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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 66

PER-ERIK JANSSON, BENGT LINDBERG, JORGEN LÖNNGREN*, CARMEN ORTEGA,
Department of Organic Chemistry, University of Stockholm, S-106 91 Stockholm (Sweden)

AND WOLFGANG NIMMICH
Institut für Medizinische Mikrobiologie und Epidemiologie Wilhelm-Pieck-Universität Rostock, DDR-25 Rostock 1 (German Democratic Republic)

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ABSTRACT

The structure of the capsular polysaccharide of *Klebsiella* type 66 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 pentasaccharide repeating-units having the following structure, where 4-*O*-Lac-D-Glc = 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose.

$$\begin{array}{l}\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 4\text{-}O\text{-Lac-}\beta\text{-D-Glc}p\end{array}$$

INTRODUCTION

All of the capsular polysaccharides from *Klebsiella* species are acidic[1,2]. Most of them contain a uronic acid component, usually D-glucuronic acid but sometimes D-galacturonic acid. Several of them also contain acetal-linked pyruvic acid; in the type 32, 56, and 72 polysaccharides, this is the only acidic component[3]. Some polysaccharides contain less-common acidic components. Thus, K 22 contains a sugar tentatively identified as 4-deoxy-*threo*-hex-4-enosyluronic acid[4], K 37 contains 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid[5], and K 38 contains 3-deoxy-L-*glycero*-pentulosonic acid[6]. D-Glucuronic acid, D-galactose, and D-mannose were observed in a hydrolysate of K 66, but preliminary studies indicated that it also contained a second acidic component. We now report more-detailed studies of this polysaccharide.

*Present address: Pharmacia Fine Chemicals AB, Box 175, S-751 04 Uppsala, Sweden.

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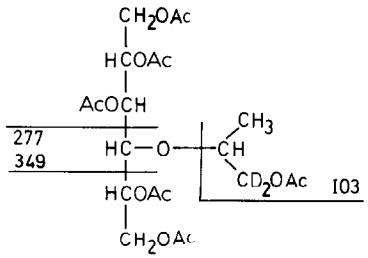
RESULTS AND DISCUSSION

The polysaccharide, isolated as previously described, had $[\alpha]_{578}$ +86°. A sugar analysis, involving methanolysis, carboxyl-reduction with sodium borodeuteride, hydrolysis with acid, reduction with sodium borohydride, acetylation, and analysis of the product by g.l.c.–m.s., showed that the polysaccharide contained mannose, galactose, glucuronic acid (analysed as glucitol-*6,6-d*$_2$ hexa-acetate), and a fourth component in the percentages 40:22:23:15. The D configuration of the three former sugars was demonstrated by the method of Gerwig *et al.*[7,8]. The mass spectrum of the product derived from the fourth component contained, *inter alia*, peaks at *m/z* 349, 277, and 103, suggesting that it was a fully acetylated 3-*O*- or 4-*O*-[2-(1-hydroxy)propyl-*1,1-d*$_2$]hexitol, *e.g.*, **1**. The origin of these fragments is indicated in formula **1**. These results suggest that the unknown sugar component of K 66 is a hexose, etherified with lactic acid at O-3 or O-4, and that the polysaccharide is composed of this component, D-mannose, D-galactose, and D-glucuronic acid in the proportions 1:2:1:1.



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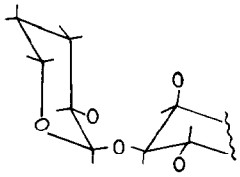
The absolute configuration of the unknown sugar was determined by treatment of a hydrolysate with boron tribromide followed by butanolysis. In addition to D-mannose and D-galactose, the presence of D-glucose was demonstrated. As discussed below, the mass spectrum of a component obtained on methylation analysis of K 66 and derived from this sugar demonstrated that the lactic acid residue is linked to O-4 of D-glucose. From studies of the *Aerococcus viridans* var. *homari*[9] capsular polysaccharide, 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose was available and the alditol acetate obtained from carboxyl-reduced K 66 was well separated from that derived from this polysaccharide. Consequently, the second acidic component of K 66 is 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose.

The ^{1}H-n.m.r. spectrum of K 66 showed, *inter alia*, a signal for the methyl group of the lactic acid residue at δ 1.31 (J 6.8 Hz) and signals for five anomeric protons at δ 4.66 (J 7.6 Hz), 5.00 (not resolved), 5.12 (not resolved), 5.26 (J 3.9 Hz), and 5.48 (J 4.0 Hz). The unresolved signals should be given by the D-mannopyranosyl residues, which, from the observed chemical shifts, should be α-linked. The observed values further indicate that two of the three sugars having the

gluco or *galacto* configuration are α-pyranosidic and that the third is β-pyranosidic.

In agreement with these conclusions, the ^{13}C-n.m.r. spectrum showed, *inter alia*, a signal for the same methyl group at δ 20.6 and signals for five anomeric carbons at δ 95.6 ($^1J_{CH}$ 170 Hz), 97.8 ($^1J_{CH}$ 171 Hz), 98.4 ($^1J_{CH}$ 171 Hz), and 104 (2 C). The $^1J_{CH}$ values of the last two signals could not be determined because of overlapping peaks and the noise level. Values of ~170 Hz are typical for glycopyranosides in which H-1 is equatorial[10], or, for the present sugars, α-glycopyranosides.

The signal at δ 95.6 belongs to one of the α-linked sugars, as seen from the coupling constant, but appears at an unusually high field. Similar high-field signals have been observed previously, for example, for α-kojibiose[11] and methyl 3-*O*-α-D-galactopyranosyl-β-D-galactopyranoside[12] each of which has a typical steric arrangement of substitutents around the glycosidic oxygen as depicted in **2**. In K 66, this arrangement can be found in the disaccharide unit **3**, and therefore the signal at δ 95.6 can be assigned to the anomeric carbon of the α-D-galactopyranosyl residue.



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→3)-α-D-Gal*p*-(1→2)-α-D-Glc*p*A-(1→

3

Fully methylated K 66 was subjected to methanolysis, reduction with sodium borodeuteride, hydrolysis with acid, reduction with sodium borodeuteride, and acetylation. Analysis of the product by g.l.c.–m.s. showed (Table I, column A) that each sugar is pyranosidic, and that the two D-mannosyl residues and the D-galactosyl residue are linked through O-3, that the D-glucuronosyl residue is linked through O-2 and O-3, and that the carboxyethyl-D-glucose is terminal. That the carboxyethyl group is linked to O-4 is evident from the mass spectrum of its derivative (**4**).

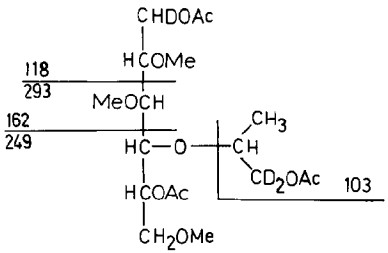

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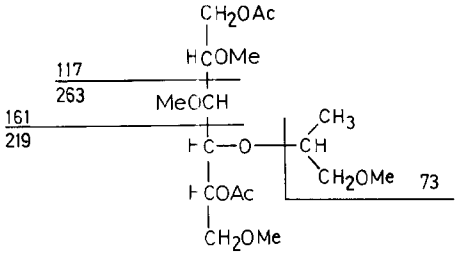
TABLE I

METHYLATION ANALYSES OF K 66 AND MODIFIED PRODUCTS[a]

Sugar[b]	T[c]	T[d]	*Mole %*				
			A	B	C	D	E
2,3*,4,6-Man	1.00	1.00					19
3,4,6-Glc	1.68	1.83			11		
2,4,6-Man	1.87	1.90	44	61	60	61	51
2,4,6-Gal	1.94	2.03	22	29	29	39[e]	30
4,6-Glc	2.92	3.49		4			
4-Glc-*6,6-d*$_2$	5.95	8.4	9				
2,3,6,R-Glc	3.6	5.05		6			
2,3,6,R′-Glc	3.6	5.94	25				

[a]*A*, After methanolysis and $NaBD_4$-reduction of methylated K 66; *B*, carboxyl-reduced K 66; *C*, carboxyl-reduced and Smith-degraded K 66; *D*, uronic acid-degraded and $NaBD_4$-reduced K 66; *E*, uronic acid-degraded and trideuteriomethylated K 66. [b]2,3*,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, OCD_3 at C-3; *etc.* R = 4-*O*-[(*R*)-2-(1-methoxy)propyl]. R′ = 4-*O*-[(*R*)-2-(1-hydroxy)propyl-*1,1-d*$_2$]. [c]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 column at 220°. [d]Same as *c*, but on an OV-225 column at 175°. [e]40% Deuterium labelling at C-1.



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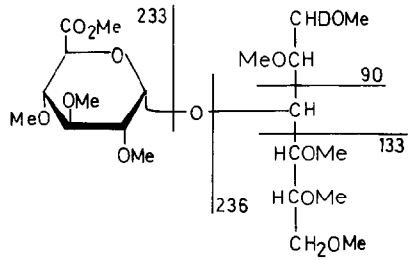

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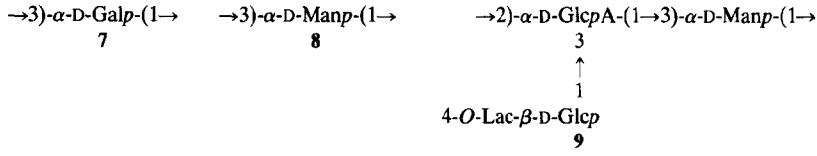
A sample of K 66 was carboxyl-reduced[13] and subjected to methylation analysis. In agreement with the results noted above, 2,4,6-tri-*O*-methyl-D-mannitol, 2,4,6-tri-*O*-methyl-D-galactitol, 4,6-di-*O*-methyl-D-glucitol, and the D-glucitol derivative **5** were obtained (Table I, column B). The carboxyl-reduction, however, was incomplete, as indicated by the stoichiometry.

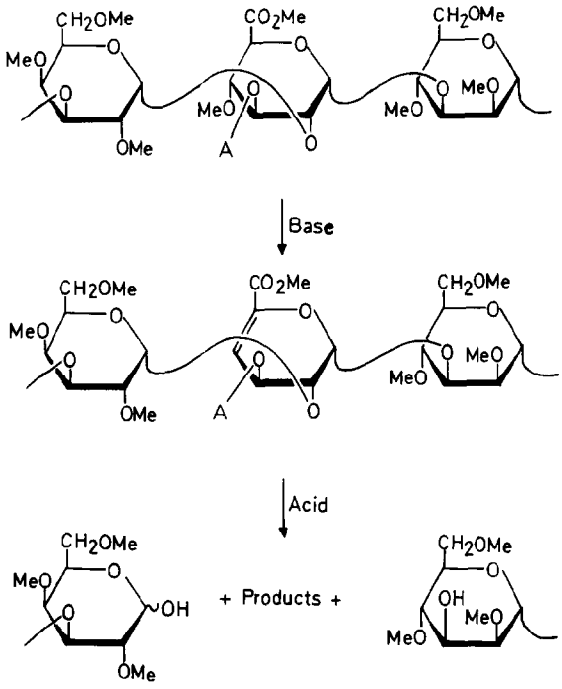
Partial hydrolysis of K 66 yielded a mixture of monomeric sugars and an aldobiouronic acid composed of D-glucuronic acid and D-mannose. The ^{13}C-n.m.r. spectrum of this acid contained signals at δ 102.2 (C), 95.8, and 95.3 (the last two signals corresponding to one carbon). The derived alditol-*1-d* gave a signal for the anomeric proton at δ 5.13 (J 3.4 Hz), demonstrating that the D-glucuronosyl group is α-linked. The mass spectrum of the permethylated alditol (**6**) confirmed that the glucuronosyl group is linked to O-3 of D-mannose. Thus, the fragment *m/z* 133 contains C-4, C-5, and C-6, all of which are methoxylated.

As indicated by the methylation analyses of K 66, only the terminal 4-*O*-(1-carboxyethyl)-D-glucosyl group should be oxidised by periodate. The carboxyl-reduced K 66 was therefore subjected to a Smith degradation (periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions), when only acetalic linkages of modified residues should be hydrolysed. Methylation analysis of the polymeric product demonstrated that the 4-*O*-(1-carboxyethyl)-D-glucose had been completely eliminated. Signals for four anomeric carbons of α-glycosides were observed at δ 97.7 ($^1J_{CH}$ ~170 Hz), 97.9 ($^1J_{CH}$ ~170 Hz), 99.4 ($^1J_{CH}$ ~169 Hz), and 103.7 ($^1J_{CH}$ ~172 Hz). In agreement with this, the ^{1}H-n.m.r. spectrum contained signals for four anomeric protons at δ 5.03 (not resolved), 5.13 (not resolved), 5.22 (not resolved), and 5.41 (J 4.0 Hz). The spectrum did not contain signals in the region for *C*-methyl protons. As all of the sugar residues in the modified polymer are α-linked, the 4-*O*-(1-carboxyethyl)-D-glucosyl group in K 66 is consequently β-linked.

The low yield of 3,4,6-tri-*O*-methyl-D-glucose obtained on methylation analysis of the carboxyl-reduced and degraded K 66 (Table I, column C) indicates that the carboxyl reduction was incomplete. However, the results clearly indicate that the 4-*O*-(1-carboxyethyl)-D-glucose is linked to O-3 of the D-glucuronic acid. In agreement with this conclusion, carboxyl-reduced K 66 showed two signals of comparable intensities for a methyl group in the ^{1}H-n.m.r. spectrum at δ 1.11 [assigned to 2-(1-hydroxy)propyl residues] and 1.18 [assigned to 1-carboxyethyl residues]. Methanolysis of the methylated, degraded product and processing as described for the methylation analysis of K 66 therefore gave 3,4-di-*O*-methyl-D-glucose-*6,6-d*$_2$ in addition to the sugars mentioned above.



From the results discussed above, the structural elements **7**, **8**, and **9** of the pentasaccharide repeating-unit of K 66 have been demonstrated. In order to determine the sequence of these elements, fully methylated K 66 was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, and the product was treated with aqueous 50% acetic acid at 100° for 16 h and then reduced with sodium borodeuteride. Part of this product was hydrolysed, and the components were analysed as their alditol acetates by g.l.c.–m.s. (Table I, column D). Another part was remethylated, using trideuteriomethyl iodide, and the product was hydrolysed and analysed as described above (Table I, column E). In the former analysis, the partial monodeuteration of the 2,4,6-tri-*O*-methyl-D-galactose demonstrates, as indicated in Scheme 1, that the D-galactopyranosyl residue in K 66 is linked to the uronic acid residue and, in part, liberated during the treatment of the alkali-degraded product with aqueous acetic acid. The finding of tetra-*O*-methyl-D-man-



Scheme 1. Uronic acid degradation of permethylated K 66. A = 4-*O*-Lac-β-D-Glc.

nose, with a trideuteriomethyl group at O-3, inthe latter analysis confirms that the uronic acid is linked to O-3 of the mannose residue.

From the combined results, it is concluded that K 66 is composed of pentasaccharide repeating-units having the structure **10**.

$$\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$

4-*O*-Lac-β-D-Glc*p*

10

4-*O*-[(*R*)-1-carboxyethyl]-D-glucose is also a component of the *Shigella dysenteriae* type 3 O-antigen[14], and the corresponding (*S*)-derivative is a component of the capsular polysaccharide of *Aerococcus viridans* var. *homari*[9]. Some other 1-carboxyethyl derivatives of monosaccharides have been observed in bacterial polysaccharides, although they do not occur as frequently as the biogenetically related acetals of pyruvic acid.

EXPERIMENTAL

General methods. — Concentrations were performed at reduced pressure at bath temperatures not exceeding 40°. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. For g.l.c., Perkin–Elmer 990 and Hewlett–Packard 5830 A instruments equipped with flame-ionisation detectors were used. Separations were performed on glass columns (180 × 0.15 cm, with 100/120 mesh Gas Chrom Q as support material) containing *A*, 3% of OV-225 (for alditol acetates and partially methylated alditol acetates); and *B*, 3% of OV-17 (for alditol acetates). Partially methylated alditol acetates and the alditol acetates of the 4-*O*-[2-(1-hydroxy)propyl]-D-glucose derivatives were separated on an SP-1000 W.C.O.T. column (25 m × 0.25 mm). Trimethylsilylated 2-butyl glycosides were separated on an SE-30 W.C.O.T. column.

G.l.c.–m.s. was performed with a Varian MAT 311 instrument equipped with the appropriate g.l.c. columns. For n.m.r. spectroscopy, JEOL FX-100 and GX-400 instruments were used. The spectra were recorded for solutions in D_2O at 85°. Chemical shifts are given in p.p.m. relative to external tetramethylsilane (^{13}C) or internal sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H).

Methylations were performed, according to Hakomori[15,16], with sodium methylsulfinylmethanide/methyl iodide in dimethyl sulfoxide. Methylated products were recovered by dialysis against water, followed by freeze-drying. For low-molecular-weight materials, or when partial depolymerisation was suspected, dimethyl sulfoxide was removed by vacuum distillation and the product was isolated by partition between chloroform and water.

Methanolyses were done with methanolic M hydrogen chloride at 60° for 5 h. The acid was then removed by repeated co-distillations with methanol. Hydrolyses were performed with 4M hydrochloric acid at 100° for 2 h. The acid was removed by repeated co-distillations with water.

Carboxyl-reduction of the polysaccharide was performed according to the procedure of Taylor *et al.*[13]. Three consecutive treatments were performed, but the ^1H-n.m.r. spectra showed that the reduction was not complete.

The absolute configurations of mannose, galactose, and glucuronic acid were determined by the procedure devised by Gerwig *et al.*[7,8]. Treatment of a hydrolysate with boron tribromide[17] yielded D-glucose, identified by g.l.c. of its (−)-2-butyl glycosides[7].

Determination of the absolute configuration of lactic acid. — A sample of K 66 was methanolysed, and the product was reduced with sodium borohydride, hydrolysed, and converted into the alditol acetates. G.l.c. of the products gave a peak at *T* 2.52 (*T* 1 for D-glucitol hexa-acetate) that was well separated from the corresponding *S*-isomer from *Aerococcus viridans*, which had *T* 2.44.

Isolation and purification of the polysaccharide. — The polysaccharide, which was isolated as previously described[1], had $[\alpha]_{578}$ +86° (*c* 1, water).

Smith degradation. — Carboxyl-reduced polysaccharide (30 mg) was dissolved in water (100 mL), and 0.2M sodium metaperiodate (50 mL) was added. The solution was kept in the dark at 4° for 120 h. Excess of periodate was reduced with ethylene glycol (3 mL), and the mixture was dialysed overnight. The solution was concentrated to 30 mL and sodium borohydride (450 mg) was added. After 9 h at room temperature, the excess of borohydride was decomposed by the addition of aqueous 50% acetic acid. The solution was then dialysed and freeze-dried. Part of the residue was subjected to sugar analysis, showing the complete absence of 4-*O*-[(*R*)-2-(1-hydroxy)propyl]-D-glucose. The remainder was hydrolysed with 0.5M trifluoroacetic acid for 32 h at room temperature, neutralised, dialysed, and freeze-dried. Part of the material was subjected to methylation analysis (Table I, column C).

Uronic acid degradation. — Carefully dried, methylated polysaccharide (3 mg) and toluene-*p*-sulfonic acid (trace) were dissolved in a mixture (1 mL) of dimethyl sulfoxide and 2,2-dimethoxypropane (19:1), using a serum vial sealed with a rubber cap. The vial was flushed with nitrogen and kept in an ultrasonic bath for 30 min. Sodium methylsulfinylmethanide in dimethyl sulfoxide (2M, 0.5 mL) was added, and the solution was sonicated for another 30 min, kept at room temperature overnight, and neutralised. The product was partitioned between chloroform and water, the chloroform phase concentrated, and the residue treated with aqueous 50% acetic acid at 100° for 16 h. The solution was concentrated, and the residue was dissolved in water (1 mL) and reduced with sodium borodeuteride (3 mg) overnight. The mixture was then acidified with acetic acid, and the boric acid was removed by distillation of methanol (3 × 2 mL) from the residue. Part of the recovered material was hydrolysed, and the resulting sugars were analysed as alditol acetates by g.l.c.–m.s. (Table I, column D). Another part was subjected to methylation analysis using trideuteriomethyl iodide (Table I, column E).

Partial, acid hydrolysis. — The polysaccharide (300 mg) was hydrolysed in 0.13M sulfuric acid (30 mL) at 100° for 3 h. The hydrolysate was neutralised with barium carbonate. The solution was added to the top of a column of Dowex 1 (AcO^-) resin, and elution first with water and then with a gradient (0–6%) of acetic acid yielded a series of acidic oligosaccharides. The aldobiouronic acid was purified by paper chromatography (ethyl acetate–acetic acid–formic acid–water, 18:3:1:4).

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