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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K74*

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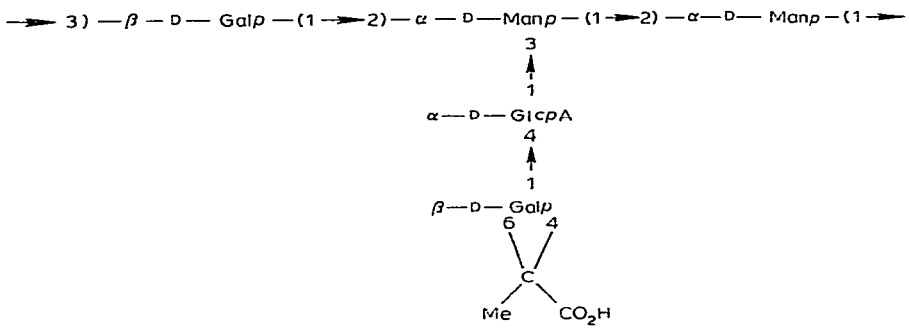
ABSTRACT

The capsular polysaccharide of *Klebsiella* serotype K74 belongs to a chemogroup consisting of seven strains, of which four contain 1-carboxyethylidene groups (pyruvic acid acetals). The polysaccharide from K74 is demonstrated to be of the "3 + 2" type, and to be based on the repeating unit shown.



INTRODUCTION

The capsular polysaccharide from *Klebsiella* K74 is composed[1] of D-glucuronic acid, D-galactose, and D-mannose, and this chemotype includes a total of seven strains[2], of which four have 1-carboxyethylidene groups (pyruvic acid acetals). In this chemogroup, the structures of the capsular polysaccharides of the serotypes K20 (ref. 3) and K21 (ref. 4) have been published.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — The polysaccharide was homogeneous as indicated by electrophoresis, and had $[\alpha]_D$ +66°, which compares very well with the

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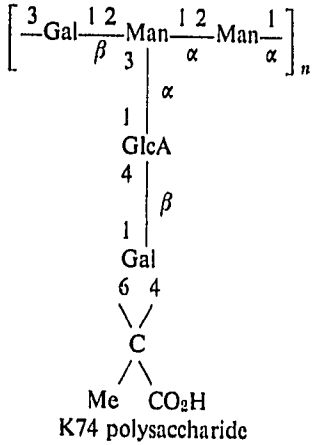
TABLE I

N.M.R. DATA FOR *Klebsiella* K74 CAPSULAR POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	*A*[b]	$J_{1,2}$[c] *(Hz)*	*^{1}H-N.m.r. data*		*^{13}C-N.m.r. data*	
			Integral proton	*Assignment*[d]	*p.p.m.*[b]	*Assignment*[e]
GlcA$\underset{\alpha}{\overset{1\,3}{---}}$Man-OH	5.34	3	1	α-GlcA	101.42	α-GlcA
	5.18	s	0.6	α-Man-OH	94.82	α-Man-OH
1	4.94	s	0.4	β-Man-OH	94.34	β-Man-OH
					61.73	C-6 of Man
GlcA$\underset{\alpha}{\overset{1\,3}{---}}Man\underset{\alpha}{\overset{1\,2}{---}}$Man-OH	5.37	2	2.1	α-Man-OH	102.85	α-Man-Man
	5.34	3		α-GlcA	101.37	α-GlcA
2	5.17	2	0.3	unknown origin	93.38	α,β-Man-OH
	5.08	2	1.0	α-Man-Man	61.94	C-6 of Man
	4.94	1	0.3	β-Man-OH		
GlcA$\underset{\alpha}{\overset{1\,3}{---}}Man\underset{\alpha}{\overset{1\,2}{---}}Man\underset{\alpha}{\overset{1\,3}{---}}$Gal-OH	5.34	3	2	α-GlcA	103.07	α-Man-Man
	5.29	4		α-Man-Gal	101.33	α-GlcA
3	5.17	s	0.3	α-Gal-OH	97.21	β-Gal-OH



K74 polysaccharide

5.07	2	1	α-Man-Man	95.33	α-Man-Gal
4.64	7	0.9	β-Gal-OH	95.08	
				93.09	α-Gal-OH
				61.92	C-6 of Man,Gal
5.37[f]	b	3	α-unassigned	103.94	β-Gal
5.26	b			103.26	
4.55	b	2	β-unassigned	100.96	α-GlcA
1.51	s	3	CH_3 of pyruvate	100.50	β-Man-Man
				96.16	α-Man-Gal
				62.19	
				62.00	C-6 of Man,Gal
				61.85	
				26.12	CH_3 of pyruvate

[a]For origin of compounds **1**, **2**, and **3**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 for ^{1}H-n.m.r., and 31.07 p.p.m. for ^{13}C-n.m.r., downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [c]Key: b = broad, s = singlet. [d]For example, α-Man-OH = proton on C-1 of α-linked D-Man residue; for disaccharides, the data refer to the glycosidic proton. [e]As for [d], but for anomeric, ^{13}C nuclei. [f]Spectra recorded without an internal standard; chemical shift assigned relative to those of CH_3 of pyruvic acetal; δ 1.51 for ^{1}H-n.m.r.

specific rotation (+65°) calculated by using Hudson's rules of isorotation[5], and a molecular weight of 4.7×10^6, determined by gel chromatography.

The ^{1}H-n.m.r. spectrum[6,7] of the original polysaccharide exhibited a sharp singlet at δ 1.51, characteristic of the methyl protons of the pyruvic acetal. The absence of a signal at δ 2.20 indicated that the polymer does not contain any acetate groups. The anomeric region (δ 4.5–5.5), although poorly resolved, showed one broad signal at δ 4.55, assigned to β-linkages, and two broad signals at δ 5.26 and 5.37, attributable to α-linkages. By integration, the ratio of the α- to the β-anomeric protons was 3:2.

Comparison of the integrals also indicated that there is one pyruvic acetal group per pentasaccharide repeating-unit, a result confirmed by courtesy of Dr. S. C. Churms, University of Cape Town, South Africa, who found that the polysaccharide contains 11.9% of pyruvic acid (calculated, 11.7%). However, Nimmich reported[2] that K74 contains only 4% of pyruvic acetal.

The results of the ^{1}H-n.m.r. analysis were confirmed by ^{13}C-n.m.r. spectroscopy of the polysaccharide. The spectrum showed five peaks in the anomeric region, at 103.94, 103.26, 100.96, 100.50, and 96.16 p.p.m., and three in the region associated with -CH_2OH groups, at 62.19, 62.00, and 61.85 p.p.m. Another peak, characteristic of the methyl group of the pyruvic acetal, appeared upfield of the acetone signal, at 26.12 p.p.m. According to observations reported by Garegg *et al.*[8], the acetal carbon atom of the pyruvic acetal group is thus assigned to be of the *R* configuration. The n.m.r. data are presented in Table I.

Total hydrolysis of *Klebsiella* K74 capsular polysaccharide, and subsequent

TABLE II

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED, K74 CAPSULAR POLYSACCHARIDE, AND DERIVED OLIGOSACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	*T*[b] *(ECNSS-M)*	*Mole %*[c]				
		I[d]	*II*	*III*	*IV*	*V*
3,4,6-Man	1.93 (1.98)[e]	23.3 (21.2)[e]		+[f]	23 6	58.6
2,4,6-Man	2 08		51.5	+	32.5	
2,4,6-Gal	2.25 (2.34)	25.9 (23 2)			20 2	35.0
2,3,4-Glc	2.45		48.5	+	23.7	
4,6-Man	3.24 (3.34)	22 0 (22.0)				6 4
2.3-Glc	5 27 (4.53)	13.4 (13.9)				
2,3-Gal	5.71 (4.72)	15.4 (19.7)				

[a]3,4,6-Man = 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol, etc. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an ECNSS-M column operated at 170°, isothermal. [c]Values corrected by use of effective, carbon-response factors given by Albersheim *et al.*[22]. [d]I, methylated, original polysaccharide; II, aldobiouronic acid, 1; III, aldotriouronic acid, 2; IV, aldotetraouronic acid, 3; V, uronic acid degradation-product. [e]Numbers in parentheses refer to data obtained when temperature programming was used; 160° for 13 min and then 2°/min to 190°. + = present, but not quantitated.

g.l.c. analysis of the corresponding alditol acetates, confirmed the presence of mannose, galactose, and glucose in the approximate molar ratios of 2 : 2 : 1. Glucose and mannose were demonstrated to be of the D configuration by circular dichroism (c.d.) measurements of their alditol acetates[9]. Galactose was shown also to be of the D configuration, based on the c.d. spectra of the acetate derivatives of 2,3-di-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol.

Methylation analysis. — Complete methylation of K74 polysaccharide in its sodium salt form, as well as in its free-acid form, proved to be difficult, owing to the low solubility of the polymer in dimethyl sulfoxide. One Hakomori methylation[10,11] and one Purdie treatment[12] were necessary in order to obtain a product that showed no hydroxyl absorption in the infrared spectrum. Reduction of the permethylated sample with lithium aluminum hydride, total hydrolysis with 2M trifluoroacetic acid, and subsequent g.l.c. analysis of the corresponding alditol acetates gave the results listed in Table II, column I.

It may be noted that, whereas quantitative data are often more precise when a g.l.c. column is operated isothermally, this procedure may cause problems with compounds having long retention-times; such is the case with the dimethylgalactose examined here. Under isothermal conditions, a low value was obtained for this compound, on account of the width of the peak; when the temperature was programmed after elution of the tri-*O*-methyl sugars, an essentially quantitative value resulted. The low figure for the dimethylglucose was due to incomplete reduction of the glucuronic ester. In general, the methylation results corroborate those obtained by n.m.r. spectroscopy, and show that the repeating unit is a pentasaccharide As demonstrated by further studies (see later), the presence of 4,6-di-*O*-methylmannose is due to a branch point, and the 1-carboxyethylidene group is present as an acetal spanning O-4 and O-6 of the terminal 2,3-di-*O*-methylgalactose.

Partial hydrolysis. — Partial, acid hydrolysis of the native K74 polysaccharide was followed by separation of the acidic and neutral fractions by ion-exchange chromatography. The neutral fraction was not studied further, but the acidic one was separated by gel-filtration chromatography, followed by descending paper-chromatography. Three pure oligosaccharides (**1**, **2**, and **3**) were thus collected.

These oligosaccharides were examined by n.m.r. spectroscopy (see Table I) and by methylation (see Table II). From these results, it follows that **3** is

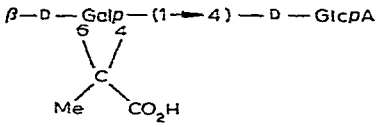
α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-D-Gal

3

and that **2** and **1** are the corresponding aldotrio- and aldobio-uronic acids.

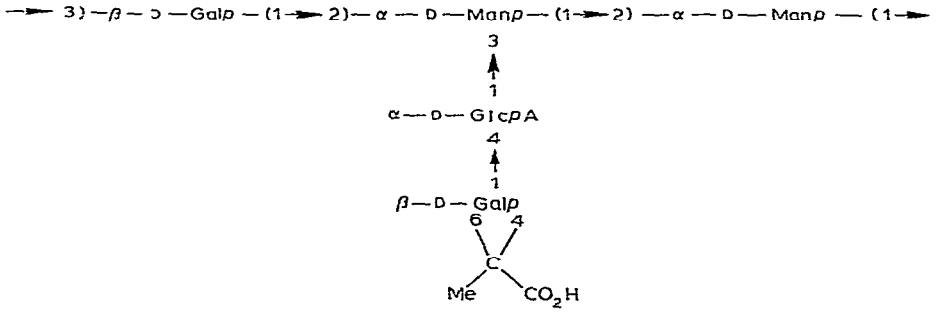
The ^{13}C-n.m.r. spectrum of oligosaccharide **3** exhibited a feature that had been observed in that of the similarly constituted aldotetraouronic acid isolated from *Klebsiella* K53 polysaccharide, namely, splitting of the anomeric signal of the manno-pyranosyl residue linked to O-3 of the (reducing) galactose residue, probably caused by the mutarotational equilibrium, that it was not due to a mixture of oligosaccharides was verified by analysis by the aldononitrile method of Morrison[13].

Uronic acid degradation. — In a single operation, the permethylated polysaccharide was treated with dimethylsulfinyl anion and subsequently alkylated with methyl iodide[14,15]. A polymeric product was obtained which, on analysis, gave the results shown in Table II, column V; these indicated that the uronic acid is in the side chain, which is thus composed of the pseudo-aldobiouronic acid shown, linked to O-3 of the D-mannosyl branch-point.



CONCLUSION

The results given, in particular those from the uronic acid degradation, taken in conjunction with the nature of 3, demonstrate that the structure of the repeating unit of the capsular polysaccharide from *Klebsiella* K74 is as follows.



The capsular antigen from *Klebsiella* K74 is known[16] to cross-react weakly in antipneumococcal sera PnII, PnVIII, and PnXX. However, little useful information can be obtained from these reactions, as there is no structural feature common to K74 and the pneumococcal capsular polysaccharides PnII, PnVIII, and PnXX.

EXPERIMENTAL

General methods. — Instrumentation and chromatographic solvents were as described in the preceding paper[17]. Photocopies of the n.m.r. spectra are available[18]. Analytical, g.l.c. separations were performed by using the following, stainless-steel columns (1.8 m × 3 mm): (*1*) 3% of SP-2340 on Supelcoport (100–130 mesh); (*2*) 5% of ECNSS-M on Gas Chrom Q (100–120 mesh); and (*3*) 3% of OV-225 on the same support. Preparative g.l.c. separations were performed by using a column (1.8 m × 6.3 mm) (*4*) 5% of Silar 10C on Gas Chrom Q (100–120 mesh).

The ^{13}C-n.m.r. spectrum of the native polysaccharide was obtained by courtesy

of Dr. M. Vignon, CERMAV/CNRS, Grenoble, France, by means of a Bruker Spectrospin instrument.

Isolation and purification of Klebsiella *K74 capsular polysaccharide.* — A culture of *Klebsiella* serotype K74 (371), obtained by courtesy of Dr. I. Ørskov, was grown as described[17] for *Klebsiella* K53. The polysaccharide (10 g), isolated in the sodium salt form, had $[\alpha]_D$ +66° (*c* 0.21, water). The purity of the polysaccharide was checked by electrophoresis, using a 1% solution on cellulose acetate strips (Sepraphore III; 15 × 2.5 cm) in Veronal buffer, pH 8.6 (LKB-Produkter AB, Stockholm 12, Sweden) at 300 V for 60 min, and then developed in Alcian Blue in citrate–buffered ethanol. Homogeneity was confirmed by gel chromatography by courtesy of Dr. S. C. Churms, University of Cape Town, South Africa, and the molecular weight of the K74 polysaccharide was determined to be 4.7×10^6.

Analysis of sugar constituents. — Sugar analysis was performed as described[17] for K53. The alditol acetates of mannose, galactose, and glucose were identified by g.l.c. (column *1*; programmed at 195° for 4 min, and then 2°/min to 260°) by comparison with authentic samples, and found to be present in the molar ratios of ~2:2:1. Paper chromatography (solvents *A* and *B*) of a hydrolyzate of the polysaccharide confirmed the presence of mannose, galactose, glucuronic acid, and pyruvic acid

The group configuration of each of the constituent sugars was determined by measurement of the c.d. curve of the alditol acetates of mannose and glucose, and of the partially methylated alditol acetates of galactose. Samples were isolated by preparative g.l.c. (column *4*; 240°, isothermal). Comparison with authentic standards confirmed the D configuration of all of the sugar constituents.

Methylation analysis. — A dried sample (127 mg) of K74 polysaccharide was dissolved in anhydrous dimethyl sulfoxide (100 mL) by using ultrasonic agitation, and methylated[10,11] by treatment overnight with dimethylsulfinyl anion (12 mL), and then with methyl iodide (8 mL) for 1.5 h. The mixture was dialyzed against running tap-water for three days, and freeze-dried. The product was taken up in chloroform, the suspension filtered, and the filtrate evaporated; Purdie treatment[12] of the residue (153 mg) with methyl iodide and silver oxide gave a product that showed no hydroxyl absorption in the infrared spectrum.

The permethylated sample was reduced with lithium aluminum hydride in refluxing oxolane for 5 h, and the reaction was continued overnight at room temperature. The excess of hydride was decomposed with water, and the product was acetylated[19]. Hydrolysis of the reduction product with 2M trifluoroacetic acid overnight at 95°, reduction with sodium borohydride, and g.l.c.–m.s. analysis[20,21] of the partially methylated, alditol acetates gave the results listed in Table II, column I.

Partial hydrolysis. — The K74 polysaccharide (~540 mg) was partially hydrolyzed with 2M trifluoroacetic acid for 2.5 h at 95°. The solution was evaporated to dryness under diminished pressure, and several portions of water were added to, and evaporated from the residue, to eliminate the excess of acid. The product was applied to a column (30 × 1.5 cm) of Bio-Rad AG1-X2 (formate) resin, and the

neutral fraction was eluted with water (600 mL), and freeze-dried; yield 254 mg. The acidic fraction was eluted with 10% formic acid (500 mL), and the eluate was evaporated to dryness under diminished pressure (several times with water), and freeze-dried; yield 293 mg. Paper chromatography (solvent *C*) of the acidic fraction showed that it contained a disaccharide and higher oligosaccharides.

The acidic oligomers were separated by gel-filtration chromatography on a column (100 × 3 cm) of Bio-Gel P-2, using, for irrigation, a buffer composed of 500:5:2 water–pyridine–acetic acid at a flow rate of 10 mL/h. Fractions (2.0–2.5 mL) were collected in tared tubes, freeze-dried, and weighed. Fractions 41–47, as well as fractions 35–40 and 29–34, were pooled, and purified by descending paper-chromatography for three d, using solvent *C*. Three pure oligosaccharides were thus collected: Compound **1**, 52 mg, $[\alpha]_D$ +80.7° (*c* 2.41, water); **2**, 60 mg, $[\alpha]_D$ +89.4° (*c* 1.61, water); and **3**, 72 mg, $[\alpha]_D$ +130° (*c* 2.10, water). Spectral data for these compounds are recorded in Table I, and methylation analyses are presented in Table II, columns II, III, and IV.

Uronic acid degradation[14,15]. — A dried sample (15 mg) of methylated K74 polysaccharide and *p*-toluenesulfonic acid (1 mg) were dissolved in 19:1 (v/v) dimethyl sulfoxide–2,2-dimethoxypropane (10 mL), and the solution was treated with dimethylsulfinyl anion (5 mL) overnight. An excess of methyl iodide (7 mL) was added with external cooling, and the mixture was stirred for 1 h, and then dialyzed against running tap-water for two days. The polymeric material was extracted with chloroform (5 × 10 mL), and the extracts were combined, and evaporated to dryness under diminished pressure. Hydrolysis of the product with 2M trifluoroacetic acid, reduction with sodium borohydride, acetylation with 1:1 (v/v) acetic anhydride–pyridine overnight at room temperature, and g.l.c. analysis of the alditol acetates, gave the results listed in Table II, column V.

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