Carbohydrate Research, 83 (1980) 351–362
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

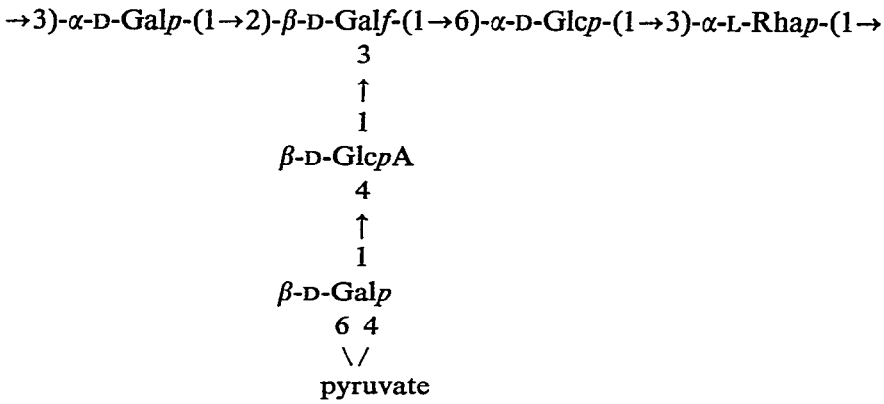
STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K12

GUY G S DUTTON AND ANGELA V SAVAGE
Department of Chemistry, The University of British Columbia, 2036 Main Mall, Vancouver, B C V6T 1Y6 (Canada)

(Received January 22nd, 1980, accepted for publication, February 25th, 1980)

ABSTRACT

Klebsiella K12 capsular polysaccharide has been investigated by the techniques of methylation, Smith degradation–periodate oxidation, uronic acid degradation, and partial hydrolysis, in conjunction with ^{1}H-n m r spectroscopy at 100 and 220 MHz, and ^{13}C-n m r spectroscopy at 20 MHz The structure has been found to consist of the hexasaccharide repeating-unit shown, having a D-galactofuranosyl residue at the branch point In this series, a D-galactofuranosyl residue has previously only been found in the polysaccharide from *Klebsiella* K41



INTRODUCTION

The genus *Klebsiella* has been classified by Ørskov[1] into approximately 80 serotypes, based on their antigenic, capsular polysaccharides Nimmich[2,3] qualitatively analyzed the polysaccharide from each strain, K12 polysaccharide was found to contain glucose, galactose, rhamnose, glucuronic acid, and pyruvic acid As part of our continuing investigation of the relationship between primary, chemical structure and immunological activity, we now report on the elucidation of the structure of the K12 polysaccharide

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This structure is in agreement with the predictions made by Heidelberger and co-workers, based on the cross-reactions of the polysaccharide with anti-*Pneumococcal* and anti-*Klebsiella* sera, of the occurrence of a (1→3)-linked L-rhamnosyl residue[4] and of a (nonreducing) 4,6-*O*-(1-carboxyethylidene)-D-galactosyl group[5] in the repeating unit

RESULTS AND DISCUSSION

Composition and n m r spectra

Klebsiella K12 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon The product had $[\alpha]_D$ +24 2°

Paper chromatography of an acid hydrolyzate of the polysaccharide showed the presence of glucose, galactose, glucuronic acid, and rhamnose Carboxyl-reduced K12 polysaccharide was hydrolyzed, and the presence of glucose, galactose and rhamnose in the ratios of 2 3 1 was determined by gas–liquid chromatography (g l c) of their alditol acetates (see Table I) Rhamnose was shown to be of the L, and glucose of the D, configuration by circular dichroism (c d) measurements of the derived alditol acetates[6]

Arabinitol pentaacetate was similarly shown to have the L configuration, indicating that the galactofuranose unit from which it was derived by loss of C-6 has the D configuration An acid hydrolyzate of the polysaccharide gave a positive reaction with D-galactostat reagent, thus confirming the D configuration of the galactose.

The 220-MHz, ^{1}H-n m r spectrum of the polysaccharide, after mild hydrolysis to lower the viscosity, showed a sharp singlet at δ 1 6, indicative of a (1-carboxyethyl-

TABLE I

G L C ANALYSIS OF NATIVE AND PERIODATE-OXIDIZED[a] POLYSACCHARIDES

Sugars (as alditol acetates)	T[b] *(in column C[c], SP 2340)*	*Mole %*	
		I[a]	*II*[a]
Glycerol	0 09		11 7[e]
Erythritol	0 26		12 4[e]
Threitol	0 33		9 9[e]
Rhamnose	0 45	17 3	21 8
Arabinose	0 65		22 5
Galactose	0 95	51 7	21 7
Glucose	1 00	31 0	—

[a]On carboxyl-reduced polysaccharide [b]Retention time relative to that of glucitol hexaacetate [c]Programmed for 8 min at 180°, and then at 4°/min to 240° [d]I, native polysaccharide, uronic acid reduced, II, periodate oxidation, reduction, and total hydrolysis of the carboxyl-reduced polysaccharide [e]Some loss of volatile components during derivatization

idene) group This signal was present in 1 1 ratio with a doublet at δ 1 3 attributable to the methyl group of rhamnose[7-9] Six discernible signals were observed in the anomeric region, at δ 5 22 (1 H, $J_{1,2}$ weak), 5 16 (1 H, $J_{1,2}$ 3 Hz), 5 13 (2 H, $J_{1,2}$ 2 Hz), 4 66 (1 H, $J_{1,2}$ 8 Hz), and 4 48 (1 H, $J_{1,2}$ 6 Hz) The ^{13}C-n m r spectrum of the polysaccharide[10,11] (150 mg/2 mL) showed high-field peaks at 17 57 (rhamnose CH_3) and 22 13 p p m (pyruvate CH_3) In the anomeric region, five signals, in the ratios of 1 1 2 1 1, were seen at 108 39, 106 99, 102 64, 99 43, and 97.18 p p m Interpretation of these p m r and ^{13}C-n m r data initially caused some difficulty, as the p m r data suggested four α-linked and two β-linked residues, whereas the ^{13}C-n m r data indicated three α-linked and three β-linked residues This problem was resolved when the methylation data showed the presence of a furanosyl sugar (see later)

The signals at 63 85 and 61 77 p p m were assigned to the C-6 atoms of hexoses, and the signal at 66 44 p p m to a C-6 atom involved in a linkage

Methylation of original polysaccharide

Methylation[12,13] of K12 polysaccharide, followed by reduction of the uronic ester, hydrolysis, derivatization as alditol acetates, and g l c–m s analysis[14a,14b], indicated that K12 polysaccharide is composed of a hexasaccharide repeating-unit consisting of five pyranose residues and one furanose, namely, galactofuranose, which constitutes a branch point (see Table II) These data also indicate that the (1-carboxyethylidene) group is linked to O-4 and O-6 of a (terminal) galactopyranosyl group Analysis of a re-methylated sample of the reduced product showed the formation of 2,3,6-tri-*O*-methylglucose and the disappearance of the 2,3-dimethyl ether, thus establishing that the uronic acid is glucuronic acid

TABLE II

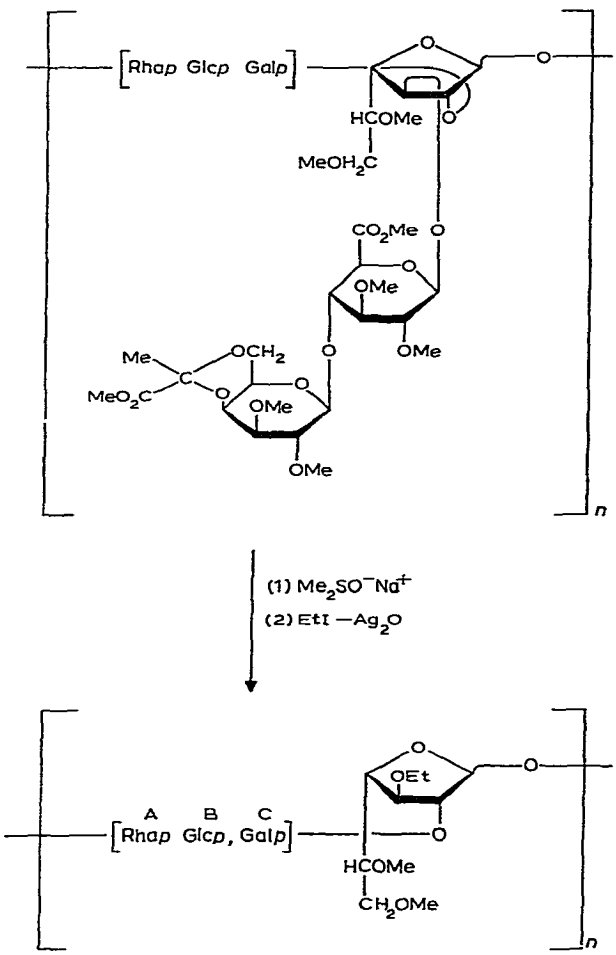
METHYLATION ANALYSIS OF NATIVE, AND DEGRADED, *Klebsiella* K12 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] (*as alditol acetates*)	T[b]		*Mole %*[c]		
	Column A[d] *(OV-225)*	*Column B*[e] *(ECNSS-M)*	*I*[f]	*II*	*III*
2,4-Rha	1 00	1 00	20 02	18 17	22 48
2,4,6-Gal	1 88	1 55	21 66	21 88	26 46
2,3,4-Glc	2 05	1 63	16 96	31 10	25 25
2,3,6-Glc	2 05	1 63			
5,6-Gal	2 28	1 75	15 88	13 11	
2,3-Glc	3 30	2 31	13 38		
2 3-Gal	3 42	2 40	12 10	15 74	25 82
3,5,6-Gal[g]		1 46			

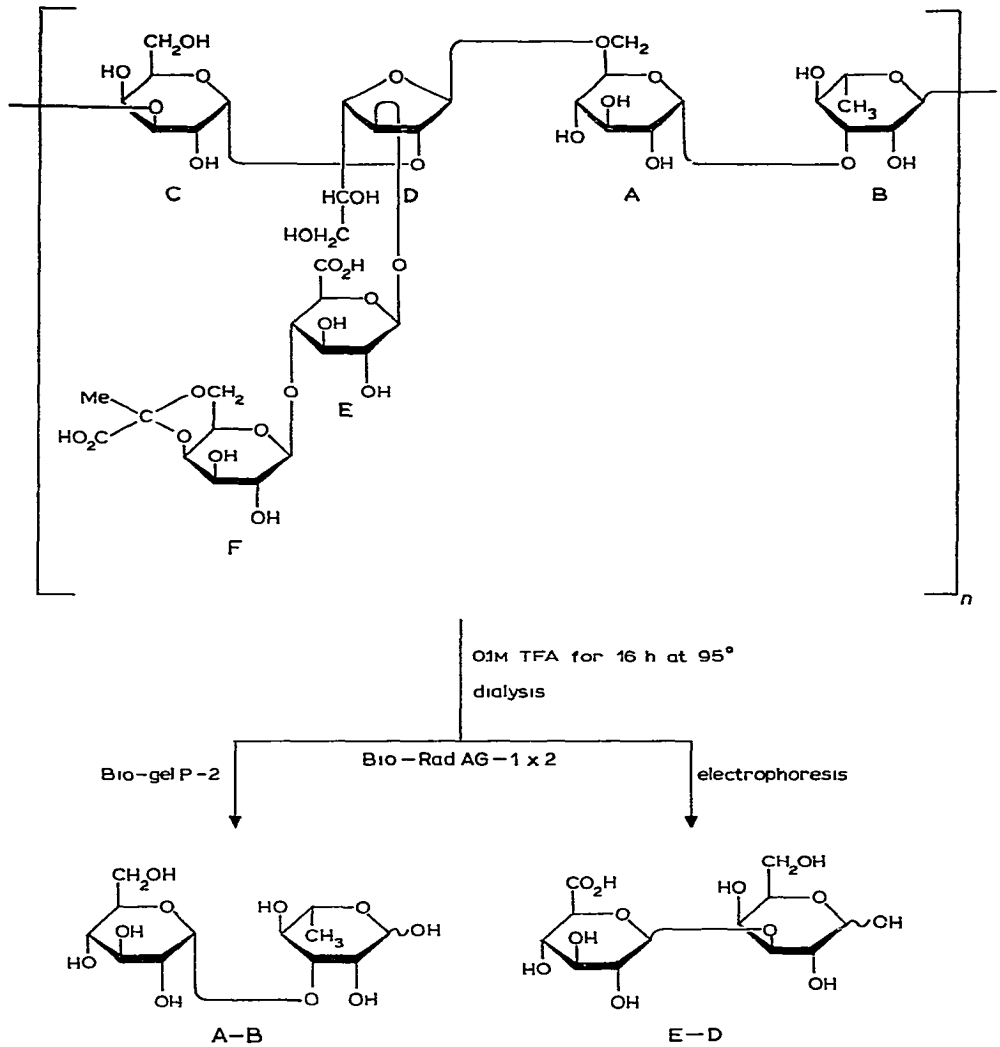
[a]2,4-Rha = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol, etc [b]Retention time relative to that of the alditol acetate derivative of 2,4-Rha [c]Values corrected by using effective, carbon-response factors[25] [d]Programmed for 4 min at 180° and then at 2°/min to 200° [e]Programmed for 4 min at 165°, and then at 2°/min to 200° [f]I, original polysaccharide methylated, and uronic ester reduced, column B, II, as in I, but remethylated, column A, III, after uronic acid degradation, and ethylation [g]1,2,4-Tri-*O*-acetyl-3-*O*-ethyl-5,6-di-*O*-methylgalactitol

Base-catalyzed degradation[15]

To determine the location of the glucuronic acid, the methylated polysaccharide was subjected to base-catalyzed degradation, and the product was directly ethylated[16] The isolation of a polymeric, degraded product indicates that the glucuronic acid is in the side chain (see Scheme 1). On hydrolysis, and derivatization for g l c–m s, the compounds shown in Table II were obtained, indicating that the glucosyluronic acid residue is attached to O-3 of the galactofuranosyl residue, and that the only other sugar in the side chain is a 4,6-*O*-(1-carboxyethylidene)-D-galactosyl group The ^{1}H-n m r spectrum indicated the absence of two β-linkage signals in the anomeric region attributable to the sugars of the side chain



Scheme 1 Base-catalyzed degradation of *Klebsiella* K12 polysaccharide



Scheme 2 Partial hydrolysis of *Klebsiella* K12 polysaccharide, and isolation of neutral and acidic disaccharides

Partial hydrolysis

A sample of K12 polysaccharide in the free-acid form was hydrolyzed for 10 h with 0·1M trifluoroacetic acid in an apparatus similar to that described by Galanos and colleagues[17], yielding a mixture of oligomers and monosaccharides which was separated by means of AG-1 X2 ion-exchange resin into acidic and neutral fractions (see Scheme 2) Preparative, paper electrophoresis of the acidic fraction gave an aldobiouronic acid (**1**) which, after hydrolysis and paper chromatography, was

TABLE III

N M R DATA FOR *Klebsiella* K12 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES

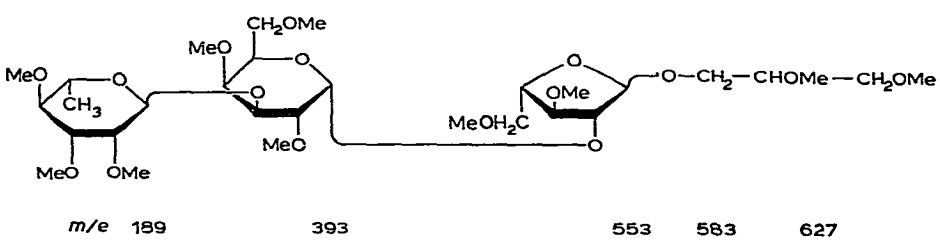
Compound[a]	^1H *N m r data*				^{13}C-*N m r data*	
	δ[b]	$J_{1,2}$ *(Hz)*[c]	*Integral (H)*	*Assignment*[d]	*p p m*[e]	*Assignment*[f]
1 3 GlcA——Gal∼OH (1) β E–D	5 30	2	0 6	α Gal∼OH	104 46	β GlcA
	4 76	7 5	1	β GlcA	96 98	β Gal∼OH
	4 65	8	0 4	β Gal∼OH	93 01	α-Gal∼OH
					61 79	C-6 of Gal
1 3 Glc——Rha∼OH (2) α A–B	5 15	s	0 6	α-Rha∼OH	96 41	α-Glc[g]
	5 10	b	0 6	α-Glc[g]	96 13	α-Glc[g]
	5 08	b	0 4	α-Glc[g]	94 52	α- + β-Rha∼OH
	4 88	s	0 4	β-Rha∼OH	61 12	C-6 of Glc
	1 30	6 ($J_{5,6}$)	3	CH_3 of Rha	17 76	CH_3 of Rha
1 3 1 2 1 1 Rha——Gal——Ara——glycerol (3) α α α B–C–D–A	5 19	1	1	α-Rha	106 93	Ara
	5 10	2	1	α-Ara	103 10	Rha
	5 05	1	1	α-Gal	99 14	Gal
	1 30	4	3	CH_3 of Rha	17 46	CH_3 of Rha
					186 47	C-6 of β GlcA
[3 1 2 1 6 1 3 1 ——Gal*p*——Gal*f*——Glc——Rha—— α 3 β α α 1 β GlcA C–D–A–B 4 1 β E Gal 6 4 F pyruvate]$_n$	5 22	s	1	α-Rha	108 39	β-Gal*f*
	5 16	3	1	α-Glc	106,99	β-Gal*p*
	5 13	s	2	α-Gal*p*	102 64	α-Rha + β-GlcA
	5 13	s		β-Gal*f*	99 43	α-Gal
	4 66	7	1	β-GlcA	97 18	α-Glc
	4 48	6	1	β-Gal*p*	85 71	C-2
	4 3–4 5	b	2	H-2,H-3 β-Gal*f*	84 24	C-3 of β-Gal*f*
	1 66	s	3	CH_3 of acetal	83 00	C-4
	1 34	6	3	CH_3 of Rha	66 44	C-6 of α-Glc
					63 85	C-6 of β-Gal*f*
					61 77	C 6 of α-Gal
					22 13	CH_3 of acetal
					17 57	CH_3 of Rha

[a]For the origin of compounds **1–3**, see text [b]Chemical shift relative to internal acetone, *δ* 2 23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D S S.) [c]Key b = broad, unable to assign accurate coupling-constant, s = singlet [d]For example, α-Gal = proton on C-1 of α-linked D-Gal residue [e]Chemical shift in p p m downfield from Me_4Si, relative to internal acetone, 31 07 p p m downfield from D S S [f]As for footnote *d*, but for anomeric, ^{13}C nuclei [g]This glycosidic atom resonates as two doublets, because of the anomeric equilibrium of the reducing unit

shown to consist of glucuronic acid and galactose Spectroscopy (^{1}H- and ^{13}C-n m r) indicated that the reducing galactose residue was now in the pyranose form, and verified that the side-chain linkage is β (see Table III) The structure of the aldobiouronic acid (E–D, **1**) is thus β-D-Glc*p*A-(1→3)-D-Gal*p*-OH Separation of the mixture of neutral oligomers by gel chromatography on Bio-Gel P-2, followed by purification by paper chromatography, gave a disaccharide (A–B, **2**) which, after hydrolysis and paper chromatography, was shown to consist of glucose and rhamnose The ^{1}H- and ^{13}C-n m r spectra are consistent with the structure α-D-Glc*p*-(1→3)-L-Rha∼OH

Periodate oxidation of the carboxyl-reduced polysaccharide

To determine the sequence of the sugars in the backbone of the polymer, the carboxyl-reduced polysaccharide[18] was oxidized with periodate After 90 h, the consumption of oxidant[19] was 5 4 mol per mol of repeating unit The theoretical consumption is 5 mol if the (1-carboxyethylidene) group remains intact, the higher consumption indicates loss of some of the acetal groups The polyol obtained by reduction with sodium borohydride was subjected to total hydrolysis, followed by derivatization as the alditol acetates, which gave the results shown in Table I The low proportion of threitol is consistent with some loss of the pyruvic acetal Smith hydrolysis[20] of the polyol, followed by sodium borohydride reduction yielded a mixture of oligosaccharides which was separated by gel chromatography Oligomer **3** was obtained pure by preparative, paper chromatography, and was shown to consist of rhamnose, galactose, arabinose, and glycerol in equal proportions The ^{1}H- and ^{13}C-n m r spectra were in agreement with these data To determine the sequence of sugars in **3**, the oligosaccharide was permethylated by the Purdie method[13] and the product purified by g l c on OV-1 and examined by electron-impact, mass spectrometry Detection of peaks at *m/e* 189 and 393, among others, indicated that the deoxyhexose is linked to the hexose, not to the pentose The source of some pertinent fragments is illustrated The anomeric nature of the linkages was determined by ^{1}H- and ^{13}C-n m r spectroscopy Oligomer B–C–D–A is thus established as having structure **3**

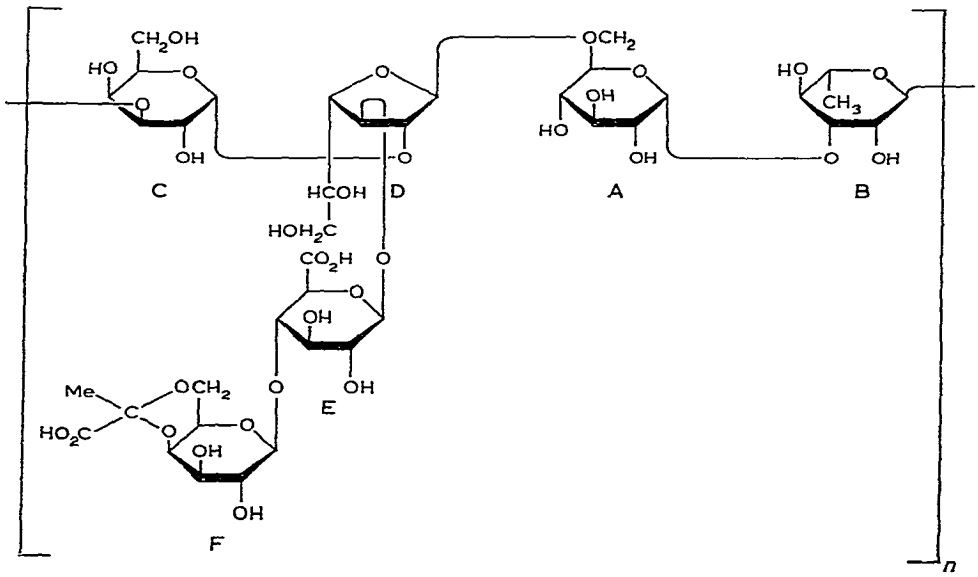


α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→2)-α-L-Ara*f*-(1→1)-glycerol

3

CONCLUSION

It thus follows that *Klebsiella* K12 capsular polysaccharide has the following structure



After the realization, from the methylation g l c –m s data, that a furanosyl residue was present, the ^{1}H- and ^{13}C-n m r spectra were more easily interpreted. In the former, the β-Gal*f* anomeric signal appears at δ 5 13, the region normally attributed to α-linked pyranoses In the ^{13}C spectrum, however, the anomeric signal occurs in the unambiguous, β-linkage region at 108 39 p p m (see Table III)

Interestingly, the only other *Klebsiella* polysaccharide reported[21] to have a furanosyl unit is K41, which has a very similar structure in which the terminal 4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl group of K12 is replaced by β-D-Glc*p*-(1→6)-α-D-Glc*p*- As expected, no cross-reaction occurs between these two polysaccharides, because the side chain is usually the immunodominant group Cross-reaction does, however, occur with anti-K11 (ref 5), which has a 4,6-*O*-(1-carboxyethylidene)-α-D-galactopyranosyl side-chain[22], and with anti-Pn-VI (ref 4), which has an in-chain, -α-D-Glc*p*-(1→3)-α-L-Rha*p*- unit

EXPERIMENTAL

General methods — Concentrations were conducted under diminished pressure at bath temperatures not exceeding 40° The equipment for m s , n m r spectroscopy, g l c , and g l c –m s was the same as that used in the investigation of *Klebsiella* K23 polysaccharide[23] Paper electrophoresis was performed in a Savant high-voltage (5 kV) system (model LT-48A), with kerosene as the coolant The buffer used was

5 2 743 (v/v) pyridine–acetic acid–water, pH 5 3 Strips (77 × 20 cm) of Whatman No 1 paper were used for all runs, with application of 25–50 mA for 1 5 h For descending, paper chromatography, the following solvent systems (v/v) were used. (*1*) freshly prepared 2 1 1 1-butanol–acetic acid–water, and (*2*) 8 2 1 ethyl acetate–pyridine–water Sugars and oligosaccharides were detected, after electrophoresis and after descending, paper chromatography, with an alkaline silver nitrate reagent[24]. Analytical g l c separations were performed in stainless-steel columns (1 8 m × 3 mm), with a carrier-gas flow-rate of 20 mL/min Columns used were (*A*) 3% of OV-225 on Gas Chrom Q (100–120 mesh), (*B*) 5% of ECNSS-M on the same support, and (*C*) 3% of SP-2340 on Supelcoport (100–120 mesh) Analogous columns (1 8 m × 6 3 mm) were used, along with a column of 5% of OV-1 on Gas Chrom Q (100–120 mesh), for preparative-g l c separations

Preparation and properties — A culture of *Klebsiella* K12 (313) was obtained from Dr. I Ørskov (Copenhagen) The polysaccharide was isolated as previously described[22], and showed $[\alpha]_D$ +24 2° (*c* 1, water)

Analysis of constituent sugars — Methanolysis of a sample (20 mg) of K12 polysaccharide with 3% methanolic hydrogen chloride, and subsequent treatment with sodium borohydride in anhydrous methanol, reduced the uronic ester Hydrolysis with 2M trifluoroacetic acid (TFA) overnight at 95°, followed by reduction ($NaBH_4$) and acetylation, gave galactitol hexaacetate, glucitol hexaacetate, and rhamnitol pentaacetate in the ratios 3 2 1 (column *C*, programmed for 8 min at 180°, and then at 4°/min to 240°) Circular dichroism (c d) of the last two components, isolated by preparative g l c , showed positive and negative curves, respectively, confirming that the glucose had the D, and the rhamnose the L, configuration The configuration of the galactose was deduced to be D from the negative c d of the pentaacetate of its (reduced) oxidation product, namely, arabinitol pentaacetate, this was confirmed by the positive action of D-galactostat (Worthington Biochemical Co) on the hydrolysis product of the polysaccharide

Methylation of the native polysaccharide — Methylation of K12 polysaccharide under the Hakomori[12] conditions, followed by a Purdie[13] treatment, yielded a product that showed no hydroxyl absorption in the i r spectrum This material was reduced overnight with sodium borohydride in 1 1 (v/v) oxolane–ethanol, and a portion of the product was hydrolyzed with 2M trifluoroacetic acid for 16 h at 95°, the resulting mixture was reduced with sodium borohydride, and the product acetylated G l c –m s gave the results shown in Table II

Another portion of the material (reduced uronic ester) was remethylated under Purdie conditions for 2 days, and the product derivatized for g l c –m s , which indicated the compounds shown in Table II

Uronic acid degradation[15] — A solution of carefully dried, methylated polysaccharide (100 mg) and *p*-toluenesulfonic acid (a trace) in 19 1 dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) was prepared in a serum vial which was then sealed with a rubber cap. The vial was flushed with nitrogen, and the solution was stirred for 3 h Sodium methylsulfinylmethanide (2M) in dimethyl sulfoxide (10 mL) was

then added with the aid of a syringe, and the solution was stirred overnight at room temperature, cooled to 10°, and ethyl iodide (3 mL) was added slowly by using a syringe[16] The solution was stirred for a further 30 min, the excess of ethyl iodide was removed by use of a rotary evaporator, and the solution was dialyzed overnight against tap water, and lyophilized, the product (65 mg) was purified by precipitation into petroleum ether (b p 30–60°), yielding 60 mg of polymeric material Subsequent hydrolysis and derivatization for g l c –m s gave the results shown in Table II

Partial hydrolysis — A sample of *Klebsiella* K12 polysaccharide was changed to the free-acid form with Amberlite IR-120 (H^+) ion-exchange resin, and lyophilized This material (1 g) was dissolved in water (100 mL, pH 3 2) and the solution auto-hydrolyzed on a steam bath for 16 h in an apparatus similar to that described by Galanos *et al*[17] Very little hydrolysis occurred, therefore, the solution was made 0 1M in TFA, and the reaction continued for a further 16 h After removal of the TFA, the products (700 mg) were separated into neutral and acidic fractions by using Bio-Rad AG-1 X2 ion-exchange resin Portions (200 mg) of the acidic fraction were applied to a column (100 × 2 5 cm) of Sephadex G-25, which was irrigated with a 500 5 2 (v/v) water–pyridine–acetic acid buffer at a flow rate of 10 mL/h Separation of components was poor Fractions containing components having R_{Glc} >0 2 (solvent *1*) were combined, and separated by preparative, paper electrophoresis A component (**1**) having R_{GlcA} 0 69, was obtained pure (30 mg), and was shown by total hydrolysis to consist of glucuronic acid and galactose (E–D) Spectroscopy (^{1}H- and ^{13}C-n m r.) indicated the presence of a β-linked glucuronic acid and a reducing galactose (see Table III) Portions (200 mg) of the neutral fraction were separated by gel chromatography on a column (100 × 2 5 cm) of Bio-Gel P-2 Irrigation with the same buffer at a flow rate of 10 mL/h, lyophilization of the fractions, and examination by paper chromatography (solvent *2*) revealed that separation was incomplete Purification of a component having R_{Glc} 0 61 by paper chromatography yielded compound **2** (30 mg) which, on hydrolysis, was found to consist of glucose and rhamnose (A–B) Spectroscopy (^{1}H- and ^{13}C-n m r) indicated the presence of an α-linked glucosyl group and a (reducing) rhamnose residue (see Table III)

Periodate oxidation of carboxyl-reduced polysaccharide — A sample of the polysaccharide was reduced by the procedure of Taylor and Conrad[17], two treatments were needed in order to achieve complete reduction Reduced, capsular polysaccharide (200 mg) was dissolved in water (40 mL), and 0 1M sodium metaperiodate (40 mL) was added The solution was stirred in the dark at 3°, and the periodate consumption was monitored spectrophotometrically[18] After three days, consumption had reached 5 4 molecules per repeating unit Ethylene glycol (10 mL) was then added, the mixture was stirred for a further 30 min, and dialyzed overnight against running tap water, and the product reduced with sodium borohydride The polyol was isolated by dialysis and lyophilization.

A portion (5 mg) of the polymeric product was hydrolyzed with 2M TFA overnight at 95° Paper chromatography (solvent *2*) then showed the presence of glycerol, a tetrose, rhamnose, arabinose, and galactose Conversion of the hydrolysis

products into the corresponding alditol acetates gave the g l c results shown in Table I Smith hydrolysis of the polyol with 0 5M TFA overnight at room temperature gave a mixture which was separated on Bio-Gel P-2 An oligomer (B–C–D–A, 3) (R_{Glc} 0 46, solvent *1*) was purified by paper chromatography The ^{1}H- and ^{13}C-n m r data are shown in Table III The mass spectrum of the permethylated (Purdie[13] method) oligomer showed significant peaks at *m/e* 627, 583, 553, 540, 527, 467, 393, 375, 361, 290, 289, 273, 272, 260, 259, 217, 189, 187, and 103 Total hydrolysis of the oligomer with 2M TFA for 16 h at 90° gave galactose, rhamnose, arabinose, and glycerol (paper chromatography, solvent *2*) G l c analysis of the derived alditol acetates showed that the constituents were present in equimolar proportions By ^{1}H- and ^{13}C-n m r. spectroscopy (see Table III), the anomeric nature of all of the linkages was shown to be α

ACKNOWLEDGMENTS

We thank Dr I Ørskov for a culture of *Klebsiella* K12 (313), Christiane Martel for growing the bacteria and for the initial investigation, Dr A A Grey and A Lee for recording the 220-MHz spectra, and Drs J -P Joseleau and M Vignon for helpful discussion of n m r data We are indebted to Merck, Sharp & Dohme, and NSERC, for financial support, and one of us (A V S) is grateful to MacMillan Bloedel for a graduate scholarship

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