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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K36 POLYSACCHARIDE*

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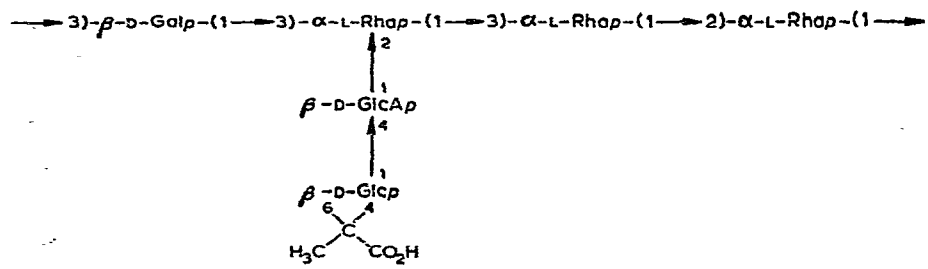
ABSTRACT

Klebsiella K36 capsular polysaccharide has been investigated by methylation, Smith-periodate oxidation and partial hydrolysis techniques. The structure was found to consist of a hexasaccharide repeating unit as shown. The anomeric configurations of the sugar residues were determined by ^{1}H and ^{13}C n.m.r. spectroscopy on isolated oligomers obtained during the degradative studies and on the intact polysaccharide.



INTRODUCTION

The bacterial genus *Klebsiella* is divisible into approximately 80 different serotypes, which are distinguished by the structure of their capsular polysaccharides. Nimmich[1,2] has analyzed qualitatively the polysaccharide from each strain and Heidelberger and Nimmich[3] have summarized the structures of the capsular polysaccharides that have been determined to date.

Many of the strains thus far examined possess capsules of different qualitative compositions. There are, however, several large groups of *Klebsiella* bacteria that have capsules having the same qualitative analysis but displaying different serological reactions. One such group includes the strains of K-types K12, K36, K45, K55, and

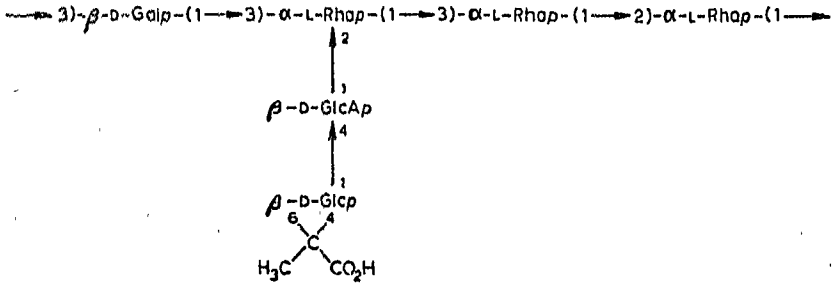
*Dedicated to the memory of Professor J. K. N. Jones, F.R.S.

TABLE I

P.M.R. DATA ON KLEBSIELLA K36 CAPSULAR POLYSACCHARIDES AND DERIVED OLIGOSACCHARIDES

Compound	*Repeating unit of compound*	τ^a (H_3)	*Ratio integrals*	*Proton assignment*
3	β-D-GlcA-(1→2)-Rha-OH	5.37(7)	1	β-GlcA
		4.63(1.8)	0.6	α-Rha-OH
		5.17(1)	0.4	β-Rha-OH
		8.70(6)	3	CH_3 of Rha
4	β-D-GlcA-(1→2)-α-L-Rha-(1→3)-Rha-OH	5.34(7)	1	β-GlcA
		4.65(2)	1	α-Rha
		4.89(2)	0.6	α-Rha-OH
		5.13(1)	0.4	β-Rha-OH
		8.70(6)	6	CH_3 of Rha
5	β-D-Glc-(1→4)-β-D-GlcA-(1→2)-α-L-Rha-(1→3)-Rha-OH	5.19(7.5)	1	β-Glc
		5.35(7)	1	β-GlcA
		4.63(2^b)	1	α-Rha
		4.89(1.8)	0.6	α-Rha-OH
		5.13(1)	0.4	β-Rha-OH
		8.70(6)	6	CH_3 of Rha
6b	β-D-Gal-(1→3)-α-L-Rha-(1→3)-Rha-OH $\uparrow^2_1$ β-D-GlcA	5.21(7.5)	1	β-Gal
		5.32(7)	1	β-GlcA
		4.71(2^b)	1	α-Rha
		4.91(2)	0.6	α-Rha-OH
		5.13(1)	0.4	β-Rha-OH
		8.70(6)	6	CH_3 of Rha

TABLE I (*continued*)

Compound	*Repeating unit of compound*	τ (H_3)	*Ratio integrals*	*Proton assignment*
1	β-D-Gal-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-glycerol	5.34(7.5)	1	β-Gal
		5.01(1.8)	1	α-Rha
		4.92(1.8)	1	α-Rha
		8.70(6)	6	CH_3 of Rha
2	α-L-Rha-(1→3)-α-L-Rha-(1→2)-glycerol	4.94(2)	1	α-Rha
		5.03(2)	1	α-Rha
		8.70(6)	6	CH_3 of Rha
		5.50(7[b])	1	β-Gal
		5.35(7)	1	β-GlcA
		5.15(7.5)	1	β-Glc
		5.02(b)	1	α-Rha
		4.84(b)	2	
		8.41(s)	3	CH_3 of pyruvate
		8.67(b)	9	CH_3 of Rha

[a]Shifts quoted relative to acetone as the internal standard (τ 7.77). $J_{1,2}$ coupling constants in Hz are given in parentheses. Singlets are designed as s, and where it was not possible to get an accurate $J_{1,2}$ value, this is indicated by b.

K70 whose capsules are composed of the sugars D-glucuronic acid, D-galactose, D-glucose, and L-rhamnose. In an attempt to explain these serological differences on a structural basis, this group is now being examined in detail, and the structure for K-type K36 here presented represents the first strain examined in this series.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K36 polysaccharide was prepared on an agar medium and purified by one precipitation with Cetavlon. The product had $[\alpha]_D$ −56°. Electrophoresis showed the material to be homogenous.

The p.m.r. spectrum of the polysaccharide in D_2O at 90° showed a sharp singlet at τ 8.41 indicative of a pyruvate acetal[4]. This signal was present in 1:3 ratio with the methyl-group signal of rhamnose (at τ 8.67). Five discernible signals were observed in the anomeric region, at τ 4.76 (2 H, $J_{1,2}$ broad), τ 4.98 (1 H, $J_{1,2}$ 2 Hz), 5.13 (1 H, $J_{1,2}$ 7 Hz), 5.30 (1 H, $J_{1,2}$ 7.5 Hz), and 5.50 (1 H, $J_{1,2}$ broad). These chemical shifts indicate[5,6] that the repeating unit contains six monosaccharide residues, three of which are α-linked and three β-linked. More-precise assignment of these signals was achieved after studying the p.m.r. spectra of the oligosaccharides obtained by partial hydrolysis; see later and Table I.

The p.m.r. analysis was confirmed by ^{13}C n.m.r. spectroscopy of the polysaccharide (160 mg/2 ml), which showed high-field peaks at 17.6 p.p.m. (rhamnose CH_3) and 25.5 p.p.m. (pyruvate CH_3); in the anomeric region only five signals could be distinguished, at 101.4, 101.8, 102.7, 103.9, and 105.1 p.p.m. Two other signals were attributable to the C-6 carbon atoms of hexoses and a further two signals, at 173.2 and 174.2 p.p.m., were due to the carboxyl groups of the pyruvate acetal and the glucuronic acid. It is thus clear that ^1H and ^{13}C spectra provide complementary information[7]. The two sets of data show the K36 polysaccharide to be composed of three residues of rhamnose, two of hexose, one of uronic acid, and one pyruvate acetal group, and to have three α- and three β-glycosidic linkages.

Paper chromatography of an acid hydrolysate of the polysaccharide showed the presence of glucose, galactose, glucuronic acid, and rhamnose. Carboxyl-reduced[8] K36 was hydrolysed, and the presence of D-glucose, D-galactose and L-rhamnose in the ratio of 2:1:3 was confirmed by gas–liquid chromatography (g.l.c.) of their alditol acetates. The configurations of the glucitol hexaacetate and rhamnitol pentaacetate, as determined by circular dichroism (c.d.)[9], were shown to be D and L respectively, and that of the galactose was also determined to be D, as based on the c.d. of the partially methylated 2,4,6-tri-*O*-methyl-D-galactitol triacetate obtained during methylation studies (see later).

Methylation of original and autohydrolyzed polysaccharides. — Methylation[10] of K36 polysaccharide and subsequent reduction with lithium aluminum hydride, hydrolysis, derivatization as alditol acetates and g.l.c.–m.s. analysis[11,12] indicated that K36 is composed of a hexasaccharide repeating unit (Table II, column I). The presence of a mono-*O*-methylrhamnose residue is attributable to a branch point, but

TABLE II

METHYLATION ANALYSIS OF ORIGINAL AND DEGRADED K36 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	T[b]			*Mole %*[c,d]		
	Column A[e] *(HIEFF-1B)*	*Column B*[f] *(OV-17)*	*Column C*[g] *(OV-225)*	I	II	III
3,4-Rha	0.87	0.79	0.90	16.6	17.0	20.9
2,4-Rha	1.00	0.91	0.96	16.3	19.7	20.6
2,3,4,6-Glc	1.00	1.00	1.00		10.6	8.4
4-Rha	1.45	1.24	1.40	16.9	20.0	19.0
2,4,6-Gal	1.77	1.59	1.68	20.3	25.0	17.5
2,3,4-Glc	1.77	1.59	1.77			6.2
2,3-Glc	2.32	2.00	2.56	30.0	7.5	7.4

[a]3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol etc. [b]Retention time relative to the alditol acetate derivative of 2,3,4,6-tetra-*O*-methyl-D-glucose. [c]I, original polysaccharide, column A; II, degraded polysaccharide (see text for details), column B; III, degraded polysaccharide, column C. [d]Values corrected by using "Effective carbon response" molar-response factors[13]. [e]Programme; 165° for 8 min, and then 2° per min to 200°. [f]Programme; 175° for 8 min and then 2° per min. to 210°. [g]Programme; 180° for 8 min and then 2° per min to 200°.

the absence of any terminal residue (either tetra-*O*-methylhexose or tri-*O*-methylrhamnose) indicates that the pyruvate acetal must be present on a terminal, side-chain sugar.

A sample of *Klebsiella* K36 polysaccharide was autohydrolyzed at pH 2.2 overnight. A p.m.r. spectrum of the recovered, non-dialyzable polymer showed the absence of pyruvate acetal and of reducing protons, consistent with a product having still a high degree of polymerization. In the anomeric region, the signals observed had the same chemical shifts as for the undergraded K36 polysaccharide, but the signal at τ 5.13 ($J_{1,2}$ 7 Hz) did not integrate as a whole-unit proton; the reason for this is explained later.

Methylation of this degraded material and reduction with lithium aluminum hydride, followed by derivatization and g.l.c.–m.s. analysis (Table II, columns II, III), gave a complex mixture that could not be resolved completely on any one g.l.c. column. Analysis of the mixture on columns of OV-17, OV-225 and HIEFF-1B (see Fig. 1) enabled all peaks to be resolved. The presence of a terminal glucosyl group demonstrates that the pyruvate acetal is attached to this residue in the original polysaccharide. The relative molar ratios of 2,3,4-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose indicate that the D-glucuronic acid residue is also present in the side chain, and that the terminal glucosidic bond undergoes partial cleavage (~40%) during the autohydrolysis; this interpretation is consistent with the p.m.r. spectrum.

Periodate oxidation. — Periodate oxidation[14] of K36 polysaccharide proceeded rapidly with 3 mol. of periodate being consumed per mol. of repeating unit after 25 h (theoretical = 3 mol.). The consumption gradually increased to a value of 4.2 mol. per mol. of repeating unit during a further 48 h. By p.m.r. spectroscopy of the sodium

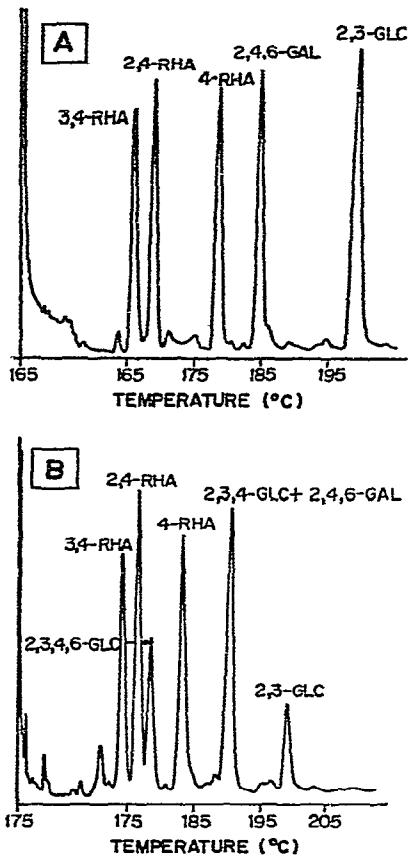


Fig. 1. A. — G.l.c. trace (HIEFF-1B) of partially methylated alditol acetates obtained from fully methylated *Klebsiella* K36 following reduction by lithium aluminum hydride, hydrolysis, and derivatisation (see text for details). Peak areas and column conditions are given in Table II. Code: 3,4-Rha ≡ 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol, etc.
B. — G.l.c. trace (OV-17) of partially methylated alditol acetates obtained from fully methylated degraded *Klebsiella* K36 (see text for details). Peak areas and column conditions are given in Table II. Code: same as for trace A.

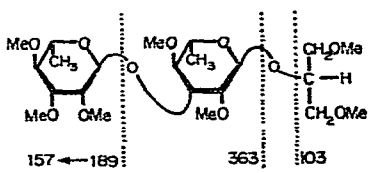
borohydride-reduced, periodate-oxidized product, it was evident that part of this slow increase was due to the hydrolysis of the pyruvate acetal at the reaction pH of 3.0.

Smith hydrolysis[15] of the sodium borohydride-reduced, periodate-oxidized product (3 mol. uptake) by using 0.5M trifluoroacetic acid for 16 h at room temperature, and subsequent reduction again with sodium borohydride, yielded a mixture that was resolved by gel chromatography (Bio-Gel P-4). A significantly large amount of polymeric material was eluted shortly after the void volume, followed by an oligosaccharide derivative, oligomer **1**. This incomplete hydrolysis, upon using trifluoroacetic acid to effect the Smith degradation, of a polyol derived from these capsular polysaccharides, was observed first[16] during studies on K18, and has been

noted subsequently[17] in the case of K55. There are earlier reports[18] on incomplete hydrolysis attributed to uronic[19,20] acid or ester sulfate[21] groups, but it is particularly surprising in the three bacterial examples cited that in each instance it is an oxidized rhamnose residue that is resistant to the acid conditions used.

Oligomer **1** ($[\alpha]_D$ −60°) was obtained pure and, after hydrolysis and paper chromatography, was shown to contain rhamnose, galactose, and glycerol. P.m.r. spectroscopy of **1** indicated (Table I) the presence of three nonreducing anomeric signals; one β-hexose signal at τ 5.34 ($J_{1,2}$ 7.5 Hz) attributable to the D-galactose residue, and two further signals at τ 5.01 ($J_{1,2}$ 1.8 Hz) and 4.92 ($J_{1,2}$ 1.8 Hz) attributable to two α-L-rhamnose groups. ^{13}C n.m.r. spectroscopy of **1** showed 21 carbon signals, indicating that, in addition to the three hexose sugars, a three-carbon fragment (glycerol) was present on the "reducing" terminus. Attempted acetolysis of **1** in an effort to remove the small aglycon (glycerol) preferentially[22] was unsuccessful.

Smith periodate degradation of **1** and gel chromatography of the resulting material yielded oligomer **2** ($[\alpha]_D$ −67°), which gave only rhamnose and glycerol on hydrolysis. In the p.m.r. spectrum, the only anomeric signals corresponded to two α-L-rhamnose anomeric protons (non-reducing), at τ 4.94 ($J_{1,2}$ 2 Hz) and 5.03 ($J_{1,2}$ 2 Hz). Methylation analysis of **2** gave 2,3,4-tri-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose (the volatile 1,3-di-*O*-methylglycerol derivative was lost under diminished pressure during processing). Mass spectra of fully methylated **2** by electron impact (e.i.), chemical ionisation (c.i.), and field desorption (f.d.) modes[23] also confirmed the presence of two 6-deoxy hexoses and a glycerol moiety. A parent peak at *m/e* 482 was obtained in the f.d. spectrum. The c.i. spectrum also gave a parent peak (M − 1) at *m/e* 481, and the origin of other major peaks in the spectrum is shown as follows.



The structure of **2** is thus established as:

α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-glycerol, (2)

and that of **1** as:

β-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-glycerol. (1)

The methylation analysis of the original and autohydrolyzed polymers indicates that the side chain is the disaccharide group β-D-Glc-(1→4)-D-GlcA→ and it is the glucosyl group that has the 4,6-*O*-(1-carboxyethylidene) substituent. It is thus now possible to deduce that the glycerol fragment in **1** originated from a

2-substituted L-rhamnose residue. Hence. the backbone of K36 is established as:

→3)-β-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ .

Attachment of side chain. — Having determined the sequence of the tetrasaccharide backbone, the nature of the side chain, and that the sugar on which branching occurs yields 4-*O*-methyl-L-rhamnose in the methylation analysis, the only remaining problem is to identify which of the three rhamnose residues constitutes the branch point. This was achieved by examination of the products of partial hydrolysis.

Characterization of oligosaccharides from partial hydrolysis. — Partial hydrolysis of K36 polysaccharide (2M trifluoroacetic acid, 3 h, 95°) and separation of acidic and neutral components by ion exchange yielded a mixture of acidic oligomers that were separated by gel-permeation chromatography (Bio-Gel P-4). Three pure oligomers (**3**, **4**, **5**) were obtained in decreasing quantities.

Compound *3*, $[\alpha]_D$ −12° was shown by ^1H and ^{13}C n.m.r. spectroscopy (see Table I) to contain one nonreducing, β-anomeric signal and two reducing anomeric signals attributable to a 6-deoxyhexose. Methylation, reduction with lithium aluminum hydride, hydrolysis, and g.l.c. of the alditol acetates gave 2,3,4-tri-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-L-rhamnose. The structure of **3** in thus established as:

β-D-Glc*A*p-(1→2)-L-Rha*p* . (3)

Compound **4**, $[\alpha]_D$ −31.3°; p.m.r. (see Table I) indicated in the anomeric region the presence of one nonreducing β-glycosyl signal, one nonreducing α-glycosyl signal, and two signals due to a reducing 6-deoxyhexose. Methylation of **4** and subsequent reduction with lithium aluminum hydride, hydrolysis, and derivatisation as alditol acetates gave 2,4-di-*O*-methyl-L-rhamnose, in addition to the two components obtained from **3**. Compound **4** is therefore established as:

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

Compound **5** was obtained in only very small quantity. P.m.r. (see Table I) indicated the presence of two nonreducing, β-anomeric signals, one nonreducing, α-anomeric signal, and two signals attributable to a reducing 6-deoxyhexose. Methylation analysis (as described for compounds **3** and **4**) yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-L-rhamnose, and 3,4-di-*O*-methyl-L-rhamnose in equal proportions. Having established the structure of **4** it is possible to assign the structure of **5** as:

β-D-Glc*p*-(1→4)-β-D-Glc*A*p-(1→2)-α-L-Rha*p*-(1→3)-L-Rha*p* . (5)

Partial hydrolysis of K36 with 0.3M trifluoroacetic acid for 3 h at 95° was also performed. After separation of acidic and neutral components, the acidic components were separated by gel-permeation chromatography. Successful resolution over the entire range of oligomers, that is, residual polymeric material to monomers, was not

achieved initially but, after removal of most "polymeric" fractions and re-chromatography of the smaller oligomers (Bio-Gel P-4), good separation was achieved. In addition to compounds **3** and **4**, a small amount of another oligomer **6** was obtained.

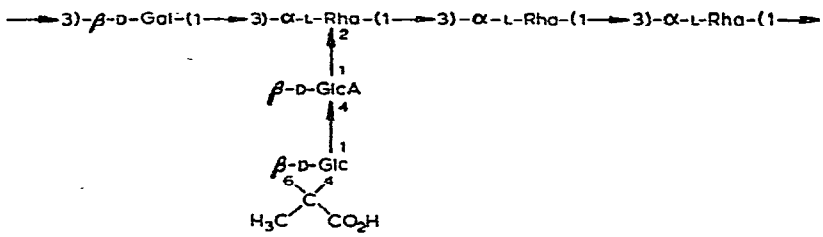
The p.m.r. spectrum of **6** indicated the presence of six sugar components. Reduction of **6** with $NaBD_4$ and subsequent methylation gave two components (R_F 0.73 and R_F 0.57) that were separated on silica gel (92:8 ethyl acetate–ethanol). The component having R_F 0.73 was analysed and established as being identical to the permethylated derivative of **5**.

The compound having R_F 0.57 was analysed similarly and yielded 2,3,4-tri-*O*-methyl-D-glucose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 4-*O*-methyl-L-rhamnose, and 1,2,4,5-tetra-*O*-methyl-L-rhamnitol. Again, the latter component was monodeuterated at C-1. The structure of this component (**6b**) is therefore shown to be:

$$\beta\text{-D-Gal}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow3)\text{-L-Rha}p$$
$$\begin{matrix}3\uparrow\\1\end{matrix}$$
$$\beta\text{-D-GlcA}p\ . \qquad \textbf{(6b)}$$

Hence the isolated oligomer **6** was not one pure hexasaccharide as originally thought, but was a mixture of two tetrasaccharides that co-eluted in gel-permeation chromatography and had identical R_F values on paper chromatography with the two solvent systems used.

The isolation of five different, yet compatible, oligomers from *Klebsiella* K36 is in agreement with the structure shown. On partial acid hydrolysis the cleavage



pattern of this polysaccharide is markedly influenced, as indicated by the very low yields of any oligomers having the galactosyl bond intact, by the lability of the 3-linked galactosyl bond.

Of the *Klebsiella* structures known to this time, K28, having a six-sugar repeating unit with a 2-unit side chain of a terminal glucose and a nonterminal glucuronic acid residue[24], is the closest analogue to K36.

2-substituted L-rhamnose residue. Hence. the backbone of K36 is established as:

→3)-β-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ .

Attachment of side chain. — Having determined the sequence of the tetrasaccharide backbone, the nature of the side chain, and that the sugar on which branching occurs yields 4-*O*-methyl-L-rhamnose in the methylation analysis, the only remaining problem is to identify which of the three rhamnose residues constitutes the branch point. This was achieved by examination of the products of partial hydrolysis.

Characterization of oligosaccharides from partial hydrolysis. — Partial hydrolysis of K36 polysaccharide (2M trifluoroacetic acid, 3 h, 95°) and separation of acidic and neutral components by ion exchange yielded a mixture of acidic oligomers that were separated by gel-permeation chromatography (Bio-Gel P-4). Three pure oligomers (**3**, **4**, **5**) were obtained in decreasing quantities.

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β-D-GlcA*p*-(1→2)-L-Rha*p* . (3)

Compound **4**, $[\alpha]_D$ −31.3°; p.m.r. (see Table I) indicated in the anomeric region the presence of one nonreducing β-glycosyl signal, one nonreducing α-glycosyl signal, and two signals due to a reducing 6-deoxyhexose. Methylation of **4** and subsequent reduction with lithium aluminum hydride, hydrolysis, and derivatisation as alditol acetates gave 2,4-di-*O*-methyl-L-rhamnose, in addition to the two components obtained from **3**. Compound **4** is therefore established as:

β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-L-Rha*p* . (4)

Compound **5** was obtained in only very small quantity. P.m.r. (see Table I) indicated the presence of two nonreducing, β-anomeric signals, one nonreducing, α-anomeric signal, and two signals attributable to a reducing 6-deoxyhexose. Methylation analysis (as described for compounds **3** and **4**) yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-L-rhamnose, and 3,4-di-*O*-methyl-L-rhamnose in equal proportions. Having established the structure of **4** it is possible to assign the structure of **5** as:

β-D-Glc*p*-(1→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-L-Rha*p* . (5)

Partial hydrolysis of K36 with 0.3M trifluoroacetic acid for 3 h at 95° was also performed. After separation of acidic and neutral components, the acidic components were separated by gel-permeation chromatography. Successful resolution over the entire range of oligomers, that is, residual polymeric material to monomers, was not

for 6 h at 30,000 r.p.m. in a Beckman T4 zonal rotor. After this time, the clear supernatant was separated and concentrated to ~400 ml. Crude polysaccharide, obtained by precipitation into ethanol (2 l), was redissolved in 400 ml of water, precipitated with 10% Cetavlon, redissolved in 4M sodium chloride (500 ml) and then dialysed against tap water overnight. Lyophilization of this solution yielded 10 g of the polysaccharide, $[\alpha]_D$ −56° (*c* 3.4, water).

Carboxyl reduction of the native polysaccharide. — A sample of the polysaccharide was reduced by the procedure of Taylor and Conrad[8]. Two treatments were required to achieve complete reduction, as estimated by titration.

Sugar and methylation analysis of native polysaccharide. — Hydrolysis of a sample (20 mg) of carboxyl-reduced K36 with 2M trifluoroacetic acid overnight at 95° and subsequent derivatisation of the liberated monosaccharides as alditol acetates gave L-rhamnitol pentaacetate, galactitol hexaacetate (m.p. 168°), and D-glucitol hexaacetate (m.p. 99°) in the ratio of 2.98:1.00:2.01 (Column D; programmed at 120° for 8 min and then at 1°/min to 200°). Circular dichroism of the rhamnitol pentaacetate showed ε_{213}^{MeCN} −1.12 and the glucose hexaacetate ε_{213}^{MeCN} +1.83. Circular dichroism of 2,4,6-tri-*O*-methyl-D-galactitol triacetate, obtained by total hydrolysis of methylated K36, was shown to be positive. Methanolysis of K36 polysaccharide overnight in refluxing 3% methanolic hydrogen chloride yielded methyl α-L-rhamnopyranoside (m.p. 108–110°).

Methylation of K36 under the Hakomori conditions, followed by subsequent Purdie[26] treatment, yielded a product that showed no hydroxyl absorption in the i.r. spectrum. This material was reduced overnight with lithium aluminum hydride in refluxing tetrahydrofuran and, following hydrolysis with 2M trifluoroacetic acid for 8 h at 95°, the mixture was reduced with sodium borohydride and then acetylated. G.l.c. (column A; programmed at 160° for 4 min and then at 1° per min to 190°) and m.s. of the collected components allowed the assignments given in Table II, column I.

Methylation analysis of degraded polysaccharide. — Autohydrolysis at pH 2.2 for 16 h at 95° gave a very soluble polymer from K36 which, after dialysis overnight against running tap-water and subsequent lyophilization, was examined by p.m.r. (D_2O, 90°). The degraded material was methylated and subsequently reduced overnight with lithium aluminum hydride in refluxing tetrahydrofuran. Hydrolysis, reduction, and acetylation, as performed on the native polysaccharide, followed by g.l.c. analysis, showed the presence of seven components (see Table I, columns II and III). A column of OV-17 (column B; programmed at 170° for 4 min and then at 1°/min to 190°) achieved complete separation of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-L-rhamnose, and the separation of 2,3,4-tri-*O*-methyl D-glucose and 2,4,6-tri-*O*-methyl-D-galactose was achieved on OV-225 (column C; programmed at 170° for 8 min and then at 2° per min to 200°).

Periodate oxidation. — Capsular polysaccharide (1 g) was dissolved in 250 ml of a solution of sodium periodate (0.05M) and sodium perchlorate (0.2M)[27]. The pH of this solution was 2.7. The solution was kept in the dark at 4°. After 20 h, 3 mol.

of periodate per repeating unit had been consumed (theoretical = 3), rising to 4.5 mol. after 172 h.

Following the addition of ethylene glycol (2 ml) after 25 h, reduction with sodium borohydride, dialysis, deionisation, lyophilization, and removal of borate, the product was hydrolysed (0.5M trifluoroacetic acid) overnight at room temperature. After removal of the acid and subsequent reduction with sodium borohydride, the material (2 × 175 mg) was applied to the top of a Bio-Gel P-4 column (2.5 × 120 cm). Elution with water gave a range of polymeric products appearing soon after the void volume (Blue Dextran), followed by pure oligomer **1**, which had R_{Glc} 1.0 (solvent A).

Oligomer **1**, 35 mg, had $[\alpha]_D$ −60.7° (*c* 2.5, water) and was examined both by p.m.r. (D_2O, 90°) and ^{13}C n.m.r. spectroscopy. Hydrolysis of **1** (0.5M trifluoroacetic acid, 4 h, 95°) showed by paper chromatography the presence of galactose, rhamnose, and glycerol.

Periodate oxidation of **1** (12 mg) with 0.05M $NaIO_4$ overnight at 4° and subsequent addition of ethylene glycol, reduction with sodium borohydride, Smith hydrolysis (0.5M trifluoroacetic acid overnight at room temperature), and reduction with sodium borohydride yielded material that was applied to the top of a Bio-Gel P-4 column (2.5 × 120 cm). Component **2** was isolated (4 mg) having R_{Glc} 0.73 (solvent A) and $[\alpha]_D$ −67° (*c* 0.35, water). P.m.r. spectroscopy of **2** showed anomeric signals at τ 4.94 (1 H, $J_{1,2}$ 2 Hz) and 5.03 (1 H, $J_{1,2}$ 2 Hz). Hydrolysis of a small portion of oligomer **2** gave only L-rhamnose and glycerol by paper chromatography. Methylation of **2** yielded 2.5 mg of the permethylated derivative having R_F 0.38 on t.l.c. (ethyl acetate). M.s. of this derivative in the f.d. mode gave major peaks at *m/e* 481, 482, 483, and 484 corresponding to M−1, M, M+1 and M+2, respectively. Chemical ionisation (methane) m.s. of this same derivative gave a spectrum that showed, among other peaks, the following fragments (relative intensities in parentheses): 88 (11), 99 (12), 125 (27), 127 (29), 129 (10), 149 (10), 159 (11), 189 (100), 190 (12), 205 (49), 233 (9), 363 (49), 364 (10), 391 (18), and 481 (2). The peak at 391 is attributed to an impurity (dioctyl phthalate). Hydrolysis of the permethylated derivative of **2** (2M trifluoroacetic acid, 6 h, 90°) and subsequent derivatisation yielded 2,3,4-tri-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose in equal amounts. The dimethyl glycerol fragment was too volatile to be isolated during processing.

Partial hydrolysis of polysaccharide. — K36 (1 g) was hydrolysed for 3 h at 95° in 2M trifluoroacetic acid. After removal of the acid by evaporation of several portions of water from the product, the mixture was neutralised with sodium hydroxide and then applied to the top of a column (2 × 20 cm) of Dowex 1-X2 (formate form). The column was then washed with 1 l of distilled water. The acidic components were eluted with 10% formic acid and, after evaporation to dryness, the residue weighed 350 mg. Paper chromatography (solvent B) showed that no neutral monosaccharides were present in the mixture.

Gel chromatography (Bio-Gel P-4; 2 × 175 mg) of the neutralised (sodium hydroxide) oligomers did not give good separation of the mixture of components. Selected fractions containing small oligomers were rerun (Bio-Gel P-4), yielding pure

aldobiouronic acid **3** (60 mg), aldotriouronic acid **4** (35 mg), and an acidic tetrasaccharide **5** (4 mg).

The aldobiouronic acid **3** (R_{Glc} 1.0, solvent A) showed $[\alpha]_D$ −12° (*c* 1.2, water)[28]. P.m.r. (D_2O, 90°) showed anomeric signals at τ 5.37 (1 H, $J_{1,2}$ 7 Hz), 4.63 (0.6 H, $J_{1,2}$ 1.8 Hz), and 5.17 (0.4 H, singlet). The ^{13}C n.m.r. spectrum showed two anomeric signals; one 104.9 p.p.m. downfield from tetramethylsilane attributable to C-1 of the D-glucuronic acid and another at 93.7 p.p.m. corresponding to the C-1 of the reducing α-L-rhamnose group. No resonances for either the reducing β-L-rhamnose group or C-6 of a hexose[29] were observed. Hakomori methylation of **3** yielded permethylated aldobiouronic acid that had R_F 0.80 (ethyl acetate) in t.l.c. Reduction of this compound with lithium aluminum hydride in tetrahydrofuran yielded a compound having R_F 0.32 in t.l.c. (9:1 ethyl acetate–ethanol). Subsequent hydrolysis (2M trifluoroacetic acid for 6 h at 95°), reduction and acetylation yielded two components as their alditol acetates in 1:1 ratio, corresponding to 2,3,4-tri-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-L-rhamnose (g.l.c. column A). G.l.c.–m.s. confirmed the assignment of the foregoing components.

The aldotriouronic acid **4** (R_{Glc} 0.88, solvent A) showed $[\alpha]_D$ −31° (*c* 1.1, water)[30]. P.m.r. (D_2O, 90°) showed anomeric signals at τ 5.34 (1 H, $J_{1,2}$ 7 Hz), 4.65 (1 H, $J_{1,2}$ 2 Hz), 4.89 (0.6 H, $J_{1,2}$ 1.8 Hz), and τ 5.13 (0.4 H, singlet). The ^{13}C n.m.r. spectrum displayed signals in the anomeric region at 105.2 p.p.m., 101.6 p.p.m. and two signals (94.7 p.p.m., 94.1 p.p.m.) attributable to the α and β reducing anomeric carbon atoms of rhamnose.

Hakomori methylation of **4** yielded permethylated aldotriouronic acid having R_F 0.70 in t.l.c. (ethyl acetate). Reduction with lithium aluminum hydride in tetrahydrofuran yielded the corresponding product having R_F 0.27 in t.l.c. (9:1 ethyl acetate–ethanol). Hydrolysis (2M trifluoroacetic acid for 6 h at 95°), reduction and acetylation yielded the alditol acetates corresponding to 2,3,4-tri-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-L-rhamnose, and 2,4-di-*O*-methyl-L-rhamnose, (g.l.c. column A), in the ratio 1:1:1. G.l.c.–m.s. confirmed the methylation pattern of the foregoing components.

Compound **5** (R_{Glc} 0.64, solvent A) gave a p.m.r. spectrum having anomeric resonances at τ 5.35 (1 H, $J_{1,2}$ 7 Hz), 5.19 (1 H, $J_{1,2}$ 7.5 Hz), 4.63 (1 H, $J_{1,2}$ 2 Hz), 4.89 (0.6 H, $J_{1,2}$ 1.8 Hz), and 5.13 (0.4 H, singlet). Methylation of **5** yielded the permethylated derivative having R_F 0.75 in t.l.c. (92:8 ethyl acetate–ethanol). Reduction of this material with lithium aluminum hydride in tetrahydrofuran gave a compound having R_F 0.37 (t.l.c. 2:1 chloroform–acetone). Hydrolysis, and conversion into alditol acetates (as for compounds **1** and **2**) yielded four components in 1:1:1:1 ratio. G.l.c. (Column B) and g.l.c.–m.s. identified these as being the alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-L-rhamnose, and 2,4-di-*O*-methyl-L-rhamnose. Compound **5** is therefore β-D-Glc*p*-(1→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*.

Partial hydrolysis of K36 (200 mg) with 0.3M trifluoroacetic acid for 3 h at 95° and treatment of the hydrolyzate as described for the preceding partial hydrolysis

yielded, in addition to components **3**, **4** and **5** (above), a component **6** (8 mg; R_{Glc} 0.64, solvent A) that was initially thought to be a hexasaccharide from its p.m.r. spectrum.

Compound **6** was reduced with $LiBD_4$ and then methylated. Two components, having R_F 0.73 (compound **6a**) and R_F 0.57 (compound **6b**) in t.l.c. (92:8 ethyl acetate–ethanol) were obtained. Column separation on silica gel yielded **6a** (4.5 mg) and **6b** (2.5 mg).

Reduction of **6a** with lithium aluminum hydride in tetrahydrofuran gave a compound having R_F 0.37 (t.l.c. 2:1 chloroform–acetone). Sequential hydrolysis, reduction, acetylation, and g.l.c. separation (Column B) showed **6a** to be identical to permethylated compound **5**. G.l.c. and g.l.c.–m.s. gave the alditol acetates corresponding to 2,3-di-*O*-methyl-D-glucose, 2,4-di-*O*-methyl-L-rhamnose, and 2,3,4,6-tetra-*O*-methyl-D-glucose, together with 3-*O*-acetyl-1,2,4,5-tetra-*O*-methyl-L-rhamnitol (some of the latter component, monodeuterated at C-1, was lost during derivatisation.)

Reduction of **6b** with lithium aluminum hydride in tetrahydrofuran gave a component with R_F 0.30 (t.l.c. 2:1 chloroform–acetone). Sequential hydrolysis, reduction, and acetylation, followed by g.l.c./g.l.c.–m.s. (Column A) gave peaks corresponding to the alditol acetates of 2,3,4-tri-*O*-methyl-D-glucose, 4-*O*-methyl-L-rhamnose, and 2,3,4,6-tetra-*O*-methyl-D-galactose, together with 3-*O*-acetyl-1,2,4,5-tetra-*O*-methyl-L-rhamnitol. The ratio of the four components was 1:1:1:0.5, with 50% of the volatile tetra-*O*-methyl-L-rhamnose derivative, monodeuterated at C-1, being lost under vacuum during isolation. Compound **6b** is thus the permethylated derivative of the tetrasaccharide shown.

D-Gal-(1→3)-L-Rha-(1→3)-L-Rhamnitol

$\uparrow^{2}_{1}$

D-GlcA

Analysis of the p.m.r. spectrum of compound **6** (taking into account that the p.m.r. spectrum of compound **5** has already been obtained) allowed the assignment (as shown in Table I) of the resonances and linkages of this branched tetrasaccharide.

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REFERENCES

1 W. NIMMICH, *Acta Biol. Med. Ger.*, 26 (1971) 397–403.
2 W. NIMMICH, *Z. Microbiol. Immunol.*, 154 (1968) 117–131.
3 M. HEIDELBERGER AND W. NIMMICH, *Immunochemistry*, 13 (1976) 67–80.
4 P. A. J. GORIN AND T. ISHIKAWA, *Can. J. Chem.*, 45 (1967) 521–532.
5 Y. M. CHOY, G. G. S. DUTTON, A. M. STEPHEN, AND M. T. YANG, *Anal. Lett.*, 5 (1972) 675–681.
6 G. M. BEBAULT, Y. M. CHOY, G. G. S. DUTTON, N. FUNNELL, A. M. STEPHEN, AND M. T. YANG, *J. Bacteriol.*, 113 (1973) 1345–1347.
7 J. M. BERRY, G. G. S. DUTTON, L. D. HALL, AND K. L. MACKIE, *Carbohydr. Res.*, 53 (1977) C8–C10.
8 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
9 G. M. BEBAULT, J. M. BERRY, Y. M. CHOY, G. G. S. DUTTON, N. FUNNELL, L. D. HAYWARD, AND A. M. STEPHEN, *Can. J. Chem.*, 51 (1973) 324–326.
10 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
11 H. BIÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohydr. Res.*, 5 (1967) 433–440.
12 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
13 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225.
14 G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 357–361.
15 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
16 M. T. YANG, Ph.D. Thesis, University of British Columbia, June 1974.
17 G. M. BEBAULT, G. G. S. DUTTON, K. L. MACKIE, AND A. V. SAVAGE, *Abstr. Pap. Am. Chem. Soc. Meet.*, 172 (1976) CARB-30.
18 G. G. S. DUTTON AND K. B. GIBNEY, *Carbohydr. Res.*, 25 (1972) 99–105.
19 H. O. BOUVENG, *Acta Chem. Scand.*, 19 (1965) 953–963.
20 G. O. ASPINALL, M. J. JOHNSTON, AND R. YOUNG, *J. Chem. Soc.*, (1965) 2701–2710.
21 P. G. JOHNSON AND E. PERCIVAL, *J. Chem. Soc. (C)*, (1969) 906–909.
22 Y. C. LEE AND C. E. BALLOU, *Biochemistry*, 4 (1965) 257–264.
23 J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 42–106.
24 M. CURVALL, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 42 (1975) 95–105.
25 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444–445.
26 E. L. HIRST AND E. PERCIVAL, *Methods Carbohydr. Chem.*, 5 (1965) 287–296.
27 J. E. SCOTT AND R. J. HARBINSON, *Histochemie*, 14 (1968) 215–220.
28 M. T. YANG, unpublished results from this laboratory.
29 L. D. HALL AND L. F. JOHNSON, *Chem. Comm.*, (1969) 509–510.
30 M. CURVALL, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 42 (1975) 73–82.