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STRUCTURAL STUDIES OF AN EXTRACELLULAR POLYSACCHARIDE (K21b) ELABORATED BY *Klebsiella* TYPE 21b

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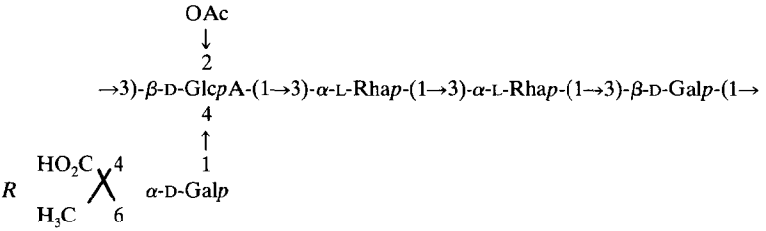
ABSTRACT

The capsular polysaccharide from a new capsular serotype of *Klebsiella*, K21b, has been investigated, using n.m.r. spectroscopy, methylation analysis, and specific degradations as the main methods. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure.



INTRODUCTION

In addition to the previously known, 80 or so different capsular serotypes of *Klebsiella*[1,2], a new serotype was recently identified by Allen *et al.*[3]. Its capsular polysaccharide showed strong cross-reactivity with that from *Klebsiella* K21. The latter, which was investigated by Choy and Dutton[4], is composed of pentasaccharide repeating-units with the structure **1**. The absolute configuration of the acetal carbon atom of the pyruvic acid acetal was determined by Garegg *et al.*[5]. Preliminary studies of the new polysaccharide showed that it was composed of glucuronic acid, galactose, rhamnose, and pyruvic acid in the proportions 1:2:2:1, and that two of the three sugars with the *gluco* or *galacto* configuration were pyranosidic and β-linked. It was further assumed, because of the high degree of cross-reactivity, that the side-chains in the two polysaccharides should be identical or closely similar.

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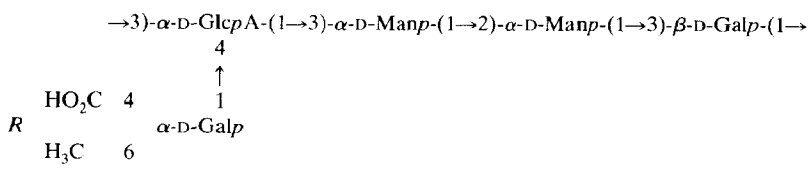
Because of this cross-reactivity, the old type was called K21a and the new K21b. We now report structural studies of K21b.



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RESULTS AND DISCUSSION

Sugar analysis of K21b confirmed the results of Allen *et al.*[3]. The absolute configurations of the sugars, D-glucuronic acid, D-galactose, and L-rhamnose, were determined by the method devised by Gerwig *et al.*[6]. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals for six protons in the region for anomeric protons, at δ 5.51 (n.r., H), 5.06 (n.r., 2 H), 4.95 (m, H), 4.83 (J 7.5 Hz, H), and 4.60 (6.9 Hz, originally broad, H), for an *O*-acetyl group at δ 2.16 (3 H), for a pyruvic acid acetal at δ 1.44 (3 H), and for methyl groups of two 6-deoxyhexoses at δ 1.28 (J 5.5 Hz, 6 H). The spectrum of *O*-deacetylated K21b contained five signals in the region for anomeric protons, at δ 5.58 (n.r., H), 5.09 (n.r., 2 H), 4.94 (7.2 Hz, originally broad, H), and 4.76 (J 7 Hz, H). One of the signals in this region in the spectrum of original K21b is thus due to a methine proton on an acetoxylated carbon. The results confirm that K21b is composed of pentasaccharide repeating-units, and that two of the three sugars with the *gluco* or *galacto* configuration are pyranosidic and β-linked.

The ^{13}C-n.m.r. spectrum of *O*-deacetylated K21b contained, *inter alia*, signals for carboxyl groups at δ 176.7 and 175.4, for anomeric carbons at δ 104.1, 103.7, 102.8, 101.4, and 99.7, for C-6 of hexosyl residues at δ 63.5 and 61.8, for the methyl group of a pyruvic acid acetal at δ 26.1, and for C-6 of rhamnosyl residues at δ 17.5 (2 C).

Methylation analysis of K21b gave the sugars listed in Table I, column *A*. Methylation analysis with carboxyl reduction of the methylated polysaccharide (Table I, column *B*) also gave 2-*O*-methyl-D-glucose, derived from the D-glucuronic acid residue. Small amounts of this sugar were also obtained in analyses performed without carboxyl reduction of the methylated polysaccharide, indicating that part of the uronic acid derivative is present as a lactone in the hydrolysate and was reduced to the alditol by sodium borohydride. A methylation analysis of K21b that had been treated with acid under mild conditions (Table I, column *C*) gave, *inter alia*, 2,3,4,6-tetra-*O*-methyl-D-galactose, demonstrating that the pyruvic acid acetal is linked to O-4 and O-6 of a D-galactopyranosyl group.

From these results, it is concluded that K21b is composed of pentasaccharide repeating-units containing two 3-linked L-rhamnopyranosyl residues, one 3-linked

TABLE I

METHYLATION ANALYSIS OF K21B AND SOME DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	*Detector response (%)*				
		A	B	C	D	E
2,3,4-Rha	0.40					45[d]
2,4-Rha	0.93	44	39	39	36	23
2,3,4,6-Gal	1.12			8		
2,4,6-Gal	1.84	33	25	25	32	32
2,4-Glc	3.20				32	
2,3-Gal	3.90	23	21	14		
2-Glc	5.27		15	14		

[a]Key: *A*, original K21b, *B*, original K21b, carboxyl reduction of the methylated polymer; *C*, K21b subjected to hydrolysis with acid under mild conditions, carboxyl reduction of the methylated product; *D*, Smith-degraded K21b, carboxyl reduction of the methylated product; *E*, uronic acid degradation of methylated K21b. [b]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [c]Retention time of the corresponding alditol acetate on a DB-225 column at 200°, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [d]Trideuteriomethylated at O-3.

D-galactopyranosyl residue, one terminal D-galactopyranosyl group with pyruvic acid acetal-linked to its 4- and 6-positions, and a D-glucopyranosyluronic acid residue linked through O-3 and O-4. It further contains an *O*-acetyl group. The n.m.r. results indicate that the two L-rhamnopyranosyl residues and one of the three other residues are α-linked. The signal in the ^{13}C-n.m.r. spectra given by the methyl group of the pyruvic acid acetal, at δ 26.1, demonstrates[5] that the configuration at the acetalic carbon is *R*.

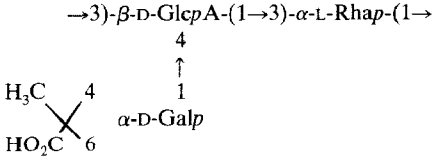
O-Deacetylated K21b was subjected to a Smith degradation[7]. Methylation analysis of the product (Table I, column *D*) showed that the terminal, pyruvylated D-galactopyranosyl group had been lost, leaving a linear polysaccharide composed of two L-rhamnopyranosyl residues, one D-galactopyranosyl residue, and one D-glucopyranosyluronic acid residue, all of which are pyranosidic and linked through O-3.

The ^{1}H-n.m.r. spectrum of the Smith-degraded K21b contained, *inter alia*, signals for anomeric protons at δ 5.01 (n.r., 2 H), 4.69 (*J* 8 Hz, H), and 4.67 (*J* 8 Hz, H). The ^{13}C-n.m.r. spectrum contained, *inter alia*, signals for four anomeric carbons at δ 104.1, 103.7, and 102.8 (2 C). The D-galactopyranosyl and D-glucopyranosyluronic acid residues are thus β-linked and the L-rhamnopyranosyl residues α-linked. The terminal D-galactopyranosyl group in K21b is consequently α-linked.

Further evidence for the α-anomeric configuration of the rhamnosyl residues was provided from the ^{13}C-n.m.r. spectrum of the linear polysaccharide obtained on Smith degradation of K21b. This spectrum was analysed by the computer program CASPER[10,11], which calculates the ^{13}C-n.m.r. spectra of all possible structures and then evaluates the fit to the experimental spectrum. It was evident that structures containing a β-L-rhamnopyranosyl residue were not compatible with

the ^{13}C-n.m.r. data. Thus, the first suggested structure (as in **4**) was correct and the first structure with a β-L-rhamnopyranosyl residue had an ~50% higher ranking value than the correct structure.

In order to determine the sequence of the sugar residues in the main chain, K21b was subjected to a uronic acid degradation[8]. Fully methylated K21b was treated with strong base in dimethyl sulfoxide, followed by hydrolysis with acid under mild conditions and remethylation using trideuteriomethyl iodide. During this treatment, the glycosidic linkage of the uronic acid residue should be cleaved, and the hydroxyl group in the released sugar residue trideuteriomethylated. The methylation analysis (Table I, column *E*) showed that the D-glucosyluronic acid group was linked to O-3 of an L-rhamnopyranosyl residue. From the results discussed above, the partial structure **2** has been demonstrated. The mutual order of the remaining β-D-galactopyranosyl and α-L-rhamnopyranosyl residues could not be determined by uronic acid degradation. The sugar residue linked to O-3 of the D-glucuronic acid should be partly released during the treatment with base, but should also be further degraded. Attempts to label the released reducing sugar residues by reduction with sodium borodeuteride therefore gave inconsistent results.



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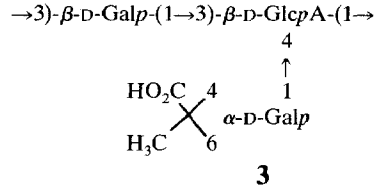
Signals for three anomeric protons, δ 4.76 and 5.09 (2 H), in the spectrum of *O*-deacetylated K21b appear at almost the same fields in the spectrum of the Smith-degraded product. However, one signal in the spectrum, at δ 4.94 (*J* 7.2 Hz), is shifted to δ 4.67 (*J* 8 Hz) after Smith degradation. From studies of 3,4-diglycosyl-substituted methyl α-D-galactopyranosides[9], it was evident that steric interactions could significantly shift the signals of anomeric protons in the substituting groups. However, the anomeric proton signal of the substituted residue, *i.e.*, the galactopyranosyl residue, was not significantly shifted. The β-D-glucuronic acid in K21b should behave similarly and therefore the signal at δ 4.67 is assigned to the β-D-galactopyranosyl residue, which should be linked to O-3 of the β-D-glucopyranosyluronic acid residue, as in **3**. This signal was broad in the spectrum of K21b, but resolved in the spectrum of the Smith-degraded product.



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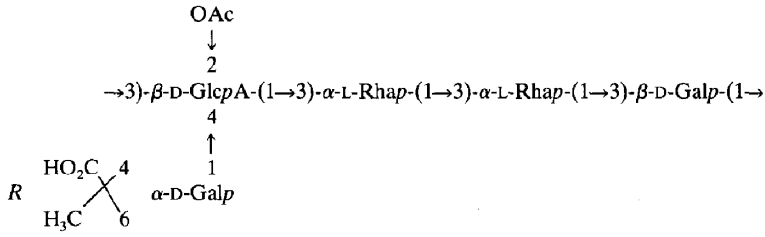
In the ^{1}H-n.m.r. spectrum of original K21b, the signal of the methine proton on the acetoxylated carbon appears at δ 4.98. Coupling between this proton and H-1 of one of the β-D-glycopyranosyl residues at δ 4.83 (J 7.5 Hz), as evident from a COSY spectrum, demonstrated that the acetyl group is linked to O-2 in this residue. Coupling constants of ~9 Hz ($J_{2,3}$; $J_{3,4}$) observed in the cross-peak between H-3 (δ 4.32) and H-4 (δ 4.02) demonstrated that the residue was β-D-glucuronic acid. It is noteworthy that a significant shift of the signal from H-1 in the β-D-galactopyranosyl residue occurs on *O*-deacetylation of K21b.

From the results discussed above, it is concluded that K21b is composed of pentasaccharide repeating-units with the structure **4**.



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The difference between this structure and that of K21a (**1**) is considerable, but nevertheless K21a and K21b show strong cross-reactivity, demonstrating the dominant role of the side-chain.

The structures of most of the 80 or so type-specific *Klebsiella* capsular polysaccharides have been determined. Some polysaccharides, namely, K1 and a subtype of K1[12], and K22[13] and K37[14], have the same carbohydrate backbone, but only one of each pair contains an *O*-acetyl group. K30[15] and K33[16] only differ in the degree of *O*-acetylation. K69[17] has the same carbohydrate backbone as K30 and K33, but pyruvic acid is acetal-linked to O-4 and O-6 of a D-galactopyranosyl group in the former and to O-3 and O-4 of this group in the last two. Others, *e.g.*, 21a[4] and 21b[3], and K55[18] and K83[19], have different carbohydrate backbones but identical side-chains and show strong cross-reactivity. The classification of the *Klebsiella* capsular serotypes is therefore confusing, and a revised classification, with groups and types, as has been done for *Streptococcus pneumoniae*[20], is needed.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument, fitted with a flame-ionisation detector, was used. Separation of alditol acetates and trimethylsilylated glycosides was performed on an HP-54 fused-silica capillary column, using the temperature programs 185° (8 min) → 250° at 5°/min, and 150° (2 min) → 250° at 1°/min, respectively. For partially methylated alditol acetates, a DB225 fused-silica capillary column at 200° was used.

G.l.c.–m.s. was performed on a Hewlett–Packard 5970 instrument. All interpretations of mass spectra were unambiguous and are not discussed. Absolute configurations were determined by the method of Gerwig *et al.*[6]. Methylation analyses were performed as previously described[21]. Methylated products were recovered by reversed-phase chromatography on Sep-Pak C_{18} cartridges[22].

N.m.r. spectra of solutions in deuterium oxide were recorded at 70° (^{13}C) and 85° (^{1}H), with a JEOL GX 270 instrument. Chemical shifts are reported in p.p.m. relative to internal acetone (δ 31.0) for ^{13}C, and internal sodium trimethylsilylpropanoate-d_4 (δ 0.00) for ^{1}H. The COSY experiment was performed with JEOL standard software.

Isolation of K21b. — A culture of *Klebsiella pneumoniae* capsular serotype K21b (1L 918) was grown as described for *Klebsiella* K55, and the capsular polysaccharide was isolated and purified analogously[16].

O-*Deacetylation.* — A solution of K21b (30 mg) in 0.1M aqueous sodium hydroxide (50 mL) was kept at room temperature for 16 h, then neutralised, dialysed against distilled water, and freeze-dried (yield, 24 mg).

Carboxyl reduction. — A solution of the methylated polysaccharide (2 mg) and lithium borohydride (20 mg) in dry tetrahydrofuran (2 mL) was boiled under reflux for 3 h, followed by conventional work-up.

Hydrolysis with acid under mild conditions. — A solution of K21b (8 mg) in 0.1M aqueous trifluoroacetic acid was kept at 100° for 30 min. The product, obtained by conventional work-up, was subjected to methylation analysis.

Smith degradation. — A solution of *O*-deacetylated K21b (25 mg) in 0.3mM sodium metaperiodate (20 mL), buffered to pH 3.9 with 0.1M sodium acetate buffer, was kept in the dark at 4° for 96 h. Excess of periodate was reduced with ethylene glycol, and the product was isolated by dialysis and freeze-drying. A solution of the product and sodium borohydride (60 mg) in water (10 mL) was kept at room temperature for 2 h, excess of reagent was decomposed with acetic acid, and the polyalcohol was isolated by dialysis and freeze-drying. A solution of the polyalcohol in 0.5M aqueous trifluoroacetic acid was kept for 48 h at room temperature and the product was isolated by dialysis and freeze-drying (yield, 18 mg).

Uronic acid degradation. — 2,2-Dimethoxypropane (0.1 mL) and a trace of *p*-toluenesulfonic acid were added to a solution of methylated K21b in dimethyl sulfoxide in a sealed vial, which was then flushed with nitrogen. Sodium methylsulfinylmethanide, in dimethyl sulfoxide (2M, 0.5 mL), was added, the solution was kept overnight and then worked-up, and the product was subjected to hydrolysis with 50% aqueous acetic acid for 16 h at 100° and then reduced with sodium borodeuteride. Methylation analysis using trideuteriomethyl iodide was then performed as described above.

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