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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* K19 BY CHEMICAL AND N.M.R. ANALYSES*†

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ABSTRACT

Sugar analysis of the capsular antigen K19 from *Klebsiella* and of the carboxyl-reduced derivative confirmed its classification into the chemotype containing rhamnose, galactose, glucose, and glucuronic acid residues. Partial acid hydrolysis and phage depolymerization of K19 provided respectively a modified, linear form of the polysaccharide and oligosaccharides of the repeating unit, these were used for the structural elucidation of the original polymer. Methylation analysis, Smith degradation, and ^{1}H- and ^{13}C-n.m.r. spectroscopy of the polysaccharide and derivatives permitted formulation of the following structure for K19:

→2)-α-L-Rha*p*-(1→2)-α-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Glc*p*A-(1→2)-α-L-Rha*p*-(1→
4
↑
1
α-L-Rha*p*

INTRODUCTION

The capsular polysaccharide K19 from *Klebsiella* has been classified[1] in the chemotype containing D-glucose, D-galactose, L-rhamnose and D-glucuronic acid as the component monosaccharides. This chemotype, which was recently revised, contains the serotypes K12, K18, K36, K41, K55, K70, and K79. In studies of the immunochemical relationships between *Streptococcus pneumoniae* and *Klebsiella*, and in particular from the cross reactions in *anti-Pn XXIII*, Heidelberger[2,3] predicted that K19 should have a non-reducing end group of L-rhamnose in its structure. This is confirmed in the present study.

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TABLE I

SUGAR ANALYSIS OF *Klebsiella* K19 POLYSACCHARIDE AND DERIVED PRODUCTS

Polysaccharide	*Sugars*[a]			$[\alpha]_D^{20}$ *(degrees)*
	Rha $T^b = 0.20$	*Gal* *0.84*	*Glc* *1.00*	
Original (K19)	2.5	1.0	0.9	+51.6 (+32.1)[c]
Carboxyl-reduced (K19-CR)	3.0	1.0	1.8	[d]
Partially hydrolyzed (K19-L)	1.8	1.0	1.1	+64.6 (+51.0)[c]
Periodate-oxidized K19 (K19-Ox)	—	1.0	0.19	[d]
Periodate-oxidized K19-CR (K19-CR-Ox)	—	1.0	0.65	[d]

[a]In molar proportions of the alditol acetate derivatives. [b]Retention times relative to glucitol hexaacetate; conditions C (see Experimental section). [c]Calculated value. [d]Not determined.

RESULTS AND DISCUSSION

Sugar analysis. — *Klebsiella* K19 bacteria grown on an agar medium gave a capsular polysaccharide which was purified by precipitation of its cetyltrimethylammonium salt[4]. The purified original polysaccharide (K19) had $[\alpha]_D^{20}$ +51.6°. Total hydrolysis of K19 revealed that it was composed of residues of L-rhamnose, D-galactose, and D-glucose in the molar proportions 2.5:1.0:0.9, respectively. These proportions of the neutral sugars were modified in the total hydrolyzate of carboxyl-reduced[5] K19 (K19 CR), where the three sugars were in the molar proportions: 3.0:1.0:1.8, respectively (Table I). The uronic acid in K19 was therefore glucuronic acid and the concomitant increase of rhamnose suggested that this sugar was linked to glucuronic acid as an aldobiouronic acid which was only partly hydrolyzed in K19. Only rhamnose was released in the first steps of mild-acid hydrolysis of K19, leaving a polymeric, nondialyzable residue, K19-L, $[\alpha]_D^{20}$ +64.6° (calculated: +51.0°), suggesting that labile rhamnose groups must exist as terminal groups.

N.m.r. spectra of K19 and modified K19 polysaccharide. — Examination of the ^1H- and ^{13}C-n.m.r. spectra of the original K19 polysaccharide showed that it did not contain any of the pyruvate or acetate groups found in many other *Klebsiella* capsular antigens. Subsequent studies employed slightly hydrolyzed samples in order to improve the resolution.

The ^1H-n.m.r. spectrum of K19 showed incompletely resolved signals in the anomeric region that integrated for six protons at 5.18 (E) (1 H), 5.07 (B) and 5.04 (A) (2 H), 4.97 (D) (1 H), and 4.79 (F) and 4.76 p.p.m. (C) (2 H) (Table II). A group of three signals at higher field was characteristic of the methyl protons of the rhamnose residues at 1.24 (A,E) (6 H) and 1.21 p.p.m. (F) (3 H). From the values of the coupling constants associated with anomeric signals, and in particular the

doublet at 4.76 (C) ($^3J_{1,2}$ 7.5 Hz) it may be concluded that one of the hexose residues or the uronic acid has the β configuration. The signal at 4.79 (F) resonating in the region of β anomeric configurations is more difficult to assign with certainty. The high-field shift of this signal, together with its low coupling-constant, corresponds to the *manno* configuration and therefore was assigned to a rhamnosyl residue, but on these ^{1}H-n.m.r. data alone it is not possible to assign its anomeric configuration with certainty[6,7].

All of the other anomeric signals resonating above 4.9 p.p.m. indicate the α configuration[8] of the corresponding sugar residues. Among these signals, the two doublets having coupling values of 3.5 (B) and 4.0 Hz (D) may be assigned to hexoses having the α configuration. Another characteristic feature of the ^{1}H-n.m.r. spectrum of K19 is the existence at 4.28 p.p.m. (D) of a doublet with a splitting of 10 Hz ($^3J_{4,5}$) which could be assigned to H-5 of the glucuronic acid residue[9]. The modifications observed in the spectrum of the partially hydrolyzed K19-L corresponded to the loss of only a rhamnosyl substituent. The main difference in the ^{1}H-n.m.r. spectrum of K19-L was the strong diminution of the signal at 4.79 p.p.m. (F), which may thus be assigned to the nonreducing, side-chain rhamnose groups. This assignment is further characterized by the concomitant diminution of the methyl doublet at 1.21 p.p.m. (F). It may be noted that concurrently the C-6 signals of the two remaining rhamnosyl residues (A′, E′) are now completely separated in the spectrum of K19-L. Another consequence of the partial hydrolysis of K19 was in the shift of the signal at 4.97 p.p.m. (D), which appeared in K19-L at 5.03 (D′) overlapped by a rhamnose signal at 5.04 p.p.m. (A′), and also in the shift of the signal at 4.76 p.p.m. (C) which moved up to 4.68 p.p.m. (C′). The two residues undergoing these shifts are therefore at the branch point of K19 or directly attached to it. The remaining doublet at 4.76 p.p.m. (C) arises from incomplete removal of the rhamnosyl side-chain.

The ^{13}C-n.m.r. spectra of K19-CR confirmed the presence of six sugars in the repeating unit of the capsular polysaccharide, as shown by the six well-resolved signals in the anomeric region (Table III). Confirmation of the existence of a β-linked hexose was provided by the signal at 103.8 p.p.m. (C). No conclusion could be drawn at this stage about the anomeric configuration of the rhamnose residues. The two signals at 62.0 and 61.5 p.p.m., in the spectrum of K19, assigned to unsubstituted CH_2OH indicated that the two hexoses, galactose (C) and glucose (B), are not linked through C-6. After carboxyl reduction, a third signal of unsubstituted C-6 appeared in the spectra of K19-CR at 61.2 p.p.m. (D″). Concurrently, the nearly complete disappearance of the signal at 100.000 p.p.m. (F) in the spectrum of K19-L allows assignment of this signal to the terminal rhamnosyl groups of the side chain.

Methylation analysis of the capsular polysaccharide K19 and modified K19. — Methylations of K19, of its carboxyl-reduced (K19-CR), and of its partially hydrolyzed–carboxymethyl reduced (K19-L-CR) forms were performed by one treatment according to Hakomori[10] followed by two Purdie treatments[11]. Analysis

TABLE II

^{1}H-N.M.R. DATA FOR *Klebsiella* K19 FOR POLYSACCHARIDE AND DERIVED PRODUCTS

Compound	δ^a (p.p.m.)	$^3J^b$ (Hz)	*Integral* (proton)	*Assignment*[c]	
Original polysaccharide (K19)	5.18	n.r.	0.9	H-1 E	→2αRha-
	5.07	3.5	2.1	H-1 B	→2αGlc-
A B C D E	5.04	s		H-1 A	→2αRha-
→2αRha1→2αGlc1→3βGal1→3αGlcA1→2αRha→	4.97	4.0	0.9	H-1 D	→3αGlcA
4					4
↑					↑
1					1
αRha					αRha-
F	4.79	n.r.	1.9	H-1 F	αRha
	4.76	7.5		H-1 C	→3βGal-
	4.28	10.0	2.3[d]	H-5 D	→3αGlcA
					4
					↑
	1.24	6.0	6.0	H-6 A,E	→2αRha-
	1.21	6.0	3.0	H-6 F	αRha-
Partially hydrolyzed polysaccharide (K19-L)	5.20	n.r.	0.9	H-1 E′	→2αRha-
A′ B′ C′ D′ E′	5.07	4.2	0.9	H-1 B′	→2αGlc-
→2αRha1→2αGlc1→3βGal1→3βGlcA1→2αRha→	5.04	n.r.	2.0	H-1 A′	→2αRha-
	5.03	~4.0		H-1 D′	→3αGlcA-
	4.76[e]	7.5	0.4	H-1 (C)	→3βGal-
	4.68	7.5	0.7	H-1 C′	
	1.25	6.0	6.0	H-6 A,E′	→2αRha-
	1.24	6.0			
	1.21[e]	6.0	~0.5	H-6(F)	αRha-
Mixture of oligosaccharides from partial hydrolysis of K19					
B′ C′	5.06	3.6	1.0	H-1 B′	αGlc-
4 αGlc1→3Gal-OH					
	4.97	3.6	0.5	H-1 C′α	→3αGal-OH
	4.58	7.8	0.6	H-1 C′β	→3βGal-OH

[a]Chemical shift relative to internal acetone: 2.17 p.p.m. downfield from tetramethylsilane. [b]Coupling constants: $^3J_{1,2}$ for H-1; $^3J_{4,5}$ for H-5; $^3J_{5,6}$ for H-6, n.r., not resolved. [c]See text and ref. 12. [d]High value due to overlapping by nearby signals. [e]Residual signal due to incomplete hydrolysis of the terminal non reducing rhamnose (F).

TABLE III

^{13}C-N.M.R. DATA FOR *Klebsiella* K19 POLYSACCHARIDE AND DERIVED PRODUCTS

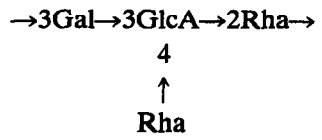
Compound	δ^a (p.p.m.)	$^1J_{C\text{-}1,H\text{-}1}{}^b$ (Hz)	*Assignment*[c]	
Original polysaccharide (K19)	176.2		C-6 D	→3αGlcA-
				4
				↑
A B C D E	103.8	163.9	C-1 C	→3βGal-
→2αRha1→2αGlc1→3βGal1→3αGlcA1→2αRha→	101.2	172.9	C-1 A	→2αRha-
	100.5	173.8	C-1 E	
4	99.9	172.9	C-1 F	αRha-
↑	98.7	174.0	C-1 D	→3αGlcA
1				4
αRha				↑
F	96.2	171.1	C-1 B	→2αGlc-
	62.0		C-6 B	
	61.5		C-6 C	→3βGal-
	17.5		C-6 A,E	→2αRha-
			C-6 F	αRha-
Carboxyl-reduced polysaccharide (K19-CR)	103.7		C-1 C″	→3βGal-
A″ B″ C″ D″ E″				
→2αRha1→2αGlc1→3βGal1→3αGlc1→2αRha→	101.1		C-1 A″	→2αRha-
	100.5		C-1 E″	
4	100.0		C-1 F″	αRha-
↑	98.7		C-1 D″	→3αGlcA-
1				4
αRha				↑
F″	96.2		C-1 B″	→2αGlc-
	62.1[d]		C-6 B″	
	61.6[d]		C-6 C″	→3βGal-
	61.2		C-6 D″	→3αGlc-
				4
				↑
	17.6		C-6 A″,E″	→2αRha-
			C-6 F″	αRha-
Partially hydrolyzed polysaccharide (K19-L)	103.9		C-1 C′	→3βGal-
A′ B′ C′ D′ E′	103.8[e]		C-1 (C)	
→2αRha1→2αGlc1→3βGal1→3αGlcA1→2αRha→	101.1		C-1A′	→2αRha-
	100.5		C-1E′	
	100.0[d]		C-1(F)	αRha-
	98.8		C-1 D′	→3αGlcA-
				4
				↑
	96.2		C-1B′	→2αGlc-
	61.9[d]		C-6 B′	
	61.6[d]		C-6 C′,(C)	→3βGal-
	17.6		C-6 A′,E′	→2αRha-
	17.5[e]		C-6 (F)	αRha-

[a]Chemical shift relative to internal acetone: 31.07 p.p.m. downfield from Me_4Si. [b]Measured by gated-decoupling. [c]See text and ref. 12. [d]C-6B and C-6C, C-6B′ and C-6C′, C-6B″ and C-6C″ can be exchanged respectively. [e]Residual signals due to incomplete hydrolysis of the lateral nonreducing rhamnose (F), indicated in parentheses.

of the products of hydrolysis by g.l.c. and g.l.c.–m.s. of their partially acetylated alditol derivatives gave the results listed in Table IV. The main structural features of the repeating unit of the polysaccharide involve a main chain comprising two rhamnose residues linked through O-2, a galactose residue linked through O-3, a glucose residue linked through O-2, and a rhamnosyl group occupying a terminal, side-chain position. The branch point may deduced from the analysis products of K19-CR as being the glucuronic acid residue. The presence of 2,6-di-*O*-methylglucose in the products from K19-CR shows that glucuronic acid was linked through O-3 and O-4. The position of the rhamnosyl side-chain on O-4 of the glucuronic acid branch-points accounts for the loss by β-elimination of 2,3,4-tri-*O*-methylrhamnose that was observed when K19 was methylated in the presence of an excess of dimethylsulfinyl anion (Table IV).

Periodate oxidation and Smith degradation. — The oxidation was performed on K19-CR. Whether galactose is directly attached to glucuronic acid or not, two results may be anticipated from Smith degradation followed by methylation analysis of the resulting glycosides. The low proportion of glucose observed after periodate oxidation shows that the carboxyl reduction was not complete. After methylation of the dialyzable material (Table IV), it may be seen that all of the galactose gave 2,3,4,6-tetra-*O*-methylgalactose and a major proportion of 2,4,6-tri-*O*-methylglucose, which agrees with the hypothesis of galactose being directly attached to glucuronic acid. The presence of some tetra-*O*-methylglucose might have resulted from the cleavage of the galactosyl bond during Smith hydrolysis and/or could have originated from underoxidation of K19-CR.

The Smith degradation therefore suggests the following sequence of galactose and glucuronic acid, which was further confirmed by the results of β-elimination degradation carried out on the repeat unit isolated by phage hydrolysis of K19 (see Part II, following paper[12]):



At this stage, the 2-linked rhamnose and the 2-linked glucose residues remained to be located in the sequence. The answer was provided by partial acid hydrolysis and moreover by the specific phage degradation of K19.

Partial hydrolysis. — Treatment of K19 with 0.1M trifluoroacetic acid for 90 min at 100° furnished, after dialysis, a mixture of mono- and oligo-saccharides which was partly resolved by paper chromatography. Separation in the acidic solvent-system *A* gave, *inter alia*, two spots **2** and **4** moving at R_{Glc} 0.09 and 0.44. The products were collected and rechromatographed in the neutral eluant *B*. The fact that only the second spot moved in this solvent (R_{Glc} 0.22), showed that it corresponded to a neutral compound. The ^{1}H-n.m.r. spectrum of this oligosaccharide **4**

TABLE IV

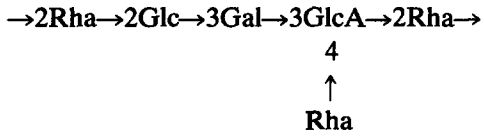
METHYLATION ANALYSIS OF *Klebsiella* K19 POLYSACCHARIDE AND DERIVATIVES

Methylated sugars[a]	*T*[b] *program A*[c]	*T program B*	*Original (K-19)*	*Carboxyl-reduced (K19-CR)*	*Partially hydrolyzed and carboxyl-reduced (K19-L-CR)*	*Periodate-oxidized K19-CR (K19-CR-Ox) after Smith hydrolysis*
2,3,4-Rha[d]	0.53	0.50	0.1	0.8	0.2[e]	—
3,4-Rha	0.94	0.94	1.5	1.9	1.5	—
2,3,4,6-Glc	1.00	1.00	—	—	—	0.6[f]
2,3,4,6-Gal	—	1.17	—	—	—	1.0
2,4,6-Glc	—	1.61	—	—	0.4[g]	0.8
3,4,6-Glc	1.50	1.63	1.0	1.0	1.0	—
2,4,6-Gal	1.56	1.76	1.0	1.0	1.0	—
2,6-Glc	2.06	2.56	—	0.9	0.2[e]	—

[a]In molar proportions of the alditol acetate derivatives. Values are corrected by use of the effective, carbon-response factors given by Sweet *et al.*[24]. [b]Retention times relative to 2,3,4,6-Glc. [c]See Experimental section. [d]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc. [e]Residual value due to uncomplete hydrolysis of the lateral non reducing rhamnose. [f]See text. [g]Low value due to incomplete reduction of the carboxyl group. See also note *e*.

showed signals at 5.06 (B'), 4.97 (Cα) and 4.58 p.p.m. (Cβ) (Table II). Signals of low intensity at 5.24 and 1.25 p.p.m. showed that some rhamnose-containing oligomer was present as an impurity. The signal at 5.06 p.p.m. (B') integrated for one proton and had a $^3J_{1,2}$ coupling constant of 3.6 Hz, characteristic of a hexose in the α configuration. The doublets at 4.97 p.p.m. (Cα) ($^3J_{1,2}$ 3.6 Hz) and at 4.58 p.p.m. (Cβ) ($^3J_{1,2}$ 7.8 Hz) integrated for 0.5 and 0.6 proton respectively, and thus corresponded to a hexose residue occupying a reducing-end position. These characteristics demonstrate that compound **4** was a disaccharide made up of two hexoses. Since in K19 the galactose residue was shown to have the β configuration (see chromium trioxide oxidation, later, and Part II), it could be concluded that **4** corresponded to the sequence αGlc1→3Gal.

This component, which was later confirmed by the structural study of the phage-released oligosaccharide[12] **P1**, completes the sequence of the capsular polysaccharide from *Klebsiella* K19:



Determination of the anomeric configurations. — Chromium trioxide oxidation provides a method for identifying the anomeric configurations[13]. For the capsular polysaccharide of *Klebsiella* K19, two types of sugar residues had to be considered, whether they belonged to the D series (glucuronic acid, galactose, and glucose) or to the L series (rhamnose). The distinction of equatorial *versus* axial orientation in the more-stable $^4C_1$(D) conformation is normally clear with the chromium trioxide oxidation method. With rhamnose, which has the *manno* configuration, the rate of oxidation of the α and β anomers does not exhibit enough difference to be conclusive. This oxidation was performed on peracetylated K19-CR in the presence of peracetylated *myo*-inositol as the internal standard. The kinetics of degradation of each sugar was monitored by g.l.c. (Table V) and showed that the galactose residue was immediately degraded and was thus in the β configuration. All the rhamnose was degraded after a certain lag time, and therefore the three rhamnose residues were deduced to have the same configuration, which should be α. The glucose residues resisted degradation by chromium trioxide and were therefore concluded to be α, thus demonstrating that the glucose and the glucuronic acid were α in the original K19.

Further evidence was necessary to confirm the anomeric determinations, particularly in the case of the three rhamnose residues. The configuration of the terminal rhamnosyl group was clearly proved by the value (172.9 Hz) of the $^1J_{C\text{-}1,H\text{-}1}$ coupling-constant. This result allows correction of the previous tentative assignment, which had been made from the ^{1}H-n.m.r. chemical shift. This discrepancy once again shows the difficulty of relying only on δ values in the rhamnose series[8,14].

TABLE V

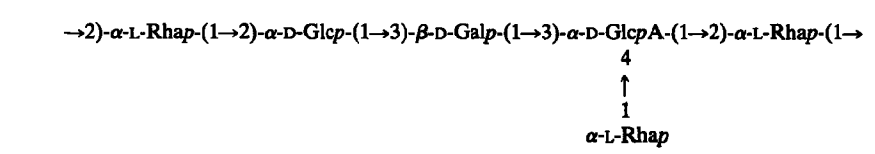
OXIDATION OF *Klebsiella* K19 CARBOXYL-REDUCED POLYSACCHARIDE BY CHROMIUM TRIOXIDE

Reaction time (min)		*0*	*15*	*30*	*60*	*120*
Sugar analysis[a]	Rha	3.1	3.2	2.0	0.6	0.5
	Gal	1.0	0.6	0.4	0.1	0.1
	Glc	2.0	N.D.	1.9	0.9	0.8

[a]In molar proportions of the alditol acetate derivatives.

The only sugar having the β configuration is thus galactose ($^1J_{C\text{-}1,H\text{-}1}$ 163.9 Hz, Table III). That the configuration of the galactose is β and that of all the other sugars is α is in good agreement with the measured and calculated value of the specific rotation of K19 and modified K19 (Table I). Thus, the difference observed in the $[\alpha]_D^{20}$ of K19 and of K19-L (where the rhamnose side-chains had been removed) shows that this rhamnose makes a negative contribution to the $[\alpha]_D^{20}$ of K19 and must therefore be α-linked.

In conclusion, from the combined evidence provided by chemical analyses and n.m.r. studies, it may be suggested that the capsular polysaccharide from *Klebsiella* serotype K19 is composed of a hexasaccharide repeating-unit having the following structure:



This sequence was further fully substantiated by study of the oligosaccharides obtained by using bacteriophage-borne enzymes[12]. The structure of K19 provides another example where Heidelberger's[2,3] immunochemical prediction of a structural feature (here the nonreducing end group of L-rhamnose) was verified. Also it is to be noted that K19 has a composition isomeric with that of K70 (ref. 15) and K36 (ref. 16) (with the exception of pyruvic acid). Moreover, not only are K19 and K70 the only reported structures of *Klebsiella* having an α-(1→2)-linked glucopyranose residue, but they also exhibit an identical, integral part of their sequence:

→2)-α-L-Rhap-(1→2)-α-D-Glcp-(1→3)-β-D-Galp-(1→

EXPERIMENTAL

General methods. — Analytical paper chromatography (p.c.) was performed on Whatman no. 1 paper, and Whatman no. 3MM paper was used for preparative

purposes. The following solvent systems (v/v) were used: (*A*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; (*B*) 8:2:1 ethyl acetate–pyridine–water. Chromatograms were developed with silver nitrate[17].

G.l.c. analyses were performed with a Packard–Becker 417 instrument coupled to a Hewlett–Packard 3380A integrator, or with a Hewlett–Packard 5710A instrument coupled with a Hewlett–Packard 3390 A integrator. Both chromatographs were fitted with dual flame-ionization detectors. A glass column (3 mm × 2 m) was used, with a carrier gas (nitrogen) flow-rate of 60 mL/min. The column was packed with 3% of SP 2340 on Chromosorb W-AW DMCS (100–120 mesh). The conditions used were: (A) from 180° for 2 min, and then at 2°/min to 220°; (B) from 190° for 4 min, and then at 1°/min to 220°; and (C) at 205°, isothermal.

G.l.c.–m.s. was performed on a Girdel 3000 instrument coupled to an AEI MS-30 mass spectrometer and a Finnigan SS 100 MS computer. Spectra were recorded at 70 eV, with a ionization current of 100 μA and a ion-source temperature of 150°. A glass capillary column (0.3 mm × 20 m) was used, with helium as carrier gas. The column was packed with OV 17 on WCOT, and programmed from 160° for 5 min, and then at 2°/min to 220°.

I.r. spectra were recorded with a Perkin–Elmer 598 spectrophotometer.

Optical rotations were measured with a Perkin–Elmer 241 polarimeter. The solutions were equilibrated during 24 h at 20° before measurements.

Isolation of the polysaccharide from Klebsiella K19. — A culture of *Klebsiella* K19 obtained from Dr. I. Ørskov (Copenhagen) was grown on Standard I Nutrient agar (Merck) for 4 days at 30°. The cells and mucus were harvested and diluted with 1% phenol in water[18]. The suspension was centrifuged for 1 h at 100,000*g* (29,000 r.p.m.) in a Beckman Spinco L50 ultracentrifuge equipped with a titanium R30 rotor. The clear supernatant solution was concentrated to 400 mL and poured into ethanol (1.5 L). The crude polysaccharide was redissolved in water (400 mL), precipitated with 3% Cetavlon[4] (200 mL), redissolved in 2M sodium chloride (250 mL), and reprecipitated by ethanol (1 L). The purified polysaccharide was then dissolved in distilled water, dialyzed, and freeze-dried, to yield K19 original, $[\alpha]_D^{20}$ +51.6° (*c* 0.042, water).

Total hydrolysis and sugar analysis. — The original K19 polysaccharide (5 mg) was totally hydrolyzed with 2M trifluoroacetic acid during 4 h at 100°. Reduction and subsequent acetylation yielded alditol acetates in the ratios expressed in Table I. The same procedure was applied to K19-CR, K19-L K19-Ox, and K19-CR-Ox (Table I).

Partial acid hydrolysis. — The rate of hydrolysis of the original K19 was monitored by p.c. (solvent systems *A* and *B*) after different times of reaction in 0.1M CF_3CO_2H at 100°. Treatment of the polysaccharide during 15 min afforded non-dialyzable material and p.c. showed only rhamnose in the dialyzate. This slightly hydrolyzed sample, $[\alpha]_D^{20}$ +57.5° (*c* 0.027, water), was used for ^{1}H-n.m.r. spectrometry.

Monitoring by p.c. showed the best yield of oligomers after a 90-min treat-

ment of the original polysaccharide. The non-dialyzable material (K19-L), $[\alpha]_D^{20}$ +64.6° (*c* 0.21, water), was analyzed by total hydrolysis, ^{1}H-n.m.r., ^{13}C-n.m.r., and methylation (Tables I–IV, respectively): the cleavage of the side-chain non-reducing rhamnose was shown to be almost complete. The dialyzable material was subjected to preparative p.c. (solvent *A*) and two main compounds were isolated: **2** (R_{Glc} 0.09, solvent *A*; R_{Glc} 0.00, solvent *B*) and **4** (R_{Glc} 0.44, solvent *A*; R_{Glc} 0.22, solvent *B*). The latter was examined by ^{1}H-n.m.r. spectroscopy (Table II).

Carboxyl reduction. — Carboxyl reduction of original K19 and K19-L was conducted according to Taylor and Conrad[5], affording K19-CR and K19-L-CR, respectively. Two treatments were necessary to attain satisfactory levels of reduction, as assayed by measuring the residual percentage of glucuronic acid in the carboxyl-reduced polysaccharides[19].

Methylation analysis. — The original K19 polysaccharide (30 mg) was dissolved in dimethyl sulfoxide and methylated in the presence of methylsulfinyl sodium by a modified Hakomori[10] method according to Sandford and Conrad[20]. Two consecutive Purdie[11] methylations were necessary to achieve complete alkylation of the polysaccharide, as determined by g.l.c. analysis. A portion of the permethylated polysaccharide was hydrolyzed with formic acid (90%, 1 h at 100°) then with CF_3CO_2H (2M, 3 h at 100°). The resulting, partially methylated sugars were analyzed as their alditol acetate derivatives by g.l.c.–m.s. (Table IV); the results indicated that some β-elimination occurred during the treatment.

Further evidence of β-elimination occurring during the methylation of original K19 was provided by a subsequent treatment under milder conditions.

Similar conditions for methylation analysis were applied to K19-CR and K19-L-CR.

Periodate oxidation of K19-CR and Smith hydrolysis. — The polysaccharide (30 mg), in 0.05M sodium metaperiodate ($NaIO_4$), was kept in the dark for 72 h at 4°. The reaction was stopped by addition of a few drops of ethylene glycol and the mixture was dialyzed against distilled water. The product was reduced with $NaBH_4$ and the resulting polyol (K19-CR-Ox) isolated by dialysis and lyophilization. A part of the polyol was totally hydrolyzed with 2M CF_3CO_2H for sugar analysis (Table I). The main portion of the polyol was subjected to mild hydrolysis in 0.5M CF_3CO_2H (for 17 h at 20°), and then dialyzed against distilled water. The dialyzate was collected (yield: 14% of the original material) and submitted to methylation analysis (Table IV).

The original K19 polysaccharide was oxidized as just described, giving a polyol (K19-Ox) that was analyzed by total hydrolysis (Table I).

Oxidation of K19-CR by chromium trioxide[13,21]. — The polysaccharide (17 mg) was dissolved in water (0.2 mL), and then 1-methylimidazol (0.4 mL) was added. The mixture was acetylated[22,23] in the presence of acetic anhydride (1 mL) for 5 h at 20°. The partially acetylated material was recovered by partition between chloroform and water. Complete acetylation was provided by treatment with pyridine–acetic anhydride (v/v 1:1, 2 mL) for 1 h at 100°.

The peracetylated polysaccharide was dissolved in acetic acid (5 mL) in the presence of peracetylated *myo*-inositol (2 mg) as the internal standard. Chromium trioxide (CrO_3, 300 mg) was added, the mixture was kept at 50°, and aliquots (1 mL) were removed at intervals. The material was recovered by partition between chloroform and water and the sugars were analyzed conventionally as their alditol acetate derivatives (Table V).

N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectra were recorded on a Cameca 250 spectrometer (250 MHz) with D_2O solutions (15 mg in 0.3 mL) at 358K. Normal ^{13}C-n.m.r. spectra were recorded with complete proton decoupling on a Bruker WP-100 spectrometer (25.2 MHz) in D_2O–H_2O solution (60 mg in 1 mL) at 353K.

Both instruments were equipped with Fourier transform. Chemical shifts (δ) are given in p.p.m. relative to internal acetone, δ = 2.17 in ^{1}H and 31.07 in ^{13}C, downfield from tetramethylsilane.

Coupling constants ($^1J_{C\text{-}1,H\text{-}1}$) were determined on a Bruker AM 400 spectrometer with a gated, ^{1}H-decoupler sequence to retain nuclear Overhauser enhancement (pulse interval: 2 s; decoupling time: 1.2 s).

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