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PROOF OF THE OCCURRENCE OF 5,6-*O*-(1-CARBOXYETHYLIDENE)-D-GALACTOFURANOSE UNITS IN THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K12

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ABSTRACT

The ^{13}C-n.m.r. spectra of the capsular polysaccharide of *Klebsiella* K41 and phage-derived oligosaccharides K41-P1 and K41-P2 were compared with spectra from the structurally similar polysaccharide of *Klebsiella* K12 and oligosaccharides K12-P1 and K12-P2. This led to the conclusion that K41 and K12 contain one and two galactofuranose residues per repeating unit, respectively, and that the terminal, lateral residue in K12 has the 5,6-*O*-(1-carboxyethylidene)-D-galactofuranose structure rather than that of a 4,6-acetal of D-galactopyranose as originally stated. This is the first reported occurrence in Nature of such a structural unit.

INTRODUCTION

Previous reports from our laboratories have stressed the practical advantages of determining the structures of bacterial polysaccharides by examining the oligosaccharides which are obtained by the action of a viral endoglycanase on the polymeric antigen and which represent the chemical repeating unit of such polysaccharides[1–3]. This approach has been further illustrated recently in the determination[4,5] of the structure of the capsular polysaccharide from *Klebsiella* K19. Considering that capsular polysaccharides have molecular weights in the range of 10^6, the resolution shown by their n.m.r. spectra (^{1}H and ^{13}C) is remarkable and attests to the regularity of these polymeric structures. Nevertheless, the spectra of phage-produced oligosaccharides which, in the *Klebsiella* series, range from tri- to hepta-saccharides, obtained with spectrometers of the greater sensitivity currently available, make possible more detailed analyses of complex polysaccharides than

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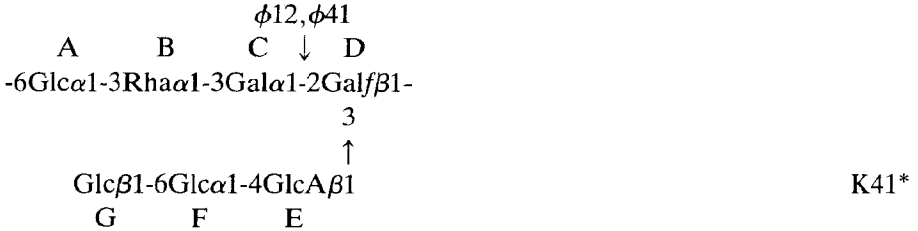
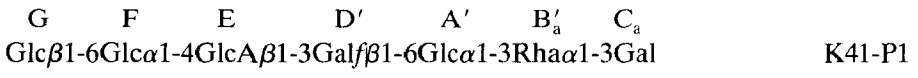
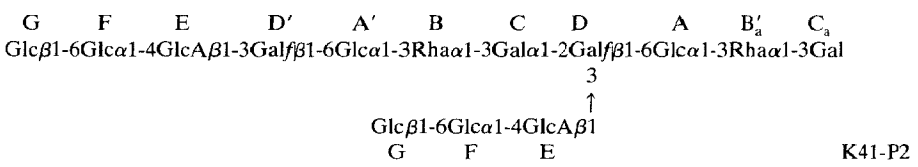
heretofore possible. In the present context, the ability to distinguish and assign ^{13}C resonances in the range of 80 to 86 p.p.m. was particularly valuable.



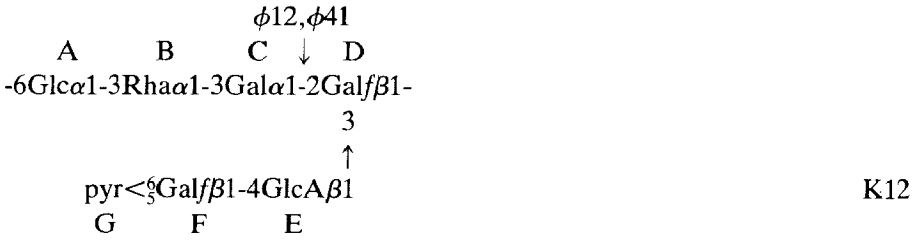
K41*



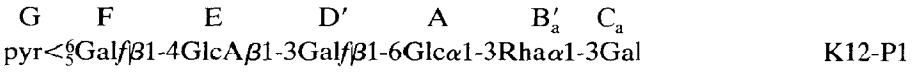
K41-P1



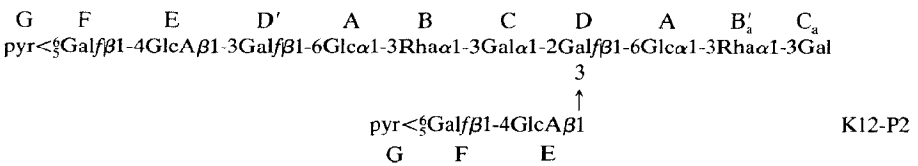
K41-P2



K12



K12-P1



K12-P2

The accompanying paper[6] describes the isolation of oligosaccharides derived from the capsular polysaccharides of *Klebsiella* serotypes K41 and K12 by the action of viral endoglycanases. The n.m.r. spectra of K41, K41-P1 and K41-P2 (each with one galactofuranose residue per repeating unit) were examined first. In the light of the results obtained, it became clear from a study of the spectra of K12, K12-P1 and K12-P2 that the structure originally reported[7] for the K12 polysacchar-

*The structures of K12 and K41 are given here in condensed notation, but the letters used to designate individual units are as in the previous paper[6]. All sugars are in the pyranose form unless otherwise indicated.

ide must be revised with respect to the nature of the acetal-galactose moiety (units F and G), and that the correct structures of K12, K12-P1, and K12-P2 are as shown on the previous page*. It should be noted that since the phage cleaves the bond between units C and D the oligosaccharides K12-P1 and K41-P1 will be linear, whereas any higher oligomers (P2, etc.) will have at least one branch point. An analysis of the spectral data which leads to this revision is the subject of the present paper.

RESULTS AND DISCUSSION

^{13}C-N.m.r. spectra of K41 and K41-P1. — The ^{13}C-n.m.r. data obtained at 25.2 MHz and presented in Table I and Fig. 1 for K41 polysaccharide (partially depolymerized) are substantially as previously determined[8], but are given here for

TABLE I

^{13}C-N.M.R. DATA FOR K41 POLY- AND K41-P1 OLIGO-SACCHARIDE

δ[a] (p.p.m.)		*Assignment*			
K41[b]	*K41-P1*	*C-Atom*	*Residue[c]*	*Position in structure*	
				K41	*K41-P1*
107.0	109.0	C-1	β-Gal*f*	D	D′
103.5	103.6	C-1	β-Glc	G	G
102.8		C-1	β-GlcA	E	
	102.7	C-1	β-GlcA, α-Rha		E, B′
102.5		C-1	α-Rha	B	
99.6		C-1	α-Gal	C	
99.1	99.1	C-1	α-Glc	F	F
97.6		C-1	α-Glc	A	
	96.8	C-1	β-Gal–OH, α-Glc		C_a, A′
86.0	80.4	C-2			
84.2	85.5	C-3	β-Gal*f*	D	D′
83.2	83.4	C-4			
68.9	69.7	C-6	α-Glc	F	F
66.8	68.2	C-6	α-Glc	A	A′
64.1	63.9	C-6	β-Gal*f*	D	D′
61.9		C-6	α-Gal, β-Glc	C, G	
	61.9	C-6	Gal–OH, β-Glc		C_a, G
17.8	17.5	C-6	α-Rha	B	B'_a

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 31.07 p.p.m. [b]The native polysaccharide was mildly hydrolyzed in order to reduce the viscosity and give a sharper spectrum. [c]All sugar residues are pyranosidic unless otherwise noted.

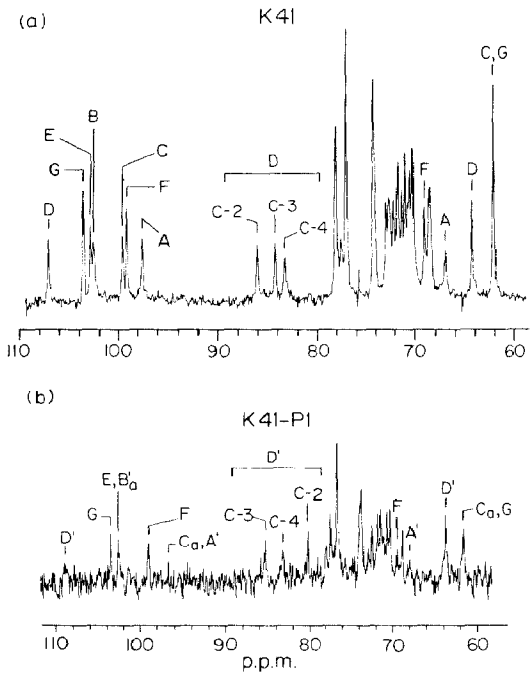


Fig. 1. Fully decoupled ^{13}C-n.m.r. spectra of (a) K41 polysaccharide, mildly hydrolyzed, (b) K41-P1 oligosaccharide. Measured at 25.2 MHz, 80°, *vs.* internal acetone (31.07 p.p.m.).

comparative purposes. The corresponding data for K41-P1 (Table I) show that the signal for C-1 of the galactofuranosyl unit (D) is shifted downfield by 2.0 p.p.m. (D′) in the oligosaccharide*. If the viral D-galactosidase cleaved this furanosyl linkage, unit D would become the reducing end of the oligosaccharide (P1) and would be expected to assume a predominantly pyranose configuration with the concomitant disappearance of the signal at ca. 108 ±1 p.p.m. With the hydrolysis of the galactopyranosyl residue (C) the galactofuranose is not longer substituted at C-2 and the continued presence of the signals in the range 80–86 p.p.m. confirms that the furanosyl linkage remains intact. Furthermore, it is the signal due to C-2 of D that exhibits a strong upfield shift in the oligosaccharide with a smaller positive β shift for C-3, confirming that unit D in the polysaccharide is substituted at position 2. Likewise, the signal for C-1 of unit C_a, now a reducing unit*, is shifted upfield from 99.6 to 96.8, corresponding to β-D-galactopyranose; the signal for α-D-galactopyranose would be expected[9] at about 93 p.p.m., but is obscured by noise.

The signals due to C-1 and C-6 of glucose residue A are both shifted to some extent. Possible explanations for this are: (*a*) the fact that this residue (A) is directly

*Primed letters refer to residues whose signals show a significant change in chemical shift by comparison with the original polymer. A subscript *a* (anomeric) indicates a reducing unit, *e.g.* C_a, or a residue assumed to be influenced by the mutarotational equilibrium, *e.g.* B'_a. This notation follows that used in ref. 5.

TABLE II

^{13}C-N.M.R. DATA FOR OLIGOSACCHARIDE K41-P2

δ^a (p.p.m.)	*Assignment*		
	C-Atom	*Residue*[b]	*Position in structure*
108.8	C-1	β-Gal*f*	D′
107.1	C-1	β-Gal*f*	D
103.6	C-1	β-Glc	G
102.9	C-1	β-GlcA	E
102.7	C-1	α-Rha	B'_a
102.6	C-1	α-Rha	B
99.6	C-1	α-Gal	C
99.1	C-1	α-Glc	F
97.4	C-1	α-Glc	A, A′
97.2	C-1	β-Gal–OH	C_a
93.3	C-1	α-Gal–OH	C_a
86.0	C-2		
84.4	C-3	β-Gal*f*	D
83.0	C-4		
80.4	C-2		
85.2	C-3	β-Gal*f*	D′
83.3	C-4		
68.9			F
67.5	C-6	α-Glc	A′
67.0			A
64.1	C-6	β-Gal*f*	D,D′
63.9			
61.9	C-6	α-Gal, β-Glc, Gal–OH	C, G, C_a
17.6	C-6	α-Rha	B, B'_a

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 31.07 p.p.m. [b]All sugar residues are pyranosidic unless otherwise noted.

linked to the galactofuranose unit, which is no longer a branch point after enzymatic cleavage, and (*b*) the effect of the reducing terminus (C_a) on A.

^{13}C-N.m.r. spectrum of K41-P2. — In order to obtain better spectral dispersion of certain signals, especially those of rhamnose (units B and B′) and glucuronic acid (E), the ^{13}C-spectrum of K41-P2 was run at 62.8 MHz. The data are presented in Table II and Fig. 2.

The galactofuranosyl units (D and D′) exhibit two signals at 108.8 and 107.1 p.p.m. for the C-1 resonances of D′, linked only at C-3, and D, linked through C-2 and C-3. Similarly, for C-2, -3 and -4 of units D and D′, separate sets of signals are seen with chemical shifts equal to those observed in K41 (for D) and in K41-P1 (for D′).

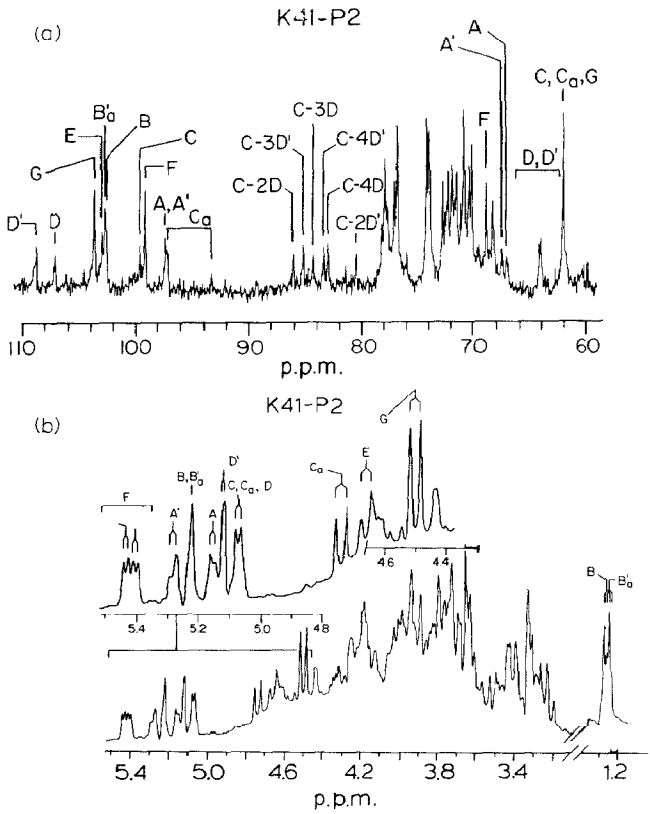


Fig. 2. N.m.r. spectra of oligosaccharide K41-P2: (a) ^{13}C as in Fig. 1 except at 62.8 MHz and 85°; (b) ^{1}H at 250 MHz, 85°, *vs.* internal acetone (δ 2.17).

TABLE III

RELAXATION TIMES T_1 FOR C-1 CARBONS OF K41-P2 OLIGOSACCHARIDE

Residue (position in structure)	*δ[a] (p.p.m.)*	*T_1^b (sec)*	*Error limit (%)*
D′	108.8	0.34	6
D	107.1	0.23	3
G	103.6	0.78	5
E	102.9	0.32	1
B'_a	102.7	0.38	3
B	102.6	0.29	7
C	99.6	0.28	4
F	99.1	0.35	1
A	97.4	0.30	0.5
C_a	97.2	0.26	3

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 31.07 p.p.m. [b]Mean of values obtained by three methods of calculation (linear regression, semilogarithmic, and exponential).

The interior galactopyranosyl unit (C) gives a signal at 99.6 p.p.m. and the terminal reducing residue (C_a) at 97.2 for the β anomer and 93.3 p.p.m. for the α anomer, this last signal being unobservable in K41-P1.

In the spectrum of K41-P2 the signals for glucuronic acid (E) and rhamnose (B and B′) are clearly distinguished; the latter pair may be assigned by considering their T_1 values, see below. The other signals presented in Table II are as expected and confirm that K41-P2 is indeed the dimer of K41-P1.

The T_1 values as measured for the C-1 carbons of K41-P2 are given in Table III and average ~0.30 sec with the following exceptions. The branch point (D) has, as expected[10], a short relaxation time (0.23 sec) while the other galactofuranosyl unit (D′), linked only through C-3, has a value of 0.34 sec. The distinct difference in the T_1 values for the two galactofuranosyl residues confirms the assignment of the C-1 signals in Table II to these two units. The nonreducing termini (G) exhibit a time of 0.78 sec, a high value accentuated by the fact that these residues are involved in 1→6 linkages. These T_1 values are consistent with the assignments in Table II and the structure of K41-P2.

^{1}H-N.m.r. spectrum. — The ^{1}H-n.m.r. data for K41-P2 are given in Table IV and Fig. 2, but the assignments are based on the spectrum of the native polymer and should be considered tentative due to the complexity of the spectrum. This is caused in part by the large number of sugar residues in the oligosaccharide, and in

TABLE IV

CHEMICAL SHIFTS OF ANOMERIC AND METHYL PROTONS IN OLIGOSACCHARIDE K41-P2

$\delta^a_{H\text{-}1}$ (p.p.m.)	$J_{1,2}$ (Hz)	*Assignment*	
		Residue[b]	*Position in structure*
5.43	3.5[c]	α-Glc	F
5.41	3.5		
5.28	4.5	α-Glc	A′
5.22	s	α-Rha	B, B'_a
5.15	3.5	α-Glc	A
5.12	1.5	β-Gal*f*	D, D′
5.07	4.0	α-Gal, β-Gal*f*, α-Gal–OH	C, D C_a
4.74	8.0	β-Gal–OH	C_a
4.66	8.0	β-GlcA	E
4.50	8.0	β-Glc	G
δ_{CH_3}	$J_{5,6}$		
1.26	6.0	α-Rha	B
1.25	6.0		B'_a

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 2.17 p.p.m. [b]All sugar residues are pyranosidic unless otherwise noted. [c]All signals are doublets except the one indicated by *s* (singlet).

part by the fact that the protons H-2 and H-3 of the galactofuranosyl residues resonate in the region associated with H-1 protons of β-D-hexopyranosyl units.

^{13}C-N.m.r. spectrum of K12. — The spectral data for a sample of K12 polysaccharide, after mild autohydrolysis, are presented in Table V and Fig. 3. The spectrum is identical to that originally reported[7] except for improved resolution in two areas. The signals for glucuronic acid (E, 102.9 p.p.m.) and rhamnose (B, 102.6 p.p.m.) are clearly resolved, and that for C-3 of the galactofuranose unit (D, 84.2 p.p.m.) is separated from a neighboring signal at 84.6 p.p.m.

The spectrum of K12 is very similar to that of K41 polysaccharide except that, as expected, the signals of the two glucose residues (F and G) in the side chain K41 are replaced in K12 by the signal of a galactose unit (F) at 108.8 p.p.m. This resonance was originally assigned[7] to the galactofuranose unit (D), but the latter

TABLE V

^{13}C-N.M.R. DATA FOR K12 POLY- AND K12-P1 OLIGO-SACCHARIDE

δ[a] (p.p.m.)		*Assignment*			
K12[b]	*K12-P1*	*C-Atom*	*Residue[c]*	*Position in structure*	
				K12	*K12-P1*
108.8	108.9	C-1	β-Gal*f*	F	F
107.1	108.6	C-1	β-Gal*f*	D	D′
102.9		C-1	β-GlcA	E	
	102.8	C-1	β-GlcA, α-Rha		E, B'_a
102.6		C-1	α-Rha	B	
99.6		C-1	α-Gal	C	
97.7	97.3	C-1	α-Glc	A	A′
	97.0	C-1	β-Gal–OH		C_a
	93.3	C-1	α-Gal–OH		C_a
85.9	80.5	C-2			
84.2	85.3	C-3	β-Gal*f*	D	D′
83.1	82.8	C-4			
82.1	82.0	C-2			
79.4	79.0	C-3	β-Gal*f*	F	F
84.6	84.8	C-4			
66.6	66.5	C-6	α-Glc	A	A′
64.1	63.8	C-6	β-Gal*f*	D	D′
	62.0	C-6	Gal–OH		C_a
	61.9	C-6	Gal–OH		C_a
61.9		C-6	α-Gal	C	
22.1	22.8	CH_3	pyruvate	G	G
17.6	17.7	CH_3	α-Rha	B	B'_a

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 31.07 p.p.m. [b]The native polysaccharide was mildly hydrolyzed in order to reduce the viscosity and give a sharper spectrum. [c]All sugar residues are pyranosidic unless otherwise noted.

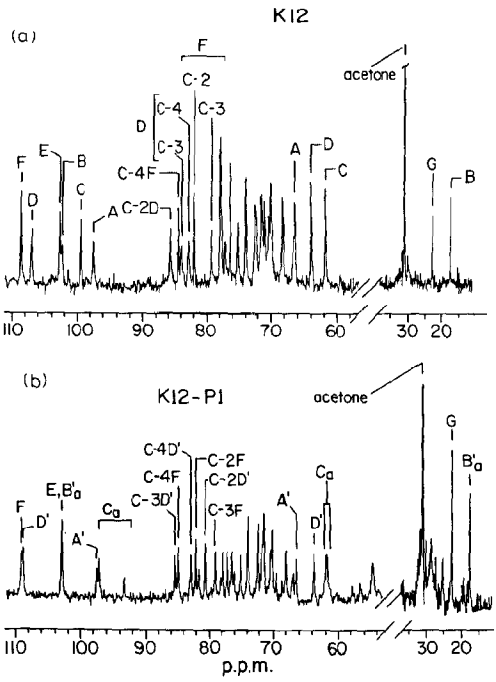


Fig. 3. ^{13}C-N.m.r. spectra of (a) K12 polysaccharide, mildly hydrolyzed, (b) K12-P1 oligosaccharide. Experimental conditions as for Fig. 1.

signal is known from the analysis of K41-P1 to have a value of 107.1 p.p.m., as for K41. This large chemical shift of 108.8 p.p.m. must, therefore, be associated with a second furanosyl residue. Furthermore, the spectrum exhibits signals at 84.6, 82.1, and 79.4 p.p.m., which values correspond closely to those published[11] for C-4, C-2, and C-3 in the model substance methyl β-D-galactofuranoside.

It is thus apparent that in the K12 polysaccharide both the lateral galactose unit and that forming the branch point are in the furanose form. This was confirmed by methylation of a deacetalated sample (see below).

^{13}C-N.m.r. spectrum of K12-P1. — In the spectrum of the oligosaccharide K12-P1 (Table V and Fig. 3) the signal for C-1 of the branch point galactofuranose (D) is shifted to low field (108.6, +1.5 p.p.m.) for the same reason as in K41-P1. That is, this unit is no longer substituted at C-2, which in turn demonstrates that the action of φ41 on K12 is the same as that on K41, and that the viral enzyme exhibits an α-D-galactopyranosidase activity. The existence of two signals at low field is further confirmation of the presence of two galactofuranosyl units in polysaccharide K12.

The signals for C-2, C-3, and C-4 of both galactofuranosyl units are clearly evident in the spectrum. The trio of peaks associated with the terminal, acetalated

galactose (F) have essentially constant chemical shifts in K12 and K12-P1. The sets of signals related to the branch point galactofuranose residues, however, undergo some marked changes. Most noticeably, the signal for C-2 of residue D′ in K12-P1 exhibits a strong upfield shift (−5.4 p.p.m.) compared to that of D in K12. Similarly, the C-3 signals exhibit a weak downfield shift (+1.1 p.p.m.) while those for C-4 in the two situations are essentially unchanged, as was observed for K41-P1. A signal at 81.8 p.p.m. was unassigned.

The signal for the galactopyranosyl unit (C) at 99.6 p.p.m. disappears to be replaced by two signals characteristic of a reducing sugar C_a; the β-anomer at 97.0

TABLE VI

^{13}C-N.M.R. DATA FOR OLIGOSACCHARIDE K12-P2

δ^a (p.p.m.)	Assignment		
	C-Atom	*Residue*[b]	*Position in structure*
108.5	C-1	β-Gal*f*	F, D′
107.1	C-1	β-Gal*f*	D
102.7	C-1	β-GlcA, α-Rha	E, B, B'_a
99.7	C-1	α-Gal	C
97.0	C-1	α-Glc, β-Gal–OH	A, A′, C_a
93.2	C-1	α-Gal–OH	C_a
86.0	C-2		
84.2	C-3	β-Gal*f*	D
83.0	C-4		
80.5	C-2		
85.1	C-3	β-Gal*f*	D′
82.6	C-4		
81.9	C-2		
79.0	C-3	β-Gal*f*	F
84.7	C-4		
72.3	C-5	β-Gal*f*	D,D′
72.1	C-5	β-Gal*f*	
67.1	C-6	β-Gal*f*	F
66.7	C-6	β-Gal*f*	
66.4	C-6	α-Glc	A, A′
63.9	C-6	β-Gal*f*	D, D′
63.8			
61.8	C-6	α-Gal, Gal–OH	C, C_a
22.7	CH_3	pyruvate	G
17.6	CH_3	α-Rha	B, B'_a

[a]Chemical shift relative to tetramethylsilane, measured from internal acetone at 31.07 p.p.m. [b]All sugar residues are pyranosidic unless otherwise noted.

and the α at 93.3 p.p.m. As a result of the mutarotational equilibrium, the signal for C-6 shows a slight degree of twinning (62.0 and 61.9 p.p.m.).

^{13}C-N.m.r. spectrum of K12-P2. — The spectrum of K12-P2 was complicated by many overlapping peaks, some of which could be assigned by reference to the spectra of K12 polysaccharide and K12-P1. Of particular interest (Table VI) was the group of signals between 79 and 86 p.p.m. Here it was possible to distinguish three sets of signals corresponding to C-2, -3, and -4 of the galactofuranose units representing (*a*) the branch point (D), (*b*) the residue adjacent to the bond cleaved (D′), and (*c*) the terminal units (F). It was also possible to discern two separate signals for C-6 of the in-chain galactofuranose residues (D and D′) due to their different environments in the oligosaccharide.

DEPT 135 experiments performed on methyl β-D-galactofuranoside and on K12-P2 confirmed many of the assignments in Table VI and identified those signals due to C-5 of units D and D′. They also demonstrated that the two residues F are nonequivalent and indicated the presence of eight C-6 signals in K12-P2.

Methylation analyses. — In the original study on K12 polysaccharide[7] the acetalated galactose unit gave rise to the 2,3-di-*O*-methyl derivative, which does not permit a distinction between a pyranosidic or furanosidic acetal. In order to confirm the deductions made from the n.m.r. data, it was necessary to obtain a modified K12 polysaccharide wherein the acetal substituent was removed selectively. The initial attempt at autohydrolysis removed not only the acetal substituent but also the galactose unit to which it was attached (product K12 − GF)[6]. A second attempt, using milder conditions, gave a modified polysaccharide, K12 − G, whose ^{13}C-n.m.r. spectrum showed the complete disappearance of the pyruvate acetal

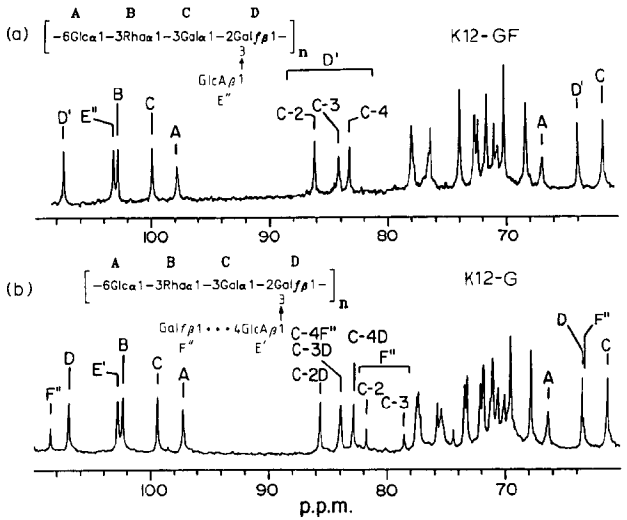


Fig. 4. ^{13}C-N.m.r. spectra of (a) polysaccharide K12 − GF, (b) polysaccharide K12 − G. Measured at 62.8 MHz, 80°, *vs.* internal acetone (31.07 p.p.m.).

TABLE VII

METHYLATION ANALYSIS OF POLYSACCHARIDES K12, K12 − GF, AND K12 − G

Methylated sugar (as alditol acetate)	T_R^a	*K12*[b]		*K12 − GF*[c]		*K12 − G*[c]	
		Mol%[d]	*Molar portion*	*Mol%*[d]	*Molar portion*	*Mol%*[d]	*Molar portion*
2,4-Rha	1.00	20	1.0	40	1.0	31	1.0
2,3,5,6-Gal[e]	1.12	0	0	0	0	8	0.3[f]
2,4,6-Gal	1.74	22	1.1	28	0.7	28	0.9
2,3,4-Glc	1.89	17	0.8	23	0.6	22	0.7
5,6-Gal	2.08	16	0.8	9	0.2[g]	10	0.3[g]
2,3-Glc		13	0.7	0	0	0	0
2,3-Gal	2.39	12	0.6	0	0	1	0.05

[a]Retention times on column A relative to 2,3,4,6-Glc; under the same conditions 2,3,4,6-Gal has a value of 1.17. [b]With reduction of acidic functions after methylation. [c]Neutral sugars only. [d]Corrected for effective carbon response (ref. 17). [e]Reference sample obtained by methylation and hydrolysis of methyl β-D-galactofuranoside (ref. 18). [f]See text. [g]Low value due to resistance of glucosyluronic bond to hydrolysis.

groups, but the loss of only a small part of the terminal galactose residues. Interestingly, the proton spectrum showed approximately 10% of the pyruvate remaining, and the methylation data (Table VII) indicated 5% intact units. It must, therefore, be appreciated that K12 − G is a mixture of (at least) three closely related structural entities, although the evidence indicates that it is primarily the K12 polysaccharide devoid of the acetal substituent.

Comparative methylation analyses were carried out on the original polysaccharide[7], with reduction of carboxyl groups after methylation, and on K12 − GF and K12 − G, neutral sugars only, and the results are presented in Table VII. It is clear that 2,3-di-*O*-methylgalactose present in K12 has been replaced almost entirely by 2,3,5,6-tetra-*O*-methylgalactose in K12 − G and eliminated completely in K12 − GF. It is likely that the low molar ratio is due to the β-elimination occurring during methylation, the effect of which is to suppress ~40% of the terminal residue, as can be seen for K12. The analysis is complicated by the fact that K12 − G is a mixture of native, deacetalated (K12 − G), and partially degraded (K12 − GF) polysaccharides.

It may be noted that while the retention times of 2,3,4,6- and 2,3,5,6-tetra-*O*-methylgalactose are close (1.17 and 1.12, respectively), they are well separated on the column used (SP 2340). The two *O*-methyl derivatives are most readily distinguished by the presence in the mass spectrum of the latter of an ion having *m/z* 89 and a weak ion at *m/z* 277. A weak signal corresponding to *m/z* 45 characteristic of methoxyl substitution on C-5 and C-6 of a hexose[12] is also observable.

CONCLUSION

The n.m.r. spectra, especially the ^{13}C spectra, and the methylation analyses

demonstrate clearly that *Klebsiella* K12 polysaccharide contains two galactofuranose residues per repeating unit, and that the terminal moiety is 5,6-*O*-(1-carboxyethylidene)-D-galactofuranose. This represents the first record of occurrence of such a structural unit, not only in the *Klebsiella* antigens, but in polysaccharides in general.

Garegg and colleagues[13] showed that the *R* and *S* forms of 4,6-*O*-(1-carboxyethylidene)-D-galactopyranose exhibit different ^{13}C chemical shifts for the methyl group of the acetal substituent, and it was noted[14] at the time that the reported[7] value for K12 did not fit with either; the reason is now clear.

EXPERIMENTAL

Gas chromatography was carried out using a Packard and Becker 417 instrument coupled to a Hewlett-Packard 3380A integrator or a Hewlett-Packard 5710A chromatograph equipped with a Hewlett-Packard 3390A integrator. Glass columns (3 mm × 2 m) packed with (*a*) 3% SP 2340 on Chromosorb W-AW DMCS (100–120 mesh), or (*b*) 3% OV 17 on the same support, were used. G.l.c.-m.s. was performed as described previously[4].

N.m.r. — All oligo- and poly-saccharides were used as their Na^+ salts. A Bruker WP 100 instrument was used at 100 MHz for ^1H and at 25.2 MHz for ^{13}C spectra. The corresponding 250-MHz and 62.8-MHz spectra were obtained on a Cameca 250 machine; both spectrometers operated in the Fourier transform mode. For proton spectra, sample concentrations were ~50 mg/mL in D_2O (0.3 mL) containing 1 drop of acetone (5% in D_2O) as internal reference (δ 2.17 with respect to Me_4Si). Samples were exchanged thrice with D_2O, the HOD peak was eliminated by saturation, and spectra were obtained at 80°. For ^{13}C spectra, sample concentrations of ~60 mg/mL in 0.5 mL D_2O and 0.5 mL H_2O were used, with the addition of acetone as internal standard (δ 31.07 with respect to Me_4Si). For K41-P1 a microcell (0.5 mL) was used. Spectra were obtained at ambient temperature (~30°), with ~75,000 acquisitions for a polymer sample (1 night) and ~25,000 (0.5 d) for an oligosaccharide sample when the concentration permitted (K12 − P2, 120 mg/mL).

Autohydrolysis of polysaccharide K12. — Polysaccharide K12 (150 mg) was dissolved in water (45 mL) and deionized with Amberlite IR 120 H^+ to give a solution at pH ~4, and this was heated for 38 h on a steam bath. N.m.r. examination showed the product to be K12 − GF. A second sample heated for 7 h gave K12 − G.

Methylation analysis of K12 − GF and K12 − G. — Modified polysaccharide (30 mg) was dissolved in 4 mL of Me_2SO and methylated according to Hakomori[15]. The product, recovered by dialysis and lyophilization, was permethylated with methyl iodide and silver oxide[16]. The methylated polysaccharide, without reduction of the uronic acid groups, was hydrolyzed with M TFA for 4 h at 100°. The monosaccharides were converted into alditol acetates by successive treatments with $NaBH_4$ and pyridine–acetic anhydride.

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