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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 59

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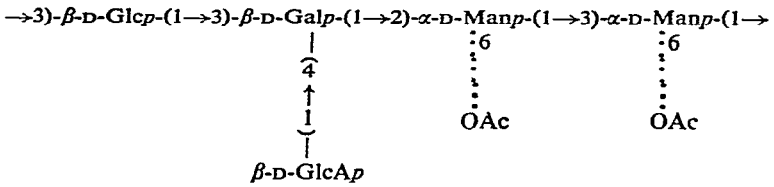
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type 59 has been investigated by methylation analysis, a modified Smith-degradation procedure, and uronic acid degradation followed by oxidation and elimination of the substituents of the oxidized residue. The oligomeric fragments produced by these degradative procedures were isolated and characterized. *O*-Acetyl groups were identified and their position determined. The polysaccharide consists of the following pentasaccharide repeating-unit (dotted lines indicate that only some of the residues carry the *O*-acetyl substituent).



INTRODUCTION

The capsular polysaccharide (K59) from *Klebsiella* type 59 contains[1] mannose, galactose, glucose, and glucuronic acid residues. Several of the *Klebsiella* capsular polysaccharides contain these sugars[1,2], and the structure of one of these, from *Klebsiella* type 7, has been investigated[3]. We now report structural studies of the K59 polysaccharide.

RESULTS AND DISCUSSION

The K59 polysaccharide, isolated as previously described[1], had $[\alpha]_{578}$ +26°. As was evident from its i.r. and n.m.r. spectra, it contained *O*-acetyl groups but no

acetal-linked pyruvic acid residues, another common component of bacterial polysaccharides. Acid hydrolysates of the original and the carboxyl-reduced[4] polysaccharide contained mannose, galactose, and glucose in the proportions 58:11:31 and 41:19:39, respectively. These figures indicate that K59 contains mannose, galactose, glucose, and glucuronic acid residues in the proportions 2:1:1:1. The sugars were isolated from a hydrolysate of the carboxyl-reduced polysaccharide and, from their optical rotations, all belonged to the D series. The content (5.5%) of *O*-acetyl groups, determined as described by Bethge and Lindström[5], corresponds to approximately one *O*-acetyl group per 5 sugar residues.

Methylation analyses[6,7] of the original and carboxyl-reduced polysaccharide (Table I, columns *A* and *B*) strongly suggest that K59 is composed of pentasaccharide repeating-units with one terminal D-glucuronic acid residue, two D-mannose residues linked to O-2 and O-3, respectively, one D-glucose residue linked to O-3, and one branching D-galactose residue, linked to O-3 and O-4. The results also demonstrate that, except for the D-galactose residue, these sugars are pyranosidic. The formation of 2,4,6-tri-*O*-methyl-D-galactose in the uronic acid-degradation experiments (see below) demonstrates that the D-galactose residue is also pyranosidic.

TABLE I

METHYLATION ANALYSES OF ORIGINAL AND MODIFIED *Klebsiella* TYPE 59 CAPSULAR POLYSACCHARIDES

Methylated sugar[a]	T[b]	T[c]	T[d]	*Mole %*[e]						
				A	*B*	*C*	*D*	*E*	*F*	*G*
1,2,4,5,6-Glc[f]	0.33	0.28	0.64	—	—	—	—	12[h]	—	—
2,3,4,6-Man	1.00	0.99	1.00	—	—	—	—	34[i]	—	32[j]
2,3,4,6-Glc	1.00	1.00	1.00	—	19	—	—	—	3	3
3,4,6-Man	1.95	1.82	1.67	27	21	27	34	9	2	—
2,4,6-Glc	1.95	1.82	1.74	25	21	26	34	11	32	28
2,4,6-Man	2.09	1.90	1.86	26	20	25	32	26	32	5
2,4,6-Gal	2.28	2.03	2.01	—	—	23[g]	—	8[g]	—	32[g]
2,6-Gal	3.65	3.14	2.82	22	19	—	—	—	32	—

[a]1,2,4,5,6-Glc = 1,2,4,5,6-penta-*O*-methyl-D-glucitol; 2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, etc. [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column at 170°. [c]As in *b*, but on an OV-225 column at 170°. [d]As in *b*, but on an SP-1000 column at 220°. [e]Polysaccharide: *A*, original; *B*, carboxyl-reduced before methylation; *C*, degraded, trideuteriomethylated polysaccharide (uronic acid degradation, see Text); *D*, degraded and oxidized polysaccharide (see Text); *E*, polysaccharide *D*, treated with base and acid, reduced with sodium borodeuteride, and trideuteriomethylated (see Text); *F*, carboxyl-reduced, periodate-oxidized, and reduced polysaccharide (see Text); *G*, degraded, trideuteriomethylated polysaccharide (Smith degradation, see Text). [f]Part of this volatile ether and derivatives was probably lost during work-up. [g]Trideuteriomethylated at O-4. [h]Monodeuterated at C-1, and trideuteriomethylated at O-1 and O-5. [i]Trideuteriomethylated at O-2. [j]Trideuteriomethylated at O-3.

The methylated sugars were identified by g.l.c.–m.s. of their derived alditol acetates[7–9]. K59 contains three different hexoses, and although the methylation

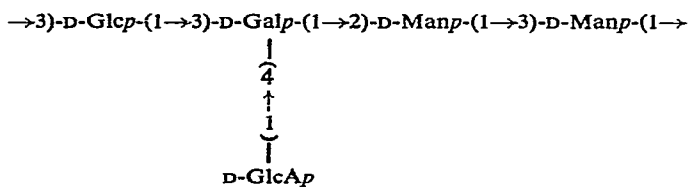
patterns are evident from m.s., this method does not distinguish between stereoisomers. The identification of the components therefore requires some comment. The five components (Table I, column *B*), in equimolecular amounts, were well separated on a SP-1000 capillary column; however, information about relative retention times (*T* values) on this column is lacking in many cases. Therefore, the mixtures were also analysed on ECNSS-M and OV-225 columns. The 2,3,4,6-tetra-*O*-methylhexose, obtained only after carboxyl-reduction, obviously derives from the D-glucuronic acid. All possible three 2,6-di-*O*-methylhexose and also the 2,4,6-tri-*O*-methylhexose derivatives are separated on the ECNSS-M and OV-225 columns, and can be identified from their *T* values. The two components that were not separated by either the ECNSS-M or OV-225 columns were shown by m.s. to be 3,4,6- and 2,4,6-tri-*O*-methylhexose derivatives. The *T* value of the latter compound indicated it was a D-glucose derivative. The former compound could be either a D-glucose or a D-mannose derivative. Since all the D-glucose, but only half of the D-mannose, found in the sugar analysis has been accounted for by the other components, this component must be a D-mannose derivative. Furthermore, the relative retention times of the five components on the SP-1000 capillary column were the same as those of authentic samples, available as pure substances or present in mixtures obtained from methylation analyses of different polysaccharides containing only one sugar.

Fully methylated K59 was subjected to a uronic acid degradation[10], thereby eliminating the terminal D-glucuronic acid residue. The product was remethylated using trideuteriomethyl iodide and hydrolysed, and the product mixture was analysed (Table I, column *C*). All of the 2,6-di-*O*-methyl-D-galactose, obtained in the analysis of the original polysaccharide, had been replaced by 2,4,6-tri-*O*-methyl-D-galactose having a trideuteriomethyl group at O-4, demonstrating that the uronic acid was linked to position 4 of the branching D-galactose residue. From the optical rotations of methylated, original K59 ($[\alpha]_{578}$ +27°) and of the methylated degradation-product ($[\alpha]_{578}$ +27°), it may be concluded that the D-glucuronic acid residue is β-linked (**1**), since this residue must have made a low, negative contribution to the optical rotation.

β-D-GlcA*p*-(1→4)-D-Gal*p*-1→
3
↑
1

The product of the uronic acid degradation is methylated in all positions except O-4 of the D-galactose residues. This material was subjected to controlled degradation by oxidation of the alcohol function, treatment with base, and acid hydrolysis under mild conditions. This degradative method has been tested on methylated polysaccharides having HO-3 unsubstituted[11]. Model experiments with simple oxidized glycosides, containing a carbonyl function at C-4, have demonstrated that the aglycon and the substituents at O-2, O-3, and O-6 are eliminated on this treatment[12]. Most of the aglycon and the substituent at O-3 are liberated during the acid treatment and not

during the treatment with base. The oxidation of the product obtained after uronic acid degradation was performed with chlorine–methyl sulphoxide in dichloromethane[13]. Following the oxidation step in the above procedure, the product contained only three methylated sugars (Table I, column *D*), demonstrating that all of the D-galactose residues had been oxidized. The oxidized product was then treated with sodium ethoxide followed by aqueous acetic acid, reduced with sodium borodeuteride, and remethylated using trideuteriomethyl iodide. A hydrolysate of this product was converted into the alditol acetates, and analysed by g.l.c.–m.s.[7–9] (Table I, column *E*). It is evident that the degradation had not gone to completion, and the presence of some 2,4,6-tri-*O*-methyl-D-galactose having a trideuteriomethyl group at O-4 is difficult to explain. However, the formation of 2,3,4,6-tetra-*O*-methyl-D-mannose having a trideuteriomethyl group at O-2 demonstrates that the oxidized D-galactose residue was linked to this position. The formation of 1,2,4,5,6-penta-*O*-methyl-D-glucitol, trideuteriomethylated at O-1 and O-5, and monodeuterated at C-1 demonstrates that the D-glucose residue was linked to the oxidized residue. Thus, in conjunction with the results of the uronic acid degradation, four of the five sugars in the repeating unit are accounted for and the sequence **2** of sugars is determined. The course of the two consecutive degradations is summarized in Scheme 1, where the correct anomeric configurations determined as described below are also given.



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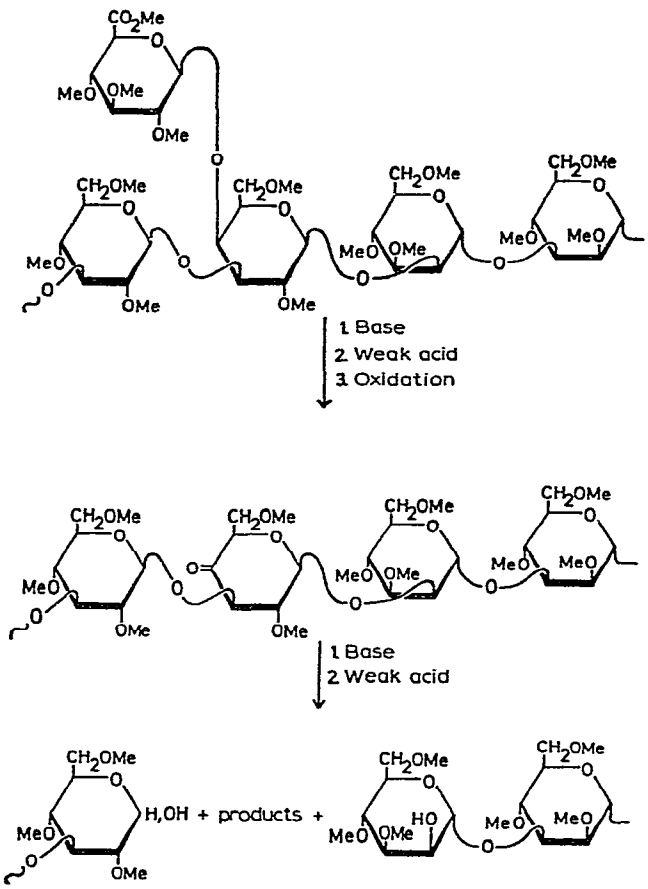
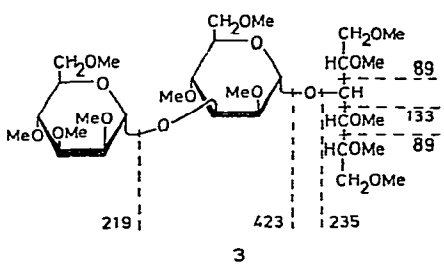
This degradation was repeated on a larger scale and the product, after borohydride reduction and remethylation using methyl iodide, was isolated by high-speed liquid chromatography. On hydrolysis, this trisaccharide derivative (**3**) yielded, as expected, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-mannose, and 1,2,4,5,6-penta-*O*-methyl-D-glucitol. The mass spectrum was also in agreement with the postulated structure[8]. The origins of some pertinent fragments are indicated in the formulae. From the optical rotation ($[\alpha]_{578}$ +60°) and the presence of two signals in the region for anomeric protons in the n.m.r. spectrum [5.10 p.p.m. ($J_{1,2}$ 1.5 Hz) and 4.96 p.p.m. ($J_{1,2}$ 1.9 Hz)], it is inferred that both D-mannose residues are α-linked.

In order to determine the anomeric natures of the D-glucose and the D-galactose residues, carboxyl-reduced K59 was subjected to a modified Smith-degradation, *i.e.*, periodate oxidation, borohydride reduction, methylation, and hydrolysis under mild conditions[14,15]. Analysis of the methylated polyalcohol (Table I, column *F*) showed that periodate oxidation was almost complete. The methylated polyalcohol was hydrolysed under mild conditions and reduced, and a portion was subjected to



Scheme 1



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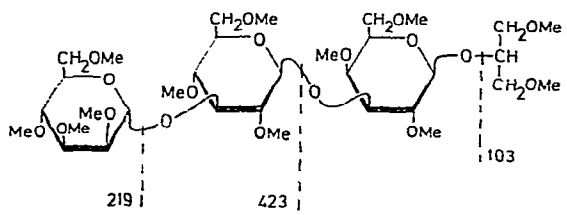
remethylation using trideuteriomethyl iodide. Complete hydrolysis of this material yielded the sugars reported in Table I, column *G*. This analysis shows that essentially all non-cyclic acetals have been hydrolysed without affecting non-oxidized sugar residues. The major part of the degraded and reduced material was remethylated using unlabelled methyl iodide, and the product was isolated by high-speed liquid chro-

matography. Hydrolysis of this product yielded, as expected, equimolecular amounts of 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-galactose. The mass spectrum[8] was also in agreement with that expected for the glycerol glycoside **4**. The origins of some pertinent fragments are depicted in the formulae. The glycoside **4** had $[\alpha]_{578}$ +13° and gave three resonances in the region for anomeric protons of the n.m.r. spectrum [δ 5.31 ($J_{1,2}$ 1.5 Hz), 4.60 ($J_{1,2}$ 7.0 Hz), and 4.41 p.p.m. ($J_{1,2}$ 7.0 Hz)]. Although individual assignment of the two latter signals was not possible, the coupling constants indicate that they derive from the D-glucose and D-galactose residues, which are consequently β-linked. The former signal derives from an α-D-mannose residue, thus confirming the results discussed above. The result of the Smith degradation confirms that the two D-mannose residues are adjacent, and that the D-glucuronic acid residue is linked to O-4 of the branching D-galactose residue, in agreement with results obtained by using the other degradative procedures.



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O-Acetyl groups were located by the method devised by De Belder and Norrman[16]. Free hydroxyl groups in K59 were protected by reaction with methyl vinyl ether under acid catalysis. On trideuteriomethylation of this product, *O*-acetyl groups were replaced by *O*-trideuteriomethyl groups. Part of the product was hydrolysed to completion, and the resulting sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[7–9] (Table II). A considerable amount of 6-*O*-trideuteriomethyl-D-mannitol was obtained, demonstrating that 6-positions of D-mannose are *O*-acetylated in K59. The identification of 6-*O*-trideuteriomethyl-D-mannitol was unambiguous, as the mannitol derivative was well separated from the corresponding D-galactitol and D-glucitol derivatives on g.l.c. The structural significance of the other methyl ethers, present in smaller amounts, is less certain. The formation of these compounds (except 4-*O*-trideuteriomethyl-D-galactose) most probably indicates incomplete reaction with the methyl vinyl ether. The identification of these components was also less certain, as all the necessary reference compounds were not available. However, the assignments were confirmed by experiments reported below. The amount of *O*-acetyl groups found linked to the D-mannose residues is in good agreement with the amount found by *O*-acetyl determination (see above)[5].

In order to locate the *O*-acetyl groups in the individual sugar residues, the acetalated and trideuteriomethylated material was subjected to hydrolysis under mild

TABLE II

SUGARS OBTAINED AFTER SUBSTITUTION OF THE *O*-ACETYL GROUPS BY *O*-TRIDEUTERIOMETHYL GROUPS AND HYDROLYSIS OF *Klebsiella* TYPE 59 CAPSULAR POLYSACCHARIDE

Sugar[a]	T[b]	*Mole %*
6-Man	3.6	31
2-Glc	5.6	8
4-Gal	6.2	4
Man (+trace of 4-Glc)	6.7	28
Gal	7.4	13
Glc	8.4	17

[a]6-Man = 6-mono-*O*-trideuteriomethyl-D-mannose, etc. [b]As in Table I, footnote *c*, but at 190°.

conditions, which removed essentially only the acetal groups, and then remethylated using methyl iodide. This technique has been used before, *e.g.*, in studies of the *Klebsiella* O group 9 lipopolysaccharide[17]. Although it is difficult to determine the exact amount of trideuteriomethyl *versus* methyl groups by m.s., the results (Table III) demonstrate unambiguously that 6-positions of both D-mannose residues are *O*-acetylated in K59. The formation of 2,4,6-tri-*O*-methyl-D-galactose, with and without a trideuteriomethyl group at O-4, demonstrates that uronic acid degradation had taken place. These results indicate that some uronic acid residues are eliminated before the acid-hydrolysis step, as has also been demonstrated in other connections[18]. Removal of uronic acid residues from polysaccharides treated subsequently with methyl vinyl ether, base, and acid under mild conditions has recently been reported[19].

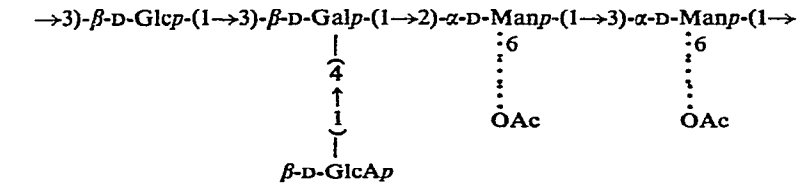
TABLE III

METHYL ETHERS, AFTER ACETALATION, TRIDEUTERIOMETHYLATION, MILD HYDROLYSIS WITH ACID, REMETHYLATION, AND HYDROLYSIS OF *Klebsiella* TYPE 59 CAPSULAR POLYSACCHARIDE

Methylated sugar[a]	T[b]	*Mole %*	*Location of* $-CD_3$	*Analogue with* $-CD_3$ (~%)
3,4,6-Man	1.67	26	O-6	80
2,4,6-Glc	1.74	24	O-2	30
			O-4	20
2,4,6-Man	1.86	26	O-6	60
2,4,6-Gal	2.01	14	O-4	40
2,6-Gal	2.82	10	—	—

[a]As in Table I, footnote *a*. [b]As in Table I, footnote *d*.

As a result of the studies reported above, it is proposed that the capsular polysaccharide from *Klebsiella* type 59 is composed of pentasaccharide repeating-units having the structure **5**. The dotted lines indicate that only some of the residues carry the substituent.



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EXPERIMENTAL

General methods. — These were essentially the same as in the investigation of the *Klebsiella* type 81 capsular polysaccharide[20]. G.l.c. separations were performed on glass columns (180 × 0.15 cm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100/120 mesh) at 170° (for partially methylated alditol acetates) or 190° (for alditol acetates); (*b*) 3% of OV-225 on the same support at 170° or 190° (for partially methylated alditol acetates); (*c*) 3% of OV-1 on the same support (for oligosaccharide derivatives). The quantitative evaluation of mixtures of partially methylated alditol acetates was performed on a wall-coated glass-capillary column (25 m × 0.25 mm) containing SP-1000 as the stationary phase at 220° (LKB-products, Bromma, Sweden). For g.l.c.–m.s. of alditol acetates and derivatives, an OV-225 S.C.O.T. column was used. High-speed liquid chromatography separations were performed on (*a*) two Waters Microporasil columns (30 × 0.5 cm) connected in series and eluted with ethyl acetate, or (*b*) two columns (21 × 0.9 cm) of Merck silica gel 60 (size A) connected in series and eluted with hexane–acetone (2:3).

Isolation of the polysaccharide from Klebsiella *K-type 59 (strain 2212/52).* — This was performed as earlier described[1]. The polysaccharide had $[\alpha]_{578}$ +26° (*c* 0.1, water). In the i.r. spectrum (KBr), a strong absorption was observed at 1735 cm^{-1} (*O*-acyl). In the n.m.r. spectrum of K59 (≃ 80 mg/ml of D_2O at 90°), an absorption from *O*-acetyl was observed at δ 2.18 p.p.m. (broad singlet). The percentages of nitrogen (0.22%) and phosphorus (0.17%) were insignificant.

Determination of O*-acetyl content.* — This was performed on a 10-mg sample of K59, according to the method of Bethge and Lindström[5].

Carboxyl-reduction of the native polysaccharide. — This was performed by the procedure of Taylor and Conrad[4]. Two consecutive treatments were needed to obtain complete reduction.

Sugar and methylation analyses. — These were performed essentially as described before[6–10,21]. The partially methylated and trideuteriomethylated alditol acetates gave mass spectra in which fragments containing a trideuteriomethoxyl group were recognized by the shift of three mass-units. Mannose, $[\alpha]_{589}^{20}$ +14°; galactose, $[\alpha]_{589}^{20}$ +51°; and glucose, $[\alpha]_{589}^{20}$ +36° (all rotations in water, *c* 1) were isolated from a hydrolysate of carboxyl-reduced polysaccharide by paper chromatography.

Uronic acid degradation of methylated polysaccharide. — In a typical experiment, a solution of carefully dried, methylated K59 (210 mg) in a mixture (30 ml) of methyl sulphoxide and 2,2-dimethoxypropane (19:1) was prepared in a serum vial sealed with

a rubber cap. The vial was flushed with nitrogen and kept in an ultrasonic bath for 30 min. Methylsulphinyl anion in methyl sulphoxide (2M, 15 ml) was added, and the solution was sonicated for another 30 min and then kept at room temperature overnight. 50% Aqueous acetic acid (20 ml) was added, the solution was dialysed, and the recovered material was treated with 10% aqueous acetic acid (50 ml) for 1 h at 100°. The reaction mixture was freeze-dried, and the recovered material was purified on a Sephadex LH-20 column (35 × 3 cm) by elution with chloroform–acetone (3:1). The eluate was monitored polarimetrically. The yield of degraded polysaccharide, $[\alpha]_{578}^{23}$ +27° (*c* 0.2, chloroform), was 150 mg. Methylated, original K59 had $[\alpha]_{578}^{22}$ +27° (*c* 0.2, chloroform). Part (6 mg) of the degraded material was subjected to trideuteriomethylation, dialysis, and hydrolysis, and the resulting sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[7–9] (Table I, column *C*).

Oxidation and degradation of degraded polysaccharide. — The oxidation agent[13] was prepared, under anhydrous conditions, at −45° by dropwise addition of methyl sulphoxide (1.8 ml) to a stirred solution of chlorine in dichloromethane (M, 5 ml). Degraded polysaccharide (15 mg of the material described above) in dichloromethane (2 ml) was added dropwise with the aid of a syringe to the stirred, cooled, oxidation mixture. The reaction mixture was kept at −45° with stirring for 6 h and then triethylamine (1.6 ml) was added dropwise. The reaction mixture was warmed to room temperature and then dialysed. The recovered material was purified on a Sephadex LH-20 column (15 × 2 cm) by elution with chloroform–acetone (3:1). The elution was monitored polarimetrically. The recovered material was reoxidized and purified as described above. Part (1/3) of the recovered product was hydrolysed and the resulting sugars were analysed (Table I, column *D*). Another part (2/3) was dissolved in dichloromethane (2 ml), and sodium ethoxide in ethanol (0.8M, 2 ml) was added. The reaction mixture was kept at room temperature for 30 min, neutralised with 90% aqueous acetic acid, and evaporated to dryness. The product was treated with 50% aqueous acetic acid (10 ml) for 1 h at 100°, the solution was evaporated to dryness, and the residue was partitioned between chloroform and water. The resulting material was reduced with sodium borodeuteride (50 mg) in *p*-dioxane–ethanol (3:1, 5 ml) overnight. The reaction mixture was treated with Dowex-50(H^+, prewashed with *p*-dioxane–ethanol) resin, and boric acid was removed by repeated distillations with methanol. This material was trideuteriomethylated, partitioned between chloroform and water, and hydrolysed, and the products were converted into alditol acetates for analysis (Table I, column *E*).

In order to isolate an oligomeric product, a larger amount (135 mg) of degraded K59 was oxidized twice and purified as described above, using appropriate amounts of reagents. The yield of oxidized, purified material was 110 mg. This product was treated with sodium ethoxide (0.4M in ethanol–dichloromethane, 20 ml), neutralised, treated with 50% aqueous acetic acid (30 ml), and reduced with sodium borohydride (500 mg) in *p*-dioxane–ethanol (30 ml) as described above. The resulting material was methylated, and recovered by partition between chloroform and water. The material from the chloroform phase was fractionated by liquid chromatography on

Microporasil columns, yielding permethylated α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-D-glucitol (**3**, 2.5 mg). On t.l.c. (silica gel; ethyl acetate–ethanol, 6:1), **3** had R_F 0.53; and on g.l.c. (OV-1 column at 240°), T_{MEL} 6.4 (retention time relative to permethylated melibiitol). The mass spectrum[8] showed, *inter alia*, the following peaks (relative intensities in brackets): *m/e* 88 (43), 89 (26), 101 (100), 133 (6), 155 (14), 187 (56), 219 (31), 235 (14), and 423 (2). In the n.m.r. spectrum ($CDCl_3$), the signals from the anomeric protons were obtained at δ 5.10 ($J_{1,2}$ 1.5 Hz) and 4.96 p.p.m. ($J_{1,2}$ 1.9 Hz). The compound had $[\alpha]_{578}^{22}$ +60° (*c* 0.3, chloroform). Part of the material was hydrolysed, and the resulting sugars were analysed. 1,2,4,5,6-Penta-*O*-methyl-D-glucitol, 2,3,4,6-tetra-*O*-methyl-D-mannose, and 2,4,6-tri-*O*-methyl-D-mannose were obtained in comparable amounts.

Smith degradation of the polysaccharide. — Carboxyl-reduced polysaccharide (125 mg) was dissolved in 0.1M sodium acetate buffer of pH 3.9 (350 ml), and 0.2M sodium metaperiodate solution (175 ml) was added. The reaction mixture was kept in the dark at 4° for 120 h. Excess periodate was destroyed with ethylene glycol (10 ml), and the mixture was dialysed overnight. The solution was concentrated to ~50 ml, and sodium borohydride (1 g) was added. After stirring overnight at room temperature, the excess of borohydride was decomposed with 50% acetic acid. The solution was then dialysed. Sugar analysis (on ~1% of the material) indicated incomplete oxidation, and therefore the oxidation–reduction procedure was repeated, as described above, and the product was recovered by freeze-drying. Sugar analysis (on ~1% of the material) indicated that essentially all of the periodate-labile sugar residues had been destroyed in the twice oxidized–reduced material. This material was methylated, part (~5%) of it was hydrolysed, and the sugars were analysed (Table I, column *F*). The main part was treated with 90% formic acid (10 ml) for 1 h at 40°. The solution was evaporated to dryness, the residue suspended in water, and the suspension freeze-dried. The recovered product was dissolved in a mixture (20 ml) of *p*-dioxane–ethanol (3:1). Sodium borohydride (200 mg) was added and the reaction mixture was stirred overnight. After conventional work-up, a portion (~5%) was subjected to trideuteriomethylation, hydrolysis, transformation into alditol acetates, and analysis (Table I, column *G*). The main portion was methylated with methyl iodide and partitioned between chloroform and water, and the product was fractionated by liquid chromatography (Merck silica gel columns), yielding permethylated α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→2)-glycerol (**4**, 3.2 mg). On t.l.c. (silica gel; hexane–acetone, 2:3), **4** had R_F 0.62; and on g.l.c. (OV-1 column at 250°), T_{MEL} 18.0. The mass spectrum[8] contained, *inter alia*, the following peaks: *m/e* 88 (69), 101 (100), 103 (13), 155 (29), 187 (61), 219 (27), and 423 (1). In the n.m.r. spectrum, the signals from the anomeric protons were obtained at δ 5.31 ($J_{1,2}$ 1.5 Hz), 4.60 ($J_{1,2}$ 7.0 Hz), and 4.41 p.p.m. ($J_{1,2}$ 7.0 Hz). The compound had $[\alpha]_{578}^{22}$ +13° (*c* 0.3, chloroform). A hydrolysate of the compound contained equimolecular amounts of 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-galactose.

Location of O-*acetyl groups.* — The acetalation (on a 17-mg sample of native K59) and fractionation of the product was performed essentially as previously

described[17]. The acetalated polysaccharide (42 mg) was trideuteriomethylated, half of the product was hydrolysed, and the resulting sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[7–9] (Table II). The other half of the acetalated, trideuteriomethylated material was treated with 50% aqueous acetic acid (5 ml) for 2 h at 100°. The solution was concentrated to dryness, the residue dissolved in water, and the solution freeze-dried. The recovered product was subjected to methylation analysis, using methyl iodide (Table III).

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REFERENCES

1 W. NIMMICH, *Z. Microbiol. Immunol.*, 154 (1968) 117–131.
2 W. NIMMICH, *Acta Biol. Med. Ger.*, 26 (1971) 397–403.
3 S. C. CHURMS, A. M. STEPHEN, AND G. G. S. DUTTON, *S. A. Med. J.*, 47 (1973) 946–947.
4 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
5 P. O. BETHGE AND K. LINDSTRÖM, *Sv. Papperstidn.*, 76 (1973) 645–649.
6 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
7 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
8 J. LÖNNGREN AND S. SVENSSON, *Advan. Carbohyd. Chem. Biochem.*, 29 (1974) 41–106.
9 J. LÖNNGREN AND Å. PILOTTI, *Acta Chem. Scand.*, 25 (1971) 1144–1145.
10 B. LINDBERG, J. LÖNNGREN, AND J. L. THOMPSON, *Carbohyd. Res.*, 28 (1973) 351–357.
11 L. KENNE, J. LÖNNGREN, AND S. SVENSSON, *Acta Chem. Scand.*, 27 (1973) 3692–3698.
12 P. E. JANSSON, L. KENNE, AND S. SVENSSON, unpublished results.
13 E. J. COREY AND C. U. KIM, *Tetrahedron Lett.*, (1973) 919–922.
14 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 361–370.
15 B. LINDBERG, J. LÖNNGREN, W. NIMMICH, AND U. RUDÉN, *Acta Chem. Scand.*, 27 (1973) 3787–3790.
16 A. N. DE BELDER AND B. NORRMAN, *Carbohyd. Res.*, 8 (1968) 1–6.
17 B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohyd. Res.*, 23 (1972) 47–55.
18 Unpublished results from the authors' laboratory.
19 M. CURVALL, B. LINDBERG, AND J. LÖNNGREN, *Carbohyd. Res.*, 41 (1975) 235–239.
20 M. CURVALL, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohyd. Res.*, 42 (1975) 73–82.
21 B. LINDBERG, J. LÖNNGREN, W. NIMMICH, AND J. L. THOMPSON, *Carbohyd. Res.*, 25 (1972) 49–57.