Acta Chemica Scandinavica B 29 (1975) 593—598

Structural Studies of the *Klebsiella* Type 57 Capsular Polysaccharide

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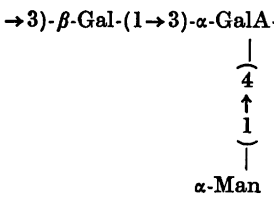
Dedicated to Professor Fritz Micheel on the occasion of his 75th birthday July 3, 1975.

The structure of the capsular polysaccharide from *Klebsiella* type 57 has been investigated. Methylation analysis, uronic acid degradation, modified Smith degradation and graded acid hydrolysis were the principle methods used. Pure oligomeric fragments were isolated using the three methods of degradation and characterized by chemical and physical methods. These studies show the structure to consist of a tetrasaccharide repeating unit (all sugar residues have the D-configuration and are pyranosidic).



The carbohydrate composition of the capsular polysaccharide from *Klebsiella* type 57 was investigated by Dudman and Wilkinson, who found that it contained mannose, galactose, and hexuronic acid residues in the proportions 44:21:28.[1] Nimmich found the same sugars and demonstrated that the acidic component was galacturonic acid.[2] We now report on structural studies of this polysaccharide.

RESULTS and DISCUSSION

The polysaccharide, isolated as previously described,[2] showed $[\alpha]_{589}$ +104°. As was evident from its IR and ^{13}C NMR spectra, it did not contain *O*-acyl groups. Acid hydrolysates of the original and the carboxyl-reduced [3] polysaccharide contained mannose and galactose in the approximate proportions 1.8:1 and 1:1, respectively. The sugars were isolated from a hydrolysate of carboxyl-reduced polysaccharide and from their optical rotations all belonged to the D-series.

Methylation analyses [4,5] of the original polysaccharide, with and without carboxyl-reduction of the fully methylated product, and of the carboxyl-reduced polysaccharide, are given in Table 1, columns C, A, and B, respectively. In these and other methylation analyses to be described later, the methylated sugars were transferred into the alditol acetates and analysed by GLC-MS.[5,6] Unambiguous identification of the partially methylated alditol acetates was based on retention times on GLC and mass spectra. Carboxyl-reduction of the methylated polysaccharide with lithium aluminium deuteride distinguished D-galactitol derivatives originating from D-galacturonic acid residues from those originating from D-galactose residues. Furthermore, 2,6-di-*O*-methyl- and 2-mono-*O*-methyl-D-galactoses were not present in the original polysaccharide but were found in carboxyl-reduced samples (Table 1, columns B and C). This indicated that these sugars originated from D-galacturonic acid. The results of the analyses reported in Table 1, columns A, B, and C, strongly suggest that the polysaccharide is composed of tetrasaccharide repeating units containing a terminal D-mannopyranose residue,

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Table 1. Methylation analyses of original and modified *Klebsiella* type 57 capsular polysaccharides.

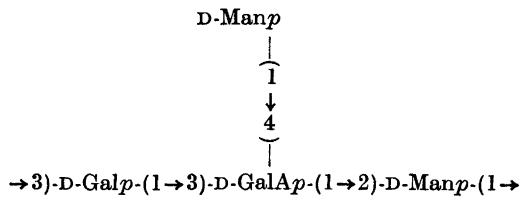
Methylated sugar [a]	T [b]	Mol % [c]						
		A	B	C	D	E	F	G
1,2,4,5,6-Gal [d]	0.42	–	–	–	–	12 [e]	–	–
2,3,4,6-Man	1.00	27	23	25	5 [f]	63 [g]	–	–
2,3,4,6-Gal	1.14	–	–	–	–	–	–	56 [h]
3,4,6-Man	1.67	34	28	29	52	8	–	–
2,4,6-Gal	1.99	39	27	29	43 [i]	17	46	44 [j]
2,6-Gal	2.82	–	22	–	–	–	54	–
2-Gal	5.55	–	–	17 [k]	–	–	–	–

[a] 1,2,4,5,6-Gal = 1,2,4,5,6-penta-*O*-methyl-D-galactitol, 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 that of 2,3,4,6-Glc on a SP-1000 column at 220 °C. [c] A, original polysaccharide; B, polysaccharide, carboxyl-reduced before methylation; C, polysaccharide, carboxyl-reduced after methylation; D, degraded, original polysaccharide (uronic acid degradation); E, degraded, trideuteriomethylated polysaccharide (uronic acid degradation); F, degraded, carboxyl-reduced polysaccharide (Smith degradation); G, degraded, trideuteriomethylated carboxyl-reduced polysaccharide (Smith degradation). [d] Part of this volatile compound and its acetate was probably lost during concentrations. [e] Monodeuterated at C-1 and trideuteriomethylated at O-1 and O-5. [f] Determined from 3 % OV-225 column. [g] ≃90 % triduteriomethylated at O-2. [h] Trideuteriomethylated at O-3. ≃33 % monodeuterated at C-1. [i] ≃80 % trideuteriomethylated at O-4. [k] Dideuterated at C-6.

a D-mannopyranose residue linked to O-2, a D-galactopyranose residue linked to O-3, and a branching D-galactopyranosiduronic acid residue, linked to O-3 and O-4. The latter could, according to the methylation analyses, also be furanosidic and linked to O-3 and O-5, but this was disproved by Smith degradation and partial acid hydrolysis studies, as discussed below. The presence of a tetrasaccharide repeating unit was also supported by the ^{13}C NMR spectrum which showed four well resolved signals in the region for anomeric carbons and one signal in the carboxyl region.[7]

The fully methylated polysaccharide was degraded by treatment first with base and then with acid under mild conditions. This uronic acid degradation has recently been described [8] and applied to other *Klebsiella* capsular polysaccharides.[9–12] The product was reduced with sodium borodeuteride and a part was hydrolysed to completion, giving mainly 3,4,6-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-galactose, deuterium labelled at C-1 indicating that the D-galactose residue becomes a reducing terminal in the degraded material (Table 1, column D). Another part was re-methylated, using trideuteriomethyl iodide. A hydrolysate of this material (Table 1, column E) contained 2,3,4,6-tetra-*O*-methyl-D-mannose with a trideuteriomethyl group at O-2, demonstrating that the D-galacturonic acid residue was linked to this position which was released during the acid treatment. As expected 1,2,4,5,6-penta-*O*-methyl-D-galactitol, with a deuterium atom at C-1 and trideuteriomethyl groups at O-1 and O-5, was also obtained. As evident from the analytical figures in Table 1, columns D and E, the elimination of the substituent at O-4 in the uronic acid residue was almost complete, as was also the cleavage of the glycosidic linkage in the thus modified residue during the subsequent mild acid hydrolysis. The cleavage of the linkage to O-3 was, however, less complete. Nevertheless, it was sufficient for establishing which sugar residue that was linked to this position.

From the result of this degradation the sequence of the sugar residues in the repeating unit (*1*) could be deduced. The course of the degradation is depicted in Scheme 1 and the correct anomeric configurations, determined as described below, are also given.



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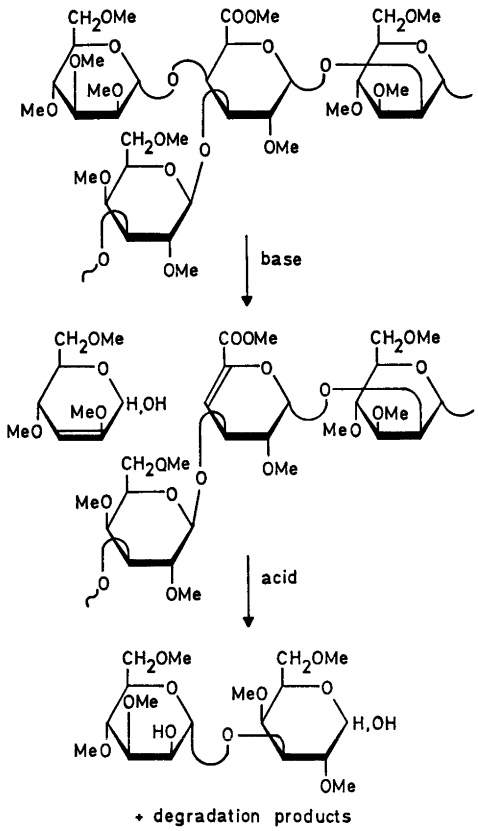


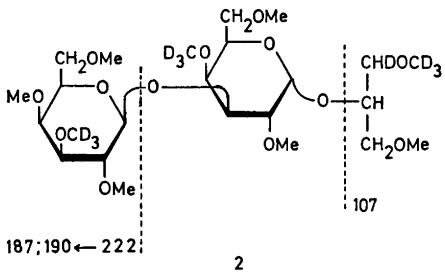
Scheme 1.

In order to determine the anomeric natures of the sugar residues, the polysaccharide was subjected to different degradations and the oligomeric products formed were isolated and characterized. High speed liquid chromatography on silica gel proved to be useful for the isolation of the lipophilic products. These degradations also furnished additional evidence for the proposed sequence (*1*).

The uronic acid degradation was repeated and the oligomeric product isolated after reduction using sodium borodeuteride and remethylation using methyl iodide. MS [6] of the product as well as analysis of its hydrolysate demonstrated that it was the expected nona-*O*-methyl ester of 3-*O*-D-mannopyranosyl-D-galactitol. In the ^{1}H NMR spectrum the anomeric proton appeared at δ 5.09, $J_{1,2}$ 1.5 Hz. The optical rotation, $[\alpha]_{578}$ +15° indicated an α-D-mannopyranosidic linkage. The partial structure α-D-Man*p*-(1→3)-D-Gal is consequently established.

Two acidic components, an aldobiouronic acid and an aldotriouronic acid, were isolated after graded hydrolysis of the polysaccharide. The former was shown to be D-GalA*p*-(1→2)-D-Man by methylation analysis of the carboxyl-reduced disaccharide alditol and by MS of the fully methylated, esterified alditol.[6] The optical rotation of the acid, $[\alpha]_{589}$ +66°, and the ^{1}H NMR spectrum of its alditol with the anomeric proton at δ 5.10, $J_{1,2}$ 3.0 Hz, demonstrate that the D-galacturonic acid residue has the α-configuration. The aldotriouronic acid, investigated by the same methods, proved to be α-D-GalA*p*-(1→2)-α-D-Man*p*-(1→3)-D-Gal. From its optical rotation, $[\alpha]_{589}$ +124°, and the appearance of two anomeric protons in the ^{1}H NMR spectrum of its alditol at δ 5.17, $J_{1,2}$ 3.2 Hz and at δ 5.22, $J_{1,2}$ 1.5 Hz, it was inferred that both the D-galacturonic acid residue and the D-mannose residue have the α-configuration.

The carboxyl-reduced polysaccharide was subjected to a modified Smith degradation procedure, as previously described for other bacterial polysaccharides.[10–13] In this procedure, acetal migration during the mild hydrolysis step is excluded and the positions liberated by this hydrolytic procedure are easily recognized. The product obtained after periodate oxidation–borohydride reduction was fully methylated. Complete acid hydrolysis of this product yielded 2,4,6-tri-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose demonstrating that the periodate oxidation had gone to completion (Table 1, column F). Mild hydrolysis with acid followed by reduction using sodium borodeuteride and remethylation using trideuteriomethyl iodide yielded a product which on hydrolysis yielded 2,3,4,6-tetra-*O*-methyl-D-galactose, with a trideuteriomethyl group at O-3, and 2,4,6-tri-*O*-methyl-D-galactose, with a trideuteriomethyl group at O-4 (Table 1, column G). The latter obviously originates from the D-galacturonic acid residue. GLC-MS [6] of the product also demonstrated the presence of a main component with the MS expected for *2*. Some pertinent fragments are depicted in the formula. The glycerol moiety, with a deuterium atom at C-1 and a trideuteriomethyl group at O-1, obviously derives from the D-mannose residue substituted



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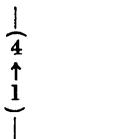
at O-2. The experiment was repeated on a larger scale and the nondeuterated analogue of *2* isolated. It showed $[\alpha]_{578}$ +41° and two anomeric protons in the ^{1}H NMR spectrum at δ 5.25, $J_{1,2}$ 3.5 Hz and δ 4.65, $J_{1,2}$ 7.0 Hz, respectively. It consequently contains one α- and one β-D-galactosidic linkage. As it was already demonstrated that the D-galacturonic acid residue is α-linked, the D-galactose residue is consequently β-linked. The partial structure β-D-Gal*p*-(1→3)-α-D-GalA*p*-(1→2)-D-Man is thereby established.

The terminal D-mannose residue in the repeating unit does not appear in any of the degradation products discussed above. The calculated values for the optical rotation of the polysaccharide, using Hudson's rules of isorotation and assuming this D-mannose residue to be α- or β-linked are $[\alpha]_{589}$ +93° and $[\alpha]_{589}$ +50°, respectively. The actual value (+104°) agrees much better with the former, which strongly indicates that this residue is α-linked. From the combined evidence presented above, it is therefore inferred that the *Klebsiella* type 57 capsular polysaccharide is composed of tetrasaccharide repeating units with the structure *3*.

→3)-β-D-Gal*p*-(1→3)-α-D-GalA*p*-(1→2)-α-D-Man*p*-(1→



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EXPERIMENTAL

General methods. Concentrations were carried out under reduced pressure at bath temperatures which did not exceed 40 °C. For GLC, a Perkin-Elmer 900 instrument fitted with flame-ionisation detector was used. Separations were performed on glass-columns (180 × 0.15 cm) containing (a). 3 % OV-225 on Gas Chrom Q (100/120 mesh) at 170 °C (for partially methylated alditol acetates) or 190 °C (for alditol acetates); (b) 3 % OV-1 on the same support (for permethylated oligosaccharide derivatives). Partially methylated alditol acetates were also separated at 220 °C on a wall-coated glass-capillary column (25 m × 0.25 mm) containing SP-1000 as the stationary phase (LKB-products, Bromma, Sweden). For quantitative evaluation of the GLC a Hewlett-Packard 3370 B integrator was used. For mass spectrometry a Perkin-Elmer 270 instrument (for alditol acetates and derivatives) or a Varian MAT 311-SS 111 MS MS-computer system (for oligosaccharide derivatives) were used. Spectra were recorded at 70 eV, at an ionisation current of 80 μA (Perkin-Elmer) and 1000 μA (Varian MAT) and ion source temperatures of 80 and 250 °C, respectively. For analytical and preparative paper chromatography Whatman No. 1 or 3 MM paper eluted with the solvent system ethyl acetate-acetic acid-water, 3:1:1, was used. The compounds were detected with 3 % *p*-anisidine hydrochloride in ethanol at 120 °C. High speed liquid chromatography was performed with Waters Solvent delivery system 6000 constant flow pump and for monitoring the columns effluent a Waters R401 differential refractometer was used. Separations were performed on two Merck silica gel 60 size A columns connected in series with the solvent system hexane: acetone (2:3). NMR spectra were recorded on Varian XL-100 or HA-100 instruments. The proton-decoupled ^{13}C NMR spectrum was measured at 25.2 MHz on a solution containing ≃100 mg native polysaccharide/ml D_2O. External TMS was used as standard and the spectrum was recorded at 85 °C. An FID acquisition time of 0.8 s was used. The Fourier-transform was computed from 290 000 transients. ^{1}H NMR spectra were recorded by the c.w.- or PFT-mode on $CDCl_3$ solutions using TMS and on D_2O solutions using sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate as internal standard, respectively. IR was recorded on a Perkin-Elmer 257 instrument and optical rotations on a Perkin-Elmer 141 instrument using 100 mm semi-microcells.

Isolation of the polysaccharide from *Klebsiella* K-type 57 (strain 4425/51) was performed as earlier described.[2] The polysaccharide showed $[\alpha]_{589}^{20}$ +104° (*c* 0.3, water). In the IR spectrum (KBr), no significant absorptions around 1735 cm^{-1} (*O*-acyl region) were observed. The percentages of nitrogen (0.78 %) and phosphorus (0.67 %) in the material were insignificant. In the ^{13}C NMR spectrum the signal at δ_{TMS} 176.1 was attributed to the carboxyl carbon and the four signals δ_{TMS} 106.1, 102.5, 101.6, and 96.8 to the four anomeric carbons. Skeleton carbons appeared in the region δ_{TMS} 62.0 – 81.0.

Sugar and methylation analyses and carboxyl-reduction of the methylated polymer were performed essentially as described before.[4,5,8,14] The partially methylated and trideuteriomethylated alditol acetates gave mass spectra in which fragments containing a trideuteriomethoxy group were recognized by the shift of three mass units. Mannose and galactose were isolated by paper chromatography from a hydrolysate of carboxyl-reduced polysaccharide and showed $[\alpha]_{578}^{27}$ +11° (*c* 0.4, water) and $[\alpha]_{578}^{27}$ +43° (*c* 0.4, water), respectively.

Carboxyl-reduction of the native polysaccharide was performed by the procedure of Taylor and Conrad.[3] Three consecutive treatments were needed to obtain complete reduction.

Uronic acid degradation of the methylated polysaccharide. A solution of carefully dried, methylated polysaccharide (23 mg) and toluene-*p*-sulfonic acid (≃0.5 mg) in a mixture (4 ml) of methyl sulfoxide and 2,2-dimethoxypropane (19:1) was prepared in a serum vial which was sealed with a rubber cap. The vial was flushed with nitrogen and kept in an ultrasonic bath for 30 min. Methylsulfinyl anion in methyl sulfoxide (2 M, 2 ml) was added and the solution sonicated for another 30 min. The vial was kept at room temperature overnight. The reaction mixture was added dropwise to ice-water (10 ml) and 50 % aqueous acetic acid (10 ml) was added. The aqueous solution was extracted with chloroform (4 × 15 ml) and the combined chloroform extracts were washed with water (5 × 15 ml) and concentrated to dryness. The product was treated with 10 % aqueous acetic acid (20 ml) for 1 h at 100 °C and then concentrated to dryness and dissolved in a mixture (10 ml) of *p*-dioxane and ethanol (8:3). Sodium borodeuteride (65 mg) was added and the mixture was stirred overnight. The mixture was then treated with Dowex 50 (H^+, prewashed with *p*-dioxane-ethanol), and boric acid was removed by repeated distillations with methanol (4 × 5 ml). Part (1/2) of this material was hydrolysed, and the resulting sugars were analysed, as their alditol acetates, by GLC-MS [5,6] (Table 1, column D). Another part (1/2) of the material was trideuteriomethylated, hydrolysed and the sugars analysed (Table 1, column E).

In order to isolate an oligomeric product a larger amount (200 mg) of methylated polysaccharide was degraded as described above using appropriate amounts of reagents. The degraded material was reduced with sodium borodeuteride in *p*-dioxane-ethanol and remethylated using methyl iodide. The remethylated product was recovered by partition between chloroform and water and purified by two successive injections on the liquid chromatography columns yielding permethylated 3-*O*-α-D-mannopyranosyl-D-galactitol (8 mg). On TLC (silica gel, hexane-acetone, 2:3) the compound showed R_F 0.71 and on GLC (OV-1 column at 200 °C) T_{mel} 0.73 (retention time relative to permethylated melibiitol). The MS [6] showed, *inter alia*, the following peaks (relative intensities in brackets): 88(100), 89(32), 101(84), 133(7), 155(7), 187(34), 219(13), 236(8). The compound showed $[\alpha]_{578}^{24}$ +15° (*c* 0.4, chloroform). In the ^{1}H NMR spectrum the signal from the anomeric proton was obtained at δ 5.09, $J_{1,2}$ 1.5 Hz. Part of the material was hydrolysed, and the sugars analysed, as their alditol acetates, by GLC-MS.[5,6] 2,3,4,6-Tetra-*O*-methyl-D-mannose and 1,2,4,5,6-penta-*O*-methyl-D-galactitol (monodeuterated at C-1) were obtained in comparable amounts in this analysis.

Partial acid hydrolysis of the polysaccharide, isolation and characterization of oligosaccharides. Native polysaccharide (150 mg) was hydrolysed with 0.25 M sulfuric acid (35 ml) for 8 h at 100 °C. After neutralisation with barium carbonate the hydrolysate was fractionated on a Sephadex G-15 column (90 × 2.5 cm) eluted with water. The fractionation was monitored by differential refractometry. The fractions eluted with the volumes expected for mono-, di-, and trisaccharides were further investigated. They were purified by paper chromatography yielding mannose (30 mg), galactose (15 mg), galacturonic acid (5 mg), the aldobiouronic acid (10 mg), and the aldotriouronic acid (13 mg). On paper chromatography the latter two compounds showed R_{man} 0.60 and 0.33, respectively. The aldobiouronic acid showed $[\alpha]_{589}^{20}$ +66° (*c* 0.3, water) and the aldotriouronic acid showed $[\alpha]_{589}^{20}$ +124° (*c* 0.4, water). The aldobio- and triouronic acids were reduced to their alditols with sodium borodeuteride. In the ^{1}H NMR spectrum (D_2O, 25 °C) of the aldobiouronic acid alditol the signal from the anomeric proton was obtained at δ 5.10, $J_{1,2}$ 3.0 Hz. The corresponding figures for the aldotriouronic acid alditol were δ 5.17, $J_{1,2}$ 3.2 Hz and δ 5.22, $J_{1,2}$ 1.5 Hz. Part of the alditol preparations was methylated using methylsulfinyl anion-methyl iodide and recovered by partition between chloroform and water. On GLC (OV-1 column at 200 °C) the permethylated aldobiouronic acid alditol showed T_{mel} 1.2 and the MS [6] contained, *inter alia*, the following fragments: 45(100), 46(26), 88(34), 89(41), 101(68), 201(24), 233(9), 236(16). The corresponding figures for the permethylated aldotriouronic acid alditol were T_{mel} 6.4 (OV-1 column at 230 °C) and 45(96), 46(18), 88(52), 89(27), 90(12), 101(100), 201(60), 233(44), 236(20), 405(5). The permethylated samples were carboxyl-reduced with sodium borodeuteride in *p*-dioxane-ethanol (8:3), hydrolysed, transformed into alditol acetates and analysed by GLC-MS.[5,6] The aldobiouronic acid derivative yielded 1,3,4,5,6-penta-*O*-methyl-D-mannitol (monodeuterated at C-1) and 2,3,4-tri-*O*-methyl-D-galactose (dideuterated at C-6). The aldotriouronic acid derivative yielded 1,2,4,5,6-penta-*O*-methyl-D-galactitol (mono-deuterated at C-1), 3,4,6-tri-*O*-methyl-D-mannose, and 2,3,4-tri-*O*-methyl-D-galactose (dideuterated at C-6).

Smith degradation of the polysaccharide. Car-

boxyl-reduced polysaccharide (29 mg) was dissolved in 0.1 M sodium acetate buffer pH 3.9 (20 ml) and sodium metaperiodate (0.2 M, 5 ml) was added. The reaction mixture was kept in the dark at 4 °C for 120 h. Excess of periodate was destroyed by adding ethylene glycol (1 ml) and the mixture was dialysed overnight. The solution was concentrated to 25 ml and sodium borohydride (300 mg) added. After 9 h at room temperature the excess of borohydride was destroyed by adding 50 % aqueous acetic acid (50 ml) under external cooling with ice-water. The solution was then dialysed. Part of the product (1/15) was subjected to sugar analysis; the only sugar detected in the hydrolysate was galactose. The main part of the periodate oxidized-reduced polysaccharide was methylated by two successive treatments with methylsulfinyl anion-methyl iodide. The methylated material was recovered by dialysis and freeze-drying. Part (1/3) of the material was hydrolysed and the sugars were analysed, as their alditol acetates, by GLC-MS [5,6] (Table 1, column F). Another part (2/3) was treated with 90 % formic acid (10 ml) for 1 h at 40 °C. The solution was evaporated to dryness and the residue was dissolved in a mixture (10 ml) of *p*-dioxane-ethanol (8:3). Sodium borodeuteride (60 mg) was added and the reaction mixture was stirred overnight. The reaction was worked up by treatment with Dowex 50-methanol and subjected to trideuteriomethylation. The methylated material was recovered by partition between chloroform and water. Part of this product was investigated by GLC-MS [6] using an OV-1 column at 210 °C. A peak showing T_{mel} 2.6 was obtained. The MS showed, *inter alia*, the following peaks: 45(100), 49(32), 91(66), 101(40), 104(87), 107(25), 187(47), 190(25), 222(29), 377(3). Another part of the trideuteriomethylated material was hydrolysed and the sugars were analysed, as their alditol acetates, by GLC-MS [5,6] (Table 1, column G).

In order to isolate an oligomeric product a larger amount (120 mg) of carboxyl-reduced polysaccharide was periodate oxidized and reduced with borohydride as described above, using appropriate amounts of reagents. The degraded material was methylated twice, hydrolysed with formic acid and reduced with borohydride in *p*-dioxane-ethanol. The product was finally remethylated using methylsulfinyl anion/methyl iodide. The remethylated product was recovered by partition between chloroform and water and purified by two successive injections on the liquid chromatography column yielding permethylated β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→2)-glycerol (3 mg). On TLC (silica gel, hexane-acetone, 2:3), the compound showed R_F 0.65. The retention time on GLC and the MS (allowing for shifts due to deuterium labelling) were the same as for the deuterated analogue described above. The compound showed $[\alpha]_{578}^{25}$ +41° (*c* 0.1, chloroform). In the ^1H NMR spectrum the signals from the anomeric protons were obtained at δ 5.25, $J_{1,2}$ 3.5 Hz and δ 4.65, $J_{1,2}$ 7.0 Hz. Part of the material was hydrolysed and the sugars analysed, as their alditol acetates, by GLC-MS.[5,6] 2,3,4,6-Tetra-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose were obtained in equimolecular amounts.

Acknowledgements. This work was supported by grants from Statens Naturvetenskapliga Forskningsråd, from Statens Medicinska Forskningsråd (B75-03X-2522-07C), from Stiftelsen Sigurd och Elsa Goljes Minne, and from Harald Jeanssons Stiftelse. Travel grants to one of us (J. P. K) from The Netherlands Organisation for the Advancement of Pure Research (ZWO) and from the European Molecular Biology Organization (EMBO) are gratefully acknowledged. We are indebted to Mr. Jan Rosenblom, Dr. M. J. A. de Bie and Miss T. Volp for measuring the NMR spectra.

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Received December 10, 1974.