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Re-investigation of the structure of the capsular polysaccharide of *Klebsiella* K15 using bacteriophage degradation and inverse-detected NMR experiments

Haralambos Parolis, Lesley A.S. Parolis and Darryl V. Whittaker

School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140 (South Africa)

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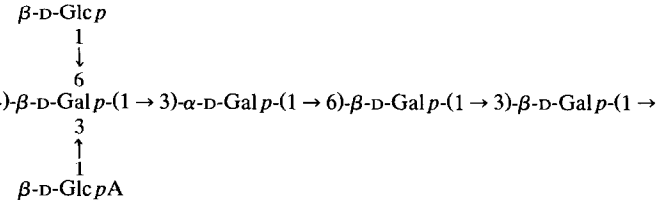
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* K15 has been re-investigated, principally by 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopy of the oligosaccharide-alditol obtained by depolymerisation of the polysaccharide with a viral-borne endoglycanase followed by borohydride reduction of the isolated repeating oligosaccharide. The capsular polysaccharide was shown to have the repeating unit:



INTRODUCTION

Klebsiella K15 formed part of a programme in our laboratory to screen for the antigenically important[1], yet often unreported, presence of *O*-acetyl groups in capsular polysaccharides. A preliminary NMR study[2] in this regard on the hexasaccharide-alditol (P1-ol), derived from the hexasaccharide produced by bacteriophage depolymerisation of the polysaccharide, did not accord with the structure (**1**) reported[3] for the repeating unit and prompted the present study.

Correspondence to: Professor H. Parolis, School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140, South Africa.

$$\begin{array}{l} \beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Gl}p\text{A} \\ \qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\quad 2 \\ \rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Gal}p(1 \rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow \end{array}$$

1

RESULTS AND DISCUSSION

Composition and 1D NMR spectra.—Klebsiella K15 bacteria were grown on sucrose-rich agar, and the acidic polysaccharide (PS) was isolated and purified by standard procedures[4]. GLC of the derived peracetylated aldononitriles[5], with and without prior reduction of the uronic acid, confirmed the presence of GlcA, Glc, and Gal in the molar ratios 1:1:4. The D configuration for the residues was established by GLC analysis of the derived acetylated (−)-2-octyl glycosides[6].

The ^{1}H-NMR spectrum of PS (Fig. 1) contained an H-1 signal at δ 5.02 for an α linkage, together with H-1 signals at δ 4.82, 4.75, and 4.48 and two overlapping H-1 signals at $\delta \sim 4.65$ for β linkages. The data are in good agreement with those

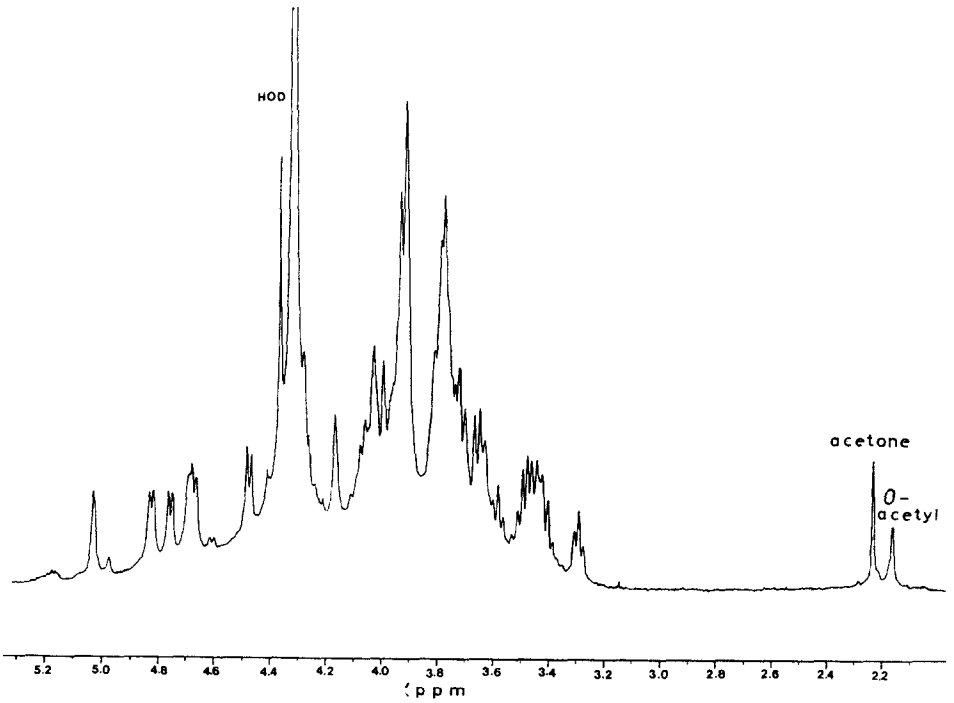


Fig. 1. 500-MHz ^{1}H-NMR spectrum of *Klebsiella* K15 polysaccharide (PS) at 67°.

TABLE I

Methylation analyses of *Klebsiella* K15 capsular polysaccharide (PS) and the derived P1-ol

Methylated sugar [a] (as alditol acetate)	T [b] on DB-225	Molar ratios [c]			
		1	*2*	*3*	*4*
1,2,4,5,6-Gal	0.60			0.63	0.54
2,3,4,6-Glc	1.00	1.00	1.00	1.00	1.00
2,4,6-Gal	1.61	1.93	2.04	1.03	0.93
2,3,4-Glc	1.73		0.63	0.62	
2,3,4-Gal	2.05	0.98	1.00	0.86	1.46
2,4-Gal	3.16			0.78	
2-Gal	3.81	0.91	1.23		

[a] 1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, etc. [b] Retention times relative to 2,3,4,6-Glc on DB-225 at 210° (see Experimental). [c] *1*, methylated PS: *2*, methylated reduced PS; *3*, methylated carboxyl-reduced P1-ol; *4*, base-degraded methylated P1-ol.

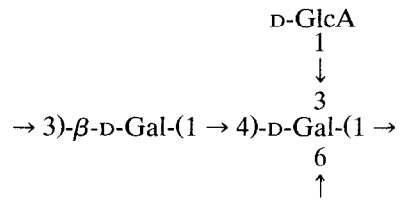
reported[3] for the 90-MHz ^{1}H-NMR spectrum of the polysaccharide at 70°. In addition, a signal at δ 2.15 for *O*-acetyl indicated that the PS was ~15% *O*-acetylated. These results indicated a hexasaccharide repeating unit for PS.

A bacteriophage isolated from sewage and propagated on the bacteria was used to depolymerise PS. The hexasaccharide repeating oligosaccharide (P1) isolated had a reducing galactose residue. Comparison of the ^{1}H-NMR spectra of PS and P1 clearly established that a β-linked residue in PS had been cleaved by the bacteriophage. These data establish the bacteriophage endoglycanase as a β-galactosidase.

Methylation analyses.—PS was methylated by a modified Hakomori procedure[7], using potassium dimsyl. GLC and GLC–MS analysis of the partially methylated alditol acetates derived from a hydrolysate of PS, without and with carboxyl reduction, gave the results shown in Table I (columns *1* and *2*, respectively). P1 was reduced with sodium borohydride, the derived oligosaccharide-alditol P1-ol was methylated, the carboxymethyl group was reduced, and the derived partially methylated alditol acetates were examined by GLC–MS (Table I, column *3*). The methylation results indicated that PS contained terminal Glc and GlcA, a 6-linked Gal, two 3-linked Gal, and a 3,4,6-linked Gal. The methylation results of P1-ol also established the reducing end of P1 as a 3-linked Gal and that this residue was linked to O-4 of the 3,4,6-linked Gal in the polysaccharide.

Base-catalysed degradation of P1-ol.—Methylated P1-ol was degraded with methylsulphinyl carbanion and then alkylated with methyl iodide[8]. The partially methylated alditol acetates derived from a hydrolysate of the product were examined by GLC–MS. The results (Table I, column *4*) established that the terminal GlcA was linked to O-3 of the 3,6-linked Gal in P1-ol and the 3,4,6-linked Gal in PS.

The results thus far established the partial structure **2** for the repeating unit of PS. Further information for the sequence of the residues in PS was obtained from 2D-NMR experiments on P1-ol.



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2D-NMR studies of P1-ol.—Complete assignment of the ^{1}H and ^{13}C resonances of the sugar residues and the alditol were made from COSY[9], two-step RELAY COSY[10], ^{1}H-detected $^{1}H-^{13}C$ heteronuclear multiple-quantum-coherence (HMQC) spectroscopy[11,12], and ^{1}H-detected heteronuclear-RELAY (HMQC-TOCSY) spectroscopy[11,13].

The H-1 resonances for the five sugar residues in P1-ol were labelled **a**–**e** in order of decreasing chemical shifts. COSY and two-step RELAY COSY contour

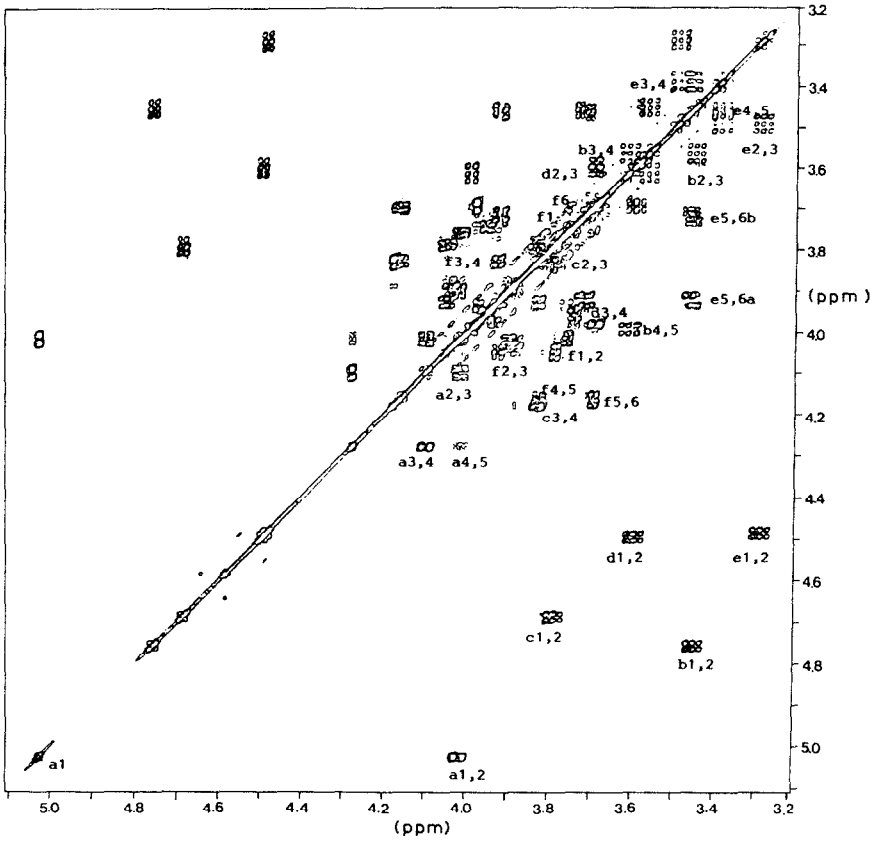


Fig. 2. COSY contour plot of the region δ 5.1–3.2 for P1-ol. The ^{1}H resonances of the *J*-coupled spin systems are labelled **a**–**f**: **a**1 connotes H-1 of residue **a**, and **a**1,2 connotes the cross-peak between H-1 and H-2 of residue **a**, etc.

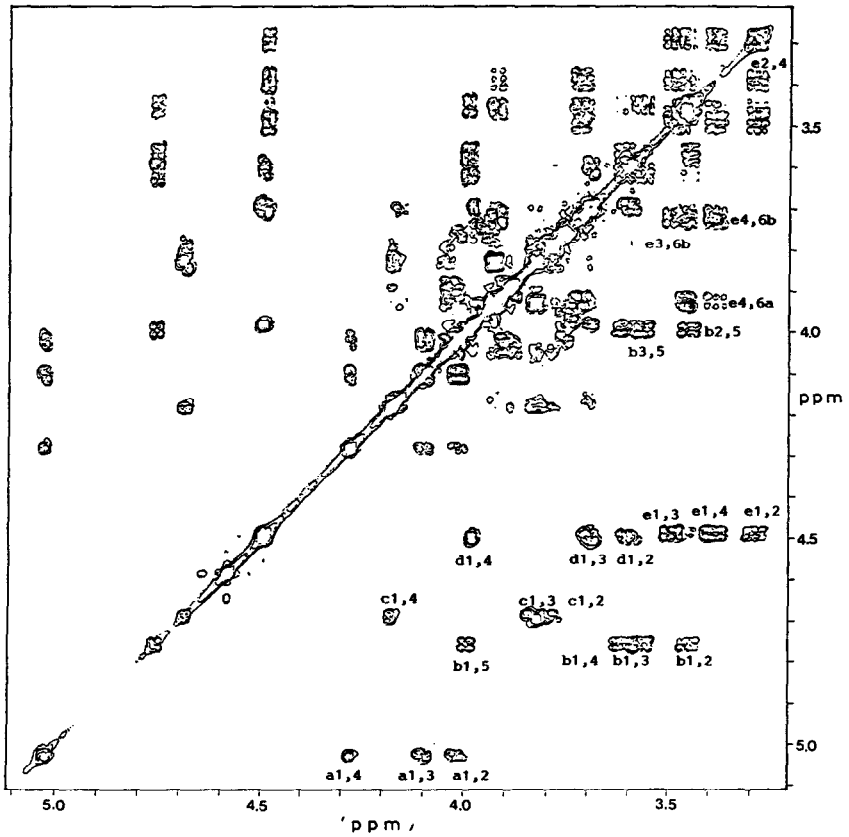


Fig. 3. Two-step RELAY COSY contour plot of the region δ 5.1–3.2. Correlated resonances are labelled **a**–**e**: **a**1 connotes H-1 of residue **a**, and **a**1,3 connotes the cross-peak between H-1 and H-3 of residue **a**, etc.

plots of P1-ol are shown in Figs. 2 and 3 , and the ^{1}H assignments are presented in Table II. Commencing from the resonance for H-1, the chemical shifts of all the ^{1}H resonances for residues **a**, **b**, and **e** were traced via the cross-peaks on the COSY contour map. All ambiguities for these residues could be resolved by referring to the two-step RELAY COSY spectrum. In the case of residues **c** and **d**, only the resonances for H-1 to H-4 could be assigned from the ^{1}H–^{1}H correlation spectra.

The ^{1}H resonances assigned for residues **a**–**e** were then compared with the ^{13}C–^{1}H correlation data obtained from the HMQC experiment (Fig. 4 and Table II). This comparison permitted the unambiguous assignment of all the ^{13}C resonances for residues **a**, **b**, and **e**, and those of C-1 to C-4 for residues **c** and **d**. The assignment of the chemical shifts for the C-5/H-5 and C-6/H-6a,6b resonances for residues **c** and **d** and the chemical shifts for the ^{13}C/^{1}H resonances for the alditol **f** was accomplished as follows. On the basis of literature values, which show

TABLE II

NMR data [a] for *Klebsiella* K15 oligosaccharide P1-ol

Residue		1a	1b	2	3	4	5	6a	6b
a	H	5.02		4.01	4.10	4.28	4.02	3.77	3.77
→ 3)-α-Gal	^3J [b]	3.9		10.2	3.2	0.9			
	C	99.44		68.00	*80.35*	69.80	71.59	62.00	
b	H	4.75		3.45	3.57	3.62	4.00		
β-GlcA	^3J	7.9		8.9	8.9	9.4			
	C	104.49		73.77	75.89	71.90	75.40		
c	H	4.68		3.79	3.83	4.18	3.89	3.91	4.04
→ 3,6)-β-Gal	^3J	7.6		10.0	3.8				
	C	104.70		70.95	*82.80*	69.00	74.00	*69.70*	
d	H	4.49		3.59	3.70	3.98	3.95	3.98	3.76
→ 6)-β-Gal	^3J	7.8		9.4	3.6				
	C	104.70		71.93	73.14	69.42	73.70	*67.80*	
e	H	4.48		3.29	3.49	3.40	3.46	3.93	3.73
β-Glc	^3J	7.9		9.3	9.2	9.2	9.7	2.0/11.8	7.8
	C	103.64		73.80	76.59	70.50	76.72	61.64	
f	H	3.80	3.80	4.06	3.94	3.84	4.16	3.70	3.70
→ 3)-Gal-ol	C	63.10		71.62	*78.70*	70.00	70.60	63.64	

[a] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm, respectively, for ^1H and ^{13}C. [b] ^1H–^1H coupling constants in Hz.

that the chemical shifts for primary carbon atoms of alditols occur approximately 1–1.5 ppm to lower field than those of hexopyranoses, the sets of $^{13}C/^1H$ resonances (HMQC, Fig. 4 and Table II) at 63.10 ppm/δ 3.80 and 63.64 ppm/δ 3.70 were assigned to the methylene groups in the alditol **f** and are labelled **f**1 and

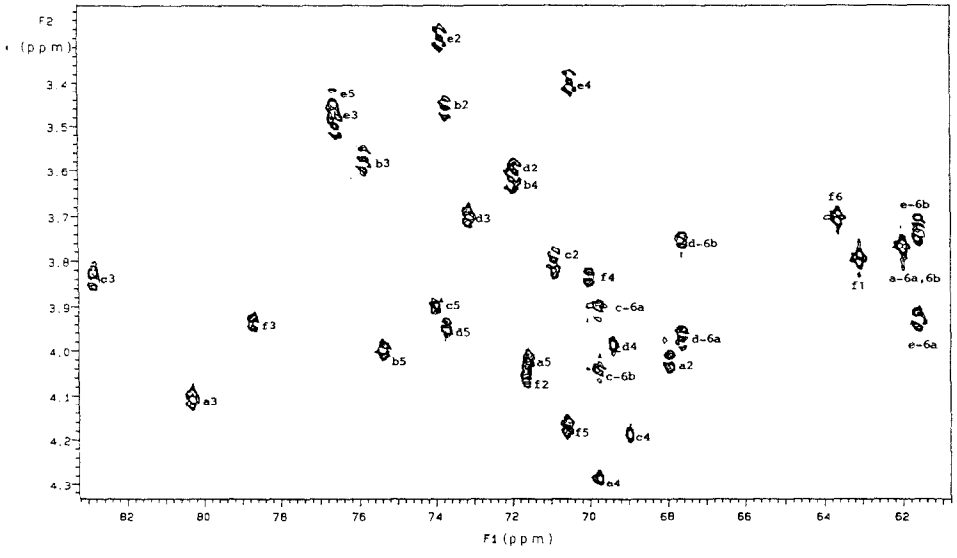


Fig. 4. HMQC ^1H–^{13}C shift correlation map of the spectral region F1 83–61 ppm (^{13}C) and F2 3.2–4.3 ppm (^1H) for P1-ol. The correlated resonances are labelled **a**–**f**.

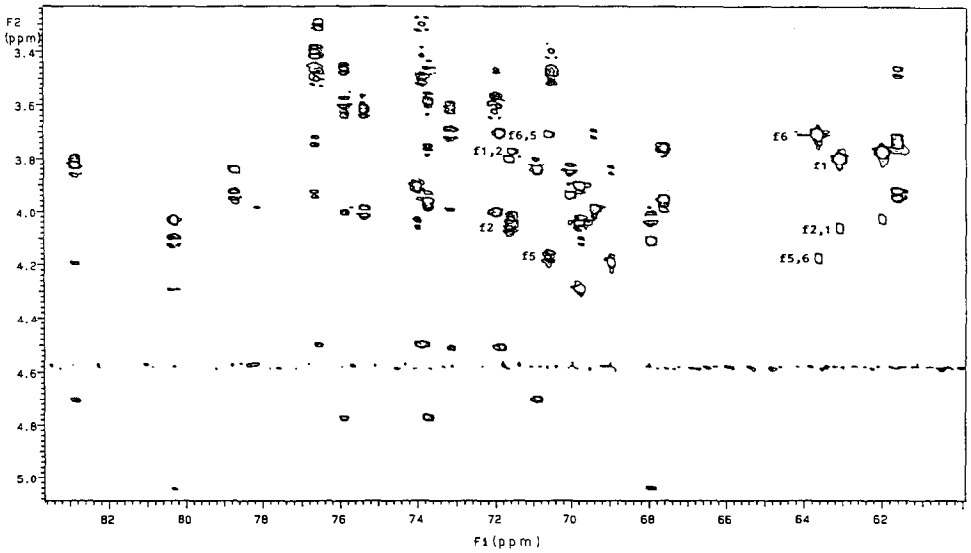


Fig. 5. HMQC-TOCSY $^{1}H-^{13}C$ contour plot of the spectral region F1 83–61 ppm (^{13}C) and F2 3.2–4.3 ppm (^{1}H) for P1-ol. The respective connectivities between **f6** and **f5**, and **f1** and **f2** are indicated, where **f1** = C-1/H-1a, H-1b, etc.

f6 (Figs. 2 and 4). The chemical shifts for C-1/H-2 (**f2**) and C-5/H-5 (**f5**) could now be established from their respective relayed connectivities to C-1/H-1a,1b (**f1**) and C-6/H-6a,6b (**f6**) in the HMQC-TOCSY spectrum (Fig. 5 and Table II). Returning to the COSY spectrum (Fig. 2), all the ^{1}H chemical shifts for the alditol **f** could be traced. Comparison of these data with the $^{13}C-^{1}H$ correlation data from the HMQC experiment permitted the assignment of all the ^{13}C resonances for **f**. Finally, the connectivities between the two sets of C-6/H-6a,6b resonances and their respective C-5/H-5 neighbours were obtained from the HMQC-TOCSY spectrum (Fig. 5). As no connectivity between H-4/C-4 and H-5/C-5 in these residues was observed in any of the correlation spectra, a long-range COSY[14] experiment was performed with the delay optimised for small couplings[15]. A connectivity between H-4 and H-5 for residue **c** was observed, which allowed the assignment of the remaining chemical shifts for residues **c** and **d**.

The $^{1}H-^{1}H$ coupling constants for the sugar residues **a**–**e** were obtained from a resolution-enhanced 1D spectrum (Fig. 6) and from 1D HOHAHA[11,16] subspectra obtained by selectively inverting a well-separated multiplet for each spin system.

Comparison of the NMR data for residues **a**–**f** with those for model compounds[17–19] identified the residues in P1-ol, as indicated in Table II. The significant deshielding of C-3 of **a**, C-3,6 of **c**, C-6 of **d**, and C-3 of **f** identified the linkage positions of the residues; these accord with the methylation results for P1-ol.

In order to establish the sequence of the residues in P1-ol, a heteronuclear, multiple-bond, correlation spectrum (HMBC) was obtained. In this ^{1}H-detected

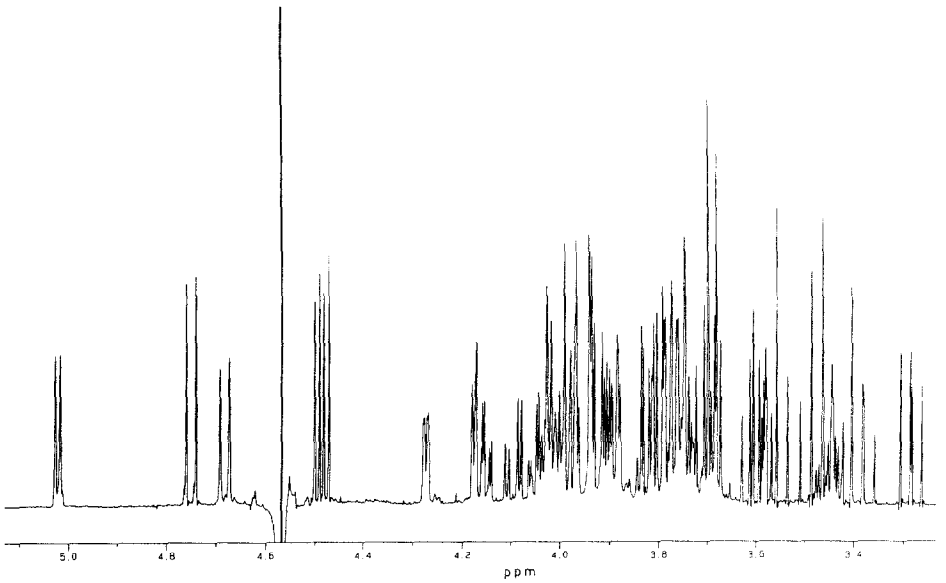


Fig. 6. Resolution-enhanced ^{1}H-NMR spectrum of P1-ol at 45°.

experiment[11,18], correlations are established through long-range $^{1}H-^{13}C$ scalar couplings. The strong inter-residue connectivities are summarised in Table III. These data establish structure **3** for P1-ol.

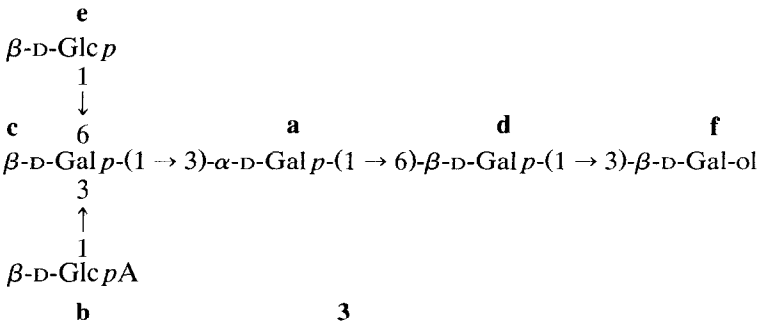
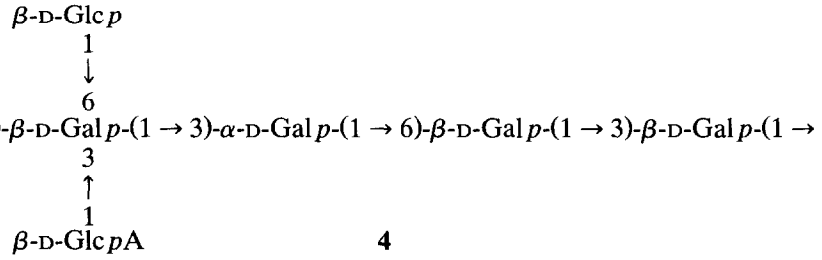


TABLE III

Observed three-bond inter-residue $^{13}C-^{1}H$ scalar coupling

Anomeric proton (residue)	Long-range connectivity (ppm)
a	67.80 (**d**, C-6)
b	82.80 (**c**, C-3)
c	80.35 (**a**, C-3)
d	78.70 (**f**, C-3)
e	69.70 (**c**, C-6)

The combined NMR and chemical data for P1-ol and PS permit the structure of the repeating unit of *Klebsiella* K15 capsular PS to be written as **4**. Structure **4** differs considerably from that reported previously[3]. The location of the *O*-acetyl groups was not investigated.



4

EXPERIMENTAL

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph, fitted with a flame-ionisation detector and a 3392A recording integrator, with He as the carrier gas. A J&W Scientific fused-silica DB-225 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm and operated isothermally at 210° was used for separating partially methylated alditol acetates. GLC–MS was performed with a Hewlett–Packard 5988A instrument in order to confirm the identities of the methylated derivatives. Acetylated 2-octyl glycosides were prepared according to the procedure of Leontein et al.[6] and were separated by GLC at 230° on the above column. Methylation analyses were carried out on the acid form of the polysaccharide or borohydride-reduced oligosaccharide, P1-ol, in a 1:1 mixture of Me_2SO and 1,1,3,3-tetramethylurea with potassium dimsyl and MeI according to the method of Narui et al.[7]. Base-catalysed degradation[8] of the methylated P1-ol was carried out using potassium dimsyl. Hydrolyses were performed with 4 M trifluoroacetic acid for 1 h at 125°. Methanolysis was carried out with methanolic 3% HCl at 80° for 16 h. Methylated PS and P1-ol were carboxyl-reduced with $NaBH_4$ in dry MeOH after methanolysis.

Isolation and purification of the Klebsiella *K15 polysaccharide.*—An authentic culture of *Klebsiella* K15 (Mich 61) was obtained from Dr. I. Ørskov (Copenhagen) and the bacteria were propagated on sucrose-rich agar[4]. The harvested bacteria were suspended in aq 1% phenol and stirred at 4°, the cellular suspension was ultracentrifuged, and the polysaccharide was precipitated by the addition of EtOH to the supernatant solution. Purification of the polysaccharide was accomplished via its cetyltrimethylammonium complex and by GPC on a column (100 × 2.5 cm) of Sephacryl S500, using aq NaOAc buffer (0.1 M) as eluent.

Bacteriophage depolymerisation of K15 polysaccharide.—A bacteriophage that could be propagated on *Klebsiella* K15 bacteria was isolated from sewage and was

used to depolymerise the capsular polysaccharide. The bacteriophage titre was increased by successive tube and flask lyses until a solution containing 1.2×10^{13} plaque-forming units was obtained. The polysaccharide (600 mg) was dissolved in the bacteriophage solution and was incubated at 37°. After 4 days, the mixture was concentrated and dialysed (mol wt cut-off, 3500) against distilled water (6×50 mL). The combined diffusate was treated with Amberlite IR-120 (H^+) resin, applied to a column (100×2.6 cm) of Biogel P4, and eluted with aq NaOAc buffer (0.1 M) to afford the hexasaccharide **P1** (130 mg).

NMR spectroscopy.—Samples were deuterium-exchanged with D_2O, and then examined as solutions in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded on either a Bruker WM 500, Bruker AMX 400, or Varian Unity 500-MHz spectrometer, using standard Bruker or Varian software. All 2D experiments were carried out at 45°. The following parameters were used. COSY and COSY LR: 512×2048 data matrix, 16 scans per t_1 value, 1-s recycle delay, unshifted sine-bell filtering in t_1 and t_2; two-step RELAY COSY: 512×2048 data matrix, zero-filled to 1024 data points in t_1, 64 scans per t_1 value, 1-s recycle delay, fixed delays of 0.036 s; HMQC: 256×2048 data matrix, 8 scans per t_1 value, 1-s recycle delay, zero-filled to 1024 data points in t_1; HMQC-TOCSY: 256×2048 data matrix, 16 scans per t_1 value, 25-ms mlev-17 mixing time, 1-s recycle delay; HMBC: 256×2048 data matrix, 32 scans per t_1 value, $\Delta 1$ and $\Delta 2$ durations of 3.45 and 60 ms, respectively, 1-s recycle delay, and a sine-bell filter.

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