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The use of bacteriophage-mediated depolymerisation in investigations of the structure of the capsular polysaccharide from *Klebsiella* serotype K71*

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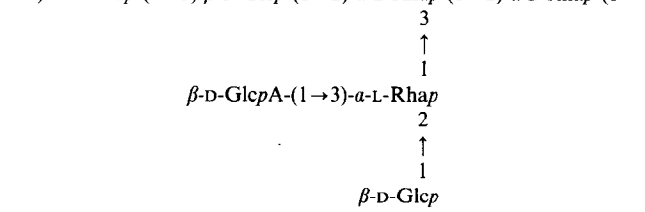
ABSTRACT

The structure (**1**) of the heptasaccharide repeating-unit of the capsular polysaccharide from *Klebsiella* serotype K71 follows from methylation analysis and n.m.r. and mass-spectrometric studies of the oligosaccharides obtained on depolymerisation of the polysaccharide with a bacteriophage-borne endorhamnosidase.



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INTRODUCTION

The capsular polysaccharide produced by *Klebsiella* serotype K71 has been classified in a chemotype containing D-glucose, L-rhamnose, and D-glucuronic acid as the component monosaccharides. This chemotype includes polysaccharides of K17, K23, K44, and K45, the structures of which have been published[1,2]. Heidelberger and Nimmich[3], on the basis of serological cross-reactions, predicted the presence in the K71 polysaccharide of terminal or (1→2)-linked D-glucuronic acid residues, and Rieger-Hug and Stirm[4] have shown that bacteriophage Φ71 cleaves the polymer into oligosaccharides having rhamnose at the reducing end. The stucture of the K71 polysaccharide has now been elucidated by chemical and spectroscopic investigation of the oligomers obtained by phage Φ71-mediated depolymerisation.

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RESULTS AND DISCUSSION

Polysaccharide capsular material, isolated from *Klebsiella* serotype K71 and purified by precipitation with Cetavlon, had $[\alpha]_D$ $-40°$ (*c* 0.1, water), contained 12.5% of uronic acid units, and was shown by steric-exclusion chromatography to have M_W $\sim 1.5 \times 10^6$. P.c. of an acid hydrolysate revealed rhamnose, glucose, and glucuronic acid. G.l.c. of the derived alditol acetates and acetylated aldononitriles indicated that glucose and rhamnose were present in the molar ratio 2.0:3.6. Methanolysis of the polysaccharide, followed by reduction with $NaBD_4$, then hydrolysis, conversion of the products into the alditol acetates, and g.l.c. gave glucose and rhamnose in the ratio 3.0:3.9, indicating the uronic acid to be glucuronic acid.

Methylation analysis — The K71 polysaccharide was methylated by the Hakomori method, then hydrolysed, and the products were converted into alditol acetate derivatives. G.l.c.–m.s. then gave the results shown in Table I, column 1. After reduction of the methylated polysaccharide with $LiAlD_4$, similar analysis gave an additional peak (containing the deuterium label) derived from the carboxyl-reduced uronic acid (Table I, column 2). Re-methylation of the carboxyl-reduced methylated polymer was followed by analysis as described above (Table I, column 3).

These data indicated single branch-points on each of two L-rhamnosyl residues and the other rhamnosyl residues to be chain units linked through O-2 and O-3, respectively. One glucosyl residue was a chain unit linked through O-3, whereas the other and the glucosyluronic acid group (*cf.* ref. 3) were terminal units.

The methylated polysaccharide was subjected to base-catalysed β-elimination by treatment with methylsulphinylmethanide, followed by re-alkylation with methyl iodide. This degradation caused loss of the glucuronic acid residue (the absence of which was shown by p.c. of a hydrolysate) and, after re-methylation, an increase in the proportion of the 3,4-di-*O*-methylrhamnosyl derivative was observed with a concom-

TABLE I

Methylation analysis of *Klebsiella* K71 polysaccharide and its derivatives

Partially methylated alditol acetate[a]	T[b]	*Mole%*[c]			
		1	*2*	*3*	*4*[d]
3,4-Me_2-Rha	0.89	14	14	17	26
2,4-Me_2-Rha	0.95	12	12	12	19
2,3,4,6-Me_4-Glc	1.00	17	18	30[e]	15
4-Me-Rha	1.46	25	26	24	15
2,4,6-Me_3-Glc	1.65	18	17	17	25
2,3,4-Me_3-Glc	1.94	[f]	13[g]	—	—

[a]3,4-Me_2-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, *etc.* [b]Retention times on column *A* at 190° relative to that of 2,3,4,6-Me_4-Glc. [c]Values corrected by use of carbon-response factors[5]. [d]1, Methylated native polysaccharide; 2, carboxyl-reduced, methylated polysaccharide; 3, re-methylated 2; 4, re-methylated, base-degraded methylated polysaccharide. [e]Peak contains ~50% of 6,6-d_2 derivative according to g.l.c.–m.s. [f]Assumed 14%, sugar ratios calculated accordingly. [g]6,6-d_2 according to g.l.c.–m.s.

TABLE II

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

Partially methylated alditol acetate[a]	T[b]	*Mole%*[c]			
		1	*2*	*3*	*4*[d]
1,3,4,5-Me_4-Rha	0.49	6	3	4	3
2,3,4-Me_3-Rha	0.53	14	7	12	10
3,4-Me_2-Rha	0.89	—	10	—	6
2,4-Me_2-Rha	0.95	—	7	—	—
2,3,4,6-Me_4-Glc	1.00	12	15	18	34[e]
4-Me-Rha	1.46	35	29	38	32
2,4,6-Me_3-Glc	1.65	19	15	15	13
2,3,4-Me_3-Glc	1.94	[f]	[f]	13[g]	—

[a]1,3,4,5-Me_4-Rha = 2-*O*-acetyl-1,3,4,5-tetra-*O*-methylrhamnitol, *etc.* [b]Retention times on column *1* at 190° relative to that of 2,3,4,6-Me_4-Glc. Assignments confirmed by g.l.c.–m.s. [c]Values corrected by use of carbon-response factors[5]. [d]1, Methylated **P1-ol** ($NaBH_4$-reduced); 2, methylated **P2-ol** ($NaBH_4$-reduced); 3, carboxyl-reduced methylated **P1-ol**; 4, re-methylated 1. [e]Contains ~ 50% of the 6,6-d_2 derivative according to g.l.c.–m.s. [f]Assumed 14%, sugar ratios calculated accordingly. [g]6,6-d_2 according to g.l.c.–m.s.

itant decrease in the amount of the 4-*O*-methylrhamnosyl derivative (Table 1, column 4). This result indicates that the terminal glucosyluronic acid group is attached to O-3 of a rhamnosyl branch-point.

Oligosaccharides **P1** *and* **P2**. — The action of bacteriophage *Φ*71 on the K71 polysaccharide and exclusion chromatography of the products gave monomeric (**P1**) and dimeric (**P2**) fractions. Reduction of **P1** with $NaBH_4$ (→**P1-ol**) followed by methylation analysis (Table II, column 1) yielded components not present among the partially methylated alditol acetates of the original polymer (Table 1, column 1) which corresponded to the acetylated derivatives of 1,3,4,5-tetra-*O*-methylrhamnitol and 2,3,4-tri-*O*-methylrhamnitol. These results suggest that cleavage occurred between the O-2 and O-3 linked rhamnosyl residues. This finding is supported by the absence of 2- and 3-linked Rha*p* units in **P1-ol**, although these residues are present in **P2-ol** (Table II, column 2) and the polysaccharide.

G.l.c–m.s. of the partially methylated alditol acetates derived from carboxyl-reduced, methylated **P1-ol** and the re-methylated product (Table II, columns 3 and 4) verified the presence of terminal glucosyluronic acid groups. These data confirmed **P1** to be a heptasaccharide, **P2** the dimer thereof, and that **P1** and **P2** each had a 2-linked rhamnose residue as the terminal reducing unit.

The identification of rhamnose as the reducing terminal group was supported by n.m.r. evidence (see below) and by the presence of rhamnitol penta-acetate among the acetylated aldononitrile (PAAN) derivatives formed[6] upon analysis of **P1**. This result accords with the findings of Rieger-Hug and Stirm[4].

Detailed n.m.r. and mass-spectrometric studies were conducted on **P1** and **P2** in order to determine their structures without recourse to further chemical methods.

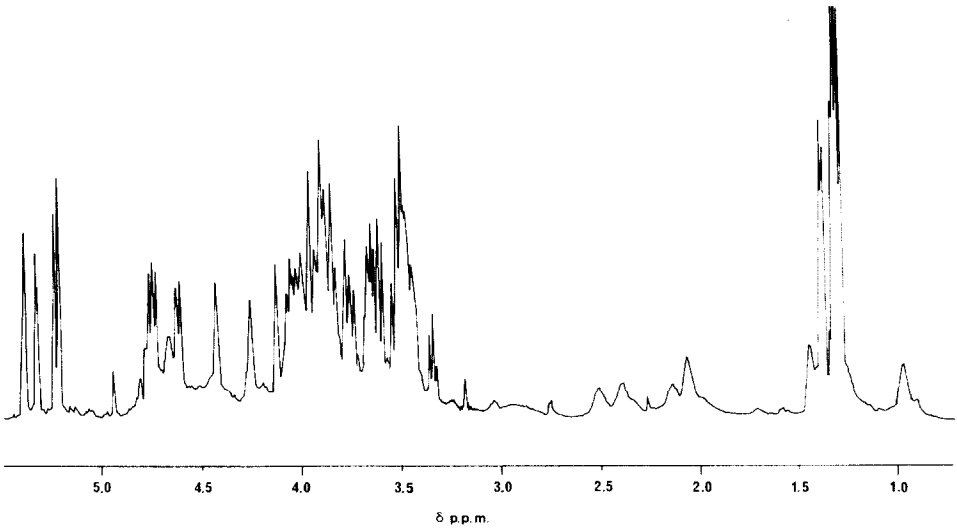


Fig. 1. ^{1}H-N.m.r. spectrum (500 MHz) of **P1** at 30°.

Assignment of the resonances of the anomeric protons and carbons of **P1**. — The ^{1}H-n.m.r. spectrum of **P1** (Fig. 1) contains signals (4 s, δ 5.4–5.1) attributable to H-1α of rhamnosyl residues, whereas the spectrum recorded at 80° revealed signals (δ 4.8–4.6, $J \sim 8$ Hz) for H-1β of hexose and uronic acid residues. The assignments were made on the basis of chemical shifts and coupling constants.

The sugar residues giving rise to the H-1 resonances were designated **a**–**g** in order of decreasing chemical shifts. The signal at δ 4.85 (s, 0.2 H) was assigned to β-Rha*p*-**c** of the reducing end. The assignments were confirmed by inspection of the ^{13}C-n.m.r. spectrum (Fig. 2), the connectivities being established using the HETCOR experiment.

The signal of H-1 of α-Rha*p*-**c** followed from the ^{13}C assignment at δ 93.58. The effect of the α and β forms of Rha*p*-**c** on adjacent residues (*i.e.*, twinning of signals[7,8]) could not be discerned in the ^{1}H-n.m.r. spectrum, although it is apparent in the ^{13}C-n.m.r. spectrum (see Fig. 2). The COSY experiment[9] aided the assignment of most of the H-2 and H-3 resonances of the sugar residues, whereas the ^{13}C assignments followed using the HETCOR diagram[10]. The ^{1}H- and ^{13}C-n.m.r. data are given in Tables III and IV, respectively.

Comparison of the chemical shift data with those for model compounds (see Tables III and IV) showed the positions of the linkages to be: 2,3-**a**, 2,3-**b**, 2-**c**, and 3-**g**, with residues **d**–**f** present as terminal groups. The assignments are consistent with the results obtained from methylation analysis (see Table II).

Examination of the ^{13}C-n.m.r. spectrum of **P1** (Fig. 2) revealed the twinning of certain signals which were tentatively attributed to those emanating from the residues at or near the reducing end. In addition to the resonances due to C-1 ($\Delta\delta$ 0.52 p.p.m.) and C-2 ($\Delta\delta$ 0.09 p.p.m.) of Rha*p*-**c**, twinning was also observed for the signals ascribed to C-1 of **b** ($\Delta\delta$ 0.45 p.p.m.) and **g** ($\Delta\delta$ 0.08 p.p.m.). The $\Delta\delta$ values for the twin signals of the

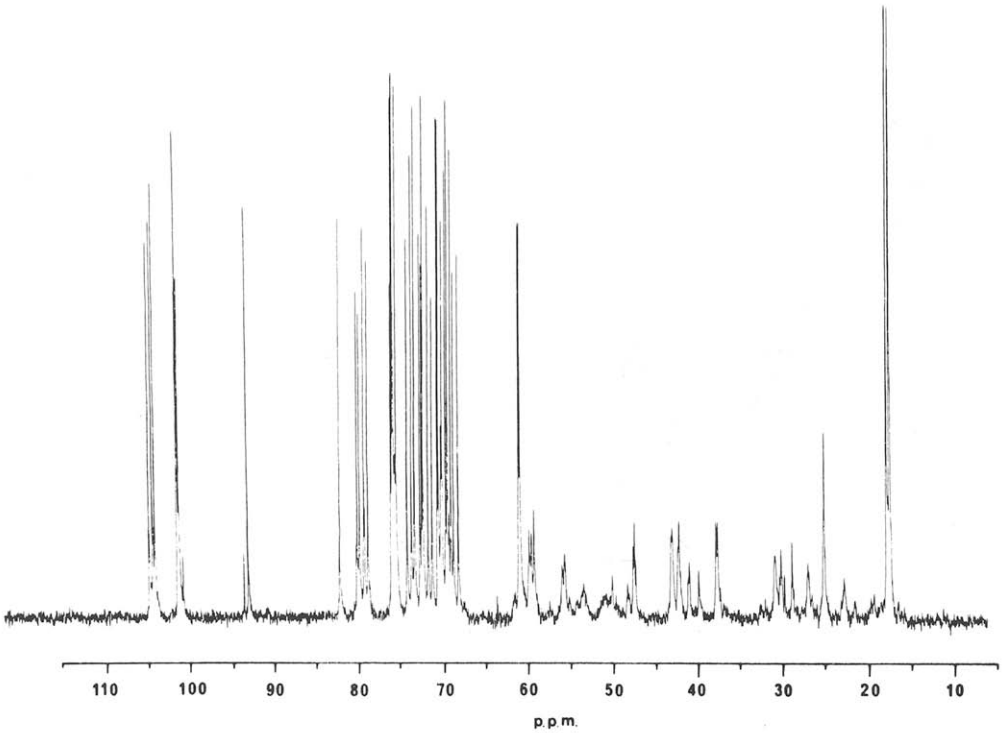


Fig. 2. ^{13}C-N.m.r. spectrum (125 MHz) of **P1** at 30°.

TABLE III

^{1}H-n.m.r. data (500 MHz) and glycosylation shifts[a] for **P-1**

Sugar residue	*Chemical shift (p.p.m.)*			*Model compound*
	H-1	*H-2*	*H-3*	
a	5.36 (+0.63)	4.40 (+0.47)	4.03 (+0.32)	α-Rha*p*-OMe[11]
b	5.30 (+0.60)	4.23 (+0.30)	3.98 (+0.27)	α-Rha*p*-OMe
c	5.12 (+0.10)	3.93 (+0.01)	n.r.[b]	α-Rha*p*[11]
d	5.19 (+0.49)	4.10 (+0.17)	3.79 (+0.08)	α-Rha*p*-OMe
e	4.73 (+0.36)	3.32 (+0.07)	3.52 (+0.04)	β-Glc*p*-OMe[12]
f	4.71 (−0.05)	3.43 (−0.02)	3.55 (−0.01)	β-Glc*p*A[13]
g	4.60 (+0.23)	3.48 (+0.23)	3.64 (+0.16)	β-Glc*p*-OMe

[a]Glycosylation shifts are given in parentheses. A downfield shift relative to the model compound is considered positive. [b]Not recorded.

TABLE IV

^{13}C-n.m.r. data (125 MHz) and glycosylation shifts[a] for **P1**

Sugar residue	*Chemical shift (p.p.m.)*			*Model compound*
	C-1	*C-2*	*C-3*	
a	102.02	79.58	80.83	α-Rha*p*[14]
	(+7.0)	(+7.7)	(+9.7)	
b	101.80[b]	80.56	76.82	α-Rha*p*
	(+6.8)	(+8.7)	(+5.7)	
c[c]	93.58	80.04	n.r.[d]	α-Rha*p*
	(−1.4)	(+8.1)		
d	101.95	71.32	70.7	α-Rha*p*
	(+7.0)	(−0.6)	(−0.4)	
e	104.68	74.41	~76.7	β-Glc*p*[14]
	(+8.0)	(−0.7)	(0.0)	
f	104.94	74.05	76.21	β-Glc*p*A[13]
	(+8.1)	(−0.9)	(−0.3)	
g	105.34[e]	74.94	82.95	β
	(+8.6)	(−0.2)	(+6.2)	

[a]Glycosylation shifts in parentheses. A downfield shift relative to the model compound is considered positive. [b]Twin signal at δ 101.35. [c]Twin signals at δ 94.10 and 79.95 due to C-1 and C-2 of β-Rha*p*-OH **c**. [d]Not recorded. [e]Twin signal at δ 105.42.

anomeric carbons suggest the sequence **g**→**b**→2**c**. Unit **g** can be linked to O-2 or O-3 of **b**, the former position of attachment being more probable because the effects of anomerisation are greater for 2- than 3-linked substituents[14].

Assignment of the ^{1}H and ^{13}C resonances of **P1-ol**. — The ^{1}H- and ^{13}C-n.m.r. spectra of **P1-ol** are presented in Figs. 3 and 4. Comparison of the ^{1}H-n.m.r. spectra of **P1** and **P1-ol** shows that the signal (δ 5.21) attributed to H-1 of **c** disappears upon reduction, whereas the resonance at δ 5.30 (**b**) is shifted upfield to δ 5.20 in **P1-ol**. These changes confirm the sequence **b**→2**c**. This result was corroborated by inspection of the ^{13}C-n.m.r. spectrum of **P1-ol** (Fig. 4), which showed the disappearance of the twin signals for residues **b** and **g**, whereas the resonances due to the reducing end (**c**) were modified as expected. These observations support the postulate that the sugar sequence **g**→2**b**→2**c** is present at the reducing end of **P1**.

The assignments of the resonances of H-1 served as the starting point for correlations established using the COSY experiment (Fig. 3), whereas the HETCOR diagram (Fig. 4) permitted identification of the attached carbon atoms. In addition, the signals for H-6 of the rhamnosyl residues **a**–**d** allowed assignment of the corresponding resonances of H-5 (and hence C-5).

Connectivities established using the long-range HETCOR experiment (Fig. 5) were corroborated by inspection of the 1D plots. The results are presented in Table V together with the data (Fig. 6) from the COLOC experiment[15]. The long-range heteronuclear correlation experiments established the connectivity between H-1 and C-3 of the α-Rha*p* units. These results confirmed the C-3 assignments and connected the assignments made from H-1 with those made from correlations established from H-6. Fig. 5

TABLE V

Assignments established using the long-range heteronuclear correlation experiments (see figs. 5 and 6)

Sugar residue	^1H *signal*	δ	*Assignment*	*Experiment*[a]
a	H-1	101.86	C-1 of **a**	LRH, COLOC
		80.64	C-3 of **a**	LRH, COLOC
		76.96	C-3 of **b**	LRH(1D), COLOC[b]
		69.98	C-5 of **a**	LRH, COLOC
	H-2	104.51	C-1 of **e**	COLOC[b]
		80.64	C-3 of **a**	COLOC
		79.36	C-2 of **a**	COLOC
		71.74	C-4 of **a**	COLOC
	H-6	71.74	C-4 of **a**	LRH, COLOC
		69.98	C-5 of **a**	LRH, COLOC
		17.72	C-6 of **a**	LRH
b	H-1	100.46	C-1 of **b**	LRH, COLOC
		80.36	C-2 of **b**	LRH, COLOC
		76,96	C-3 of **b**	LRH, COLOC
		69.85	C-5 of **b**	LRH, COLOC
	H-2	105.06	C-1 of **g**	COLOC[b]
		80.36	C-2 of **b**	COLOC
		76.96	C-3 of **b**	COLOC
		73.02	C-4 of **b**	COLOC
	H-6	73.02	C-4 of **b**	LRH, COLOC
		69.85	C-5 of **b**	LRH, COLOC
		17.24	C-6 of **b**	LRH
c	H-6	76.53	C-4 of **c**	LRH, COLOC
		68.07	C-5 of **c**	LRH, COLOC
		19.62	C-6 of **c**	LRH
d	H-1	101.81	C-1 of **d**	LRH, COLOC
		82.72	C-3 of **g**	LRH, COLOC[b]
		71.00	C-3 of **d**	LRH, COLOC
		69.56	C-5 of **d**	LRH, COLOC
	H-2	72.83	C-4 of **d**	COLOC
		~71.0	C-2 and C-3 of **d**	COLOC
	H-6	72.83	C-4 of **d**	LRH, COLOC
		69.56	C-5 of **d**	LRH, COLOC
		17.35	C-6 of **d**	LRH
e	H-1	104.51	C-1 of **e**	LRH
		79.36	C-2 of **a**	LRH[b]
	H-2	104.51	C-1 of **e**	LRH
		76.53	C-3 of **e**	LRH
f	H-1	104.77	C-1 of **f**	LRH
		80.64	C-3 of **a**	LRH[b]
	H-2	76.03	C-3 of **f**	LRH
g	H-1	105.06	C-1 of **g**	LRH
		80.36	C-2 of **b**	LRH[b]
	H-2	105.06	C-1 of **g**	LRH
		82.72	C-3 of **g**	LRH

[a]LRH, Long-range HETCOR experiment ($J_{C,H}$ 6 Hz); COLOC, Correlation spectroscopy via Long-range Couplings[15]. The experiments were conducted on a Bruker WM500 instrument at 30°. [b]Correlations showing inter-residue connectivities.

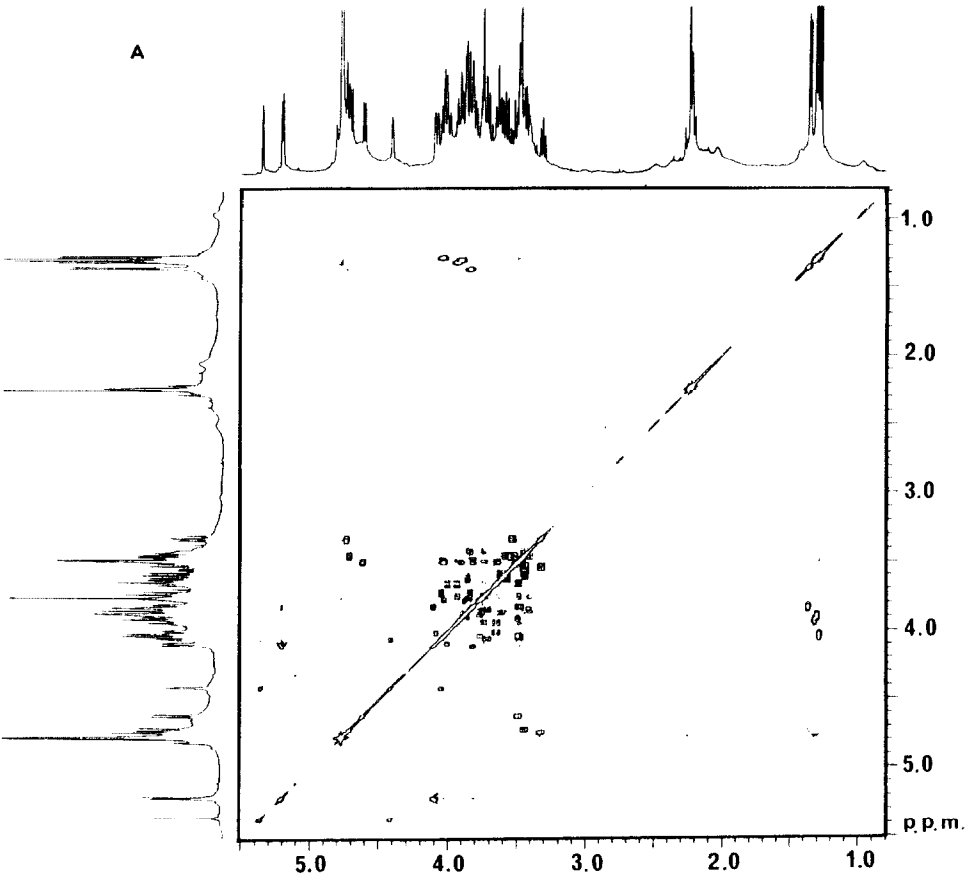


Fig. 3. Full (A) and expanded (B) (opposite page) COSY spectra of **P1-ol** at 30°.

also revealed the connectivities between H-6 of the rhamnosyl residues and C-6, C-5, and C-4 of the same residues, thus permitting identification of the resonances for H-4 (using the HETCOR diagram). These experiments made possible the full assignment of residues **a**, **b**, and **d** [see Tables VI (^{1}H) and VII (^{13}C)].

The n.m.r. assignments of residue **c** of **P1-ol** were made as follows. Inspection of the ^{13}C-n.m.r. spectra of **P1** and **P1-ol** permitted the assignments of C-2 at δ 80.25 (δ 80.04 and 79.95 in **P1**) and C-6 at δ 19.62. The C-6 assignment led to the identification of H-6 (δ 1.28), from which the C-5 and C-4 resonances of **c** were assigned (see long-range HETCOR, Fig. 5). The HETCOR experiment on **P1-ol** also revealed connectivities between H-2 of residue **c** and the ^{13}C signals at δ 60.17 and 61.88 (not present in the ^{13}C-n.m.r. spectrum of **P1**); these resonances were assigned to C-1 and possibly C-3 of **c**, respectively. However, the ^{13}C signal at δ 68.47 (also not present in the ^{13}C-n.m.r. spectrum of P1) may be attributed to C-3 of **c**, on the basis of chemical shift (*cf.* 2-linked rhamnitol[16]) and the connectivity suggested by the COSY spectrum (Fig. 3). The ^{13}C assignments as depicted in Table VII were partly verified by identification of the

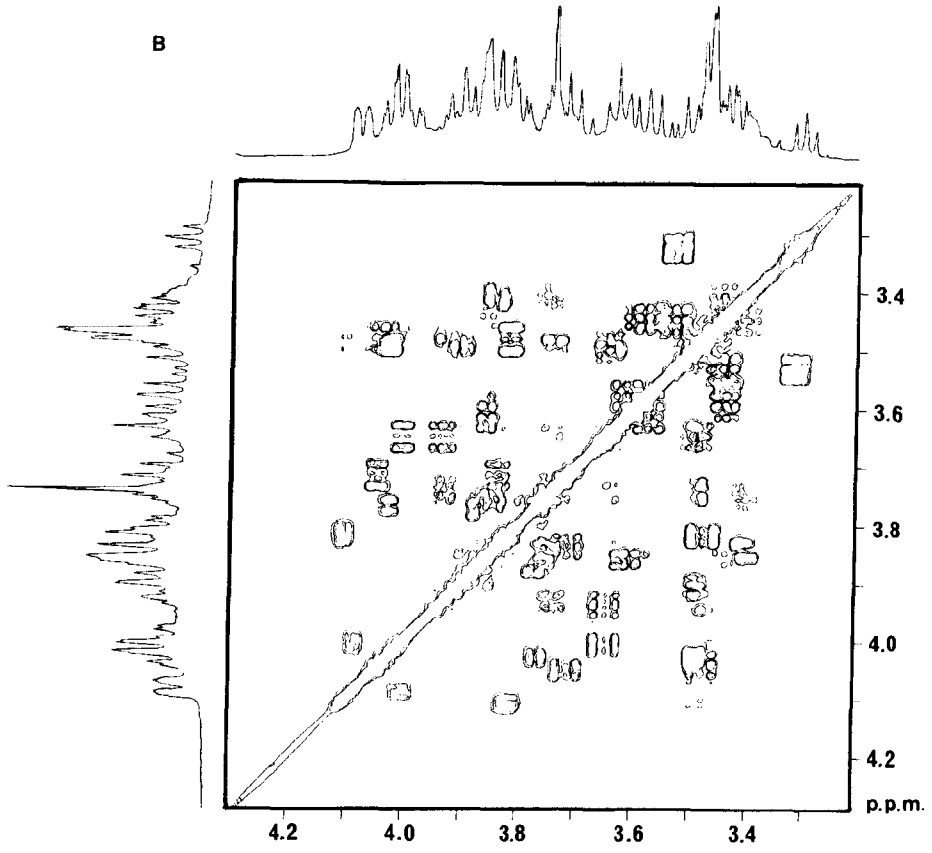


Fig. 3. (B).

attached protons (using the HETCOR diagram, Fig. 4) and inspection of the connectivities indicated by the COSY plot (Fig. 3).

The β sugars (**e**–**g**) were less amenable to n.m.r. analysis because of the small differences in chemical shift. The COSY experiment (Fig. 3) enabled identification of the H-2 and H-3 signals, whereas the attached carbon assignments followed from the HETCOR plot. The long-range HETCOR experiment (Fig. 5) showed the following couplings: H-2 to C-1 and C-3 of **e**, H-3 to C-2 of **f**, and H-2 to C-4 of **g**. Residue **e** was assigned to the terminal Glc, on the basis of the characteristic low-field triplet found for H-2 of **e** in **P1** (δ 3.32) and **P1-ol** (δ 3.30) (*cf.* δ 3.45 found for H-2 of β-Glc*p*A[13] versus δ 3.13 and 3.25 reported for β-Glc*p* and its methyl glycoside[12,17]). The assignment of **f** as the terminal glucosyluronic acid group follows from that of **e**. N.m.r. assignments together with the glycosylation shifts are shown in Tables VI (^{1}H) and VII (^{13}C).

The sequence and pattern of substitution of the sugar residues in **P1**. — The glycosylation shifts in Tables VI and VII, especially the ^{13}C shifts, confirm the linkage

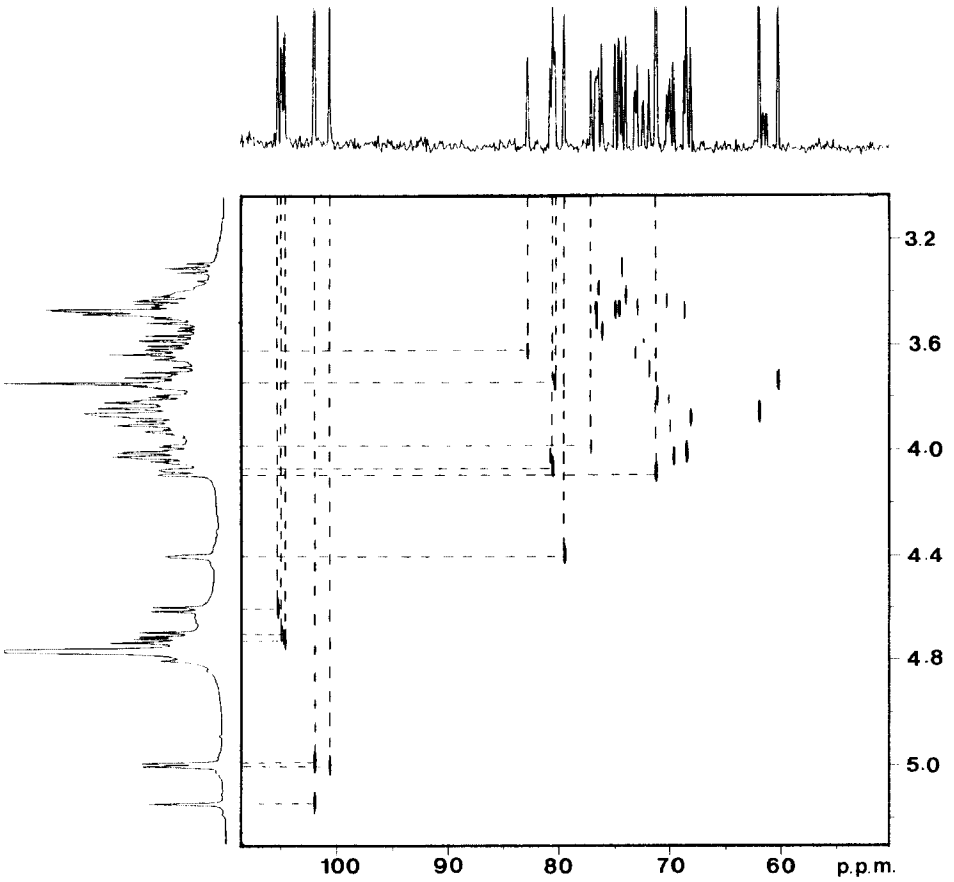
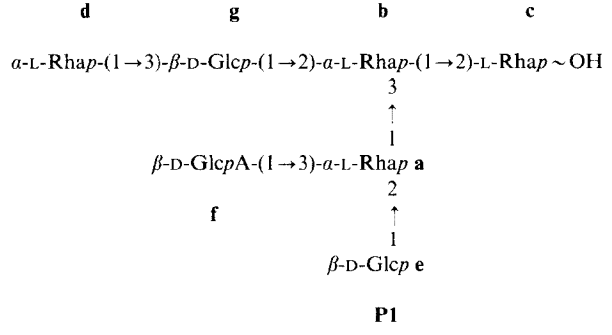


Fig. 4. HETCOR spectrum of **P1-ol** at 30° ($J_{C,H}$ 140 Hz).

assignments made for **P1**. The establishment of the sequence of sugar residues **a**–**g**, as depicted below, follows from the inter-residue connectivities revealed by the long-range ^{1}H–^{13}C correlation experiments (Table V, Figs. 5 and 6).



P1

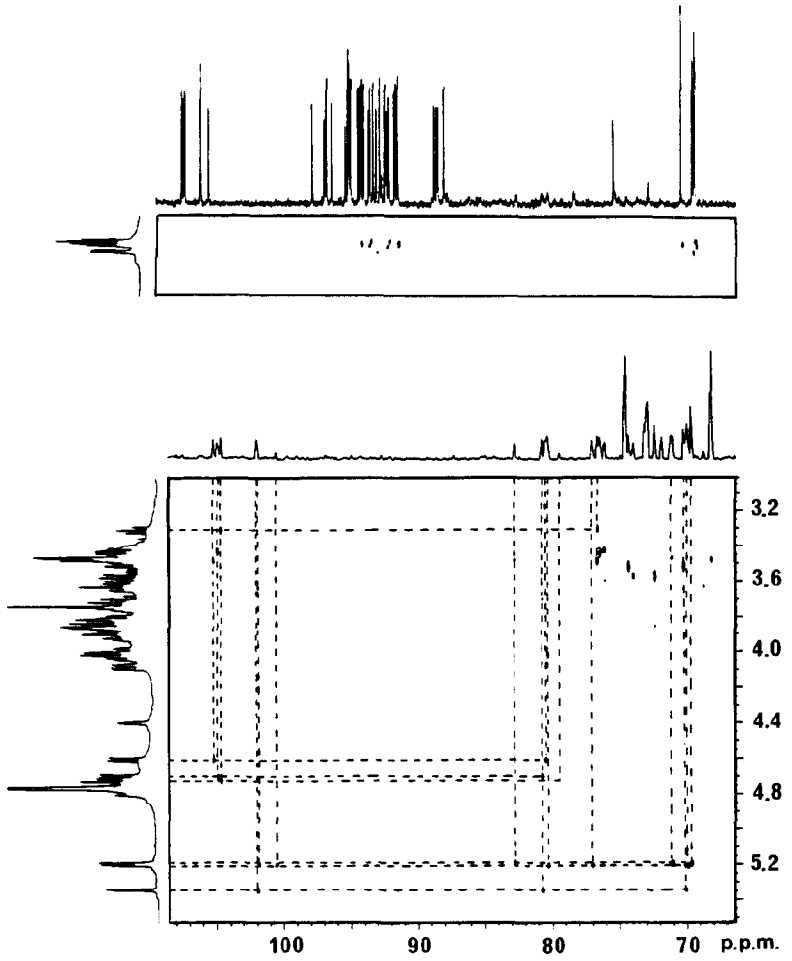


Fig. 5. Expanded long-range HETCOR spectrum of **P1-ol** at 30° ($J_{C,H}$ 6 Hz); upper section shows δ_H centred at 1.3 p.p.m. and full ^{13}C spectrum from 110–10 p.p.m.

Some of the absolute configurations of the sugar residues were also confirmed by application of correlations[18] involving ^{13}C chemical shift and configurational factors.

N.m.r. analysis of **P2**: *proof that the 2-linked Rhap unit is a.* — In order to establish the anomeric configurations of all the sugar residues in the K71 polysaccharide, the n.m.r. spectra of the dimer (**P2**) and the $NaBH_4$-reduced dimer (**P2-ol**) were examined. Assignments of the anomeric signals of **P2** were substantiated by analysis of the ^1H- and ^{13}C-n.m.r. spectra of **P2-ol** (see Table VIII).

The signals for the anomeric proton (δ 5.18) and carbon (δ 101.29) assigned to **c** are diagnostic for α-Rha*p* units, thus confirming the original assignment based on the ^1H-n.m.r. spectrum of the K71 polysaccharide, whereas the ^{13}C signal at δ 101.45 (not present in the ^{13}C-n.m.r. spectrum of **P1-ol**) was ascribed to the 3-linked Rha*p* residue (**d**).

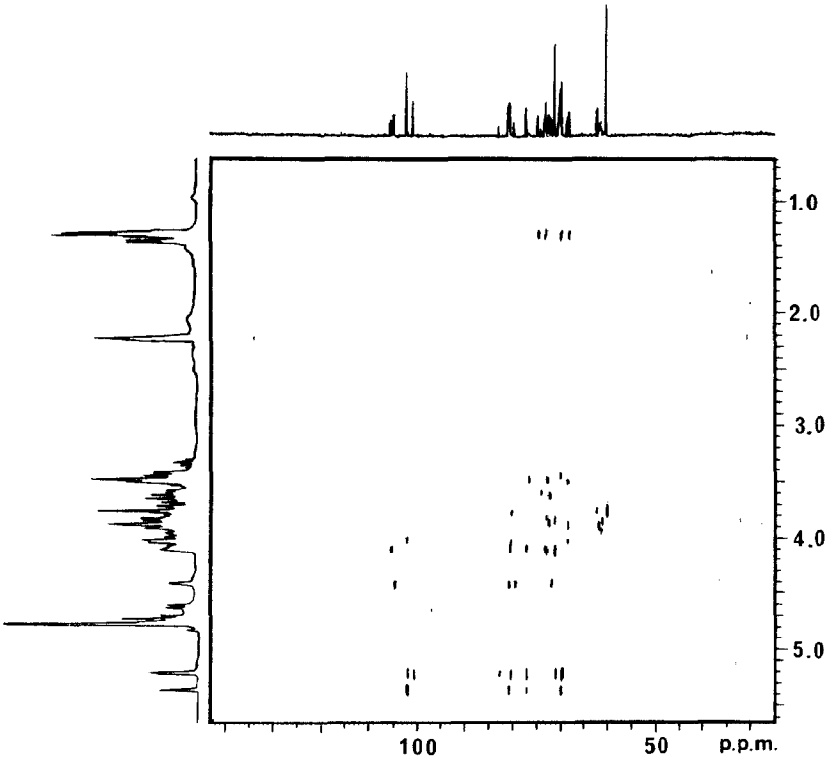


Fig. 6. COLOC spectrum of **P1-ol** at 30°.

Sequence analysis of **P1** *by mass spectrometry.* — Confirmation of structural aspects of the proposed formula for **P1** was obtained from e.i.-m.s. of methylated **P1-ol** (see Fig. 7). The fragment ions are described using the letters **a**–**g** as used in the n.m.r. study.

The A-series ions formed from the non-reducing end are characteristic of terminal deoxyhexose (**d**), hexose (**e**), and hexuronic acid (**f**) groups, whereas the fragment ions at *m/z* 393 and 361 can be attributed to a deoxyhexose–hexose sequence (gdA_1 and gdA_2 ions, respectively). The peak at *m/z* 611 was ascribed to the A-fragment formed by cleavage of the **a**→2**b** bond (*i.e.*, the $afeA_1$ ion) which gives rise to the fragment ion at *m/z* 579 by loss of MeOH. Alternatively, loss of the 2- and 3-substituents should result in fragment ions at *m/z* 375 (and 343) and 361 (and 329), as observed. The low intensity of the peak at *m/z* 375 compared to that of the *m/z* 361 ion (although the latter is also attributed to the gdA_2 ion) suggests that the larger substituent (**f**) is eliminated preferentially, thereby indicating it to be attached to O-3 of **a** (*cf.* the relative intensities of fragment ions formed by elimination of the 2- or 3-substituent[19-21]). The peak of high intensity at *m/z* 205 confirms the presence of rhamnitol (**c**) at the reducing end (cA_1); no J-series ions could be detected. Although the A-series fragment generated by cleavage of the **b**→**c** bond was not observed (calibration range of mass spectrometer ~ 750 a.m.u.),

TABLE VI

^{1}H-n.m.r. data (500 MHz) and glycosylation shifts[a] for **P1-ol**

Sugar residue	*Chemical shift (p.p.m.)*						*Model compound*
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	
α-Rha*p* **a**	5.34	4.40	4.03	3.70	3.85	1.34	α-Rha*p*-OMe[11]
	(+.64)	(+.47)	(+.32)	(+.26)	(+.21)	(+.04)	
α-Rha*p* **b**	5.20	4.07	3.99	3.63	3.92	1.29	α-Rha*p*-OMe
	(+.50)	(+.14)	(+.28)	(+.19)	(+.28)	(+.01)	
Rhamnitol **c**	3.73	3.75	4.00[b]	3.49	3.90	1.28	α-Rha*p*-OMe
	(−.97)	(−.18)	(+.29)	(+.05)	(+.26)	(−.02)	
α-Rha*p* **d**	5.19	4.09	3.79	3.45	4.02	1.24	α-Rha*p*-OMe
	(+.49)	(+.16)	(+.08)	(+.01)	(+.38)	(−.06)	
β-Glc*p* **e**	4.72	3.32	3.52	n.r.[c]	n.r.	3.77[d]	β-Glc*p*-OMe[12]
	(+.35)	(+.07)	(+.12)			—	
β-Glc*p*A **f**	4.69	3.43	3.53	n.r.	n.r.	—	β-Glc*p*A[13]
	(−.07)	(−.02)	(−.13)				
β-Glc*p* **g**	4.60	3.49	3.62	n.r.	n.r.	3.71[d], 3.92	β-Glc*p*-OMe
	(+.23)	(+.24)	(+.22)			—	

[a]Glycosylation shifts given in parentheses. A downfield shift relative to the model compound is considered positive. [b]Or δ 3.88. [c]Not recorded. [d]Assignments might be reversed.

TABLE VII

^{13}C-n.m.r. data (125 MHz) and glycosylation shifts[a] for **P1-OL**

Sugar residue	*Chemical shift (p.p.m.)*						*Model compound*
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	
α-Rha*p* **a**	101.86	79.36	80.64	71.74	69.98	17.72	α-Rha*p*[14]
	(+6.9)	(+7.5)	(+9.5)	(−1.6)	(+0.6)	(−0.3)	
α-Rha*p* **b**	100.46	80.36	76.96	73.02	69.85	17.24	α-Rha*p*
	(+5.5)	(+8.5)	(+5.9)	(−0.3)	(+0.4)	(−0.76)	
Rhamnitol **c**	60.17[b]	80.25	68.47[b]	76.53	68.07	19.62	L-Rha-ol[16]
	(−5.5)	(+6.5)	(−3.7)	(+0.6)	(−1.3)	(−1.8)	
α-Rha*p* **d**	101.81	71.14	71.00	72.83	69.56	17.35	α-Rha*p*
	(+6.8)	(−0.7)	(−0.1)	(−0.5)	(+0.2)	(−0.6)	
β-Glc*p* **e**	104.51	74.23	76.53	n.r.[c]	n.r.	61.23[d]	β-Glc*p*[14]
	(+7.8)	(−0.9)	(−0.2)			(−0.5)	
β-Glc*p*A **f**	104.77	73.88	76.03	n.r.	n.r.	174.50	β-Glc*p*A[13]
	(+8.0)	(−1.0)	(+0.4)			(−2.2)	
β-Glc*p* **g**	105.06	74.45	82.72	n.r.	n.r.	61.44[d]	β-Glc*p*
	(+8.4)	(−0.6)	(+6.0)			(−0.3)	

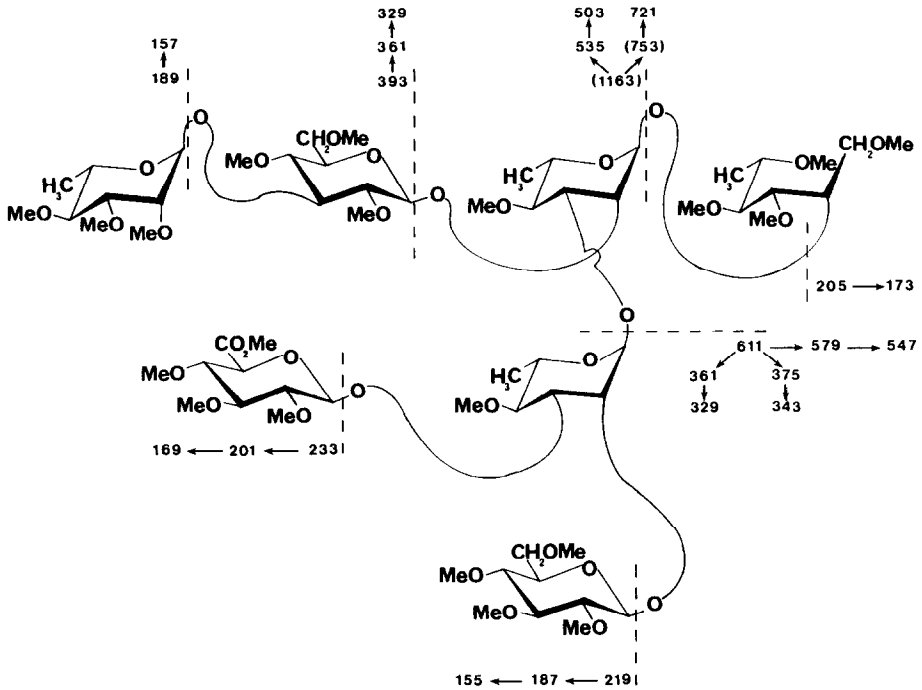
[a]Glycosylation shifts given in parentheses. A downfield shift relative to the model compound is considered positive. [b]Or δ 61.88. [c]Not recorded. [d]Assignments might be reversed.

TABLE VIII

^{1}H- and ^{13}C-n.m.r. data[a] for **P2-ol**

	^1H (200 MHz)			^{13}C (50.3 MHz)	
δ(p.p.m)	$J_{1,2}$ (Hz)	Integral	Assignment	δ(p.p.m.)	Assignment
5.34	s	2	H-1 of α-Rha*p* **a**,**a**′	105.12	C-1 of **g**,**g**′
5.28	s	1	H-2 of α-Rha*p* **b**	104.99	
5.18	s	4	H-1 of α-Rha*p* **b**′,**d**,**d**′, **c**	104.62	C-1 of **f**, **f**′
4.70[b]	~6.4	2	H-1 of β-Glc*p* **e**,**e**′	104.59	
4.67[b]	~5.4	2	H-1 of β-Glc*p*A **f**,**f**′	104.49	C-1 of **e**, **e**′
4.58[b]	~5.4	2	H-1 of β-Glc*p* **g**,**g**′	101.72	C-1 of **a**, **a**′ ,**d**
4.38	s	2	H-2 of α-Rha*p* **a**,**a**′	101.45	C-1 of **d**′
4.21	s	1	H-2 of α-Rha*p* **b**	101.29	C-1 of **c**
~1.30	—	24	H-6 of α-Rha*p* **a**–**d**,**a**′–**d**′	100.35	C-1 of **b**′

[a]Chemical shift values relative to that for internal acetone at δ 2.21 (^{1}H) and 31.0 (^{13}C). [b]Revealed when the spectrum was recorded at 80°.



the ions at *m*/*z* 535 and 503 could be assigned to the A_2 (and A_3) fragments formed by elimination of the 3-substituent (followed by the loss of MeOH). Elimination of the 2-substituent followed by the loss of MeOH would account for the fragment ion detected at *m*/*z* 721. The relative intensities of the fragments formed by elimination of the 2- and 3-substituents can be rationalised as before. The correctness of the assign-

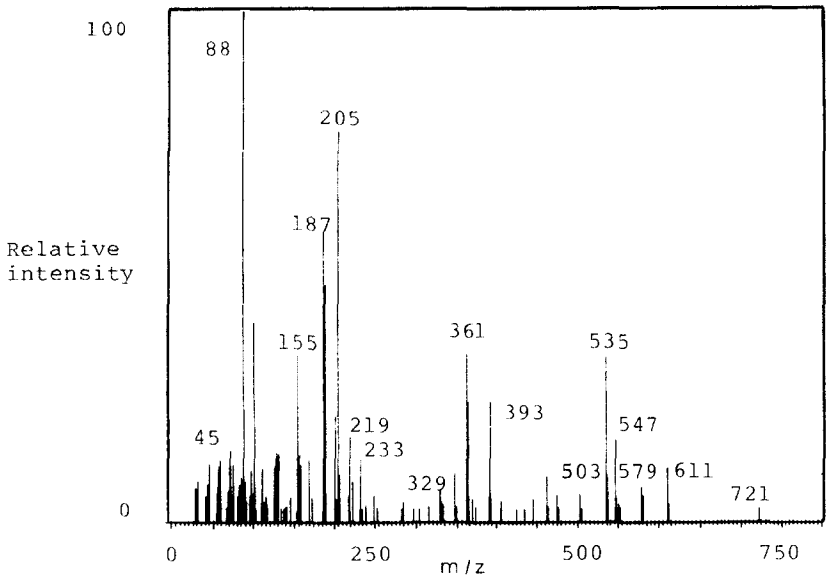
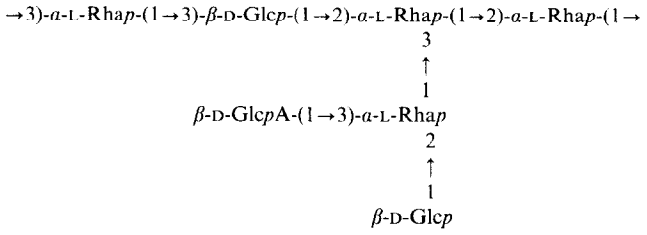


Fig. 7. E.i.-m.s. fragmentation of methylated **P1-ol** recorded at 20 eV. Interpretation above, spectrum below.

ments and interpretation described above was substantiated by m.s. of the carboxyl-reduced methylated derivative of **P1-ol**.

On the basis of the foregoing data and arguments, the capsular polysaccharide from *Klebsiella* serotype K71 can be assigned the heptasaccharide repeating-unit **1**.



1

The repeating structure **1** is of the "4+2+1" type, *Klebsiella* serotype K67 being the only other example in this series to have a capsular polysaccharide with a branched side-chain[22]. Within the chemotype, the structure of the K45 capsular polysaccharide is identical to that postulated for K71 with respect to the main chain. However, the side chain contains only a single β-D-Glc*p*A group, attached directly to O-3 of the 2-linked in-chain α-L-Rha*p* residue ("4+1" type)[2].

EXPERIMENTAL

General methods. — Solvent systems used in p.c. and t.l.c. were *A*, 20:20:7 chloroform–methanol–water; *B*, 97:3 chloroform–methanol; *C*, 8:2:1 ethyl acetate–pyridine–water; and *D*, 4:1:5 upper phase 1-butanol–ethanol–water. Sugars were detected after t.l.c. with *p*-anisaldehyde–sulphuric acid–ethanol (1:1:18) or *p*-anisidine hydrochloride (2%) in sulphuric acid–ethanol (1:20) followed by heating for 5–10 min at 110°. Sugars after p.c. were detected in the usual way[23].

Optical rotations were measured on solutions in water for underivatised samples and in chloroform for the methylated products at 20 ±3° with a Perkin–Elmer Model 141 polarimeter. I.r. spectra of solutions of methylated derivatives in chloroform were recorded with a Perkin–Elmer Model 983 spectrophotometer. U.v. spectra were recorded with a Beckman UV 5260 spectrophotometer. The ^{1}H- (200 and 500 MHz, 20° and 80–90°) and ^{13}C-n.m.r. spectra (50.3 and 125.7 MHz, 25°) were obtained with Varian VXR-200 and Bruker WM500 spectrometers, respectively. Samples were prepared by freeze-drying solutions in 99.7% D_2O thrice in succession. Chemical shifts of ^{1}H resonances were measured with reference to internal acetone, δ 2.21 (at 200 MHz) or 2.225 (at 500 MHz) downfield from the signal for Me_4Si, and those of ^{13}C were measured with reference to internal acetone, taken as δ 31.0 (at 50.3 MHz) or 31.07 (at 125.7 MHz) downfield from the signal for Me_4Si. All 2D-n.m.r. experiments were conducted at 25° or 30°, using the procedures described in the text. M.s. was performed on a VG Micromass 16F mass spectrometer, operating at 70 or 20 eV. G.l.c. and g.l.c.–m.s. were performed on *1*, a fused-silica capillary DB-225 column (30 m × 0.32 mm i.d.; film thickness, 0.25 μm; J & W Scientific, Inc.); or *2*, a glass column (2 m × 3 mm i.d.) packed with 3% of OV 17 on Chromosorb W-HP, 80–100 mesh.

Distributions of molecular weights were determined using columns of Sepharose 4B (60 × 0.9 cm) or Bio-Gel P-2 (55 × 2.5 cm). Steric exclusion chromatography (s.e.c.) was performed on a column (75 × 2 cm) of Trisacryl GF05 by elution with 0.1M pyridinium acetate buffer (pH 5.0). Fractions were monitored by the phenol–H_2SO_4 assay[24], polarimetry, or t.l.c. (solvent *A*).

Sugar analyses. — Neutral sugars were determined by g.l.c. of the derived alditol acetates[25] or aldononitrile acetates[6]. Uronic acid was determined colorimetrically[26]. Methanolysis[27] permitted simultaneous determination of acidic and neutral sugars. Methylations were carried out by the Hakomori method[28], using potassium methylsulphinylmethanide (2M), prepared[29] by addition of dry Me_2SO to KH at 0°.

Samples containing uronic acid were deionised with Amberlite IR-120 (H^+) resin and freeze-dried prior to methylation. In general, samples were dried in vacuum over P_2O_5 for at least 18 h before being dissolved in dry Me_2SO for methylation, conducted under purified N_2. The time of contact with the base ranged from 0.5–3 h, the presence of excess of base being confirmed by testing with triphenylmethane[29]. Solutions of the alkoxides were frozen in ice before addition of MeI or CD_3I, and the solutions were then stirred at room temperature for at least 0.5 h. Polymeric products were recovered by addition of water and $CHCl_3$, followed by dialysis, and recovery of the organic phase in

the non-dialysable portion. After evaporation of $CHCl_3$, products were purified by elution from a column of Sephadex LH-20 with 2:1 ethanol–chloroform. The eluate was monitored for carbohydrate with anthrone–sulphuric acid[30]. Oligomeric products were recovered from the reaction mixture by addition of chloroform, and extraction of salts and Me_2SO with water. Where methylation of uronic acid-containing polysaccharides was incomplete, further methylation was carried out by the Purdie method[31].

Carboxymethyl groups were reduced[32] with lithium aluminium hydride (LAH) or deuteride (LAD) for 30 min at 80° in dry tetrahydrofuran, and portions of the supernatant solution after centrifugation were added to the methylated glycan in dry tetrahydrofuran. The resulting solution was heated for 18 h at 60°, and the reduced products were recovered by the addition of moist ethyl acetate, filtration through Celite, washing of the residues with $CHCl_3$, and concentration of the combined filtrate and washings to dryness. Completeness of methylation and reduction procedures was assessed by i.r. spectroscopy. Methylation analyses were carried out after hydrolysis of the methylated products in 2M CF_3COOH for 8 h or 18 h at 100°, and removal of the acid by freeze-drying. Some hydrolysates were examined by p.c. (solvent *D*), using the mixture of partially methylated sugars from *Virgilia oroboides* gum[33,34] as a standard. The aldoses were then converted into partially *O*-methylated alditol acetates for g.l.c.

Isolation and analysis of the K71 polysaccharide. — A culture of *Klebsiella* K71 was grown[23] on a medium containing Müller–Hinton broth (6 g of meat infusion, 17.5 g of casein hydrolysate, and 1.5 g of starch) in 1 L of water to which 2 g of NaCl, 30 g of sucrose, and 15 g of agar were added. The polysaccharide was purified by precipitation once with cetyltrimethylammonium bromide. The polysaccharide [~5 g from three batches of six trays (each 45 × 32 × 5 cm)] typically had the properties described in the text. Sugar analyses were conducted on 10-mg samples as described above; g.l.c. was performed on columns *1* (alditol acetates at 215°) and *2* (PAANs at 220°).

The absolute configurations of the sugar components were established as follows. A portion of the polysaccharide (200 mg) was hydrolysed (2M CF_3COOH, 18 h, 100°), and p.c. (solvent *C*) gave good separation of the sugars which were extracted into water. The $[\alpha]_D$ values accorded with those for D-glucuronic acid, D-glucose, and L-rhamnose.

Methylation analysis of K71 polysaccharide. — Methylation was conducted on a portion (100 mg) of the polysaccharide as described above (see Table I, columns 1–3). A sample (40 mg) of the methylated polysaccharide was base-degraded, and then re-alkylated with methyl iodide to give results shown in Table I, column 4.

Propagation of bacteriophage Φ71. — Bacteriophage Φ71 (obtained by courtesy of Professor S. Stirm, Giessen) was propagated on host strain *Klebsiella* K71 initially on plates and then in solutions of nutrient broth until 2 L of lysate containing ~10^{13} p.f.u. had been obtained[35]. The phage solution was concentrated and dialysed against running tap-water for 2 days, then concentrated (to 200 mL).

Production and analysis of oligosaccharides **P1** *and* **P2**. — Polysaccharide (1.5 g) was stirred with the phage solution for 3 days at 37° in the presence of chloroform (3 mL). The oligomers produced were isolated by dialysis and preparative s.e.c. (Trisacryl GF05) to yield **P1** (760 mg) and **P2** (200 mg). A sample (5 mg) of each oligosaccharide

was dissolved in water (2 mL) and treated with sodium borohydride (10 mg) overnight. The reduced oligosaccharides recovered were hydrolysed (2M CF_3COOH, 18 h, 100°) and the products were converted into PAAN derivatives for g.l.c.–m.s. (column *2* at 220°).

Methylations were conducted on **P1** (40 mg) and **P1** (5 mg) after reduction with $NaBH_4$, to give the results shown in Table II. E.i.–m.s. of the methylated derivatives of **P1-ol** was performed on a VG Micromass 16F mass spectrometer operating at 20 eV and with direct insertion, to give the results presented in Fig. 7.

N.m.r. spectroscopy of **P1** (80 mg) and **P1-ol** (60 mg) was conducted with a Bruker WM500 spectrometer, using standard conditions (see text). The n.m.r. spectra of **P2** (not presented) and **P2-ol** were recorded with a Varian VXR-200 instrument.

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