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CHEMICAL AND N M R -SPECTROSCOPIC INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K41

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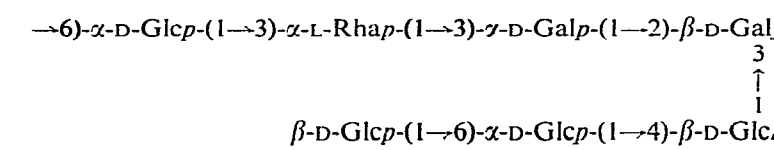
ABSTRACT

The structure of the repeating unit of the capsular polysaccharide from *Klebsiella* type 41 has been investigated by methylation analysis of the original and the carboxyl-reduced polymer, uronic acid degradation, Smith degradation, and graded acid hydrolysis Proton- and ^{13}C-n m r spectroscopy of the original polysaccharide and of the fragments obtained by various methods confirmed some structural features and allowed determination of the anomeric configuration of the glycosidic linkages This polysaccharide is shown to have the following heptasaccharide repeating-unit



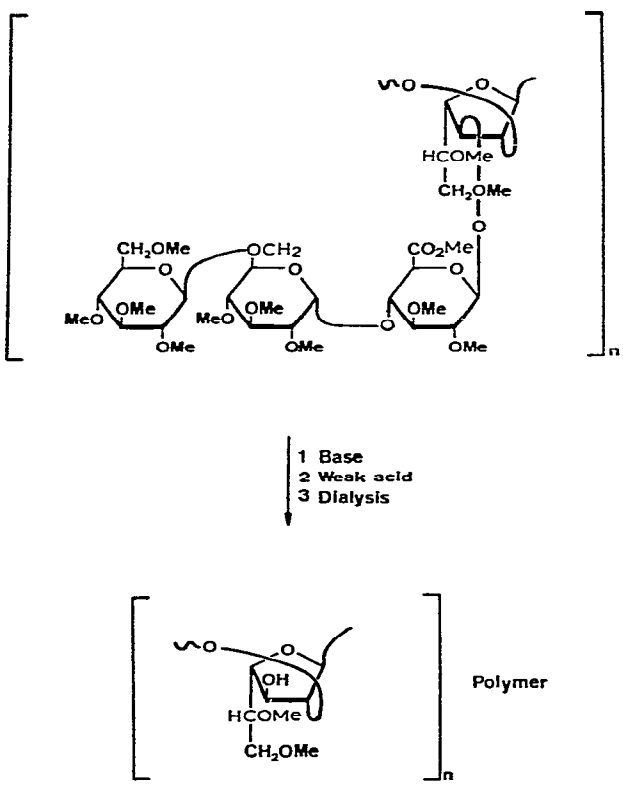
This is the first polysaccharide antigen K of the *Klebsiella* series found to have seven sugar residues in its repeating unit, and to contain a galactose residue in its furanose form

INTRODUCTION

Capsular polysaccharides from *Klebsiella* have been classified into several chemotypes according to their sugar composition[1,2] The capsular polysaccharide from *Klebsiella* type 41 is one of the eleven strains that contain rhamnose, galactose, glucose, and glucuronic acid residues In addition, some of these contain pyruvic acid acetal[3] and acetyl substituents

RESULTS AND DISCUSSION

Chemical investigations — The K41 polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide, and it had $[\alpha]_D$ +23° An equivalent weight of about 1050 was estimated by titration with sodium hydroxide Acid hydrolysis of the polysaccharide showed the presence, in the neutral fraction, of rhamnose, galactose, and glucose in 1 1 3 molar proportions, and of an aldobiouronic acid which, after carboxyl reduction followed by hydrolysis, gave glucose and galactose in 48 52 proportion Acid hydrolysis of the carboxyl-reduced polysaccharide[4] gave rhamnose, galactose, and glucose in 14 28 57 ratio These ratios indicate that the repeating unit of K41 comprises seven monosaccharide residues, namely, rhamnose, galactose, glucose, and glucuronic acid in the proportions 1 2 3 1, respectively, as the glucose residue obtained after carboxyl reduction of the aldobiouronic acid was shown to arise from glucuronic acid (see later, methylation analysis) The existence of a heptasaccharide repeating-unit was also demonstrated by examination of the anomeric region of the ^{1}H-n m r and ^{13}C-n m r spectra (see n m r section) The optical rotations of the sugars isolated from the hydrolyzate,



Scheme 1

established that galactose, glucose, and glucuronic acid had the D configuration, and rhamnose, the L.

Methylation[5] and subsequent hydrolysis, and glc–ms analysis[6] of the original polysaccharide, gave the partially methylated neutral sugars shown in Table I (column A), together with acidic compounds that, upon carboxyl reduction followed by hydrolysis, yielded two methylated sugars (Table I, column B) Reduction of the uronic acid[4] in the methylated K41 polysaccharide, prior to hydrolysis, gave the compounds shown in column C of Table I These results confirm that the repeating unit is a heptasaccharide having one L-rhamnosyl residue linked through O-3, one D-galactosyl residue linked through O-3, one D-galactosyl residue linked through O-2 and O-3, two D-glucosyl residues linked through O-6, one terminal D-glucosyl residue, and a glucuronic acid residue that was shown by methylation of the aldobiouronic acid (see later) to be pyranosidic and thus linked through O-4 The results also indicate that, except for one D-galactofuranosyl residue, all of the other sugars are pyranosidic The presence of a 2,3,4,6-tetra-*O*-methyl-D-glucose derivative demonstrates that the repeating unit carries a side chain having a terminal D-glucopyranosyl residue 5,6-Di-*O*-methyl-D-galactofuranose, obtained from the methylation analysis, is unusual and was characterized by comparison with the synthetic compound[7]. Its presence in the aldobiouronic acid shows that it is linked to the glucuronic acid residue and that it is also the branch point of the side chain

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *Klebsiella* TYPE K41 POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	T^b		*Mole %*[c,d]			
	Column A (ECNSS-M)	*Column B (OV-225)*	*A*	*B*	*C*	*D*
2,3,4,6-Glc	1 00	1 00	41 0[e]		35 3[e]	
2,4-Rha	1 00	0 94				25 6
2,4 6-Gal	2 42	2 14	17 3		14 1	21 6
2,3,4-Glc	2 54	2 34	37 0		32 3	33 4
5,6-Gal	3 22	2 78	4 4	44 6	11 3	19 4
2 3-Glc	6 07	4 68		55 4	7 0	

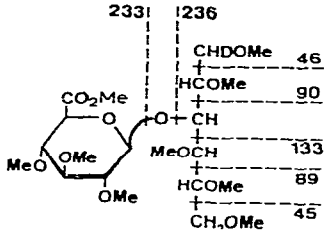
[a]2,3 4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and so on [b]Retention time relative to the alditol acetate derivative of 2,3 4,6-tetra-*O*-methyl-D-glucose [c]A, original polysaccharide, neutral sugars, B, original polysaccharide, acidic sugars (see text) C, methylated polysaccharide subsequently carboxyl-reduced and D, uronic acid-degraded polysaccharide [d]Values obtained on column A [e]Quantitative determination on column B equimolar ratio for 2 3 4 6-Glc and 2 4-Rha

Uronic acid degradation[8] performed on the fully methylated K41 polysaccharide yielded a polymer that was recovered by gel chromatography on Sephadex LH-20 The isolation of a polymeric, degraded product indicates that the uronic acid residue is contained in the side chain Hydrolysis of the degraded material, followed

by derivatization and g l c –m s analysis, gave the compounds shown in Table I, column D The results indicated that β-elimination caused the loss of one 2,3,4-tri-*O*-methyl-D-glucose residue and one tetra-*O*-methyl-D-glucose residue, and the D-glucuronic acid residue was decomposed As the uronic acid residue is linked directly to the galactofuranose residue (see later), the side chain is thus constituted of the trisaccharide component **1**

D-Glc*p*-(1→6)-D-Glc*p*-(1→4)-D-GlcA*p*-(1→ (1)

Partial hydrolysis of the K41 polysaccharide (0 5M trifluoroacetic acid, 30 min, 100°) and separation of acidic and neutral components by ion exchange yielded a mixture of acidic and neutral oligomers Paper chromatography of the acidic fraction afforded, among other products, the aldobiouronic acid **2**, which was reduced with sodium borodeuteride and then methylated and subjected to g l c –m s analysis The fragmentation in mass spectrometry was as follows



The presence of peaks at *m/e* 89, 90, and 133 demonstrates that the galactitol residue is linked through O-3 Additional evidence for the structure of **2** was obtained by examination of the products of hydrolysis of the carboxyl-reduced methylated aldobiouronic acid which showed (after derivatization and g l c –m s analysis) compounds corresponding to 1,2,4,5,6-penta-*O*-methyl-D-galactitol deuterated on position 1, and to 2,3 4-tri-*O*-methyl-D-glucitol The structure of the aldobiouronic acid moiety is thus

GlcA*p*-(1—3)-Gal*f* (2)

Separation of the mixture of neutral oligomers by gel chromatography (Bio-Gel P-2), followed by purification on paper chromatography, gave two pure oligomers, **3** and **4** Reduction of compound **3**, followed by hydrolysis and acetylation, gave acetylated glucose and rhamnitol in equimolar proportion According to the methylation-analysis data, this compound must thus be

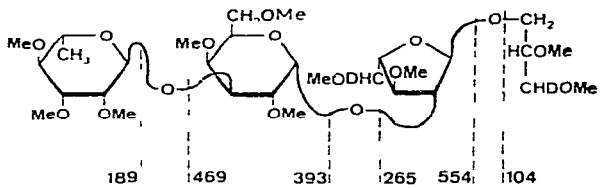
Glc*p*-(1—3)-Rha*p* (3)

On hydrolysis, compound **4** showed glucose as the only constituent sugar, and according to the results of uronic acid degradation, its structure must be

Glc*p*-(1—6)-Glc*p* (4)

Additional evidence for the structures of oligomers **2**, **3**, and **4** was obtained from their n m r spectra, which allowed determination of their anomeric linkages, as demonstrated later (see n m r study)

Knowing the structure of the side chain and the nature of the branch point, and in order to determine the sequence of sugar residues in the main chain of the repeating unit, the polysaccharide K41 was subjected to Smith degradation[9] The periodate-oxidized product (8 mol uptake) was reduced with sodium borodeuteride and hydrolyzed under mild conditions Paper chromatography demonstrated the formation of the oligomer **5**, hydrolysis of which showed glycerol, rhamnose arabinose and galactose, respectively, in equimolar proportion Compound **5** was permethylated and examined by g l c–m s As the Smith-oligosaccharide contained three different sugar derivatives (one deoxyhexose, one hexose, and one pentose), the fragmentation pattern demonstrated unambiguously the structures of the constituent sugar residues and their sequence In addition the presence of the deuterated glycerol moiety in compound **5** permitted confirmation of the complete structure of the main chain of the repeating unit



The n m r study of compound **5** (see later) established the anomeric configuration of the linkages between the sugar residues The structure of **5** is thus established as

γ-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→2)-α-L-Ara*f*-(1→1)-glycerol (5)

The results of the methylation analysis, the uronic acid degradation, together with the isolation of three oligomers, and the Smith degradation study, are compatible with the final structure **6**, wherein all of the anomeric configurations were established by n m r spectroscopy as demonstrated in the second part of this report

$$\begin{array}{l} \rightarrow 6)\text{-}\gamma\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\gamma\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\gamma\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-GlcA}p \end{array} \quad (6)$$

N m r -spectral investigations — For clarity of the following discussion, the different sugar residues constitutive of the repeating unit of K-41 are designated as in Fig 1 (for instance, H-1A will refer to the anomeric proton of sugar A, C-1A to the anomeric carbon atom of sugar A, and so on The ^{1}H- and ^{13}C-n m r spectra obtained for the original K-41 polysaccharide are depicted in Fig 2, and chemical shifts are given in Tables II and III

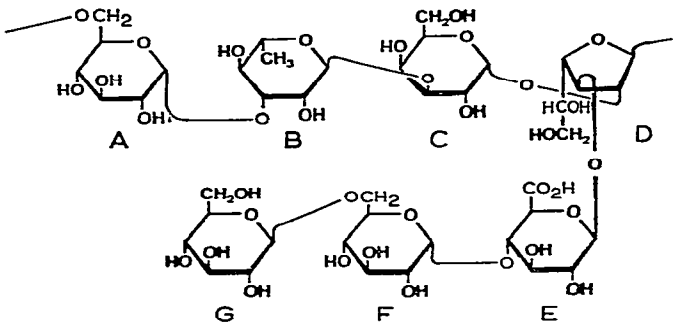


Fig 1 The repeating unit of *Klebsiella* K41 capsular polysaccharide

TABLE II

^{1}H-N M R DATA FOR *Klebsiella* K41 CAPSULAR POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound	*δ Values*[a] *(coupling constants in Hz)*[b]	*Ratio of integrals*	*Proton assignment*	*Anomeric configuration*
Original polysaccharide	5 48 (4)	1	H-1F	α
6)-α-A-(1→3)-α-B-(1→3)-α-C-(1→2)-β-D-(1→	5 22	1	H-1B	α
3	5 17 (3 5–4)	1	H-1A	α
↑	5 12	2	H-1C	α
1	5 12		H-1D	β
β-G-(1→6)-γ-F-(1→4)-E	4 63 (8)	1	H-1E	β
	4 52 (8)	1	H-1G	β
	4 3–4 5	2	H-2D, H-3D	
	1 34 (6)	3	3H-6B	
Disaccharide 4	5 24	0 35	H-1F	α
β-G-(1→6)-F	4 67 (8)	0 65	H-1F	β
	4 51 (8)	0 35	H-1G	β[c]
	4 53 (8)	0 65	H-1G	β[c]
Disaccharide 3	5 18	0 6	H-1B	α
α-A-(1→3)-B	5 12 (4)	0 4	H-1A	α[c]
	5 10 (4)	0 6	H-1A	α[c]
	4 88	0 4	H-1B	β
	1 32 (6)		H-6B	γ
	1 34 (6)		H-6B	β
Aldobiouronic acid 2	5 17 (3 5)	0 5	H-1D[d]	α
β-E-(1→3)-D′	4 60 (7 5)	1	H-1E	β
	4 53 (8)	0 5	H-1D′[d]	β
Oligosaccharide from Smith degradation 5	5 24	1	H-1B	α
	5 16	1	H-1D″[e]	α
α-B-(1→3)-α-C-(1→2)-α-D″-(1→1)-glycerol[e]	5 10	1	H-1C	α

[a]In p p m with TSP (sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate) as the internal standard in D_2O [b]Coupling constants, when available, are expressed in Hz [c]This glycosidic proton resonates as 2 doublets because of the anomeric equilibrium of the reducing unit [d]In this aldobiouronic acid, the sugar D (galactofuranosyl) of the original polysaccharide has become D′ (galactopyranose) [e]Sugar D (β-D-galactofuranosyl) of the original polysaccharide has become D″ (α-L-arabinosyl) after Smith degradation

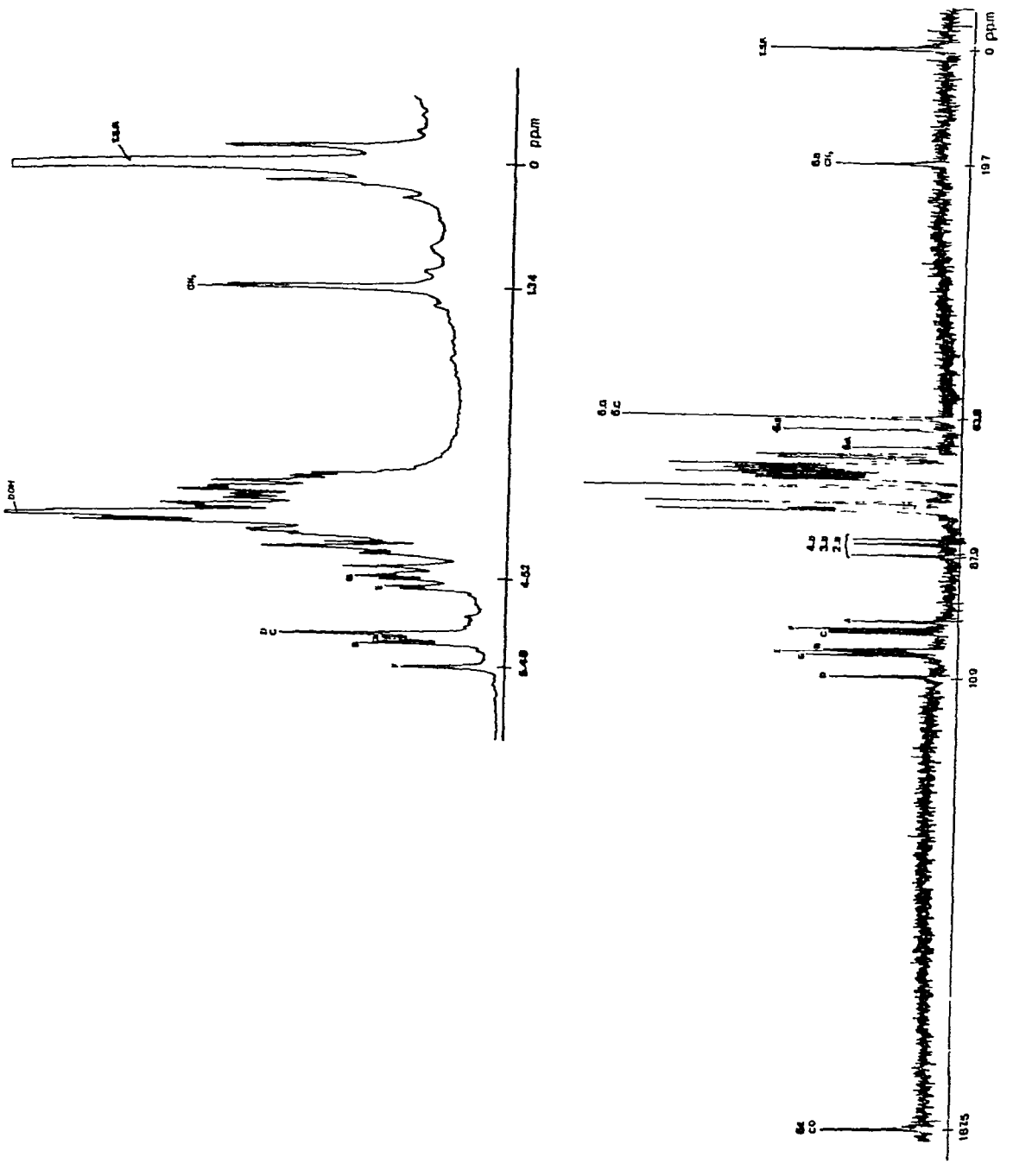


Fig 2 N m r spectra of *Klebsiella* K41 capsular polysaccharide Above, ^{1}H n m r spectrum Below, ^{13}C-n m r spectrum (See text and experimental section for conditions)

TABLE III

^{13}C-N M R DATA FOR *Klebsiella* K-41 CAPSULAR POLYSACCHARIDE

Chemical shift[a] (*p p m*)	*Carbon assignments*	*Coupling constants (Hz)*
187 75	C-6E	
109 00	C-1D	$^1J_{C\text{-}1D\ H\text{-}1D}$ 172 5
105 50	C-1G	$^1J_{C\text{-}1G\ H\text{-}1G}$ 160 15
104 80	C-1E	$^1J_{C\text{-}1E\ H\text{-}1E}$ 160 0
104 45	C-1B	$^1J_{C\text{-}1B\ H\text{-}1B}$ 171 0
101 55	C-1C	$^1J_{C\text{-}1C\ H\text{-}1C}$ 169 5
101 05	C-1F	$^1J_{C\text{-}1F\ H\text{-}1F}$ 171 0
99 50	C-1A	$^1J_{C\text{-}1A\ H\text{-}1A}$ 170 0
87 90, 86 10, 85 15	C-2D, C-3D, C-4D	$^1J_{C\text{-}2D\ H\text{-}2D}$, $^1J_{C\text{-}4D\ H\text{-}4D}$, $^1J_{C\text{-}3D\ H\text{-}3D}$ 145–148
79 90, 79 55	C-3B, C-3C, C-4E	
78 85, 78 70	C-3G C-5G	
76 00, 75 90, 75 75	C-2G	
74 60, 74 35, 74 15, 73 80, 73 45 72 95, 72 70, 72 40 72 20, 71 95	C-6F	
70 85, 70 20	C-6A	
68 75	C-6D	
65 95	C-6C C-6G	
63 75	C-6B	
19 70		

[a] See Table II footnote [a]

The 250-MHz, proton-n m r spectrum of the sodium salt of the original K41 polysaccharide[10 11] at 90° in D_2O showed characteristic resonances of the anomeric protons in the region δ 4 3–5 5, together with a doublet at δ 1 34 ($^3J_{5\ 6}$ 6 Hz) integrating for 3 protons and corresponding to the CH_3 group of rhamnose In the anomeric region, four signals (integrating for five protons) resonated beyond 5 p p m and could thus be expected to correspond to α-linked sugar residues, whereas the two signals at δ 4 63 and 4 52, each integrating for one proton and showing a coupling constant of 8 Hz, could be expected to correspond to β-linked sugar residues The ratio of integrals of the latter signals was broadened by the presence between δ 4 3–4 5 of two signals, very probably attributable to H-2 and H-3 of the galactofuranosyl residue[7]

The 62 84-MHz, ^{13}C-n m r spectrum of the sodium salt of the original K-41 polysaccharide at 70° in D_2O showed, among other features, characteristic resonances of the carbon atom of a carboxyl group at δ 187 75 p p m , corresponding to C-6 of the glucuronic acid residue, together with C-6 of the rhamnose residue resonating at δ 19 65 The resonances in the anomeric region clearly showed seven signals (Fig 2, Table III) between 99 and 110 p p m Because of the variations in the in-

tensities of the signals in the ^{13}C-n m r spectra recorded with complete proton-decoupling, an anti-gated experiment[12 13] was necessary to achieve better quantitative ratios in the resonance-intensities of all carbon atoms This afforded the most unambiguous demonstration of the heptasaccharidic nature of the repeating unit of K-41 The $^{1}J_{C\text{-}1,H\text{-}1}$ couplings were measured by the gated-decoupling technique and showed, in the anomeric region, five coupling values of ~170 Hz and two of ~160 Hz This observation, again, could be expected to correspond to five α linkages and two β linkages[14], respectively, in the repeating unit This distinction between α and β linkages from the coupling values is restricted to pyranoses, as there is a galacto-furanosidic residue in the K-41 polysaccharide, this mode of identification is not reliable In fact, coupling values for furanosides of L-arabinose[15] and D-galactose range between ~172 and ~175 Hz, according to the anomeric configurations The n m r data for the anomeric carbon atom of the methyl α- and β-D-galactofuranosides ($^{1}J_{C\text{-}1\ H\text{-}1}$ ~175 Hz and 172 5 Hz, respectively[7]) allowed assignment of the signal at δ 109 00, $^{1}J_{C\text{-}1\ H\text{-}1}$ 172 5 Hz, to the galactofuranose residue and then demonstration

TABLE IV

^{13}C-N M R DATA FOR OLIGOSACCHARIDES OBTAINED BY DEGRADATION FROM *Klebsiella* K-41 CAPSULAR POLYSACCHARIDE

Oligosaccharide **4** *β-G-(1—6)-F*		*Oligosaccharide* **3** *z-A-(1→3)-B*		*Oligosaccharide* **5** *from Smith degradation* α-B-(1→3)-α-C-(1→2)-α-D″→ glycerol[c]	
δ[a]	*C assignment*	δ[a]	*C assignment*[b]	δ[a]	*C assignment*
105 50	C-1G	98 25	C-1A_z	109 15	C-1D″
98 80	C-1F_f	97 95	C-1A_β	105 10	C-1B
95 00	C-1F_z	96 30	C-1B_z	101 55	C-1C
78 75 }	C-3G	96 10	C-1B_β	90 05	C-2D″
78 50 }	C-5G	80 20	C-3B_β	85 95	C-4D
78 50	C-3F_β	78 10	C-3B_z	80 00 }	C-3D″
77 75	C-5F_β	75 60	C-3A	77 90 }	C-3C
76 85	C-2F_β	74 70	C-5B_β	75 15	
75 95	C-2G	74 35 }	C-2A	74 15	
75 55	C-3F_z	74 05 }	C-5A	73 55	
74 20	C-2F_z	73 10		73 25 }	
73 30	C-5F_z	72 80		72 15 }	
72 45	C-4F	72 00	C-4A	72 10	
72 35 }	C-4G	71 10		70 45	
71 65 }	C-6F_β	70 50		64 05 }	C-5D
71 55	C-6F_z	70 15		63 85 }	C-6C
63 60	C-6G	62 92	C-6A	19 65	C-6B
		19 65	C-6$B_{z,\beta}$		

[a]For oligosaccharide **4**, δ values were recorded with TSP as internal reference For oligosaccharide **3** and oligosaccharide from Smith degradation δ values were recalculated with respect to the signal of C-6 of L-rhamnose at δ = +18 0/Me_4Si[22] namely, +19 65/TSP [b]The differentiation between the α and β forms was evident as they were in 2 1 ratio, respectively, as established from the corresponding proton n m r spectrum [c] D″ corresponds to the arabinofuranosyl group, see Table II, footnote

its β configuration As a result, it may be seen that there are four α linkages and three β linkages in the repeating unit of the K-41 polysaccharide The preceding indicates the need for care in the interpretation of the ^{1}H- and ^{13}C-n m r spectra of a polysaccharide, knowledge of the identities of the different sugar residues and of their conformations is necessary

In order to establish the anomeric configuration of all linkages in the repeating unit of K-41 and to confirm some structural features already ascribed by chemical evidence, a detailed n m r investigation of the available oligomeric fragments was performed The corresponding proton- and ^{13}C-n m r data are given in Tables II and IV, respectively

The disaccharide **4**, established by methylation analysis to be a glucopyranosyl-(1→6)-glucopyranose, showed its anomeric-proton resonances at δ 4 51 and 4 53, with coupling constants of 8 Hz (see Table II note *c*) indicating a β linkage Both the ^{1}H and ^{13}C spectra were indistinguishable from those of an authentic sample of gentiobiose These data allowed the assignments of the main signals belonging to the glucose residue (G) in the spectra of the polysaccharide (Tables II and IV)

The oligosaccharide **3**, identified as glucopyranosyl-(1→3)-rhamnose by chemical analysis as already described, showed signals characteristic of an α linkage at δ 5 12 and 5 10 (J 4 Hz), and at δ 98 25 and 97 95, in the ^{1}H- and ^{13}C-spectra, respectively (Tables II and IV, note *c*) Although these chemical shifts are not in exact agreement with those obtained for the original polysaccharide, the characteristic signals corresponding to residues A and B may be assigned in the spectra of K-41

Smith degradation of the K-41 polysaccharide provided the trisaccharide-glycerol derivative **5** [rhamnopyranosyl-(1→3)-galactopyranosyl-(1→2)-arabinofuranosylglycerol [-B-(1→3)-C-(1→2)-D'-glycerol] described in the chemical investigation The anomeric region of the ^{1}H-n m r spectrum showed three signals, at δ 5 24 5 16, and 5 10, demonstrating that the rhamnosyl and the galactopyranosyl residues have the α configuration No definite conclusion could be drawn for the arabinofuranosyl residue, as the resonance frequencies of the α and β anomers are too close[15] However, the ^{13}C spectrum allowed a more accurate assignment of the anomeric carbon atoms, which resonated at δ 109 15, 105 10, and 101 55 The anomeric carbon atom resonating at lower field may then be ascribed to an α-linked L-arabinosyl group[15], thus corresponding to the β-D-galactofuranosyl residue (D) of the original polysaccharide The two other signals (δ 105 10 and 101 55) are assigned to the L-rhamnopyranosyl (B) and D-galactopyranosyl (C) residues, respectively, their δ values being in good agreement with those in the corresponding original polysaccharide (Tables III and IV) It is noteworthy that the chemical shifts of C-2 and C-4 of the furanosidic arabinose and galactose residues show a strong downfield shift as compared with the usual chemical shifts of the corresponding carbon atoms of the pyranosidic sugars This is clearly shown in the spectrum of the oligosaccharide, and it is thus possible to assign the carbon frequencies at δ 90 05 and 85 95 to C-2 and C-4, respectively, of the arabinofuranose residue Similarly, by using the ^{13}C-n m r spectroscopic data obtained for the model compound (methyl 2,3-di-*O*-benzyl-β-D-

galactofuranoside) reported previously[7], it was possible to assign, in the spectrum of the original K41 polysaccharide, the signals at δ 87 90, 86 10, and 85 15 ($^1J_{^{13}C\,H}$ 145–148 Hz) to C-2, C-4, and C-3 of the β-D-galactofuranosyl residue (D) This provides further evidence for the furanosyl ring-form and the β configuration of the linkage of this galactose residue, existing as a branch point in the polymer

Confirmation that the glucuronic acid residue (E) is β-linked was given by examination of the ^{1}H-n m r spectrum of the derived aldobiouronic acid **2** (E–D'), which showed a typical chemical shift at δ 4 60 (^3J 7 5 Hz), in good agreement with that found in the spectrum of the corresponding polysaccharide

As it was not possible to isolate a fragment containing the linkage between residues F and E, the anomeric configuration of this glycosidic bond was deduced from the foregoing results It was indeed shown that there were four α linkages and three β linkages in the polymer and as the three latter have already been assigned (D E, and G), as well as three α linkages (A B, and C) it is evident that F has the α configuration

The correspondence between the ^{1}H and ^{13}C spectra of the original polysaccharide could again be ascertained by a selective, heteronuclear, double-irradiation technique[16–20] The assignment of the 7 different anomeric protons being known, this experiment provided attribution of the seven anomeric-carbon signals In addition, irradiation of the proton signals at δ 4 3–4 5 established their correspondence with the three carbon atoms resonating between 85–88 p p m and corresponding to C-2, C-3, and C-4 of the galactofuranosyl residue

Conclusion — The availability of high-resolution n m r spectrometers equipped with Fourier transform enables clear spectra to be obtained from native polysaccharides of low solubility In the present investigation, conjunction of the various ^{1}H- and ^{13}C-n m r techniques permitted unambiguous and direct determination of the most conspicuous features of the seven sugar residues constituting the repeating unit, of the presence of the carbonyl group of the uronic acid, and of the methyl group of the 6-deoxy sugar A more-precise, quantitative estimation of the ratio of the different signals in the ^{13}C spectra was achieved by using the anti-gated technique This procedure is particularly interesting with those groups characterized by long relaxation-times (namely carbonyl carbon atoms) or for those carbon atoms differing in their proton environment (such as –CHOH versus $-CH_3$) which have different sensitivities because of spin decoupling By application of the off-resonance technique, in which the multiplicity arising from the number of protons directly attached to the carbon atom observed, permits distinction between the primary and quaternary carbons, it was possible to assign all of the individual C-6 atoms as these gave rise to triplets

From the chemical shifts and the coupling constants in both ^{1}H and ^{13}C spectra ($^1J_{C\,H}$ measured by the gated decoupling technique) and with the use of derived oligosaccharides and model compounds, it was possible to assign all of the seven anomeric signals and thus to determine the α or β linkage-configuration in the repeating unit of K41 It was also possible to assign other signals, as shown in

Table III, among which the most readily available are those of the free and the linked C-6 The n m r data for the isolated oligosaccharides and the corresponding residues when they are engaged in the native polysaccharide are not always in perfect agreement[21], as may be expected because of environmental effects and possible changes in the overall conformation The ring-carbon atoms involved in interglycosidic linkages are usually expected to resonate between 78 and 90 p p m However, during this investigation, we noted that this region should be interpreted cautiously; the spectrum of the K41 polysaccharide also shows C-4 of the galactofuranosidic residue in addition to the aforementioned unsubstituted carbon atoms (see Table III)

EXPERIMENTAL

General methods — Analytical paper chromatography was performed on Whatman No 1 paper, and Whatman No 3 MM was used for preparative purposes the following solvent systems (v/v) were used (*A*) 8 2 1 ethyl acetate–pyridine–water (*B*) 18 3 1 4 ethyl acetate–acetic acid–formic acid–water, (*C*) 10 4 3 ethyl acetate–pyridine–water, and (*D*) 2 1 1 1-butanol–acetic acid–water Chromatograms were developed with silver nitrate G l c analyses were performed on a Packard–Becker 417 instrument fitted with dual flame-ionization detectors Peak areas were measured with a Hewlett–Packard 3380 A digital integrator Glass columns (3 175 mm o d) were used, with a carrier-gas flow-rate of 60 ml/min Columns were (*A*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 180° (for alditol acetates) or 150° (for partially methylated alditol acetates), (*B*) 3% of OV-225 on Chromosorb WAW-DMCS (100–210 mesh) at 150° (for partially methylated alditol acetates), and (*C*) 2% XE-60 on the same support (for oligosaccharide derivatives) G l c–m s was performed on a Girdel 3000 chromatograph coupled to an AEI MS-30 mass spectrometer Spectra were recorded at 70 eV with an ionization current of 100 μA and an ion-source temperature of 100°

Isolation of the polysaccharide from Klebsiella *K-41* — A culture of *Klebsiella* K41 was obtained from Dr I Ørskov, Copenhagen, and was grown on 4 l of Standard I Nutrient Agar Merck, No 7881, for 4 days at 30° The cells and mucus were harvested, and diluted with 400 ml of water containing 1% of phenol The suspension was centrifuged for 1 h at 29,000 r p m in a Beckman Spinco L 50 ultracentrifuge equipped with a titanium R30 rotor The clear supernatant solution was concentrated to ~200 ml and poured into ethanol (1 l), and the crude polysaccharide was redissolved in water, precipitated with 3% Cetavlon, redissolved in 2M sodium chloride (100 ml), and reprecipitated by ethanol (600 ml) The purified polysaccharide was then dissolved in distilled water (200 ml), deionized with Amberlite IR-120(H^+) resin dialyzed and freeze-dried, to yield about 1 g of the polysaccharide, $[\alpha]_D$ +22 7° (*c* 2 2, water) equivalent weight by sodium hydroxide titration ~1050

Hydrolysis of the native polysaccharide, and sugar analysis — The polysac-

charide (20 mg) in 72% sulfuric acid (0 30 ml) was diluted to 2M and kept for 6 h at 100° After neutralization with barium carbonate, one portion of the hydrolyzate was separated into neutral and acidic fractions by using ion-exchange resins (Amberlite IR-120 and Amberlite IR-45) The neutral sugars were converted into their alditol acetates and examined in g l c (column *A*) and found to contain rhamnose, galactose, and glucose in the ratio 1 00 0 98 2 93 The neutral sugars were isolated by preparative paper-chromatography (solvent *A*) and their optical rotations measured (*c* 1, water) D-glucose, $[\alpha]_D^{20}$ +45° D-galactose, $[\alpha]_D^{20}$ +40° (m p 165°) and L-rhamnose $[\alpha]_D^{20}$ +5°

The acidic fraction gave essentially one compound in paper chromatography (solvent *B*, R_{Glc} 0 28), corresponding to the aldobiouronic acid **2** Esterification with methanol containing 1% of hydrogen chloride, followed by reduction with lithium borohydride and subsequent hydrolysis (2M trifluoroacetic acid, 3 h 100°), gave glucose and galactose in equal amounts, as determined by g l c of their alditol acetates A second portion of the hydrolyzate of K41 was successively esterified reduced, and then rehydrolyzed by the preceding procedure, and the total neutral sugars were examined by g l c of their alditol acetates Rhamnose galactose, and glucose were found in the ratio of 1 1 96 3 97

Carboxyl reduction of the native polysaccharide — This reduction was achieved by two consecutive treatments by the procedure of Taylor and Conrad[4]

Methylation analysis — Methylation of K41 under the Hakomori[5] conditions followed by two consecutive Purdie treatments[2,5], yielded a product that showed no hydroxyl absorption in the i r spectrum Hydrolysis by sulfuric acid under the preceding conditions, and separation of the neutral and acidic constituents on ion-exchange resins, gave the results shown in Table I, column A, for the neutral sugars successive esterification, reduction, and rehydrolysis of the acidic fraction gave the results shown in Table I, column B

A portion of the permethylated polysaccharide was reduced overnight with lithium aluminium hydride in boiling tetrahydrofuran Successive hydrolysis reduction, and acetylation gave the results shown in Table I column C

Uronic acid degradation of methylated polysaccharide[6] — The permethylated polysaccharide (55 mg) was dissolved in a mixture (12 ml) of methyl sulfoxide and 2,2-dimethoxypropane (19 l) and the solution was kept in an ultrasonic bath for 30 min Methylsulfinyl carbanion in methyl sulfoxide (2M, 6 ml) was then added and the mixture was kept for 18 h at room temperature, after which time it was neutralized with 50% acetic acid, and then diluted with water and extracted with chloroform The chloroform extracts were washed with water and evaporated The recovered material was submitted to mild hydrolysis with 50% aqueous acetic acid (15 ml) for 1 h at 100° A portion (5 mg) of the degraded material was dissolved in a mixture (5 ml) of 1,4-dioxane–ethanol (8 3) and reduced overnight with sodium borodeuteride (5 mg) After neutralization with Dowex-50 (H^+) resin and evaporation, methanol was repeatedly re-evaporated from the residue to remove the borate The degraded material was then purified on a column (58 × 2 5 cm) of Sephadex LH-20 by elution

with acetone The elution was monitored by the phenol–sulfuric acid method[24], the main product (31 mg) being eluted with the void volume (60 ml) This fraction was evaporated, the residue hydrolyzed, and the partially methylated sugars examined by g l c –m s of their alditol acetates (Table I, column D)

When the experiment was repeated, the gel filtration on a column of Sephadex LH-20 was replaced by dialysis of the degraded material, in order to recover the polymeric material

Partial hydrolysis of the polysaccharide — The native K41 polysaccharide (250 mg) was partially hydrolyzed by trifluoroacetic acid (0 5M, 30 ml) for 30 min at 100°, and then dialyzed against distilled water The non-dialyzable material was rehydrolyzed under the same conditions The hydrolyzate and dialyzate were combined and then separated into neutral and acidic fractions on ion-exchange resins

The neutral fraction gave several components in paper chromatography (solvent *C*) The compounds migrating in the zone for disaccharides were eluted from the paper A second chromatographic separation (solvent *C*) gave oligomers **3** (17 mg, R_{Glc} 1 214) and **4** (24 mg R_{Glc} 0 545) A portion (5 mg) of each oligosaccharide was hydrolyzed in sulfuric acid (0 5M, 1 ml, 1 h, 100°), and then the hydrolyzates were neutralized and examined by g l c as their alditol acetates Compound **3** showed glucitol and rhamnitol acetates in equal proportions, and compound **4** showed only glucitol acetate A portion (5 mg) of disaccharide **3** was reduced with sodium borohydride (5 mg) and then hydrolyzed as before and acetylated G l c of the products showed acetylated glucose and rhamnitol in equimolar proportions

The acidic fraction was analyzed by paper chromatography in solvent *B*, and the two main components were isolated by using solvent system *D* The aldobiouronic acid **2** (7 mg, R_{Glc} 0 236, solvent *D*) was reduced with sodium borodeuteride (10 mg) and then permethylated by the Hakomori procedure, a part was subjected to g l c –m s analysis (column containing 2% of XE 60 at 190°) The mass spectrum showed, among other details peaks at *m/e* 45, 46, 89, 90, 133, 169, 172, 233, 236, 296, 396 and 440 (M−45) Carboxyl reduction and hydrolysis of the permethylated compound and then derivatization as the alditol acetates showed in g l c –m s (column *B*) the presence in equimolar amounts of acetylated 2,3,4-tri-*O*-methyl-D-glucitol and 1 2 4 5,6-penta-*O*-galactitol deuterated at C-1

The second compound treated as for **2**, was shown to correspond to a tetrasaccharide, but was still impure and was not further studied

Periodate oxidation of the polysaccharide — Polysaccharide K41 (250 mg) was dissolved in distilled water (50 ml), and sodium metaperiodate (0 1M, 50 ml) was added The solution was kept in the dark at 5°, and the periodate consumption was measured at intervals according to Aspinall and Ferrier[25] After 70 h, 1 2 mol of periodate per sugar residue had been consumed After the addition of ethylene glycol (2 ml) and dialysis, the recovered material was reduced with sodium borohydride (300 mg) The reduced polyalcohol was hydrolyzed with sulfuric acid (0 5M, 6 h, 100°) and the products were converted into their alditol acetates G l c analysis (column *A*)

showed, among other details, acetylated rhamnitol, galactitol, and arabinitol in the ratio 1 1 1

Smith degradation of the polysaccharide — The polysaccharide (250 mg) was oxidized as before, and then reduced with sodium borodeuteride and subsequently hydrolyzed with sulfuric acid (0 5M, overnight, room temperature) The partially hydrolyzed material was separated on preparative paper-chromatography (solvent *D*) and gave the oligomer **5** (20 mg, R_{Glc} 0 451) Compound **5** was permethylated as before and subjected to g l c–m s in a column containing 3% of DEXSIL at a temperature programmed from 180–320° (at 4° per min) The mass spectrum showed significant peaks at *m/e* 59 71, 72, 88, 89, 99, 101, 104, 113, 125, 145, 157, 173, 187, 189, 219, 235, 265, 325, 361, 393, 469, 529, and 554

N m r spectroscopy — The ^{1}H- and ^{13}C-n m r spectra were recorded with a CAMECA 250 spectrometer, with D_2O as solvent The ^{1}H-n m r spectra (250 MHz) were recorded[10] at 90° using 5-mm o d tubes The ^{13}C-n m r spectra were recorded in 5-mm tubes for oligosaccharides and 8-mm tubes for the polymers (~50 mg in 1 5 ml of D_2O) at 70° Chemical shifts (δ values) were measured relative to sodium 2,2,3,3-tetradeutero-4,4-dimethyl-4-silapentanoate (TSP) as internal reference Normal ^{13}C spectra were recorded with complete proton decoupling at 62 86 MHz with a spectrometer equipped with Fourier transform (spectral windows of 200 p p m and digitalization into 12,000 data points) pulse width 10 μsec (~70) and interval between the pulses 0 6 sec (corresponding to the acquisition time)

Coupling constants were determined with a gated ^{1}H decoupler sequence to retain nuclear Overhauser enhancements (interval between the pulses 1 6 sec, decoupling time 1 0 sec) For off-resonance experiments, irradiation was effected at the ^{1}H resonance frequency of TSP In the anti-gated experiments, the protons were irradiated during the acquisition time (0 6 sec) followed by a 5-sec delay without irradiation[12] The selective, heteronuclear, double-irradiation spectra were obtained by application of a continuous wave of fixed frequency and with a weak field of ~0 1 Gauss

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