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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K50*

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ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* K50 has been determined by using the techniques of methylation, periodate oxidation, partial hydrolysis, and β-elimination. N.m.r. spectroscopy (^1H and ^{13}C) was used to establish the nature of the anomeric linkages. The polysaccharide is comprised of repeating units of the heptasaccharide shown and is one of 19 capsular polysaccharides that are composed of D-glucuronic acid, D-galactose, D-glucose, and D-mannose residues, this is one. It has the only known, "five-plus-two", repeating unit; the structure of the polysaccharide from *Klebsiella* K50 is, therefore, unique in this series.

INTRODUCTION

The capsular polysaccharide from *Klebsiella* serotype K50 is composed of D-glucuronic acid, D-glucose, D-galactose, and D-mannose residues, and is one of those, from almost twenty strains, having the same, qualitative composition[1]. Identity of qualitative analysis, however, bears no relationship to the structural pattern of the polysaccharide, and examination of the capsular material from *Klebsiella* K50 has shown it to have a unique structure in this series. Several capsular polysac-

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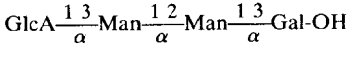
TABLE I

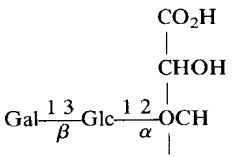
NMR DATA FOR *Klebsiella* K50 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	^{1}*H-N.m.r. data*				^{13}*C-N.m.r. data*	
	δ[b]	$J_{1,2}$ *(Hz)*	*Integral proton*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[c]
GlcA$\xrightarrow[\alpha]{1,3}$Man-OH **$A_1$**	4.93	s	0.4	$\xrightarrow{3}$Man$\underset{\beta}{}$OH		
	5.19	s	0.6	$\xrightarrow{3}$Man$\underset{\alpha}{}$OH		
	5.34	2.5	1.0	GlcA$\underset{\alpha}{}$		
GlcA$\xrightarrow[\alpha]{1,3}Man\xrightarrow[\alpha]{1,2}$Man-OH **$A_2$**	4.93	2	0.3	$\xrightarrow{2}$Man$\underset{\beta}{}$OH	103.4	$\xrightarrow{3}$Man$\underset{\alpha}{}$
	5.08	2	0.7	$\xrightarrow{3}$Man$\underset{\alpha}{}$OH	101.93	GlcA$\underset{\alpha}{}$
	5.32	2.5				
			2.0	GlcA$\underset{\alpha}{}$ $\xrightarrow{2}$Man$\underset{\alpha}{}$	93.81	$\xrightarrow{2}$Man$\underset{\beta}{}$
	5.37	2				



A_3

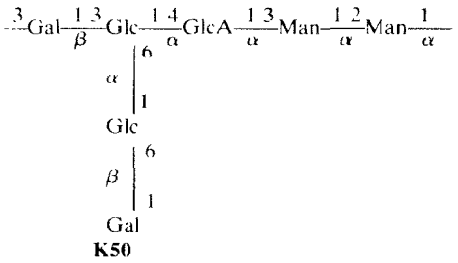
4.65	7	0.8	$\xrightarrow{3}$Gal$_{\beta}$—OH	103.1	$\xrightarrow{3}$Man$_{\alpha}$—
5.08		1	$\xrightarrow{3}$Man$_{\alpha}$—	101.34	GlcA$_{\alpha}$—
5.19			$\xrightarrow{3}$Gal$_{\alpha}$—OH	97.16	$\xrightarrow{3}$Gal$_{\beta}$—OH
			GlcA$_{\alpha}$—	95.41	$\xrightarrow{2}$Man$_{\alpha}$—Gal$_{\beta}$—OH
5.31		2	$\xrightarrow{2}$Man$_{\alpha}$—	95.10	$\xrightarrow{2}$Man$_{\alpha}$—Gal$_{\alpha}$—OH
				93.04	$\xrightarrow{3}$Gal$_{\alpha}$—OH



SH_1

4.65	8	0.9	Gal$_{\beta}$—	104.11	Gal$_{\beta}$—
				99.62	$\xrightarrow{3}$Glc$_{\alpha}$—
5.20	4	1	$\xrightarrow{3}$Glc$_{\alpha}$—		

Table I (continued)

Compound[a]	^{1}*H-N.m.r. data*				^{13}*C-N.m.r. data*	
	Δ[b]	$J_{1,2}$ *(Hz)*	*Integral proton*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[c]
 **K50**	4.50		1	^{3}Gal-β	104.04	Gal-β ^{3}Gal-β
	4.71		1	Gal-β	103.97	^{3}Man-α
	5.02		2	2-Man-α 3-Glc-α	103.17	^{6}Glc-α
	5.27		2	3-Man-α 6-Glc-α	101.30	^{4}GlcA-α
	5.47		1	^{4}GlcA-α	99.16	^{2}Man-α ^{3}Glc-α

^{3}Gal$\frac{1\,3}{\beta}$Glc$\frac{1\,4}{\alpha}$GlcA$\frac{1\,3}{\alpha}$Man$\frac{1\,2}{\alpha}$Man$\frac{1}{\alpha}$ α 6 | 1 Glc 6 **Degraded K50**	4.51	*f*	^{3}Gal$\frac{}{\beta}$	103.83	^{3}Gal$\frac{}{\beta}$ ^{3}Man$\frac{}{\alpha}$
	5.02	*f*	^{2}Man$\frac{}{\alpha}$ ^{3}Gal$\frac{}{\alpha}$	103.16	Glc$\frac{}{\alpha}$
	5.27	2	^{3}Man$\frac{}{\alpha}$ Glc$\frac{}{\alpha}$	101.30	^{4}GlcA$\frac{}{\alpha}$
	5.51	1	^{4}GlcA$\frac{}{\alpha}$	99.16	^{2}Man$\frac{}{\alpha}$
				98.80	^{3}Glc$\frac{}{\alpha}$

[a]For the source of $\mathbf{A_1}$, $\mathbf{A_2}$, $\mathbf{A_3}$, and $\mathbf{SH_1}$, see text. [b]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) [c]The numerical prefix indicates the position in which the sugar is substituted; the α or β, the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing-sugar residue. Thus 3-Gal$\frac{}{\alpha}$ refers to the anomeric proton of a 3-linked galactosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group [d]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from DSS. [e]As in *c*, but for ^{13}C nuclei. [f]See text.

charides based on a heptasaccharide repeating-unit are known, but this is the first instance where such a unit is of the "5 + 2" type. Furthermore, in those capsular polysaccharides in which the uronic acid residue is part of the main chain, only single-unit lateral substituents have previously been found. The polysaccharide of serotype K50 is thus, on two counts, unique among the some sixty structures now known in this series. The experimental evidence on which this structure is based is summarized next.

RESULTS AND DISCUSSION

The isolation and purification of the polysaccharide were achieved as previously described[1,2]. The purified product obtained after Cetavlon precipitation had $[\alpha]_D$ +102°, and was shown by gel-permeation chromatography to be homogeneous ($\bar{M}_w = 3.2 \times 10^6$).

The ^{1}H-n.m.r. spectrum of partially hydrolyzed polysaccharide indicated the presence of seven anomeric protons, corresponding to five α and two β linkages. The ^{13}C-n.m.r. spectrum confirmed these results; see Table I.

Paper chromatography of an acid hydrolyzate of the polysaccharide showed galactose, glucose, glucuronic acid, mannose, and aldobiouronic acid. Determination, as alditol acetates, of the sugars obtained from the carboxyl-reduced polysaccharide[3] gave mannose, galactose, and glucose in the molar ratios of 2.0:2.4:3.0.

Methylation analysis. — Methylation of the K50 polysaccharide, followed by hydrolysis, derivatization as alditol acetates, and g.l.c.–m.s. analysis, gave the values shown in Table II, column I. These results indicated that the polysaccharide consists of a heptasaccharide repeating-unit having a branch on glucose, with galactose as the terminal group. By reduction of the methylated polysaccharide (see col-

TABLE II

METHYLATION ANALYSES OF *Klebsiella* K50 POLYSACCHARIDE AND DERIVATIVES

Methylated sugars (as alditol acetates)	T[a] *Column B*[c]	*Mole %*[b]		
		I[d]	*II*	*III*
2,3,4,6-Man[e]	0.87	—	—	16.5
2,3,4,6-Gal	**1.00**	19.8	14.5	23.5
3,4,6-Man	1.42	12.9	10.1	25.6
2,4,6-Man	1.49	8.7	18.4	
2,4,6-Gal	1.59	15.8	14.5	8.9
2,3,4-Glc	1.66	26.7	15.2	20.9
2,4-Glc	2.52	16.0	11.1	4.4
2,3-Glc	2.59	—	15.5	—

[a]Relative retention time, referred to 2,3,4,6-Gal as 1.00. [b]Values are corrected by use of the effective carbon-response factors given by Albersheim *et al.*[13]. [c]Programmed for 4 min at 160°, and then at 2°/min to 200°. [d]I, Original polysaccharide, II, carboxyl-reduced polysaccharide, III, uronic acid-degraded polysaccharide. [e]2,3,4,6-Man = 3-*O*-ethyl-2,4,6-tri-*O*-methylmannitol

umn II), the proportion of 2,4,6-tri-*O*-methylmannose was increased, and 2,3-di-*O*-methylglucose was formed, indicating that glucuronic acid is linked at O-4, and that it is linked to mannose. Methylation–ethylation analysis of the product obtained by base-catalyzed degradation of the uronic acid showed the presence of 3-*O*-ethyl-2,4,6-tri-*O*-methylmannose derived from ethylation at O-3 of the mannose of the aldobiouronic acid (see column III).

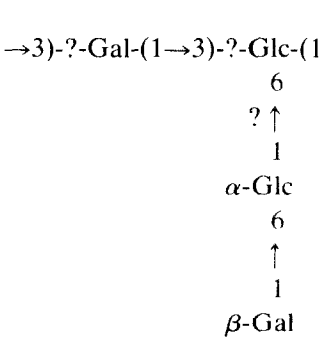
Selective, partial hydrolysis. — Treatment of K50 polysaccharide with very dilute acid, and dialysis of the products against distilled water, afforded a non-dialyzable polymeric material and a dialyzate. The dialyzate contained galactose, as was shown by paper chromatography and g.l.c. (as alditol acetate). Examination of the nondialyzable portion by gel-filtration chromatography showed the polymer to be extensively depolymerized, and this was attributed to the acid lability of the 3-substituted β-D-galactopyranosyl unit, even under these mild conditions. The ^{1}H-n.m.r. spectrum of this material lacked one signal (δ 4.71) corresponding to a β linkage, but integration of the signals for anomeric protons was unsatisfactory, due to the depolymerization (see Table I). Methylation analysis of the nondialyzable material showed that 2,3,4,6-tetra-*O*-methylglucose largely replaced the corresponding galactose compound.

Partial hydrolysis. — Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and the neutral fractions by ion-exchange chromatography. The neutral fraction contained monosaccharides. The acidic fraction contained three acidic oligosaccharides. On the basis of paper chromatography, their n.m.r.-spectral data (see Table I), and methylation analysis (see Table III), the structures of these compounds were shown to be as follows.

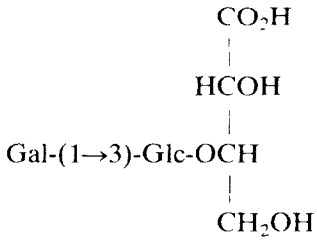
A_1 α-GlcA-(1→3)-Man
A_2 α-GlcA-(1→3)-α-Man-(1→2)-Man
A_3 α-GlcA-(1→3)-α-Man-(1→2)-α-Man-(1→3)-Gal

The aldotetraouronic acid (**A_3**) obtained from partial hydrolysis had previously been isolated from other *Klebsiella* capsular polysaccharides, namely, K21 (ref. 4), K74 (ref. 5), and K26 (ref. 6).

Periodate oxidation. — Periodate oxidation of carboxyl-reduced polysaccharide[3], followed by methylation, Smith hydrolysis, remethylation[7], and hydrolysis, gave a mixture that was found, by g.l.c. analysis of the alditol acetates, to contain derivatives of 2,3,4,6-tetra-*O*-methylmannose, 2,3,4,6-tetra-*O*-methylgalactose, and 2,4,6-tri-*O*-methylglucose, indicating that the galactose in the main chain is 3-linked to glucose, the branch point. The following structure may, therefore be proposed; in it, the configuration of three linkages had yet to be determined.



Smith degradation of the original K50 polysaccharide gave an oligosaccharide, analysis of which showed it to be



and its ^{1}H-n.m.r. spectrum indicated the presence of one α and one β linkage. Because the two monosaccharides therein exhibit similar coupling-constants, no definitive assignment could be made by such spectroscopy. Incubation with a β-D-galactosidase caused cleavage (α-D-galactosidase, negative), thus demonstrating that the in-chain galactose has the β configuration. Six of the seven anomeric linkages, including both of the β signals, having been positively assigned, it followed that the side chain must be attached to the main chain by an α-glycosidic bond.

From the sum of these experiments, the complete structure of the capsular polysaccharide from *Klebsiella* K50 may be written; it is consistent with the qualitative analysis originally reported by Nimmich[1].

EXPERIMENTAL

General methods. — The instrumentation used for infrared and n.m.r. spectroscopy, g.l.c., g.l.c.–m.s., and measurements of optical rotation has been described[8]. Paper chromatography was conducted by the descending method, using Whatman No. 1 paper and the following solvent systems (v/v): (*1*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (*2*) 8:2:1 ethyl acetate–pyridine–water, (*3*) 2:1:1 1-butanol–acetic acid–water. Chromatograms were developed with silver nitrate, or by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol and heating the papers for 5–10 min at 110°. Preparative paper-chromatography was performed by the descending method, using Whatman No. 3 MM paper and solvent

3. Analytical-g.l.c. separations were achieved in stainless-steel columns (1.8 m × 3 mm), with nitrogen as the carrier-gas at a flow-rate of 20 mL/min. The columns used were; (*A*) 3% of SP-2340 on Supelcoport (100–200 mesh), and (*B*) 5% of ECNSS-M and (*C*) 3% of OV-225, each on Gas Chrom Q (100–120 mesh).

Ion-exchange chromatography, for separation of neutral from acidic oligosaccharides, was performed in a column (2 × 28 cm) of Bio-Rad AGl-X2 (formate) resin (200–400 mesh). The neutral fraction was eluted with water, and the acidic, with 10% formic acid.

Preparation and properties of K50 capsular polysaccharide. — A culture of *Klebsiella* K50, obtained from Dr. Ida Ørskov, Copenhagen, was grown as previously described[1,2], and the polysaccharide was purified by one precipitation with Cetavlon. The isolated polysaccharide (3.4 g) had $[\alpha]_D^{25}$ +102° (*c* 0.095, water). The average molecular weight was determined by gel chromatography (courtesy of Dr. S. C. Churms, University of Cape Town, South Africa) to be 3.2×10^6. N.m.r. spectroscopy (^1H and ^{13}C) was performed on the original K50 polysaccharide, but much better spectra were obtained after treatment of the polysaccharide with 0.01M trifluoroacetic acid during 2 h at 95°, in order to lower the viscosity. The principal signals in, and their assignments for, both the ^1H- and ^{13}C-n.m.r. spectra are recorded in Table I.

Hydrolysis of the polysaccharide. — Hydrolysis of a sample (20 mg) of K50 polysaccharide with 2M trifluoroacetic acid (TFA) overnight at 95°, removal of the acid by repeated coevaporation with water, followed by paper chromatography (solvents *1* and *2*), showed D-mannose, D-galactose, D-glucose, D-glucuronic acid, and an aldobiouronic acid. The quantitative, sugar analysis of the carboxyl-reduced polysaccharide was performed as previously described[9]. The alditol acetates of mannose, galactose, and glucose were identified by g.l.c. (column *A*, programmed for 4 min at 195°, and then at 2°/min to 260°), and found to be present in the ratios of 2.0:2.4:3.0.

Circular dichroism measurements. — Glucose was proved to be of the D-configuration by circular dichroism (c.d.) measurements made on glucitol hexaacetate. Galactose and mannose were assigned the D configuration from c.d. measurements made on partially methylated derivatives[10]. Alditol acetates were separated preparatively in column *A* programmed from 195 to 240° at 2°/min. Partially methylated alditol acetates were separated in column *C* programmed from 180 to 230° at 2°/min. Circular dichroism measurements were made by using a Jasco J-500A automatic recording spectropolarimeter with a quartz cell of path length 0.01 cm. The following values of $\Delta\varepsilon_{213}^{MeCN}$ were obtained for the acetates of: glucitol, +1.3; 2,3,4,6-tetra-*O*-methyl-galactitol, +0.7; and 2,4,6-tri-*O*-methylmannitol, +0.64.

Methylation analysis. — The capsular polysaccharide (297 mg) in the free-acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H^+) resin, was dissolved in dry dimethyl sulfoxide (20 mL) and methylated by the Hakomori procedure[11]. The product (316 mg), recovered after dialysis against tap water, had been completely methylated (no hydroxyl absorption in the i.r.

spectrum). It was hydrolyzed, and analyzed as alditol acetates by g.l.c. and g.l.c.–m.s. in columns *B* and *C* (see Table II, column I). Carboxyl reduction of the fully methylated polysaccharide (103 mg) with $LiAlH_4$ in anhydrous oxolane, hydrolysis of the product with 2M trifluoroacetic acid, reduction of the sugars with sodium borohydride, and acetylation of the alditols with 1:1 acetic anhydride–pyridine gave a mixture of partially methylated alditol acetates which was analyzed by g.l.c. and g.l.c.–m.s. in columns *B* and *C* (see Table II, column II).

Uronic acid degradation[17]. — A sample (76 mg) of methylated K50 polysaccharide was dried and then, together with a trace of *p*-toluenesulfonic acid, was dissolved in 19:1 dimethyl sulfoxide–2,3-dimethoxypropane (20 mL) under N_2 in a flask that was then sealed. Dimethylsulfinyl anion (5 mL) was added and allowed to react for 16 h at room temperature, when ethyl iodide (7 mL) was added. The solution was stirred for 1 h, and the methylated, degraded product was isolated by partition between water and chloroform. Hydrolysis and g.l.c. analysis of the alditol acetate derivatives showed the presence of 3-*O*-ethyl-2,4,6-tri-*O*-methylmannose (see Table II, column III).

Selective, partial hydrolysis[6] — A solution of K50 polysaccharide (500 mg) in 0.01M TFA (50 mL) was heated on a steam-bath for 12 h. The acid was removed, and the product was dialyzed against distilled water (1 L), to afford a polymeric material (250 mg). Paper chromatography of the dialyzable fraction showed galactose, confirmed by g.l.c. as galactitol hexaacetate. Methylation analysis of the polymeric material by g.l.c. (column *C*) indicated the presence of 2,3,4,6-tetra-*O*-methylglucose, instead of the 2,3,4-tri-*O*-methylglucose found originally.

Partial hydrolysis. — A solution of K50 polysaccharide (710 mg) in M TFA (50 mL) was heated for 5 h on a steam bath. After removal of the acid by repeated coevaporation with water, an acidic and a neutral fraction were separated on a column of Bio-Rad AGl-X2 ion-exchange resin. The acidic fraction (198 mg) was separated by preparative paper-chromatography (solvent *3*), to give 51.5 mg of a pure aldobiouronic acid (A_1), 19.1 mg of a pure aldotriouronic acid (A_2), and 27.7 mg of a pure aldotetraouronic acid (A_3). Paper chromatography of the neutral fraction showed glucose, galactose, and mannose.

The analyses performed on each oligosaccharide were as follows. (*a*) Paper chromatography. Acidic oligosaccharides were treated with 2M TFA overnight, and the hydrolyzates were examined by paper chromatography (solvents *1* and *2*). (*b*) Sugar analysis. The hydrolyzates were then reduced with $NaBH_4$, and the alditols acetylated with 1:1 acetic anhydride–pyridine. The alditol acetates obtained were analyzed by g.l.c. in column *A* (195° for 4 min, and then 2°/min to 260°). Neutral oligosaccharides were hydrolyzed, and analyzed similarly. (*c*) Methylation analysis. All methylations were conducted by the method of Hakomori[11]. The products were hydrolyzed with 2M TFA, and analyzed (as alditol acetates) by g.l.c. and g.l.c.–m.s. in columns *B* and *C*.

The results obtained for each oligosaccharide are given in Table III, and the n.m.r. data, in Table I.

TABLE III

ANALYSES OF ACIDIC OLIGOSACCHARIDES FROM *Klebsiella* K50 POLYSACCHARIDE

Oligosaccharide	*Paper chromatography*[a]	*Sugar analysis*[b] *(molar proportions)*		*Methylation analysis*[c] *(molar proportions)*	
A_1	GlcA	Man	(0.8)	—	
	Man	Glc (GlcA)	(1)	—	
A_2	GlcA	Man	(1.5)	2,4,6-Man	(0.7)[d]
	Man	Glc (GlcA)	(1)	3,4,6-Man	(1.0)
A_3	GlcA	Man	(1.75)	2,4,6-Gal	(1.0)
	Man	Gal	(1.2)	2,4,6-Man	(0.7)[d]
	Gal	Glc (GlcA)	(1)	3,4,6-Man	(0.8)[d]

[a]Solvents *1* and *2*. [b]As alditol acetates. [c]As alditol acetates; neutral sugars only. [d]Ratios are low, due to incomplete hydrolysis of the glucosyluronic linkage.

Periodate oxidation. — A solution of carbodiimide-reduced[3] K50polysaccharide (46.9 mg) in water (20 mL) was mixed with a mixture (5 mL) of 0.1M $NaIO_4$ and 0.4M $NaClO_4$, and kept in the dark at 4°. After 6 d, ethylene glycol was added. The polyaldehyde was reduced to the polyalcohol with $NaBH_4$, the base neutralized with 50% AcOH, and the solution dialyzed overnight, and freeze-dried, to yield the polyalcohol (50.9 mg) which was methylated by the Hakomori procedure[11]. One-third of the methylated product was hydrolyzed[7] with 50% AcOH for 90 min, and then remethylated; the product was hydrolyzed, and the sugars characterized as alditol acetates by g.l.c. in column *B*. The rest of the material was hydrolyzed with 2M TFA overnight on a steam-bath. Conversion of the partially methylated sugars into alditol acetates, and g.l.c. thereof in column *B*, showed the presence of 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,4-di-*O*-methylglucose in equimolar proportions.

Smith degradation. — A solution of K50 polysaccharide (295 mg) in water (100 mL) was mixed with a mixture (28 mL) of 0.1M $NaIO_4$ and 0.4M $NaClO_4$, and kept in the dark at 4°. After 95 h, ethylene glycol (10 mL) was added. The solution was dialyzed overnight, the polyaldehyde reduced to the polyalcohol with $NaBH_4$ (1 g), the base neutralized with 50% AcOH, and the solution dialyzed, and freeze-dried, to yield polyalcohol (258 mg) which was subjected to Smith hydrolysis with 0.5M TFA for 20 h at room temperature. Paper chromatography (solvent *3*) of the products showed glycerol and three oligosaccharides. The chromatogram obtained indicated that the Smith hydrolysis was not complete, but 49.6 mg of a pure, acidic oligomer (**SH_1**) was isolated, $[\alpha]_D^{25}$ +59° (*c* 0.08, water) and R_{Glc} 0.55 (solvent *3*): n.m.r. data are given in Table I. Sugar analysis of the product (by g.l.c., as the alditol acetates) gave galactose and glucose in the ratio of 1:1. Methylation by the Hakomori method[11] gave the partially methylated alditol acetates corresponding to

2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylglucose.

The acidic product (**SH$_1$**, 2.3 mg) was dissolved in 1 mL of buffer, pH 7.3, and a solution of β-D-galactosidase (1.1 mg, from *E. coli*; Sigma) in 0.1 mL of the same buffer was added. The mixture was incubated for 3 d at 37°; then, the reaction was terminated by addition of 50% acetic acid. The product, isolated by lyophilization, was examined by paper chromatography (solvent 2), which showed the presence of galactose, the identity of which was confirmed by g.l.c. as galactitol hexaacetate.

In a control experiment, compound **SH$_1$** and melibiose were separately incubated with α-D-galactosidase (pH 4). Galactose was released from melibiose, but not from **SH$_1$**.

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