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ANALYSIS OF LINKAGE POSITIONS IN A POLYSACCHARIDE CONTAINING NONREDUCING, TERMINAL α-D-GLUCOPYRANOSYLURONIC GROUPS BY THE REDUCTIVE-CLEAVAGE METHOD*

SALLY A. VODONIK AND GARY R. GRAY†

Department of Chemistry, University of Minnesota, 207 Pleasant St. S.E., Minneapolis, Minnesota 55455 (U.S.A.)

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ABSTRACT

The fate of terminal (nonreducing) α-D-glucopyranosyluronic groups under reductive cleavage conditions was investigated by using the *Klebsiella* K2 (strain NCTC-418) capsular polysaccharide. Treatment of the fully methylated polysaccharide (**1**) with triethylsilane and a mixture of trimethylsilyl methanesulfonate ($Me_3SiOSO_2CH_3$) and boron trifluoride etherate ($BF_3 \cdot Et_2O$) as the catalyst, resulted in complete cleavage of all glycosidic linkages to yield the expected products, namely 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**2**), 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-mannitol (**3**), 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**4**), and methyl 2,6-anhydro-3,4,5-tri-*O*-methyl-L-gulonate. Treatment of **1** with trimethylsilyl trifluoromethanesulfonate ($Me_3SiOSO_2CF_3$) as the catalyst resulted in incomplete cleavage of the glycosidic linkage of the methylated D-glucopyranosyluronic group, to yield 4-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-3-*O*-(methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosyluronate)-D-mannitol (**9**). Reductive cleavage of **1** in the presence of $BF_3 \cdot Et_2O$ resulted in incomplete cleavage of all glycosidic linkages and gave rise to all four dimers (including **9**) that could be formed from a tetrasaccharide repeating unit. The proposed structures of these dimers are based upon their composition, as established by chemical ionization mass spectrometry and by the reported structure of the polysaccharide. A small proportion of 1,5-anhydro-2,4,6-tri-*O*-methyl-3-*O*-(methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosyluronate)-D-mannitol (**12**) was also detected in the products of the $BF_3 \cdot Et_2O$-catalyzed reductive cleavage. The presence of **12** is chemical evidence for the phase of the tetrasaccharide repeating unit in the polysaccharide. The reductive cleavage of **1** was also accomplished after reduction of its ester groups with lithium aluminum hydride. Complete cleavage of all glycosidic linkages was observed when either $Me_3SiOSO_2CF_3$ or $Me_3SiOSO_2CH_3$–$BF_3 \cdot Et_2O$ was used to

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†To whom correspondence should be addressed.

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catalyze reductive cleavage, and anhydroalditols **2, 3, 4,** and 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol were produced, as expected.

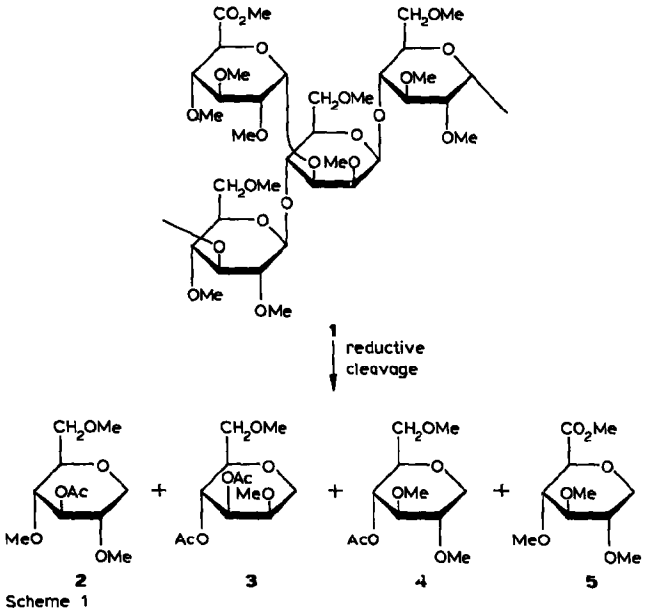
INTRODUCTION

The finding[1] that carboxylic acid esters are stable to the conditions required for reductive cleavage of the glycosidic carbon–oxygen bonds in a methylated polysaccharide suggested that it might be possible to analyze, directly, polysaccharides containing uronic acid and sialic acid residues and pyruvic acetal, lactic acid ether, or ester substituents by the reductive cleavage technique[2–11]. Indeed, studies[12] with a polysaccharide containing 4-linked α-D-glucopyranosyluronic residues demonstrated the feasibility of carrying out structural analysis of polysaccharides containing uronic acid residues. Although reductive cleavage of the glycosidic bond of the glucuronic residue was observed in these studies, the product was not the expected, pyranoid anhydroalditol, but a furanoid anhydroalditol arising as a result of isomerization and ring contraction. Direct reductive cleavage was therefore not suitable in this case as a means to establish unequivocally the position of linkage and ring form. It was ascertained, however, that an unambiguous determination of linkage position and ring form could be made by reductive cleavage after reduction of the fully methylated polysaccharide with lithium aluminum hydride.

Mechanistically, the formation of a furanoid anhydroalditol product from a methylated D-glucopyranosyluronic residue can occur only when that residue is 4-linked. We therefore expected that reductive cleavage of 2- and 3-linked D-glucopyranosyluronic residues and terminal (nonreducing) D-glucopyranosyluronic groups would proceed with retention of ring size. It is conceivable, however, that in these cases the thermodynamically favored route for reduction would involve intermediate *acyclic* oxonium ions, and thus ultimately lead to reductive cleavage of the *endocyclic* anomeric carbon–oxygen bond. Clearly, the formation of acyclic products would complicate the analysis of uronic acid-containing polysaccharides by this method. The present report describes our studies with a polysaccharide that contains terminal (nonreducing) α-D-glucopyranosyluronic groups, namely, the *Klebsiella* K2 capsular polysaccharide.

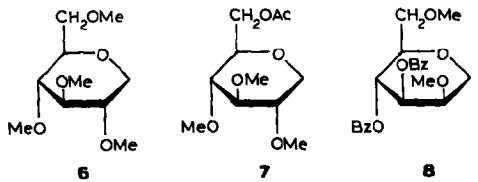
RESULTS

Reductive cleavage of the permethylated polysaccharide. — The structures of the permethylated K2 polysaccharide[13,14] (**1**) and its expected reductive-cleavage products (**2–5**) are shown in Scheme 1. The reductive cleavage of **1** was carried out in the presence of 5 equivalents each of triethylsilane (Et_3SiH) and trimethylsilyl trifluoromethanesulfonate ($Me_3SiOSO_2CF_3$), followed by *in situ* acetylation, and then by extraction with aqueous sodium hydrogencarbonate[3,8]. Analysis of the mixture of products by g.l.c. combined with both chemical ionization mass spectrometry (c.i.m.s.), with ammonia as the reagent gas, and electron impact mass



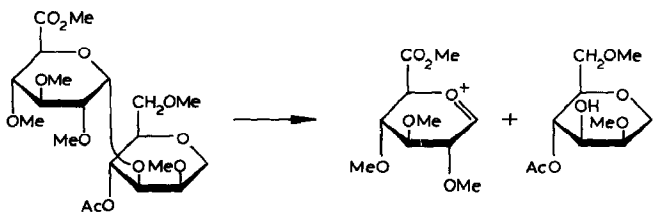
Scheme 1

spectrometry (e.i.m.s.) revealed the presence of two minor and four major anhydroalditol derivatives. The identities of components eluted first, third, fourth, and fifth were established through comparison to independently synthesized standards by g.l.c.–c.i.m.s., g.l.c.–e.i.m.s., and g.l.c. retention times. The component eluted first (11.3 min) was identified as 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**6**), and its presence was not surprising, as small proportions of terminal (nonreducing) D-glucopyranosyl groups were found by Barker *et al.*[13] to be present in the polysaccharide. The components eluted third (18.5 min) and fifth (19.9 min) were respectively identified as compounds **4** and **2**, the expected products of reductive cleavage of the 4-linked and 3-linked D-glucopyranosyl residues of the polysaccharide. The component eluted fourth (19.0 min), which was derived in only traces from the intact polysaccharide, was identified as 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**7**), and its origin is unknown. The component eluted last (23.6 min) was identified as 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-mannitol (**3**) on the basis of its c.i. and e.i. mass spectra and of the ^{1}H-n.m.r. spectrum of the corresponding dibenzoyl derivative (**8**), obtained for a sample isolated by high-performance liquid chromatography (h.p.l.c.).



The remaining component, which was eluted second (15.4 min) was identified as methyl 2,6-anhydro-3,4,5-tri-*O*-methyl-L-gulonate (**5**). The c.i. mass spectrum of this component established that it had a molecular weight of 234, as expected for **5**. Its e.i. mass spectrum displayed characteristic ions at *m/z* 175 (M − 59), due to loss of the exocyclic, methoxycarbonyl moiety, and at *m/z* 143 (M − 91), due to the further loss of methanol from the (M − 59) ion. The ^{1}H-n.m.r. spectrum of this component, obtained for a sample isolated by preparative g.l.c., was also consistent with structure **5**. The spectrum displayed one methyl ester (δ 3.80) and three methoxyl (δ 3.47, 3.49, 3.62) resonances, in addition to the expected number of ring-hydrogen resonances. Resonances were well resolved for H-2 (δ 3.73, d, J 9.1 Hz) and H-6*e* (δ 4.11, dd, J 4.0, 11.0 Hz). The chemical shift of the H-6*e* resonance, its multiplicity, and the magnitudes of its coupling constants are almost identical to those observed for H-1*e* of partially methylated derivatives of 1,5-anhydro-D-glucitol[8], as expected for the 2,6-anhydro-L-gulonate ring-skeleton.

Integration of the g.l.c. profile, and correction for molar response[5,15], gave the molar ratios listed in Table I. It is evident from these data that reductive cleavage was incomplete when $Me_3SiOSO_2CF_3$ was used as the catalyst. The molar ratio (0.39) of compound **5** is substantially lower than expected, and is attributable to failure to cleave the glycosidic linkage of the methylated D-glucopyranosyluronic group, as well as to degradation of the latter during methylation. Other studies *(vide infra)* demonstrated that degradation of the terminal D-glucopyranosyluronic groups occurred to the extent of 32% during methylation. The molar ratio (0.39) for **5** therefore represents a 57% recovery. Incomplete reductive cleavage of the methylated α-D-glucopyranosyluronic linkage is also evident from the molar ratio (0.68) of **3**, which is correspondingly low, and from the presence of two components at longer retention times (44.3 and 45.3 min) derived from the methylated D-glucopyranosyluronic and D-mannopyranosyl residues. The c.i. mass spectra of both components contained an ion at *m/z* 484, which corresponds to the $(M + NH_4)^+$ ion of a compound having a molecular weight of 466. Based upon the structure of the fully methylated K2 polysaccharide, only *one* reductive cleavage fragment (**9**) having this molecular weight is possible. In support of this proposed structure (**9**), the c.i. mass spectra of both components contained fragment ions at *m/z* 201 and 235, which are respectively interpreted as arising *via* elimination of methanol from the glycosyl oxonium ion (**10**) and protonation of the anhydroalditol fragment **11**.



9, $M + NH_4^+$ = *m/z* 484 **10**, M^+ − MeOH = *m/z* 201 **11**, $M + H^+$ = *m/z* 235

TABLE I

MOLAR RATIOS OF PRODUCTS DERIVED BY REDUCTIVE CLEAVAGE OF THE FULLY METHYLATED K2 POLYSACCHARIDE AND ITS REDUCED ($LiAlH_4$) DERIVATIVE

Catalyst	*Molar ratio*					
	2	**3**	**4**	**5**	**6**	**7**
	Before $LiAlH_4$ reduction					
$Me_3SiOSO_2CF_3$	1.00	0.68	0.73	0.39	tr[a]	0.03
$Me_3SiOSO_2CH_3 + BF_3 \cdot Et_2O$	0.97	0.48	1.00	0.64	tr	0.27
	After $LiAlH_4$ reduction					
$Me_3SiOSO_2CF_3$	1.00	0.98	1.00	—	tr	0.68
$Me_3SiOSO_2CH_3 + BF_3 \cdot Et_2O$	1.00	0.49	0.87	—	tr	0.68

[a]tr = trace.

The presence of *two* compounds of this type is most likely attributable to anomerization during reductive cleavage; *i.e.*, the components at retention times of 44.3 and 45.3 min are **9** and its β anomer.

The reductive cleavage of **1** was also carried out with a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ as the catalyst, and the product was acetylated as reported previously[11]. The gas–liquid chromatogram of the product was similar to that obtained with $Me_3SiOSO_2CF_3$ as the catalyst, except for the absence of components having retention times of 44.3 and 45.3 min. Integration of the chromatogram and correction for molar response gave the molar ratios listed in Table I. In this experiment, compound **5** was produced in a substantially greater proportion than when $Me_3SiOSO_2CF_3$ was used as the catalyst. The molar ratio (0.64) observed for **5** corresponds to a recovery of 94%, as 32% of the D-glucopyranosyluronic groups were degraded during methylation *(vide infra)*. Compound **4** was also produced in the expected molar ratio (1.00) in this experiment. For reasons unknown, the molar ratio (0.73) of **4** was lower than expected when $Me_3SiOSO_2CF_3$ was used as the catalyst. Compound **3**, arising from the branched D-mannopyranosyl residue, was produced in a proportion (molar ratio 0.48) that was lower than expected, however, when a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ was used to catalyze reductive cleavage. The reason for the low ratio of **4** is not known, but similar results in other experiments suggest that the loss occurs during workup of the reductive-cleavage reaction-mixture. Compound **7** was derived in an unexpectedly high molar ratio (0.27) with the $Me_3SiOSO_2CH_3$–$BF_3 \cdot Et_2O$ catalyst, relative to the molar ratio (0.03) obtained when $Me_3SiOSO_2CF_3$ was used as the catalyst. This appears to be an artifact, however, produced by cochromatography of an impurity.

Substantially different results were obtained when $BF_3 \cdot Et_2O$ was used to catalyze reductive cleavage of **1**. Analysis of the acetylation product by g.l.c. revealed the presence of small proportions of the expected monomers (**2–5**), but of much greater proportions of dimers eluted at longer retention times. Shown in Fig.

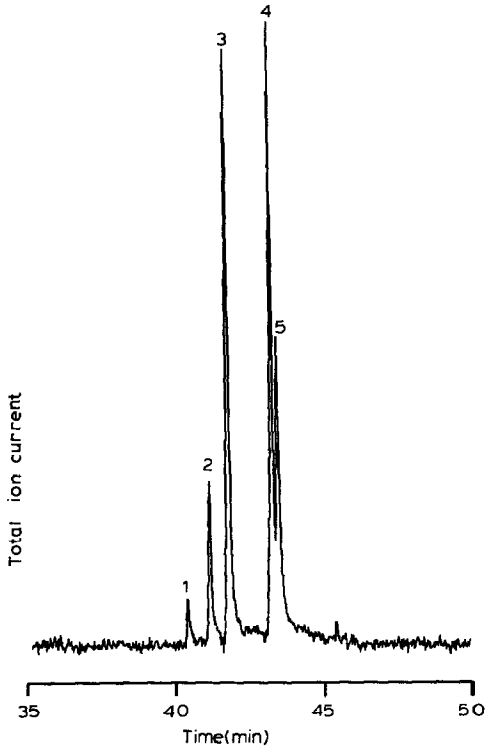


Fig. 1. Gas–liquid chromatogram of the high-molecular-weight products derived by $BF_3 \cdot Et_2O$-catalyzed reductive cleavage of the permethylated K2 polysaccharide. The products were detected by the total ion current during chemical ionization (NH_3) mass-spectral analysis. The proposed identities of the numbered peaks are as follows: (1) **12**, (2) unknown, (3) **13**, (4) mixture of **9** (major) and **14** (minor), and (5) **15**.

1 is a portion of the gas–liquid chromatogram obtained when the effluent was analyzed by c.i.m.s. The numbered peaks were identified based upon their c.i. mass spectra and the reported structure of the K2 polysaccharide.

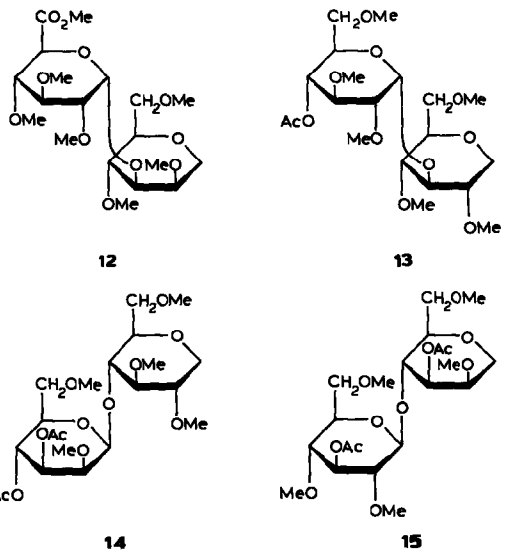
The c.i. mass spectrum of peak 1 (see Fig. 1) contained an ion at *m/z* 456, which corresponds to $(M + NH_4)^+$ for the fully methylated dimer **12**. The mass spectrum of this component also contained fragment ions at *m/z* 201 and 207, which are interpreted as arising *via* elimination of methanol from the glycosyl oxonium ion, and protonation of the anhydroalditol fragment, respectively. It should be noted that **12** can arise as a product only if the nonreducing end of the polysaccharide terminates with a D-glucopyranosyluronic group, thereby giving rise to a singly linked D-mannopyranosyl residue.

The c.i. mass spectra of Peaks 2 and 3 (see Fig. 1) were very similar. Both spectra contained an ion at *m/z* 470, which corresponds to $(M + NH_4)^+$ of a mono-*O*-acetylhexa-*O*-methyl hexosylanhydrohexitol (M_r 452). From the reported sequence of the polysaccharide, only one dimer with this composition is

possible, namely, **13**. Both c.i. mass spectra also contained characteristic fragment-ions at *m/z* 247 and 207, which are interpreted to be the glycosyl oxonium ion and the protonated anhydroalditol fragment, respectively. The presence of two components having this composition is surprising, and it may be indicative of some anomerization of **13** during reductive cleavage. It is also plausible, however, that other dimers having the same molecular weight as **13** could arise should the branching D-glucopyranosyluronic group be missing at a few sites in the repeating unit.

The c.i. mass spectrum of Peak 4 (see Fig. 1) demonstrated that it contained two components. The major component is **9** (M_r 466), as evidenced by characteristic ions at *m/z* 484 $(M + NH_4)^+$, 201, and 235. The same component was observed when $Me_3SiOSO_2CF_3$ was used to catalyze reductive cleavage, as mentioned previously. The minor component of peak 4 (see Fig. 1) gave an ion at *m/z* 498, corresponding to $(M + NH_4)^+$ for a di-*O*-acetylpenta-*O*-methyl hexosylanhydrohexitol. Based upon the structure of the repeating unit in the polysaccharide, there are two possible dimers (**14** and **15**) having this molecular weight. The minor component of Peak 4 must be compound **14** by virtue of the presence of ions at *m/z* 207 and 275 in its c.i. mass spectrum; the *m/z* 207 ion would correspond to $(M + H)^+$ of the anhydroalditol fragment, whereas the ion at *m/z* 275 would correspond to the glycosyl oxonium ion.

The remaining component (Peak 5 in Fig. 1) in the $BF_3 \cdot Et_2O$-catalyzed reductive cleavage of **1** was the dimer **15**, as expected. Although **14** and **15** possess the same molecular weight (M_r 480), they are readily distinguished by the fragment ions in their c.i. mass spectra. Peak 5 (see Fig. 1) gave the same $(M + NH_4)^+$ ion (*m/z* 498) as was observed for the minor component (**14**) in Peak 4, but fragment



ions at *m/z* 235 and 247 were observed in the c.i. mass spectrum of **15**, whereas fragment ions at *m/z* 207 and 275 were detected in the spectrum of **14**. Heterolytic cleavage of the glycosidic linkage of **15** gives rise to a glycosyl oxonium ion at *m/z* 247 and the $(M + H)^+$ ion of the resultant anhydroalditol at *m/z* 235.

Reductive cleavage of the permethylated and reduced polysaccharide. — Reductive cleavage of the permethylated K2 polysaccharide was also performed after reduction of its ester groups with lithium aluminum hydride[16]. Reductive cleavage was carried out in the presence of 5 equivalents each of Et_3SiH and $Me_3SiOSO_2CF_3$, followed by *in situ* acetylation, and extraction with aqueous sodium hydrogencarbonate. The gas–liquid chromatogram of the product was the same as obtained by direct reductive cleavage of **1**, except for the absence of compound **5**, the presence of substantially greater proportions of compound **7**, and the absence of dimers arising as a result of incomplete reductive cleavage. Integration of the g.l.c. profile, and correction for molar response, gave the molar ratios listed in Table I. In this experiment, the theoretical proportions of **2**, **3**, and **4** were obtained, but compound **7**, arising from the reduced D-glucuronic group, was present in a lower proportion (molar ratio 0.68) than expected. The low value for the molar ratio of **7** is attributed to degradation of the D-glucopyranosyluronic group during Hakomori methylation[17,18]. These results indicate that degradation of the D-glucuronic group during methylation occurred to the extent of at least 32%, or perhaps somewhat greater (35%) if the small proportion (molar ratio 0.03) of **7** produced by direct reductive cleavage of **1** is considered.

Reductive cleavage of the permethylated, $LiAlH_4$-reduced polysaccharide was also performed with a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ as the catalyst[11]. Analysis of the acetylated product by g.l.c., and correction for molar response, gave the molar ratios listed in Table I. Essentially the same results were obtained in this experiment as when reductive cleavage was catalyzed with $Me_3SiOSO_2CF_3$, with the exceptions that compound **4** was produced in a slightly lower molar ratio (0.87), and compound **3** was produced in a much lower molar ratio (0.49). Compound **3** was also derived in a substantially lower proportion when a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ was used to catalyze the direct cleavage of **1** (see Table I).

Standard methylation analysis. — The positions of linkage in the K2 polysaccharide, and the molar ratios of products derived from the constituent monomers, were checked by standard methylation analysis. The Hakomori-methylated polysaccharide was reduced with lithium aluminum deuteride, and the product hydrolyzed, and the resulting monomers were reduced with sodium borodeuteride and the products acetylated. The resulting mixture of partially methylated alditol acetates was analyzed by g.l.c.–c.i.m.s. and g.l.c.–e.i.m.s. The partially methylated alditol acetates expected were indeed observed, and the molar ratios obtained for these products were in good agreement with the respective values derived by reductive cleavage ($Me_3SiOSO_2CF_3$ as the catalyst) of the methylated and $LiAlH_4$-reduced polysaccharide. Of particular note was the good agreement between the two

methods with regard to the molar ratio of the product derived from the terminal (nonreducing) D-glucopyranosyluronic group; *i.e.*, compound **7** was derived in a molar ratio of 0.68 when reductive cleavage was employed, and the corresponding alditol acetate was derived in a molar ratio of 0.72 by standard methylation analysis.

DISCUSSION

The results reported herein demonstrate that the reductive-cleavage technique is suitable for the structural analysis of polysaccharides that contain terminal (nonreducing) D-glucopyranosyluronic groups. In contrast to standard methylation analysis, the fully methylated polysaccharide can be analyzed directly by reductive cleavage, owing to the stability of ester groups under the reaction conditions chosen. Reductive cleavage of the D-glucopyranosyluronic groups of the K2 polysaccharide was relatively slow (~60% cleavage in 8 h) when $Me_3SiOSO_2CF_3$ was used as the catalyst, but essentially complete cleavage was observed when a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ was used as the catalyst under the same conditions. Importantly, the expected product (**5**) was observed in all reactions as a consequence of reductive cleavage of the glycosidic carbon–oxygen bond of the methylated D-glucopyranosyluronic groups. In no experiment was a product observed as a consequence of reductive cleavage of the endocyclic, anomeric carbon–oxygen bond.

Satisfactory analytical data were also obtained when reductive cleavage of the fully methylated K2 polysaccharide was accomplished *after* reduction of its ester groups with lithium aluminum hydride. 6-*O*-Acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**7**) was detected as a product when reductive cleavage was accomplished with either $Me_3SiOSO_2CF_3$ or $Me_3SiOSO_2CH_3$–$BF_3 \cdot Et_2O$ as the catalyst, and the molar proportion (0.68) of **7** formed in these experiments was that expected, based upon an independent determination by standard methylation analysis.

The relatively low rate of cleavage of methylated D-glucopyranosyluronic groups can actually be used to advantage, especially when a more selective catalyst ($BF_3 \cdot Et_2O$) is used in reductive cleavage. In the $BF_3 \cdot Et_2O$-catalyzed, reductive cleavage of the fully methylated K2 polysaccharide, small proportions of the expected monomers (**2–5**) were observed, but the major products were dimers (see Fig. 1) arising as a result of incomplete reductive cleavage of the various glycosidic linkages. Based upon the structure of the repeating unit of the K2 polysaccharide, there are only 4 dimers (**9, 13, 14,** and **15**) that can arise as a result of incomplete reductive cleavage. Indeed, these four dimers were observed, based upon analysis of their c.i. mass spectra. As expected, **9** was observed as a major product in the $BF_3 \cdot Et_2O$-catalyzed reaction, owing to the relative stability of the glycosidic linkage of the D-glucopyranosyluronic group toward reductive cleavage. Unexpectedly, **13** was also formed as a major product in the $BF_3 \cdot Et_2O$-catalyzed reductive cleavage of **1**. The presence of a substantial proportion of **13** was surprising,

because, in previous work[8], $BF_3 \cdot Et_2O$ was found to catalyze reductive cleavage of 4-linked α-D-glucopyranosyl residues effectively.

In addition to the four possible dimers that could arise as a result of incomplete reductive cleavage of the fully methylated K2 polysaccharide, small proportions of two other dimers were detected by g.l.c.–c.i.m.s. analysis. One of these (Peak 2 in Fig. 1) has the same molecular weight as **13**, but its structure is not known with certainty. Of more interest is the presence of the remaining dimer (Peak 1 in Fig. 1) which, based upon c.i.m.s. analysis, must have the composition of compound **12**. This component can only arise from the nonreducing end of the polysaccharide, and its presence is therefore chemical evidence for the "phase" of the repeating unit in the polysaccharide.

It should be emphasized that the anomeric configurations of all dimers, and the sequences of dimers **13**, **14**, and **15**, are proposals based upon the reported structure of the K2 polysaccharide.

EXPERIMENTAL

General. — ^{1}H-N.m.r. spectra were recorded with an IBM NR/300 n.m.r. spectrometer for solutions in $CDCl_3$, and were referenced to internal tetramethylsilane. Analytical g.l.c.was performed with a Hewlett–Packard Model 5890A gas–liquid chromatograph equipped with a Hewlett–Packard Model 3392A integrator, a flame-ionization detector, and a J. and W. Scientific DB-5 fused silica capillary column (0.25 mm × 30 m). The temperature of the column was held at 120° for 10 min, and then programmed to 300° at 4°/min. Preparative g.l.c. was performed by using the same instrument equipped with a thermal conductivity detector and a stainless-steel column (6.4 mm × 3.53 m) of 10% of SP-2401 on 100–120 Supelcoport. G.l.c.–m.s. analyses were performed by using either a Finnigan 4000 mass spectrometer equipped with a VG Multispec data system, or a VG Analytical Ltd. Model VG 7070E-HF high-resolution, double-focusing mass spectrometer. Column effluents were analyzed by chemical-ionization mass spectrometry with ammonia as the reagent gas, whereby characteristic $(M + H)^+$ and $(M + NH_4)^+$ ions were detected, and by electron-impact mass spectrometry. High-performance liquid chromatography was carried out on an instrument consisting of two Waters Associates M-6000A pumps, a Waters model M660 solvent programmer, a Waters model 440 absorbance detector measuring absorbance at 254 nm, and a Rheodyne model 7125 injector.

Methylation was carried out by a modification[19] of the Hakomori[20] procedure. The fully methylated polysaccharide was purified by adsorption and elution from a C-18 reversed-phase Sep-Pak cartridge[21]. Prior to methylation, the polysaccharide was dissolved in water, and the solution lyophilized. Reduction of methyl ester groups in the fully methylated polysaccharide was accomplished with either lithium aluminum hydride or lithium aluminum deuteride in freshly distilled tetrahydrofuran[16]. Reductive cleavage with $Me_3SiOSO_2CF_3$ as the catalyst, and *in*

situ acetylation, were conducted as described previously[8], except that reduction and acetylation were each carried out for 8 h. Reductive cleavage with $BF_3 \cdot Et_2O$ as the catalyst and subsequent acetylation were performed as described previously[8], except that reduction was conducted for 24 h. Reductive cleavage with a mixture of $Me_3SiOSO_2CH_3$ and $BF_3 \cdot Et_2O$ as the catalyst was achieved by a slight modification of the method previously reported[11]. A pre-mixed solution of Et_3SiH (0.5M), $Me_3SiOSO_2CH_3$ (1.0M) and $BF_3 \cdot Et_2O$ (0.2M) in dry CH_2Cl_2 was prepared in a silylated (inside) 3-mL Wheaton V-vial immediately before use. After stirring for 10 min, a volume of the pre-mixed solution equal to 5 equivalents of Et_3SiH, 10 equivalents of $Me_3SiOSO_2CH_3$, and 2 equivalents of $BF_3 \cdot Et_2O$ per equivalent of acetal was then added to the dry, methylated polysaccharide. The reaction was allowed to proceed for 8 h, and the mixture was then processed in the usual way[11]. Standard methylation analysis was performed by the method of Lindberg[16], and the resultant partially methylated alditol acetates were analyzed by g.l.c.–c.i. and g.l.c.–e.i. mass spectrometry[22].

Samples of authentic 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**2**), 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**4**), 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**6**), and 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**7**) were available from previous work[8].

3,4-Di-O-acetyl-1,5-anhydro-2,6-di-O-methyl-D-mannitol (**3**). — G.l.c.–c.i.m.s. (NH_3, positive): *m/z* 277 (59) and 294 (100); g.l.c.–e.i.m.s.: *m/z* 43 (100), 45 (18), 58 (18), 69 (11), 87 (12), 97 (40), 111 (11), 129 (29), 171 (26), 172 (2), 184 (3), 185 (4), 186 (2), and 231 (6).

Methyl 2,6-anhydro-3,4,5-tri-O-methyl-L-gulonate (**5**). — Compound **5** was isolated from the reductive-cleavage products of the fully methylated K2 polysaccharide by preparative g.l.c. The temperature of the column was held for 5 min at 180° and was increased to 300° at 5°/min. ^{1}H-N.m.r. ($CDCl_3$): δ 3.47, 3.49, 3.62 (3 s, 9 H, 3 MeO), 3.80 (s, 3 H, MeO_2C), 3.23–3.56 (complex, 4 H, H-3,4,5,6*a*), 3.73 (d, 1 H, *J* 9.1 Hz, H-2), and 4.11 (dd, 1 H, *J* 4.0, 11.0 Hz, H-6*e*); g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 171 (67), 235 (100), 252 (52); g.l.c.–e.i.m.s.: *m/z* 45 (26), 58 (100), 59 (12), 71 (13), 73 (18), 75 (42), 85 (38), 87 (32), 88 (47), 101 (24), 117 (20), 143 (10), 159 (3), 175 (13), and 202 (1).

1,5-Anhydro-3,4-di-O-benzoyl-2,6-di-O-methyl-D-mannitol (**8**). — Successive reductive-cleavage ($Me_3SiOSO_2CF_3$ as the catalyst) of the fully methylated K2 polysaccharide, and benzoylation of the products, gave a mixture of benzoylated anhydroalditols that was fractionated by reversed-phase h.p.l.c. on a DuPont Zorbax ODS column (9.2 mm × 25 cm). The column was eluted with a 20-min, linear gradient increasing from 50% acetonitrile in water to 90% acetonitrile at a flow rate of 4 mL/min, and the component (**8**) eluted at 15.1 min was collected. ^{1}H-N.m.r. ($CDCl_3$): δ 3.35, 3.45 (2 s, 6 H, 2 MeO), 3.45–3.90 (complex, 5 H, H-1*a*,2,5,6,6′), 4.27 (dd, 1 H, *J* 2.0, 12.9 Hz, H-1*e*), 5.33 (dd, 1 H, *J* 3.2, 10.0 Hz, H-3), 5.71 (t, 1 H, *J* 10.0 Hz, H-4), and 7.26–8.00 (complex, 10 H, benzoyl); g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 105 (73), 157 (21), 279 (10), 401 (56), and 418 (100); g.l.c.–e.i.m.s.:

m/z 44 (19), 77 (20), 105 (100), 111 (4), 122 (3), 125 (3), 141 (2), 143 (2), 173 (2), 191 (2), 201 (4), 203 (3), 207 (4), 233 (6), 247 (6), 249 (2), and 355 (2).

Molar-response values (flame-ionization detection) of anhydroalditol derivatives **2**–**7**. — The integral values of all g.l.c. peaks were corrected for molar response by the effective carbon-response (e.c.r.) method[15], which had been shown[5] to be applicable to anhydroalditols. The e.c.r. values of anhydroalditol derivatives were normalized to **6** set at unity, and are: **2** (1.09), **3** (1.18), **4** (1.09), **5** (0.91), **6** (1.00), and **7** (1.11). Integrated areas were divided by the appropriate, normalized e.c.r. values, in order to correct for molar response.

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