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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 37

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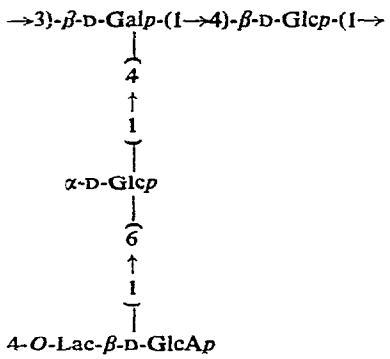
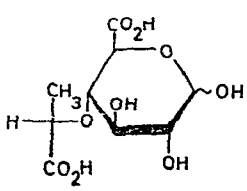
ABSTRACT

The structure of the *Klebsiella* type 37 capsular polysaccharide has been investigated Methylation analysis, various specific degradations, and n m r spectroscopy were the principal methods used It is concluded that the polysaccharide is composed of tetrasaccharide repeating-units having the structure {4-*O*-Lac-D-GlcA ≡ 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid}



INTRODUCTION

We recently demonstrated that the acidic sugar component in the *Klebsiella* type 37 capsular polysaccharide (K 37) was 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic



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acid[1] (4-*O*-Lac-D-GlcA, **1**), a sugar not previously found in Nature We now report structural studies of this polysaccharide

RESULTS AND DISCUSSION

K 37, isolated as previously described[2], had $[\alpha]_{589}$ +14°, and an acid hydrolysate of K 37 contained D-glucose and D-galactose in the ratio 1 9 1 0 The sugars in an acid hydrolysate of the carboxyl-reduced[3] polysaccharide were reduced to their alditols, acetylated, and analysed, giving the acetates of D-glucitol. D-galactitol, and 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucitol in the proportions 1 8 1 0 1 0

The ^{1}H-n m r spectrum of K 37 was not well-resolved, but the spectrum of the carboxyl-reduced polysaccharide showed, *inter alia*, signals at δ 1 10 (J 7 Hz, 3 H, CH_3 of the hydroxypropyl group), 4 3–4 5 (2 H, overlapping signals from anomeric protons with large coupling-constants), 4 65 ($J_{1,2}$ 8 Hz, 1 H, anomeric proton), and 4 94 ($J_{1\ 2}$ 3 Hz, 1 H, anomeric proton) In the ^{13}C-n m r spectrum of K 37 (sodium salt), signals given by the methyl carbon of the lactic acid moiety (δ 20 7) and by the two carboxylate carbons (δ 176 5 and 183 3) were readily identified The signals for the anomeric carbons were not well-resolved, but three signals, with relative intensities ~1 2 1, were observed at δ 100 8–106 1

TABLE I

METHYLATION ANALYSES OF ORIGINAL AND CHEMICALLY MODIFIED *Klebsiella* TYPE 37 CAPSULAR POLYSACCHARIDES

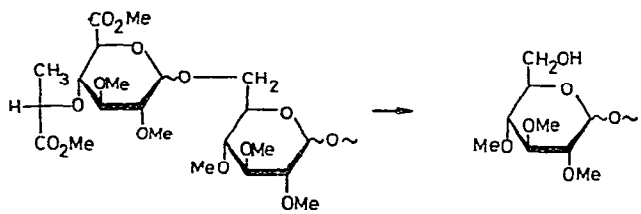
Methylated sugar[a]	T[b]	T[c]	*Mole* %[d]				
			A	B	C	D	E
2,3,4,6*-Glc	1	1	—	—	38	—	29
2,3,6-R	1 75	2 22	—	24	—	—	—
2,4*,6-Gal	1 94	2 03	—	—	—	—	22
2,3 6-Glc	1 94	2 32	34	23	31	41	34
2,3,4-Glc	2 00	2 22	31	23	trace	22	—
2,6-Gal	2 11	3 14	35	31	31	37	15

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc R = 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose, * indicates position of CD_3-group [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 SP-1000 glass-capillary column at 220°. [c]As in *b*, but on OV-225 at 170° [d]Polysaccharide (for details, see text), *A*, original, *B*, carboxyl-reduced, *C*, uronic acid-degraded, trideuteriomethylated, *D*, oxidised, *E*, polysaccharide *D*, degraded and trideuteriomethylated

K 37 and carboxyl-reduced K 37 were subjected to methylation analyses[4–6], including g l c–m s analysis of partially methylated alditol acetates (Table I, columns *A* and *B*) The identification of 1,5-di-*O*-acetyl-2,3,6-tri-*O*-methyl-4-*O*-[(*S*)-2-(1-methoxy)propyl]-D-glucitol in the analysis of carboxyl-reduced K 37 has been previously reported[1] The results of the methylation analyses, in conjunction with the

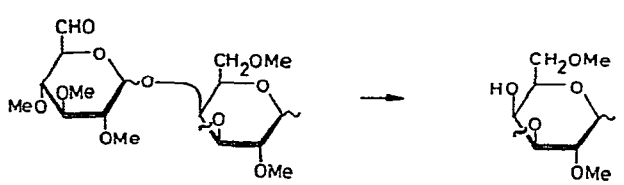
sugar analyses and the n m r evidence, indicate that K 37 is composed of tetrasaccharide repeating-units Assuming that all sugar residues are pyranosidic, in agreement with the resistance of K 37 to mild hydrolysis with acid, the repeating unit should contain one terminal 4-*O*-Lac-D-GlcA group, one D-glucopyranosyl residue linked through O-4, one D-glucopyranosyl residue linked through O-6, and one D-galactopyranosyl residue linked through O-3 and O-4

Methylated K 37 was subjected to a uronic acid degradation[7] by treatment with strong base (when the substituent in the β-position to the ester group was eliminated), followed by acid hydrolysis under mild conditions During this treatment, the terminal uronic acid group (in **2**) was eliminated, and a free hydroxyl group in the resulting polymer (**3**) was exposed On methylation of **3** (using trideuteriomethyl iodide), acid hydrolysis, and analysis of the product (Table I, column *C*), 2,3,4,6-tetra-*O*-methyl-D-glucose having a trideuteriomethyl group at O-6 was obtained The terminal uronic acid group is consequently linked to O-6 of a D-glucopyranosyl residue



2 **3**

The uronic acid-degraded polymer was further degraded by a method involving oxidation and subsequent β-elimination, by treatment with base and mild hydrolysis with acid[8 9] In the present example, with an aldehyde group at C-6 (as in **4**), the reaction was analogous to the uronic acid degradation, and the terminal, oxidised sugar residue was eliminated A hydrolysate of the oxidised material (**4**) showed that only about half of the hydroxymethyl groups in **3** had been oxidised (Table I, column *D*) The degraded product (**5**) was methylated (using trideuteriomethyl iodide), and hydrolysed, and the products were analysed (Table I, column *E*) The appearance of 2,4,6-tri-*O*-methyl-D-galactose, with a trideuteriomethyl group at O-4, demonstrated that the second sugar residue which had been eliminated was linked to O-4 of the branching D-galactopyranosyl residue As there are only four sugar residues in the repeating unit, their sequence (**6**) is consequently determined



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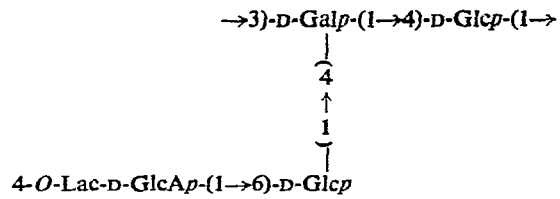

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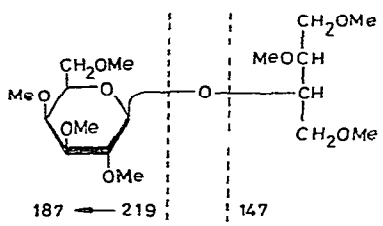
K 37 was subjected to a modified Smith-degradation[10 11], involving periodate oxidation, borohydride reduction, methylation, and hydrolysis under mild conditions Analyses of hydrolysates of the polyalcohol, before and after methylation, yielded D-galactose and 2,6-di-*O*-methyl-D-galactose, respectively, as the monosaccharide components, indicating complete oxidation of susceptible residues The product after the mild hydrolysis with acid was remethylated, and fractionated by high-speed liquid chromatography The main component, $[\alpha]_{578}$ −11°, gave a mass spectrum in agreement with that expected[5] for fully methylated 2-*O*-D-galactopyranosyl-D-erythritol (7) The origins of some pertinent fragments are indicated in the formula The ^{1}H-n m r spectrum of 7 showed, *inter alia*, a signal at δ 4 32 ($J_{1\ 2}$ 7 Hz) attributed to the anomeric proton Hydrolysis of the product yielded 2,3,4,6-tetra-*O*-methyl-D-galactose, which was identified by g l c –m s after borohydride reduction and acetylation The Smith degradation thus demonstrated that the D-galactopyranosyl residue was β-linked and confirmed the sequence 8 in K 37



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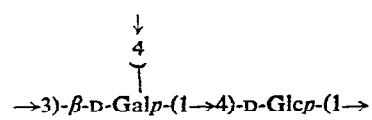

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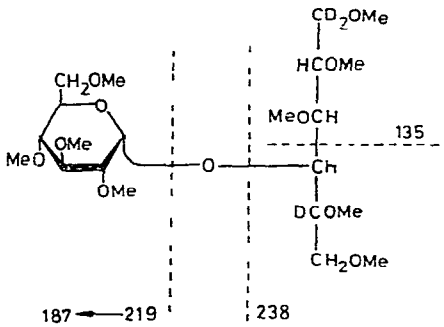
Although the optical rotation of K 37, together with ^{1}H-n m r studies, suggested the presence in the repeating unit of one α-linked and three β-linked pyranosidic residues, more conclusive and detailed evidence concerning the individual assignments of anomeric linkages was desired Carboxyl-reduced K 37 was therefore acetylated, and treated with chromium trioxide in acetic acid During this treatment, hexopyranosides in which the aglycon group occupies an equatorial position in the most-stable chair form are oxidised to 5-hexulosonates[12 13] The corresponding anomer with an axially attached aglycon group are oxidised only slowly The oxidation was performed in the presence of an internal standard (*myo*-inositol hexaacetate), and sugar analysis of the product revealed that one D-glucopyranosyl

residue had survived In order to decide which of the two D-glucopyranosyl residues was α-linked, the oxidized material was treated with borodeuteride, during which both the ketone group at the 5-position and the aldonate ester group should be reduced The deacetylated material was fractionated on a Sephadex column, yielding a main fraction eluted in the disaccharide region Hydrolysis of this material gave D-glucose, D-galactitol, and L-altritol (1 0 54 0 09) G l c –m s of the alditol acetates showed that the D-galactitol and L-altritol derivatives were, as expected, dideuterated at C-1 and monodeuterated at C-5 The α-linkage in the disaccharide alditol(s) was evident from the optical rotation, $[\alpha]_{578}$ +70°, and the signal from the anomeric proton at δ 5 06 ($J_{1\ 2}$ 3 5 Hz) in the ^{1}H-n m r spectrum (separate signals from the D-galactitol and L-altritol analogues were not detected) The disaccharide alditol(s) after methylation gave a single peak on g l c The mass spectrum of this material agreed with structure **9**, and some pertinent fragments are indicated in the formula (the isomer having the D-*galacto* configuration is given) On hydrolysis, 2,3,4,6-tetra-*O*-methyl-D-glucose and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylhexitol-*1*,*1*,*5*-d_3 were obtained The α-linked D-glucosyl residue is therefore linked through O-4 of the branching D-galactosyl residue



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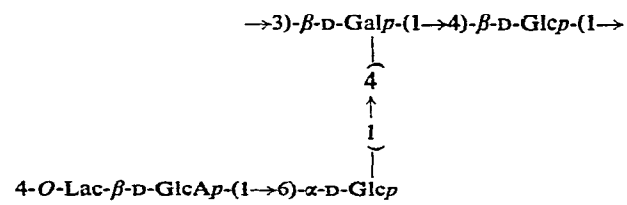
From the combined evidence, structure **10** is proposed for the repeating unit of the capsular polysaccharide from *Klebsiella* type 37 This structure is closely analogous to that[14] of the capsular polysaccharide from *Klebsiella* type 22 The main difference seems to be that the terminal unit in the latter is a 4-deoxyhex-4-enuronic acid group The two polysaccharides are also hydrolysed, at the β-D-galactopyranosidic linkage, by the same bacteriophage enzyme[14]



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EXPERIMENTAL

General methods — Equipment and columns for g l c , g l c –m s , and high-speed liquid chromatography were essentially the same as in the investigations of *Klebsiella* type 59 (Ref 15) and type 81 (Ref 16) capsular polysaccharides N m r spectra were recorded on a Varian XL-100 instrument

Isolation of the polysaccharide from Klebsiella *K-type 37* (strain 8238) — This was performed as described earlier[2] The polysaccharide had $[\alpha]_{589}^{25}$ +14° (*c* 0 5, water) The ^{1}H-n m r spectra of polysaccharides were run on solutions containing 10 mg/ml of D_2O at 85° The ^{13}C-n m r spectrum was recorded on a solution containing 50 mg/ml of D_2O at 85° Chemical shifts are given relative to Me_4Si No absorptions from *O*-acetyl groups or pyruvic acid residues were observed K 37 contained nitrogen (0 14%), but no phosphorus

Carboxyl-reduction of the native polysaccharide — This was performed by the procedure of Taylor and Conrad[3], and in order to achieve complete reduction, the procedure was performed three times

Sugar and methylation analyses — These were performed as described before[4–6 17 18] From a hydrolysate of the carboxyl-reduced K 37, D-glucose, $[\alpha]_{589}^{25}$ +49°, D-galactose, $[\alpha]_{589}^{25}$ +83°, and 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose, $[\alpha]_{589}^{25}$ +57° (all rotations in water, *c* 0 2), were isolated by paper chromatography in ethyl acetate–acetic acid–water (3 1 1) and 1-butanol–pyridine–water (6 4 3)

Uronic acid-degradation of methylated K 37 — Native K 37 (100 mg) was methylated, and recovered by partition between water and chloroform The material was dried, and dissolved (together with a trace of *p*-toluenesulfonic acid) in methanol–2,2-dimethoxypropane–dichloromethane (18 1 2, 21 ml), and the solution was boiled under reflux for 30 min Sodium (250 mg) was dissolved in the cooled solution, which was then boiled under reflux for 2 h After cooling, the pH was adjusted to 6 using 50% aqueous acetic acid, and water (150 ml) was added The mixture was extracted with chloroform (3 × 25 ml), and the organic phase was washed with water (25 ml) and evaporated The residue was treated with 50% aqueous acetic acid at 100° for 1 5 h, the solution was evaporated, and the degraded polysaccharide was suspended in water and freeee-dried Part (3 mg) of this material was subjected to methylation analysis with trideuteriomethyl iodide (Table I, column *C*)

Oxidation and degradation of uronic acid-degraded K 37 — The oxidising agent[19] was prepared, under anhydrous conditions, at −45° by dropwise addition of methyl sulfoxide (9 ml) to a stirred solution of chlorine in dichloromethane (M, 25 ml) Uronic acid-degraded K 37 (see above) in methyl sulfoxide (10 ml) was added dropwise with a syringe to the stirred oxidation mixture The reaction mixture was kept at −45° with stirring for 5 h, and then triethylamine (7 ml) was added dropwise with continued stirring After 5 min, the reaction mixture was allowed to attain room temperature and the product was recovered after dialysis by freeze-drying The oxidation procedure was repeated twice Part (1/10) of the final product was hydrolysed, and the resulting sugars were analysed (Table I, column *D*) Another part (1/5) was

dissolved in dichloromethane (10 ml), and sodium ethoxide in ethanol (M, 10 ml) was added The reaction mixture was kept at room temperature for 1 h, neutralised with 90% aqueous acetic acid, and evaporated to dryness The product was treated with 50% aqueous acetic acid (20 ml) at 100° overnight, evaporated to dryness, and partitioned between chloroform and water The product was remethylated with trideuteriomethyl iodide, and hydrolysed, and the resulting sugars were analysed (Table I, column *E*)

Smith degradation of K 37 — K 37 (250 mg) was dissolved in 0 1M sodium acetate buffer (pH 3 9, 250 ml), and 0 2M sodium metaperiodate (63 ml) was added the solution was kept in the dark at 4° for 120 h Excess of sodium metaperiodate was reduced by addition of ethylene glycol (1 ml), and the solution was dialysed and concentrated to 50 ml before addition of sodium borohydride (3 g) After stirring for 9 h, excess of borohydride was destroyed by addition of 50% aqueous acetic acid, and the solution was dialysed In order to ensure complete reaction, the oxidation–reduction cycle was repeated Part (1/100) of the product was hydrolysed and analysed, only galactose was obtained The remaining material was methylated, and recovered by dialysis Part of the methylated material (1/100) was hydrolysed, and analysis showed the presence of 2,6-di-*O*-methyl-D-galactose The remaining methylated material was treated with 90% aqueous formic acid (100 ml) for 1 h at 40° The solution was evaporated to dryness, and the residue was suspended in water and freeze-dried This product was remethylated, and recovered by partition between chloroform and water The material was purified by high-speed liquid chromatography on two Waters Microporasil columns connected in series and eluted with ethyl acetate, to give fully methylated β-D-Gal*p*-(1→2)-D-erythritol (7, 30 mg), $[\alpha]_{589}^{25}$ −11° (*c* 0 3, chloroform), R_F 0 17 (t l c , silica gel, ethyl acetate), T_{MEL} 0 29 (g l c , OV-1 column at 190°, retention time relative to permethylated melibiitol) N m r data ($CDCl_3$) δ 4 32 ($J_{1,2}$ 7 Hz) The mass spectrum showed, *inter alia*, the following fragments (relative intensities in brackets, and some assignments[5] in square brackets) *m/e* 45(46), 88(100), 89(26), 101(45), 111(12), 115(42), 147(16)[bA_1], 187(5)[aA_2], 207(17)[abJ_1], and 219(0 6)[aA_1] On hydrolysis, 2,3,4,6-tetra-*O*-methyl-D-galactose was obtained

Oxidation of carboxyl-reduced K 37 with chromium trioxide — Carboxyl-reduced K 37 (50 mg) was dissolved in formamide (30 ml), and treated with acetic anhydride (15 ml) and pyridine (15 ml) overnight at room temperature The acetylated polysaccharide was recovered by dialysis and freeze-drying

Part (1/5) of this material was dissolved in glacial acetic acid (3 ml), and *myo*-inositol hexa-acetate was added as an internal standard Part (2/3) of the acetic acid solution was treated with chromium trioxide (200 mg) on an ultrasonic bath at 50° for 1 h The material was recovered by partition between chloroform and water, and evaporation of the chloroform phase The remaining portion (1/3) was used as reference Sugar analysis of the oxidised material showed 1 4 units of D-glucose and 0 1 unit of D-galactose {no 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose was detected} per unit of *myo*-inositol hexa-acetate The reference showed 2 8 units of D-glucose,

1 6 units of D-galactose, and 1 5 units of 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose per unit of *myo*-inositol hexa-acetate

The remaining acetylated polysaccharide (4/5) was oxidised in the same way, with proportional increases of solvents and reagents After work-up, the carefully dried material was dissolved in dry 1,4-dioxane–ethanol (1 1, 20 ml) and treated with sodium borodeuteride (~100 mg) with stirring overnight Water was added and the solution was kept at room temperature for 3 h The solution was acidified to pH ~4 with Dowex 50 (H^+) resin After work-up, the residue was purified on a column (50 × 1 5 cm) of Sephadex G-25 The material in the major fraction (3 mg) had $[\alpha]_{589}^{25}$ +70° (*c* 0 3, water), ^{1}H-n m r data (D_2O) δ 5 06 ($J_{1,2}$ 3 5 Hz) Part of the material (1/3) was hydrolysed and analysed; D-glucose, D-galactitol, and L-altritol (1 0 54 0 09) were obtained The remaining material was methylated, and the product gave a single peak in g l c (OV-17 column at 210°) having T_{LAC} (retention time relative to permethylated lactitol) 0 86 The mass spectrum showed, *inter alia* *m/e* 45(50), 88(100), 101(55), 135(6), 187(72)[aA_2], 219(11)[aA_1], 238(25)[bA_1], and 298(8)[abJ_1] Hydrolysis, reduction, and acetylation gave 2,3,4,6-tetra-*O*-methyl-D-glucitol diacetate and 1,2,3,5,6-penta-*O*-methylhexitol monoacetate dideuterated at C-1 and monodeuterated at C-5

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