounted for in the analyses

In order to gain further information on sequences, the fully methylated polysaccharide was subjected to a degradation that has been applied recently to other polysaccharides containing uronic acid residues[13] This degradation involves treatment with base under anhydrous conditions, followed by a mild hydrolysis with acid During the treatment with base, the linkage to C-4 of a uronic acid residue is broken by β-elimination The methoxyl group at C-3 in a reducing sugar thus liberated is eliminated in a second β-elimination[13,14]. The unsaturated sugars formed are acid labile and new end-groups are formed during the mild hydrolysis with acid.

Thus, the methylated polysaccharide was first treated with methylsulphinyl sodium in methyl sulphoxide and subsequently with aqueous acetic acid. The product then was reduced with sodium borodeuteride, whereby reducing sugars generated during the degradation became labelled at C-1, and either hydrolysed (Table I, column *D*) or trideuteriomethylated and hydrolysed (Table I, column *E*), the sugars in the hydrolysates were analysed, as their alditol acetates, by g l c–m s [7] Part of the trideuteriomethylated material was not hydrolysed but investigated by g l c [11]. No peak was observed in the disaccharide region The implication of this will be discussed below The degradation products obtained in the eliminations are not accounted for in these analyses Parallel experiments, using sodium methoxide in anhydrous methanol as base[13], gave essentially the same results From the results of these experiments, the partial structure **4** depicted below (together with its mode of degradation) is indicated for the polysaccharide



The disappearance of the major part of the 3,4-di-*O*-methyl-L-rhamnose (Table I, columns *D* and *E*) demonstrates that the 2-substituted L-rhamnose residue is linked to C-4 in the uronic acid residue in the original polymer The appearance of 3,4,6-tri-*O*-methyl-D-galactose, trideuteriomethylated at C-3, and the concomitant decrease in the amount of 4,6-di-*O*-methyl-D-galactose demonstrates that the uronic acid residue is linked to position 3 in the branching D-galactose residue (Table I, column *E*) The formation of 2,4,6-tri-*O*-methyl-D-galactose, deuterated at C-1 (Table I, column *D*), or 1,2,4,5,6-penta-*O*-methyl-D-galactitol, trideuteriomethylated at positions 1 and 5 (Table I, column *E*), demonstrates that the 3-linked D-galactose residue is linked to position 2 of the L-rhamnose residue which was decomposed during the degradation It is evident from the analyses that neither the first nor the second β-elimination reaction is complete The results nevertheless demonstrate partial structure **5** in the polysaccharide



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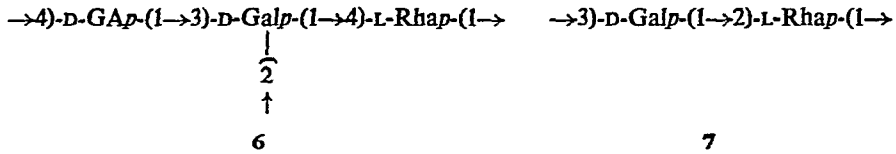
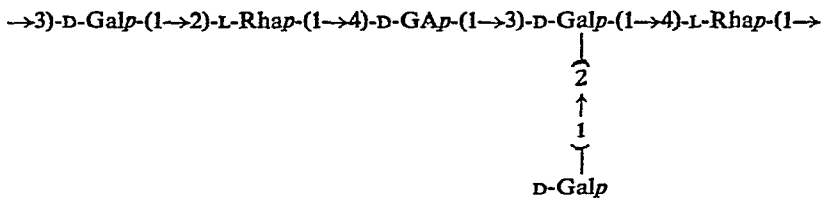
This, and the partial structures **6** and **7**, indicated by the partial hydrolysis studies, contain all the structural elements demonstrated by the methylation analyses, except the terminal D-galactose residue That all residues are pyranosidic is evident from the methylation analysis and the investigation of the methylated oligosaccharide alditols



6 **7**

Assuming that the polysaccharide is composed of hexasaccharide repeating-units, these partial structures, which in part are overlapping, can be combined to two possible structures **8** and **9**. Of these, **8** should give a tetrasaccharide derivative, and **9** a disaccharide derivative, in the degradation discussed above No disaccharide derivative was observed, which lends strong support to structure **8**



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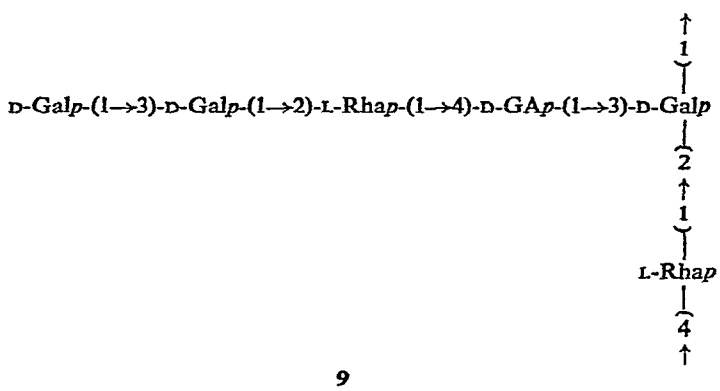

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The methods for sequence analysis used in this study require only small amounts of material but give no information on the anomeric nature of the sugar residues, which must be determined by other methods.

EXPERIMENTAL

The general methods were essentially the same as in previous investigations[1,2] In addition to the XE-60 columns, glass columns (200 × 0 15 cm) containing 3%

Dexsil 300 on Gas Chrom Q (100/120 mesh) were used for g l c and g l c–m s of permethylated oligosaccharide alditols

Isolation of the polysaccharide from *Klebsiella* Type 52 (strain 5759/50) was performed as previously described[3]. In the i r spectrum (KBr), no significant absorption around 1735 cm^{-1} was observed. The polysaccharide showed $[\alpha]_D^{20}$ +42° (*c* 0 1, water)

Sugar and methylation analyses were performed by methods previously described[1,2 4,7,15,16] The location of trideuteriomethyl groups in partially methylated alditol acetates by m s was unambiguous and will not be discussed L-Rhamnose, $[\alpha]_D^{20}$ +5° (*c* 0 1, water), and D-galactose, $[\alpha]_D^{20}$ +70° (*c* 0 1, water), were isolated from a hydrolysate by paper chromatography.

Carboxyl reduction of the polysaccharide[5]. — 1-Cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide-metho-*p*-toluenesulphonate (1 35 g) was added with stirring to a solution of polysaccharide (54 mg) in water (50 ml) Three drops of 1-octanol were added as anti-foam agent and the pH was kept at 4 75 by addition of 0 01M hydrochloric acid. After 2 h, aqueous 2M sodium borohydride (25 ml) was added during 1 h, and the pH was kept at ~7 by simultaneous addition of 4M hydrochloric acid. The solution was dialysed for 24 h against tap water and freeze-dried. Sugar analysis revealed that ~70% of the D-glucuronic acid residues had been reduced. The procedure described above was therefore repeated. In the twice-reduced product, obtained in almost quantitative yield, essentially all of the D-glucuronic acid residues had been reduced

Partial, acid hydrolysis of the methylated polysaccharide — Methylated polysaccharide (20 mg) was treated with 90% formic acid (6 ml) at 100° for 45 min or at 70° for 2 h The solution was concentrated to dryness, and the residue was suspended in water and freeze-dried A solution of the product and lithium aluminium deuteride (75 mg) in dichloromethane (4 ml) and ethyl ether (16 ml) was refluxed for 4 h. The product was worked up in the usual manner, dried, and methylated by the Hakomori procedure[6], using trideuteriomethyl iodide The methylated material was recovered by partition between chloroform and water The product was analysed by g l c.–m s [1,2,8–12] Component **1** had $T_{melibiitol}$ 0.61 (XE-60 at 200°) and 0 67 (Dexsil 300 at 200°) ($T_{melibiitol}$ = retention time relative to permethylated melibiitol) The m s showed, *inter alia*, the following peaks (relative intensities in brackets) 45(55), 49(17), 88(100), 91(30), 92(21), 96(30), 101(40), 104(43), 136(10), 160(15), 192(12), 195(79), 227(10), 245(30), and 305(7). Component **2** had $T_{melibiitol}$ 0 55 (XE-60 at 200°) and 0 57 (Dexsil 300 at 200°) The m s showed, *inter alia*, the following peaks· 49(18), 62(36), 88(18), 91(77), 101(65), 104(29), 106(23), 187(35), 190(12), 212(100), and 222(17) Component **3** had $T_{melibiitol}$ 5 5 (XE-60 at 230°) and $T_{cellotriitol}$ 0 58 (Dexsil 300 at 250°). The m s showed, *inter alia*, the following peaks 49(15), 62(45), 88(70), 91(5), 93(13), 101(40), 104(55), 137(15), 195(100), 212(42), 227(16), and 434(6) Part of the reduced and trideuteriomethylated product from the hydrolysis performed at 70° was hydrolysed, and the sugars were analysed, as their alditol acetates, by g l c –m s [7] (see Text)

Degradation of methylated polysaccharide with base — A solution of carefully dried, methylated polysaccharide (25 mg) and toluene-*p*-sulphonic acid (trace) in a mixture (4 ml) of methyl sulphoxide and 2,2-dimethoxypropane (19 1) was prepared in a serum vial which was sealed with a rubber cap The vial was flushed with nitrogen and kept in an ultrasonic bath for 30 min before addition of 2M methylsulphinyl sodium (2 ml) added by means of a syringe The solution was agitated in the ultrasonic bath for a further 30 min and kept at room temperature overnight It was then poured into water (10 ml) and an excess of 50% aqueous acetic acid was added The solution was extracted with chloroform (4 × 15 ml), and the extract was washed with water (5 × 15 ml) and concentrated The product, after treatment with 10% aqueous acetic acid (20 ml) at 100° for 1 h, was recovered by freeze-drying, and dissolved in a mixture of *p*-dioxane (8 ml) and ethanol (3 ml) Sodium borodeuteride (40 mg) was added and the solution was kept overnight The mixture was then treated with Dowex-50 (H^+), and boric acid was removed by repeated distillations with methanol. Half of the product was hydrolysed, and the resulting sugars were analysed, as the alditol acetates, by g l c–m s (Table I, column *D*) The other half was trideuteriomethylated, and recovered by partition between chloroform and water Part of this product was hydrolysed and analysed as above (Table I, column *E*), another part was analysed by g l c. using an XE-60 column at 200°

The methylated polysaccharide was also degraded by treatment with sodium methoxide in methanol, as previously described[13], hydrolysed with 10% acetic acid, and analysed as above Essentially the same results were obtained in each case

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