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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K45

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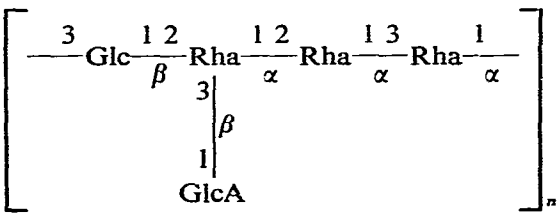
ABSTRACT

The structure of the capsular polysaccharide of *Klebsiella* K45 is of the "four plus one" type shown, and thus bears a formal analogy to those of serotypes K9 and K59. The structure was established mainly by identification of the oligosaccharides produced on partial hydrolysis, and is in agreement with predictions made on the basis of serological cross-reactions.



INTRODUCTION

The *Klebsiella* capsular polysaccharides that are composed[1] of D-glucuronic acid, D-glucose, and L-rhamnose are derived from serotypes K17 (ref. 2), K23 (ref. 3), K44 (ref. 4), K45, and K71. None of these serotypes contain acetal-bound pyruvic acid. We now report the structure of the capsular antigen from serotype K45.

RESULTS AND DISCUSSION

The polysaccharide, purified by precipitation with Cetavlon, had $[\alpha]_D$ −43.8°, and was composed of D-glucuronic acid, D-glucose, and L-rhamnose in the molar ratios of 1 : 1 : 3. In agreement with the observations of Nimmich[1], D-galactose was a contaminant in each batch of polysaccharide, but was eliminated by rigorous purification, and was not present in the methylated polymer.

The p.m.r. spectrum confirmed the concept of a pentasaccharide repeating-

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TABLE I

N.M.R. DATA FOR *Klebsiella* K45 CAPSULAR POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	Δ[b]	*$J_{1,2}$ (Hz)*	*Integral proton*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[e]
Glc$\frac{1\,2}{\beta}$Rha$\frac{1\,2}{\alpha}$Rha$\frac{1\,3}{\alpha}$Rha 3 | β 1 | GlcA **A1**	5.22	s	1	$\frac{2\,3\,\vert}{}$Rha$\frac{}{\alpha}$	104.42	Glc$\frac{}{\beta}$
	5.18	s	1	$\frac{2}{}$Rha$\frac{}{\alpha}$		GlcA$\frac{}{\beta}$
	5.11	s	0.7	$\frac{3}{}$Rha$\frac{}{\alpha}$	102.13	$\frac{2}{}$Rha$\frac{}{\alpha}$ (3 |)
	4.88	s	0.3	$\frac{3}{}$Rha$\frac{}{\beta}$	101.57	$\frac{2}{}$Rha$\frac{}{\alpha}$
	4.75	8	1	Glc$\frac{}{\beta}$	94.72	$\frac{3}{}$Rha$\frac{}{\alpha}$
	4.74	8	1	GlcA$\frac{}{\beta}$	94.17	$\frac{3}{}$Rha$\frac{}{\beta}$
					61.31	C-6 Glc
					17.49	C-6 Rha
Glc$\frac{1\,2}{\beta}$Rha$\frac{1\,2}{\alpha}$Rha 3 | β 1 | GlcA **A2**	5.23	s	1	$\frac{2\,3\,\vert}{}$Rha$\frac{}{\alpha}$	104.37	Glc$\frac{}{\beta}$
	5.21	s	1	$\frac{2}{}$Rha$\frac{}{\alpha}$		GlcA$\frac{}{\beta}$
	4.77	8	1	Glc$\frac{}{\beta}$	102.07	—Rha$\frac{}{\alpha}$ (3 |)
	4.70	8	1	GlcA$\frac{}{\beta}$	93.47	$\frac{2}{}$Rha$\frac{}{\alpha}$
					61.35	C-6 Glc
					17.69	C-6 Rha
					17.52	C-6 Rha
GlcA$\frac{1\,3}{\beta}$Rha$\frac{1\,2}{\alpha}$Rha$\frac{1\,3}{\alpha}$Rha **A3**	5.20	s	1	$\frac{3}{}$Rha$\frac{}{\alpha}$	104.35	GlcA$\frac{}{\beta}$
	5.11	s	0.7	$\frac{3}{}$Rha$\frac{}{\alpha}$	102.79	$\frac{3}{}$Rha$\frac{}{\alpha}$
	5.00	s	1	$\frac{2}{}$Rha$\frac{}{\alpha}$	101.53	$\frac{2}{}$Rha$\frac{}{\alpha}$
	4.88	s	0.3	$\frac{3}{}$Rha$\frac{}{\beta}$	94.77	$\frac{3}{}$Rha$\frac{}{\alpha}$
	4.72	8	1	GlcA$\frac{}{\beta}$	94.17	$\frac{3}{}$Rha$\frac{}{\beta}$
					17.49	C-6 of Rha

TABLE I *(continued)*

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[b]	$J_{1,2}$ *(Hz)*	*Integral proton*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[e]
Rha$\frac{1\,3}{\alpha}$Glc$\frac{1\,2}{\beta}$Rha$\frac{1\,2}{\alpha}$Gly	5.31	s	1	$\frac{2}{}$Rha$\frac{}{\alpha}$		
S1	5.16	s	1	Rha$\frac{}{\alpha}$		
	4.63	8	1	$\frac{3}{}$Glc$\frac{}{\beta}$		
	1.30	6 ($J_{5,6}$)	3	CH$_3$ of Rha		
	1.28	6 ($J_{5,6}$)	3			
$\frac{3}{}$Glc$\frac{1\,2}{\beta}$Rha$\frac{1\,2}{\alpha}$Rha$\frac{1\,3}{\alpha}$Rha$\frac{1}{\alpha}$ (3 → β, 1 → GlcA)	5.24	s	1	$\frac{2\,\vert\,3}{}$Rha$\frac{}{\alpha}$	104.38	$\frac{3}{}$Glc$\frac{}{\beta}$
K45	5.19	s	1	$\frac{2}{}$Rha$\frac{}{\alpha}$		GlcA$\frac{}{\beta}$
	5.15	s	1	$\frac{3}{}$Rha$\frac{}{\alpha}$	102.18	$\frac{2}{}$Rha$\frac{}{\alpha}$ (3 |)
	4.75	8	1	$\frac{3}{}$Glc$\frac{}{\beta}$	101.74	$\frac{2}{}$Rha$\frac{}{\alpha}$
	4.69	8	1	GlcA$\frac{}{\beta}$		$\frac{3}{}$Rha$\frac{}{\alpha}$

[a]For the source of **A1**, **A2**, **A3**, and **S1**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [c]The numerical prefix indicates the position in which the sugar is substituted; α or β gives the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing-sugar residue. Thus, 3-Gal$\frac{}{\alpha}$ refers to the anomeric proton of a 3-linked galactosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. [d]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [e]As in *c*, but for ^{13}C nuclei.

unit, and showed that all of the rhamnose was α-linked, whereas the glucose and the glucuronic acid were β-linked (see Table I).

Methylation analyses[5,6]. — The methylation results shown in Table II, column I, clearly indicate that the uronic acid is terminal, and that one rhamnose residue is the branch point. It remained to ascertain the length of the side chain and its point of attachment, as well as the sequence in the main chain. A β-elimination experiment[7,8] revealed that only the D-glucuronic acid was lost, and that 4-*O*-methylrhamnose was replaced by 3,4-di-*O*-methylrhamnose (see Table II, column II), thus showing that the uronic acid is attached to O-3 of the branch point.

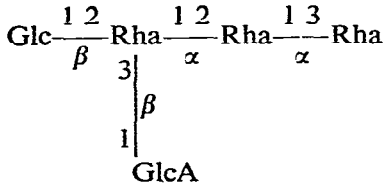
TABLE II

METHYLATION ANALYSIS OF *Klebsiella* K45 POLYSACCHARIDE AND DERIVATIVES

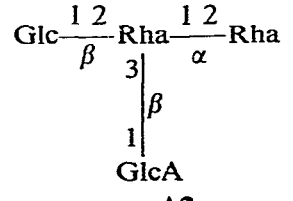
Methylated sugars[a] *(as alditol acetates)*	R_T[b]		*Mole %*[c]						
	Column A (HIEFF-1B)	*Column B (OV-17)*	*I*[d]	*II*	*III*	*IV*	*V*	*VI*	*VII*
2,3,4-Rha		0.49							1.06
3,4-Rha	0.57	0.78	0.81	1.94	1.1	1.0	1.0	tr	1.00
2,4-Rha	0.66	0.90	0.89	1.00	1.0		2.0		
4-Rha	**1.00**	1.25	1.00		0.9	0.9		0.8	
2,4,6-Glc	1.13	1.55	1.18	1.06					1.11
2,3,4-Glc	1.24	1.62	0.94		0.9	0.8	1.0	0.9	
2,3,4,6-Glc	0.67	**1.00**			1.0	1.0		1.0	

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc. [b]Relative retention times on column *A* operated at 170° for 4 min, and then at 2°/min to 200°, and on *B*, 175° for 8 min and then at 2°/min to 210°. [c]Values were corrected by use of effective, carbon-response factors given by Albersheim *et al.*[17]. [d]Key: I, original, capsular polysaccharide, methylated and uronic ester reduced; II, product of β-elimination; III–VI, compounds **A1**, **A2**, and **A3**; VII, oligosaccharide **S1** (see text for details).

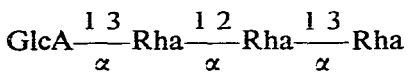
Partial hydrolysis. — The presence of three 6-deoxy sugar residues in the repeating unit made it difficult to obtain oligosaccharides higher than an aldobiouronic acid, but hydrolysis with 0.01M trifluoroacetic acid was found to be the best compromise; however, this left a large amount of polymeric material (which was reprocessed). Paper chromatography gave three pure compounds: **A1** (pentasaccharide), A_2, and **A3** (both tetrasaccharides) (and higher oligosaccharides that were not examined further). **A1** had R_{Glc} 0.41 (solvent *3*) and $[\alpha]_D$ −43° (*c* 2.4, water), calculated[9] $[\alpha]_D$ −44°; **A2** had R_{Glc} 0.55 and $[\alpha]_D$ −33° (*c* 0.98, water), calculated $[\alpha]_D$ −37°; and **A3** had R_{Glc} 0.63 and $[\alpha]_D$ −41° (*c* 1.65, water), calculated $[\alpha]_D$ −45°. The analytical results summarized in Table II, columns III–VI, together with the n.m.r. data presented in Table I established the structures shown.



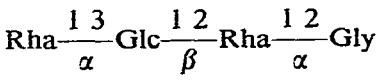
A1



A2



A3



S1

It is interesting that, in compounds **A1** and **A3**, each terminating in a 3-substituted rhamnose unit, both the ^{1}H- and the ^{13}C-n.m.r. spectra show the existence of a mutarotational equilibrium (~70% α, 30% β). However, for compound **A2**, terminated by a 2-substituted rhamnose, both spectra show signals exclusively for the α anomer.

The identification of these oligosaccharides and, in particular, of the alditol derived from **A2**, is sufficient to establish the structure of the polysaccharide from *Klebsiella* K45, but a confirmatory periodate oxidation was conducted.

Periodate oxidation and Smith degradation. — The consumption of periodate after 24 h was 2.95 mol per repeating unit (theoretical, 3.0), and Smith hydrolysis[10] yielded an oligosaccharide (**S1**), analysis of which gave glucose, rhamnose, and glycerol in the ratios of 1:2:1. Methylation of this oligosaccharide gave the products shown in Table II, column VII, consistent with the sequence

$$\text{Rha}\overset{1\,3}{\underset{\alpha}{---}}\text{Glc}\overset{1\,2}{\underset{\beta}{---}}\text{Rha}\overset{1\,2}{\underset{\alpha}{---}}\text{Glycerol.}$$

The anomeric linkages shown follow from the n.m.r. data in Table I, as well as from the optical rotation; calculated[9] $[\alpha]_D$ −50°, found $[\alpha]_D$ −48°.

CONCLUSIONS

The structure reported here confirms the prediction by Heidelberger and Nimmich[11], based on serological cross-reactions with anti-Pn II, that the antigen has side-chain units of D-glucuronic acid. The aldobiouronic acid unit had been found, earlier[12] in this series, in the polysaccharide from *Klebsiella* K47.

EXPERIMENTAL

The instrumentation used has been described previously[13]. Paper chromatography was conducted by the descending method, using Whatman No. 1 paper and the following solvent systems (v/v): (*1*) 4:1:1 ethyl acetate–pyridine–water, (*2*) 4:1:5 1-butanol–acetic acid–water, (*3*) 2:1:1 1-butanol–acetic acid–water, and (*4*) butanone–water azeotrope. Chromatograms were developed with silver nitrate, or with *p*-anisidine trichloroacetate. Preparative paper-chromatography was performed by the descending method, using Whatman No. 3 MM paper and solvent *3*. G.l.c. separations were performed in stainless-steel columns (1.8 m × 3 mm, or 1.8 m × 6.3 mm) with a carrier-gas flow-rate of 20 mL/min. Columns used were (*A*) 3% of diethylene glycol succinate (HIEFF-1B), (*B*) 3% of OV-17, and (*C*) 5% of Silar 10C, all on Gas Chrom Q (100–120 mesh).

Preparation and properties of K45 capsular polysaccharide. — A culture of *Klebsiella* K45 (8464) was obtained from Dr. I. Ørskov, Copenhagen, and was grown as previously described[13]. The polysaccharide, purified by precipitation with Cetavlon, had $[\alpha]_D$ −43.8° (*c* 0.5, water). Paper chromatography (solvents *1* and *2*) of the

neutral sugars in a hydrolyzate showed glucose and rhamnose, and the total sugar ratios[14] determined by g.l.c. (column *B*) gave glucuronic acid : glucose : rhamnose in the molar ratios of 1 : 1 : 3, with traces of galactose. Glucitol hexaacetate was obtained crystalline, m.p. 96–98° (lit.[15] m.p. 99°), and rhamnitol pentaacetate as a syrup. Measurement of the c.d. spectra[16] gave $\Delta\varepsilon_{213}$ +0.116 for the former, and $\Delta\varepsilon_{213}$ −1.52 for the latter, indicating D and L configurations, respectively.

Following hydrolysis of the K45 polysaccharide with 0.1M trifluoroacetic acid for 30 min on a steam bath, the 400-MHz spectrum showed distinct singlets at δ 5.26, 5.21, and 5.16, and doublets at δ 4.75 and 4.69: each signal integrated for 1 H (see Table I).

Methylation analysis. — The polysaccharide was methylated by the methods of Hakomori and Purdie, to give a product lacking any hydroxyl absorption in the i.r. spectrum. A sample (19 mg) was dissolved in dry oxolane (1.5 mL) and added to a stirred suspension of lithium aluminum hydride (30 mg) in the same solvent (2 mL). The slurry was stirred for 24 h at 25°; addition of water, and extraction with chloroform, gave 11 mg of product. Hydrolysis with 90% formic acid for 1 h at 100°, and with 2M trifluoroacetic acid for 6 h at 100°, and examination on paper (solvent *4*) showed five components, R_F 0.65, 0.53, 0.50, 0.47, and 0.40. G.l.c. analysis in column *A* (170° for 4 min, then 2°/min to 200°) gave the results shown in Table II, column I.

Uronic acid degradation. — To a solution of permethylated K45 polysaccharide (80 mg) and *p*-toluenesulfonic acid (1 mg) in 19 : 1 dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) was added 2M dimethylsulfinyl anion (10 mL). The solution was kept overnight at 25°; methyl iodide (7 mL) was added, and the solution cleared after 1.5 h. The excess of methyl iodide was removed *in vacuo*, the residue was dialyzed overnight, and, after lyophilization, the product was extracted with chloroform. Analysis gave the results presented in Table II, column II.

Partial hydrolysis. — A preliminary experiment was conducted with polysaccharide (25 mg) in 0.01M trifluoroacetic acid (12 mL). The solution was heated on a steam bath, and samples were withdrawn at 3.5, 8.5, 14.5, 20.5, and 28.5 h. From chromatograms in solvent *3*, a time of 30 h was judged suitable.

K45 polysaccharide (1.30 g) was dissolved in 0.01M trifluoroacetic acid (520 mL), and the solution was heated on a steam bath for 30 h. The resulting syrup was added to a column of Bio-Rad AG-1 X-2 (formate) resin, which was washed with water (550 mL), to yield neutral sugars that were discarded. Elution with 10% formic acid gave 1.20 g of acidic oligosaccharides: this material was divided into two portions.

The smaller (300 mg), in the minimum amount of water, was added to a column of Sephadex G-25 which was eluted with water to give 160 fractions (1.5–2.0 mL each). Paper chromatography (solvent *3*) of representative tubes indicated that there were three main fractions: I (tubes 10–27), 100 mg of polymeric material; II (28–47), 115 mg of high-molecular-weight oligomers; and III (48–120), 94 mg of oligosaccharides.

The larger portion (600 mg) was dialyzed in a Spectropor No. 3 bag against distilled water (6 × 250 mL). The dialyzate, fraction IV, weighed 126 mg, and was combined with fraction III. The nondialyzable material was combined with fractions I and II, and rehydrolyzed with 0.01M trifluoroacetic acid for 24 h, to give, after ion-exchange separation, a further 380 mg of acidic oligosaccharides. These, together with fractions III and IV, were separated on six sheets of Whatman 3 MM paper in solvent *3* for 40 h. Silver nitrate showed seven components, which were eluted with water. Component **A1** (88 mg), R_{Glc} 0.41, was pure; **A2** (30 mg) contained traces of **A1**; **A3** (41 mg) was judged to contain 10–20% of **A2**. Pure samples of **A2** and **A3** were obtained by a second separation on paper. Materials moving more slowly than **A3** were mixtures, and were not examined further.

The structures of, and n.m.r. data for, **A1**, **A2**, and **A3** are presented in Table I, and the results of methylation analyses are given in Table II, columns III–V. Compound **A2** was also subjected to methylation analysis after reduction to the alditol (see Table II, column VI).

Periodate oxidation. — Polysaccharide (310 mg) was dissolved in 0.05M sodium periodate (200 mL), and after 24 h at 4°, the consumption of periodate was 2.95 mol per repeating unit. Following reduction with sodium borohydride, and hydrolysis with M trifluoroacetic acid for 48 h at 25°, an oligosaccharide **S1** (220 mg) was obtained. **S1** had $[\alpha]_D$ −48° (*c* 0.76, water) and R_{Glc} 0.75 (solvent *3*). G.l.c. of hydrolyzed **S1** gave glycerol:rhamnose:glucose in the ratios of 1:2:1. The n.m.r. data are presented in Table I, and the results of the methylation analysis in Table II, column VII.

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