Carbohydrate Research, 244 (1993) 325–340
Elsevier Science Publishers B.V., Amsterdam

Structural investigation of the capsular polysaccharide produced by a novel *Klebsiella* serotype (SK1). Location of *O*-acetyl substituents using NMR and MS techniques

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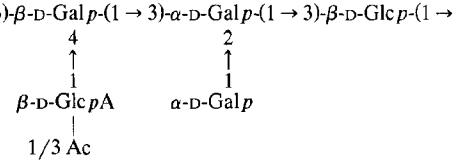
(Received May 4th, 1992; accepted in revised form November 18th, 1992)

ABSTRACT

The capsular polysaccharide of *Klebsiella* SK1 was investigated by methylation analysis, Smith degradation, and ^{1}H NMR spectroscopy. The oligosaccharides (**P1** and **P2**) obtained by bacteriophage ΦSK1 degradation of the polymer were studied by methylation analysis, and 1D- and 2D-NMR spectroscopy. The resulting data showed that the parent repeating unit is a branched pentasaccharide having a structure identical to the revised structure recently proposed for *Klebsiella* serotype K8 capsular polysaccharide.



The 2D-NMR data showed that one third of the glucuronic acid residues in the SK1 polymer are acetylated at O-2, O-3, or O-4. FABMS studies confirmed the presence of monoacetylated glucuronic acid residues. Thus, the relationship between the *Klebsiella* K8 and SK1 polymers is akin to that found for *Klebsiella* polysaccharides K30 and K33, which have been typed as serologically distinct yet their structures differ only in the degree of acetylation.

INTRODUCTION

The encapsulated SK1 strain of *Klebsiella pneumoniae* was first isolated by Rennie and Duncan from an equine source[1]. This strain shares a number of

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characteristics with other *Klebsiellae* isolated from humans, in particular resistance to antibiotics as found[2] for strains K9, K21, K22, K37, and K45. Serological analysis[3] showed that SK1 may be a new serotype which cross reacts with human antisera to *Klebsiella* K52, as well as those to *Klebsiella* K8 and K59, indicating structural similarities.

The structure of the capsular polysaccharide produced by the SK1 serotype has been investigated by chemical and spectroscopic analysis of the polysaccharide and the oligosaccharides derived from it by bacteriophage degradation.

RESULTS AND DISCUSSION

Isolation and composition of SK1 capsular polysaccharide.—*Klebsiella* SK1 bacteria were grown on a 3% sucrose-yeast extract-agar medium[4] and the capsular material was extracted with 2% aqueous phenol. The polysaccharide, after purification via its cetyltrimethylammonium bromide salt, eluted as a single peak on a Sephacryl S400 analytical column. Paper chromatography of the acid hydrolysate (solvent *A*) showed the presence of galactose, glucose, and glucuronic acid. The neutral sugars were quantitated by GLC analysis of their alditol acetate derivatives, which showed the glucose : galactose molar ratio to be 1.0 : 3.0. Methanolysis of the polysaccharide followed by acetylation and subsequent GLC confirmed the presence of glucuronic acid. All three sugars were shown to be D isomers by GLC analysis of their trimethylsilylated (−)-2-butyl glycosides[5,6].

The ^{1}H spectrum of the polysaccharide showed five signals in the region for anomeric protons. These corresponded to two α linkages (δ 5.62 and 5.27) and three β linkages ($\delta \approx 4.74$). Two signals visible at high field (δ 2.16 and 2.22) were attributed to acetyl groups present to the extent of one per three repeating units.

Methylation analysis and related experiments.—The capsular polysaccharide was methylated and the derived alditol acetates, with and without prior carboxyl-reduction, were analysed by GLC–MS (Table I). A sample of methylated, carboxyl-reduced polysaccharide was remethylated and analysed, with the results also shown in Table I. Another portion of the methylated polysaccharide was subjected to base-catalysed β-elimination followed by realkylation with ethyl iodide. GLC–MS analysis of the derived alditol acetates gave the results listed in Table I.

These methylation analyses showed that the polysaccharide is composed of terminal galactose and glucuronic acid groups together with 3-linked glucopyranosyl, and 2,3- and 3,4-linked galactopyranosyl residues. The β-elimination experiment (Table I, IV) indicated that the glucuronic acid is bound to position 4 of the 3,4-linked galactopyranosyl residue.

Smith degradation.—The capsular polysaccharide was subjected to sodium metaperiodate for 1 h (**S1**) in order to selectively oxidise the terminal galactose[7,8] and treated exhaustively for 72 h (**S2**). After reduction and mild acid hydrolysis, **S1** and **S2** were subjected to methylation analysis (Table II), which showed that the terminal galactose is bound to O-2 of the 2,3-linked galactosyl residue and

TABLE I

Methylation analysis of *Klebsiella* SK1 capsular polysaccharide

Methylated sugar [a] (as alditol acetate)	t_R [b]	Molar ratios [c]			
		I	II	III	IV
2,3,4,6-Glc [d]	0.90			0.9	
2,3,4,6-Gal	**1.00**	1.0	1.0	1.0	1.0
2,4,6-Glc	1.34	0.7	0.7	1.1	0.8
2,3,4-Glc [d]	1.39		1.1		
2,4,6-Gal [e]	1.47				0.9
2,6-Gal	1.81	0.7	0.9	0.9	
4,6-Gal	1.84	1.1	0.8	0.9	0.6

[a] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b] Retention time relative to that of 2,3,4,6-Gal. [c] I, methylated polysaccharide; II, methylated then carboxyl-reduced polysaccharide; III, methylated, carboxyl-reduced, and remethylated polysaccharide; IV, methylated, β-eliminated, and ethylated polysaccharide. [d] C-6 dideuterated according to GLC–MS. [e] 1,3,5-Tri-*O*-acetyl-2,6-di-*O*-methyl-4-*O*-ethylgalactitol (see Experimental).

confirmed that the terminal glucuronic acid is bound to O-4 of the 3,4-linked galactosyl unit.

Oligosaccharides **P1** *and* **P2**.—The action of bacteriophage ΦSK1 on the SK1 polysaccharide and exclusion chromatography of the products gave "monomeric" (i.e., one repeating unit) (**P1**) and dimeric (**P2**) fractions.

P1 and **P2** were reduced with $NaBD_4$ to **P1-ol** and **P2-ol** respectively and subsequently methylated. GLC–MS analysis of the derived alditol acetates (Table III) showed the presence of two new components: 3-linked glucitol and 4-linked galactosyl units, thus revealing that the cleavage must have occurred between the glucosyl and the 3,4-linked galactosyl residues. These results are supported by the absence of 3-linked glucopyranosyl and 3,4-linked galactopyranosyl units in **P1-ol**, although they are present in **P2-ol**.

Detailed NMR spectroscopic studies were performed on **P1, P1-ol**, and **P2-ol** in order to confirm the structural data obtained and to locate the sites of acetylation.

TABLE II

Methylation analysis of the Smith-degraded *Klebsiella* SK1 polysaccharide

Methylated sugar [a] (as alditol acetate)	t_R [b]	Molar ratios [c]			
		I	II	III	IV
2,3,4,6-Gal	0.75	0.1	0.1		
2,4,6-Glc	**1.00**	1.0	1.0	1.0	1.0
2,3,4-Glc [d]	1.04		0.8		0.4
2,4,6-Gal	1.09	1.1	1.0	1.6	1.5
2,6-Gal	1.35	0.7	0.9	0.4	0.5
4,6-Gal	1.37	0.3	0.2		

[a] 2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc. [b] Retention time relative to that of 2,4,6-Glc. [c] I, methylated **S1**; II, methylated then carboxyl-reduced **S1**; III, methylated **S2**; IV, methylated then carboxyl-reduced **S2**. [d] C-6 dideuterated according to GLC–MS (see Experimental).

TABLE III

Methylation analysis of **P1-ol** and **P2-ol**

Methylated sugar [a] (as alditol acetate)	t_R [b]	Molar ratios [c]			
		I	II	III	IV
1,2,4,5,6-Glc [d]	0.54	0.6	0.5	0.4	0.3
2,3,4,6-Gal	**1.00**	1.0	1.0	1.0	1.0
2,4,6-Glc	1.34			0.4	0.7
2,3,6-Gal	1.36	0.5	0.4	0.4	0.6
2,3,4-Glc [e]	1.39		0.9		1.0
2,6-Gal	1.80			0.3	0.7
4,6-Gal	1.83	0.2	0.3	1.1	1.2

[a] 1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, etc. [b] Retention time relative to that of 2,3,4,6-Gal. [c] I, methylated **P1-ol**; II, methylated then carboxyl-reduced **P1-ol**; III, methylated **P2-ol**; IV, methylated then carboxyl-reduced **P2-ol**. [d] C-1 deuterated according to GLC–MS. [e] C-6 dideuterated according to GLC–MS (see Experimental).

NMR studies of **P1**.—The ^{1}H NMR spectrum (Fig. 1) of **P1** shows signals for three α- and three β-linked anomeric protons. The sugar residues giving rise to these H-1 resonances are designated **a**–**f** in order of decreasing chemical shift. Assignment of residues **c** and **e** as the α- and β-anomeric forms of the reducing unit followed from inspection of the HETCOR plot. The extent of twinning[9,10] of the signals attributed to residues **a** ($\Delta\delta$ = 11 Hz) and **b** ($\Delta\delta$ = 4 Hz) suggested the sequence **b** → **a** → **c**/**e**. This twinning of signals due to anomerization of the reducing end could not be discerned in the ^{13}C NMR spectrum (Fig. 2).

High field singlets at δ 2.20, 2.19, and 2.14 indicated the presence of *O*-acetyl groups[11] in approximately equal amounts in three different locations corresponding to a total of 33% acetylation. Anomeric-region proton signals at δ 5.05, 4.90,

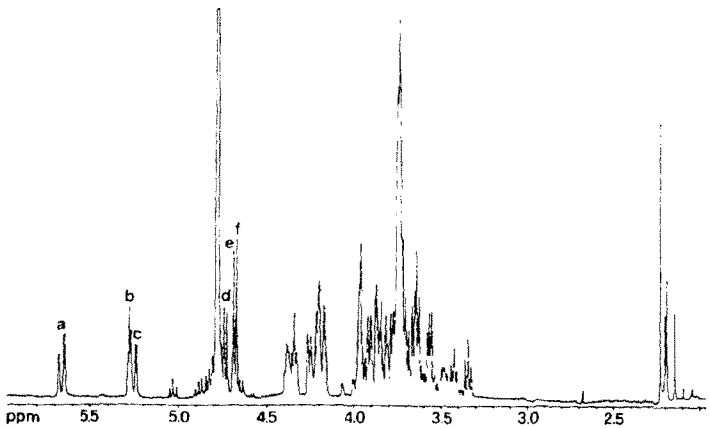


Fig. 1. 500-MHz ^{1}H NMR spectrum of **P1** at 30°C. Note peaks of small intensity due to partial acetylation.

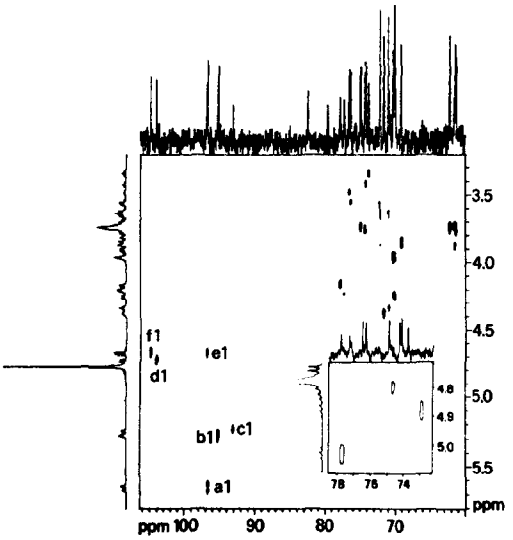


Fig. 2. $^{1}H-^{13}C$ shift correlation map of spectral regions f_2, 106–60 ppm and f_1, 5.8–3.2 ppm for **P1**. The correlated resonances are labelled **a**–**f**. The expansion of the spectral region f_2, 79–72 ppm and f_1, 5.2–4.6 ppm is shown in the insert.

and 4.80 were shown to be connected to carbons resonating at δ 77.8, 72.9, and 74.8 using the HETCOR diagram (Fig. 2) and thus ascribed to ring protons geminal to the attached *O*-acetyl groups[12]. The movement of these signals into the anomeric region results from the *O*-acetylation chemical shift effect.

The anomeric proton signals of the unacetylated **P1** served as the starting point for the ^{1}H shift-correlated experiments [COSY (Fig. 3), one- to three-step RELAY COSY, TOCSY] which allowed assignment of most of the ^{1}H resonances of residues **a** to **f**. The ^{13}C assignment followed from the HETCOR diagram (Fig. 2, Table IV) while the long-range HETCOR experiment provided useful intraresidue connectivities (e.g., H-1 to C-3 and C-5 for **a**, **b**, and **c**) thereby permitting the elucidation of spin systems containing tightly coupled resonances and small coupling constants.

The ^{1}H and ^{13}C NMR data for the unacetylated repeating unit (**P1**) are given in Table IV. Comparison of these data for residues **a**–**f** with literature values for monosaccharides[13–15] permitted the residues to be identified as indicated in the table, and suggests the position of glycosyl substituents to be: 2, 3 on **a**, 3 on **c**/**e**, and 4 on **f**, with residues **b** and **d** present as terminal groups. The glycosylation shifts found for the 2,3-branched residue **a** cannot be readily explained, for although H-2 is significantly deshielded (+0.42 ppm) the attached carbon is not (+0.6 ppm). Unusual ^{13}C shifts for branched oligosaccharides have been previously reported[13].

The long-range HETCOR plot also revealed the interresidue connectivities presented in Table V, and these assignments were corroborated by inspection of

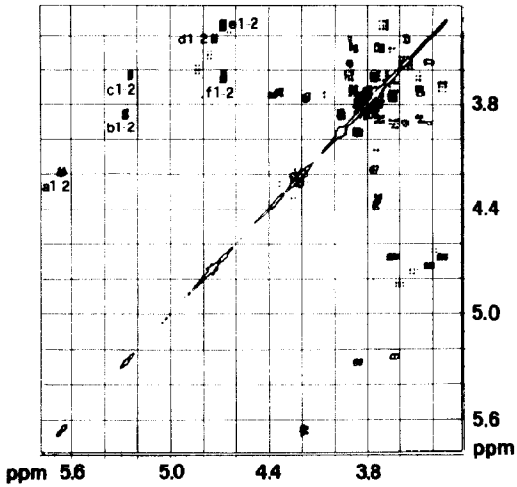


Fig. 3. COSY contour plot of the region 5.8–3.2 ppm for **P1**. The ^{1}H resonances of the *J*-coupled spin systems are designated by residue: **a**1–2 indicates the cross-peak between H-1 and H-2 of residue **a**, etc.

the 1D plots. The data permit the following sequence to be written **d**(1 → 4)**f**(1 → 3)**a**(1 → 3) with the remaining terminal group **b** attached to O-2 of **a**. This sequence of sugar residues accounts for the twinning of H-1 observed ($\Delta\delta$**a** > $\Delta\delta$**b**) and explains the larger ^{13}C glycosylation shifts found for the β-substituted **a** (C-3, +7.2 ppm) and **f** (C-4, +8.0 ppm) and the smaller shifts observed for the α-substituted **a** (C-2, +0.6 ppm) and reducing-end **c**/**e** (C-3, +5.8 for the α and +5.7 ppm for the β anomer).

Crosspeaks due to the acetylated species of **P1** can be observed in the COSY spectrum (Fig. 3), however, the complexity of the spectrum and the low intensity of

TABLE IV

^{1}H and ^{13}C NMR data for **P1** (unacetylated)

Residue		Chemical shift (δ) [a]					
	Position:	1	2	3	4	5	6
a(α-Gal)	H	5.67	4.20	4.23	4.26	4.34	3.73
	C	96.6	70.0	77.3	70.1	70.8	61.4
b(α-Gal)	H	5.28	3.86	3.96	3.95	4.38	~3.74
	C	95.2	69.0	70.1	70.3	71.6	62.1
c(α-Glc)	H	5.25	3.63	3.92	n.r.	3.57	3.73
	C	93.1	70.9	79.6	n.r.	72.1	61.6
d(β-GlcA)	H	4.72	3.42	3.55	3.58	3.91	
	C	104.1	74.1	76.3	72.2	n.r.	
e(β-Glc)	H	4.68	3.34	3.70	3.63	3.47	3.88, 3.73
	C	96.8	73.8	82.4	n.r.	76.5	61.4
f(β-Gal)	H	4.67	3.65	3.77	4.17	n.r.	~3.73
	C	104.9	72.1	74.3	77.7	n.r.	61.2

[a] In ppm downfield from the signal for Me_4Si; n.r., not resolved.

TABLE V

Assignments established for **P1** via the long-range HETCOR ($J_{C,H}$ 6 Hz) experiment

Proton	Correlation to			
a, H-1	82.4 (**e**, C-3)	79.6 [a] (**c**, C-3)	77.3 (**a**, C-3)	70.8 (**a**, C-5)
a, H-5	96.6 (**a**, C-1)	70.1 (**a**, C-4)	61.4 (**a**, C-6)	
b, H-1	71.6 (**b**, C-5)	70.1 (**b**, C-3)	69.0 [a] (**b**, C-2)	
b, H-5	95.2 (**b**, C-1)	70.3 (**b**, C-4)	62.1 (**b**, C-6)	
c, H-1	79.6 (**c**, C-3)	72.1 (**c**, C-5)		
d, H-1	77.7 (**f**, C-4)			
d, H-2	104.1 (**d**, C-1)	76.3 (**d**, C-3)		
d, H-3	74.1 (**d**, C-2)	72.2 (**d**, C-4)		
e, H-2	96.8 (**e**, C-1)	82.4 (**e**, C-3)		
f, H-1	77.3 (**a**, C-3)			
f, H-4	104.1 (**d**, C-1)	74.3 (**f**, C-3)	72.1 (**f**, C-2)	

[a] Observed in the 1D plot.

the peaks prevented assignment of these spin systems. For this reason, the oligosaccharide **P1** sample was fractionated by preparative paper chromatography into unacetylated **P1** (the slowest moving component), **P1A**, and the fastest moving component containing a mixture of **P1B** and **P1C**. The 2D spectra of the unacetylated **P1** provided support for the assignments presented in Table IV, while further confirmation of the oligosaccharide structure followed from NMR studies of reduced **P1** (see later).

NMR studies of acetylated **P1** (**P1A**, **-B**, *and* **-C**).—Detailed 1D- and 2D-NMR studies were performed on the two samples in order to locate the positions of acetylation. Despite the presence of unacetylated **P1** in the sample of **P1A**, the COSY (Fig. 4) and HETCOR (Fig. 5) spectra revealed correlations not present for **P1**. These were ascribed to signals associated with acetylation. Proton assignments for residues **d** and **f** were made using the COSY and TOCSY experiments, while some of the ^{13}C assignments followed from the HETCOR diagram. The ^{1}H and ^{13}C shift data (Table VI) show that the acetyl group is attached to O-2 of the glucuronic acid residue (**d**), leading to dramatic deshielding of H-2 (+1.38 ppm) and perturbation of the rest of the signals from this residue, as well as those emanating from the contiguous residue **f**.

^{1}H-Homonuclear shift correlated spectra [COSY (Fig. 6), relay COSY, and TOCSY] of the second sample permitted elucidation of the spin systems associated with acetylation, while some of the corresponding ^{13}C resonances were identified from the HETCOR diagram (Fig. 7). The ^{1}H and ^{13}C shift data (Table VI) show that the acetyl group is attached to O-4 (**P1B**) or O-3 (**P1C**) of the terminal glucuronic acid residue (**d**). No perturbation of the signals due to residue **f** could be discerned, in contrast to the situation found for acetylation of O-2 (**P1A**). Signals of low intensity due to the presence of unacetylated **P1** were also observed.

These NMR studies show that the 33% acetylation detected in the polymer, and in the repeating unit oligosaccharide derived from bacteriophage depolymeriza-

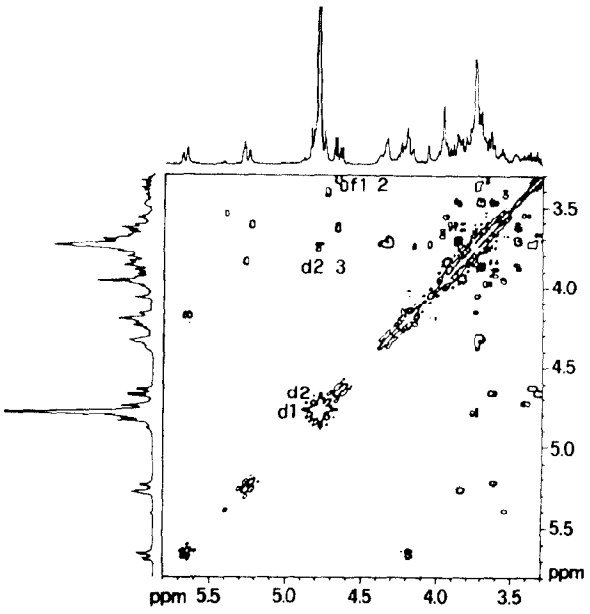


Fig. 4. COSY contour plot of the region 5.8–3.2 ppm for **P1A**: **d**1 indicates H-1 of residue **d**, and **d**2–3 indicates the cross peak between H-2 and H-3 of residue **d**, etc.

tion, is associated with the terminal glucuronic acid residue and equally involves O-2, O-3, and O-4 of this residue. The presence of the *O*-acetyl groups on the terminal glucuronic acid group only was confirmed by FABMS studies of the partially acetylated **P1** mixture (see later).

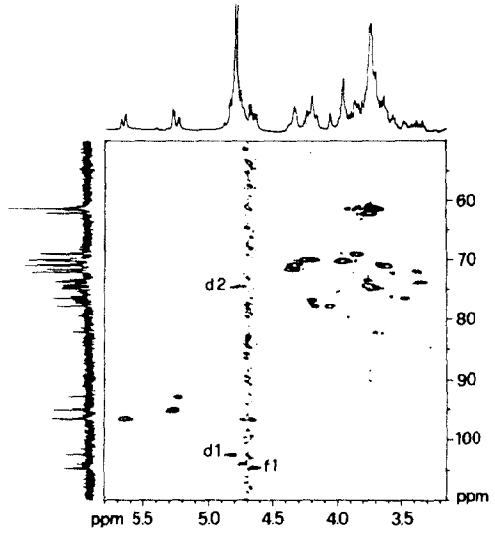


Fig. 5. ^{1}H–^{13}C shift correlation map for **P1A**. Some of the C–H correlations are shown.

TABLE VI

^1H and ^{13}C NMR data for **P1A**, **P1B**, and **P1C**

Sample		Chemical shift (δ) [a]				
	Position:	1	2	3	4	5
P1A **d**	H	4.82	4.80	3.76	3.69	n.r.
	C	102.5	74.8	n.r.	n.r.	
f	H	4.63	3.38	3.73	4.05	
	C	104.8	72.0	n.r.	77.7	
P1B **d**	H	4.79	3.53	3.81	4.90	4.12
	C	103.9	72.7	72.3	72.9	72.7
P1C **d**	H	4.87	3.61	5.05	3.78	4.09
	C	103.9	72.4	77.8	n.r.	75.2

[a] In ppm downfield from the signal for Me_4Si; n.r., not resolved.

NMR studies of **P1-ol**.—Detailed NMR studies were conducted on $NaBH_4$-reduced **P1** (**P1-ol**). Comparison of the ^1H NMR spectra of **P1** and **P1-ol** shows that the resonances at δ 5.25 and 4.68, attributed to the reducing-end residues (**c** and **e**), disappear upon reduction, whereas the signals at δ 5.68 (**a**) and 5.28 (**b**) are shifted upfield to δ 5.46 and 5.23 and no longer twinned in **P1-ol**, as expected. This result was corroborated by inspection of the ^{13}C spectrum of **P1-ol**, where the signals attributed to C-1 of the reducing-end units (**c** and **e**) are absent and the resonances at δ 96.8 (**a**) and 95.1 (**b**) are shifted downfield to 97.7 and 95.9, respectively. These results are consistent with the sequence **b** → **a** → **c**/**e**.

^1H Spin systems were identified using the COSY, relay COSY, and TOCSY (Fig. 8) experiments, while the HETCOR spectrum permitted assignment of all of the ^{13}C resonances. The ^1H and ^{13}C NMR data for **P1-ol** are given in Table VII.

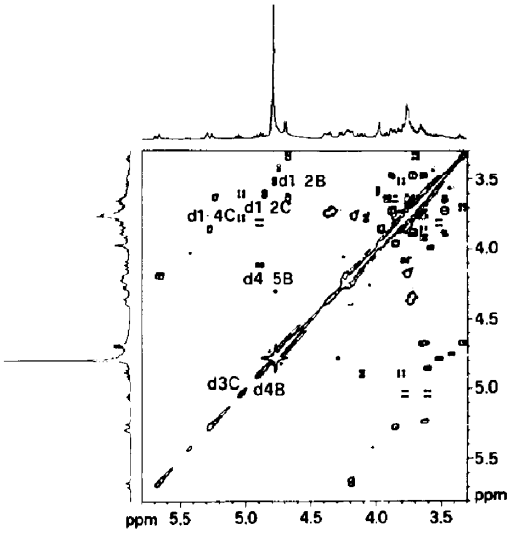


Fig. 6. COSY spectrum of the mixture **P1B**–**P1C**. Some of the spin systems are shown.

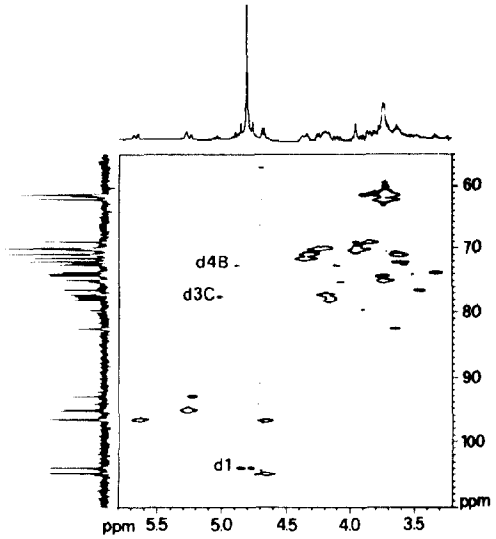


Fig. 7. $^{1}H-^{13}C$ shift correlation map of the mixture **P1B–P1C**. Some of the C–H correlations are shown.

In addition, the long-range HETCOR experiment (Table VIII) revealed inter-residue connectivities, confirming the sequence **d**(1 → 4)**f**(1 → 3)**a**(1 → 3)**c-ol**, while the spectral shifts and loss of twinning upon reduction of **P1** to **P1-ol** show that **b** is attached to O-2 of **a**. As for **P1**, this assignment is supported by the large glycosylation shift found for H-2 (+0.43 ppm), although the attached carbon experiences an unusually small glycosylation shift (+1 ppm).

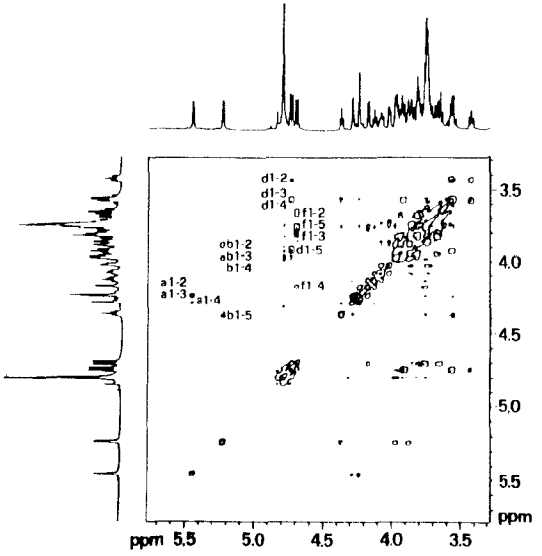


Fig. 8. TOCSY spectrum of **P1-ol**.

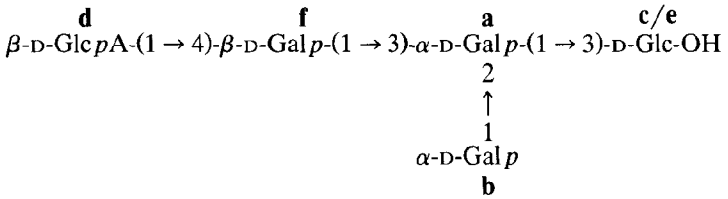
TABLE VII

^1H and ^{13}C NMR data for **P1-ol**

Residue		Chemical shift (δ) [a]					
	Position:	1	2	3	4	5	6
a(α-Gal)	H	5.46	4.24	4.24	4.29	4.13	~3.76
	C	97.7	70.5	77.3	70.2	71.9	61.8
b(α-Gal)	H	5.23	3.87	3.97	~3.97	4.37	~3.75
	C	95.9	69.0	70.0	70.2	71.6	62.0
c(e)(glucitol)	H	~3.86	4.08	4.02	3.81	3.93	~3.73
	C	63.3	73.2	79.3	70.4	71.5	63.6
d(β-GlcA)	H	4.74	3.42	3.55	3.57	3.91	
	C	104.1	74.1	76.3 [b]	72.2 [b]	75.7	
f(β-Gal)	H	4.70	3.65	3.77	4.17	3.74	~3.76
	C	104.7	72.2	74.3	77.8	74.9	61.3

[a] In ppm downfield from the signal for Me_4Si. [b] Assignments may have to be reversed.

The combined NMR data for **P1** and **P1-ol** permit the structure of the carbohydrate (**P1**) repeating unit to be written as



This sequence is corroborated by the FABMS studies described below.

TABLE VIII

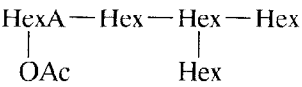
Assignments established for **P1-ol** via the long-range HETCOR ($J_{C,H}$ 6 Hz) experiment

Proton	Correlation to		
a, H-1	79.3 (**c**, C-3)	77.3 (**a**, C-3)	71.9 (**a**, C-5)
a, H-2/H-3	104.7 (**f**, C-1)	77.3 (**a**, C-3)	70.5 (**a**, C-2)
a, H-4	77.3 (**a**, C-3)	70.5 (**a**, C-2)	
a, H-5	70.2 (**a**, C-4)	61.8 (**a**, C-6)	
b, H-1	71.6 (**b**, C-5)	70.0 (**b**, C-3)	
b, H-3	69.0 (**b**, C-2)		
b, H-5	95.9 (**b**, C-1)	70.2 (**b**, C-4)	62.0 (**b**, C-6)
c, H-3	97.7 (**a**, C-1)	73.2 (**c**, C-2)	63.3 (**c**, C-6)
c, H-4	71.5 (**c**, C-5)	63.6 (**c**, C-5)	
d, H-1	77.8 (**f**, C-4)		
d, H-2	104.1 (**d**, C-1)	76.3 (**d**, C-3)	
d, H-3	74.1 (**d**, C-2)		
d, H-5	76.3 (**d**, C-3)	72.2 (**d**, C-4)	
f, H-1	77.3 (**a**, C-3)		
f, H-2	74.3 (**f**, C-3)		
f, H-4	104.1 (**d**, C-1)	74.3 (**f**, C-3)	72.2 (**f**, C-2)
f, H-5	61.3 (**f**, C-6)		

Sequence analysis of **P1** *by FABMS.*—Only molecular weight related ions were observed in the spectrum of the underivatized oligosaccharide. Ions at m/z 929 and 907, respectively, corresponded to a di- and mono-sodiated pentasaccharide consisting of four hexose (Hex) units, one hexuronic acid (HexA), and one acetyl group. A small peak at m/z 865 was from the nonacetylated oligosaccharide, and suggested that **P1** may be only partially acetylated in its native form.

After perdeuterioacetylation, most of the peaks in the mass spectra appeared as doublets differing internally by 3 amu, confirming that the native oligosaccharide was partially acetylated. Moreover, the m/z values increased to 1578 and 1583 ($[M + NH_4]^+$ and $[M + Na]^+$ respectively), further establishing that **P1** is a pentasaccharide consisting of four Hex units, one HexA, and one acetyl group. From the presence of the ion at m/z 309, representing a partially deuterioacetylated HexA oxonium ion, it was concluded that the native acetate group must reside on the terminal HexA residue. Another terminal oxonium ion, the base peak at m/z 343, corresponded to a fully deuterioacetylated Hex residue. The appearance of these two ions, m/z 309 and 343, implied that the pentasaccharide has two nonreducing terminals: a Hex and a partially acetylated HexA.

The ion of m/z 1201 implicated a Hex residue as the reducing terminal, while the ion at m/z 606 represented a partially deuterioacetylated disaccharide (HexA–Hex) oxonium ion, suggesting that the branch-point is the hexose adjacent to the reducing terminal. This is further confirmed by the presence of the oxonium ion at m/z 892, formed via cleavages at the Hex reducing end and at the partially acetylated HexA nonreducing end to give the fragment HO–Hex–Hex$^+$–Hex. Therefore, **P1** is a 4 + 1 pentasaccharide with its nonreducing terminal HexA residue partially acetylated in the native form.



NMR studies of **P2-ol**.—In order to determine the anomeric configurations of all the sugar residues in the SK1 capsular polysaccharide, the ^1H and ^{13}C NMR spectra of $NaBH_4$-reduced **P2** (**P2-ol**) were examined (Table IX). Comparison of the ^1H NMR spectra of **P1**, **P1-ol**, and **P2-ol** showed the presence of a new signal at δ 4.92 (J 7.7 Hz) which correlates to the signal at δ 102.63 in the HETCOR experiment. These results indicate that the Glc$p$ residue in the polymer has the β configuration.

Overall, the data permit the primary structure of the capsular polysaccharide produced by *Klebsiella* serotype SK1 to be formulated as follows:

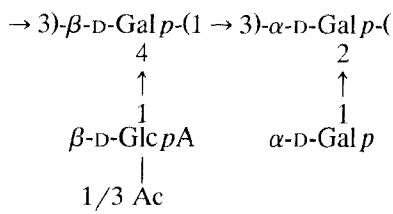


TABLE IX

Anomeric signals in the ^1H and ^{13}C NMR spectra of **P2-ol**

Signal (δ value)		Assignment	
^1H (400 MHz)	^{13}C (75.5 MHz)		
5.62	96.7	α-Gal	**a** [a]
5.45	97.8	α-Gal	**a'** [a]
5.27	95.1	α-Gal	**b**
5.22	95.7	α-Gal	**b'**
4.94	102.6	β-Glc	**c**
4.73	104.1	β-GlcA	**d**
4.71	104.7	β-GlcA	**d'**
4.66	105.1	β-Gal	**f, f'**

[a] **a** Indicates a sugar residue as in **P1**; **a'** corresponds to residue **a**, but at the reducing extremity, etc.

This branched pentasaccharide repeating unit is identical to the recently revised structure proposed for *Klebsiella* serotype K8 capsular polysaccharide[16], except for the presence of acetyl substituents. A similar example has already been reported in the literature; in fact, the *Klebsiella* capsular polysaccharides K30 and K33, although typed as serologically distinct, differ only in the amount of acetyl substitution present[17]. The anomeric chemical shift values reported for the SK1 and K8 polysaccharides are in good agreement even if the individual assignments for the β-linked residues are not. This is not surprising, as the previous study[16] did not make use of heteronuclear correlation spectroscopy.

Many capsular polysaccharides produced by *Klebsiella pneumoniae* serotypes present one single-residue side chain per repeating unit; only one (K60) has three single-residue side chain. *Klebsiella* SK1 and K8 capsular polysaccharides are the only examples having two single-residue side chains per repeating unit.

EXPERIMENTAL

General methods.—Solvent systems used in paper and thin layer chromatography were: **A**, 8:2:1 EtOAc–pyridine–H_2O; *B*, 2:1:1 butanol–AcOH–H_2O; and *C*, 20:20:7 $CHCl_3$–MeOH–H_2O. Sugars on thin-layer plates were detected by spraying with (1:1:18) *p*-anisaldehyde–H_2SO_4–EtOH followed by heating at 110°C for 5–10 min; detection on paper was by staining with $AgNO_3$.

Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph equipped with a flame-ionization detector and a DB-17 bonded-phase capillary column (15 m × 0.25 mm, 0.25-μm film), using He as the carrier gas. The following temperature programmes were used: for alditol acetates, 180 (2 min) to 240°C at 5°/min; for methylated alditol acetates, 180 (1 min) to 240°C at 2°C/min. GLC–MS was performed on a Varian Vista 6000 gas chromatograph coupled directly to a Delsi Nermag R10-10C quadrupole mass spectrometer, using a 30 m DB-17 capillary column.

Hydrolyses were carried out with 2 M CF_3CO_2H at 100°C for 18 h (polysaccharide) and 6–8 h (oligosaccharides). Alditol acetates were prepared using standard procedures[18]. Samples were methanolysed[19] with 1 M HCl in MeOH for 18 h at 85°C and the methyl glycosides were acetylated prior to GLC analysis. Gel filtration chromatography of the SK1 polysaccharide was performed on a Sephacryl S400 column (1.6 × 65 cm) with 0.1 M pyridinium acetate as eluent.

Methylation analysis and related experiments.—Methylations were carried out by the modified Hakomori[20] method using potassium methylsulfinylmethanide[21]. The methyl-esterified carboxyl groups were reduced[22] with $LiAlD_4$. Base degradation of methylated uronic acid-containing polysaccharide was done by a modification[23] of the method of Lindberg and Lönngren[24]. The methylated alditol acetates derived from these reactions were examined by GLC–MS.

Smith degradation[25].—SK1 polysaccharide (29 mg) was treated with 6.6 mL of 1.1×10^{-2} M $NaIO_4$ for 1 h at 4°C in the dark[7,8]. The consumption of periodate was followed spectrophotometrically[26] at 223 nm. Excess of periodate was decomposed and the oxidized product was reduced by $NaBH_4$ overnight. Acetic acid (50%) was added and the solution was dialysed and lyophilised. The sample was hydrolysed with 0.5 M CF_3CO_2H, dialysed, and freeze-dried. The product (**S1**) was methylated and the derived alditol acetates, with and without prior carboxyl-reduction, were analysed by GLC–MS.

SK1 polysaccharide (29 mg) was dissolved in 6.6 mL of 6×10^{-2} M $NaIO_4$ and kept 72 h at 4°C in the dark, after which further consumption of $NaIO_4$ was not observed. The sample (**S2**) was treated and analysed as described for **S1**.

Propagation of bacteriophage[27].—A bacteriophage which infects *Klebsiella* SK1 bacteria was isolated from Vancouver sewage and propagated on its host strain until $\approx 10^{12}$ PFU had been obtained. The phage solution was concentrated, dialysed against running tap-water for 2 days, concentrated to 280 mL, and centrifuged.

Depolymerization[28] *of the polysaccharide with bacteriophage SK1.*—SK1 polysaccharide (500 mg) was dissolved in 280 mL of phage solution ($\approx 10^{12}$ PFU) and the mixture, after dissolution of the polymer, was shaken for 4 days at 32°C. The product was concentrated, dialysed, freeze-dried, and subsequently separated on a Biogel P2 column (2.8 × 85 cm) to give oligosaccharides corresponding to one and two repeating units (**P1** and **P2**, respectively). **P1** and **P2** were purified through an Amberlite IR-120(H^+) resin column to yield 136 and 33 mg, respectively.

NMR spectroscopy.—Samples were deuterium-exchanged by dissolution in D_2O and freeze-drying, and then dissolved in 99.99% D_2O. Acetone was used as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 30°C with a Bruker WH-400 or AMX-500 spectrometer, equipped with an Aspect 3000 computer and an array processor using standard Bruker software.

The COSY[29] and TOCSY[30] spectra were recorded at 500 MHz using a spectral width of 1672 Hz. The initial (t_1, t_2) matrices of 256 × 1024 data points provided 1.6 Hz/point digital resolution in the second domain. For the COSY experiment,

48 transients were collected for each t_1 delay and the matrices were zero filled in the t_1 dimension, transformed in the magnitude mode by use of a nonshifted sine-bell window function in both dimensions, and symmetrised. A relaxation delay of 1.2 s was employed. In the TOCSY experiments[31] a recycle delay of 1.9 s and mixing time of 45 ms were used.

The COSY spectrum (for **P1**) and all the relay COSY experiments were recorded at 400 MHz using the conventional pulse sequences[29,32,33]. A relaxation delay of 0.035 s was used.

The $^{13}C-^{1}H$ shift correlated (HETCOR) experiments[34–36] were recorded with the Bruker AMX-500 spectrometer, once conventionally with ^{13}C detection[37] (**P1**), otherwise using the more sensitive inverse variant[35], with a recycle delay of 1.5 s. The ^{1}H-detected long-range HETCOR experiments[38] were conducted using a recycle delay of 2 s.

Mass spectrometry.—Fast atom bombardment MS was performed on a VG 70-SE instrument (VG Analytical, Manchester, UK) operated at 8 kV in the positive ion mode, with a Cs ion gun operating at 25 kV. A 1:1 mixture of thioglycerol–glycerol was used as the matrix for the underivatized material and a 1:1 mixture of thioglycerol–*m*-nitrobenzyl alcohol was used for the perdeuterioacetylated material. Current-controlled scans were acquired at a rate of 10 s/decade, and 5 scans were summed up as continuum data. The resolution was 1:2500 and CsI clusters were used for calibration. The reported m/z values correspond to chemical masses.

Perdeuterioacetylation was carried out as described by Dell and Tiller[39]. The samples (100 μg) were incubated at room temperature in 500 μL of 2:1 trifluoroacetic anhydride–acetic acid-d_3. After 20 min the perdeuterioacetylated products were recovered by three extractions into chloroform from an aqueous phase. Approximately 1 μg of each sample was used for FAB analysis.

ACKNOWLEDGMENTS

We thank Mrs. M. Austria and Mrs. L. D'Arge for recording the NMR spectra, Dr I. Ørskov for serological analysis, and N.S.E.R.C., Ottawa, for continued financial support.

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