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ANALYSIS BY THE REDUCTIVE-CLEAVAGE METHOD OF LINKAGE POSITIONS IN A POLYSACCHARIDE CONTAINING 4-LINKED D-GLUCOPYRANOSYLURONIC RESIDUES*

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ABSTRACT

The fate of 4-linked D-glucopyranosyluronic residues under reductive-cleavage conditions was investigated by using the *Klebsiella aerogenes* type 54 strain A3 capsular polysaccharide. Treatment of the fully methylated polysaccharide with triethylsilane and trimethylsilyl trifluoromethanesulfonate in dichloromethane, followed by *in situ* acetylation, yielded 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol, 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol, and 3-*O*-acetyl-1,5-anhydro-2,4-di-*O*-methyl-L-fucitol, as expected, but the expected product of reductive cleavage of the 4-linked D-glucopyranosyluronic residue, namely, methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-L-gulonate, was not observed. Instead, methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**6**) was identified as the sole product of reductive cleavage of the 4-linked D-glucopyranosyluronic residue. That compound **6** arose as a result of rearrangement during reductive cleavage rather than by reductive cleavage of a 5-linked D-glucofuranosyluronic residue, was established by reductive cleavage of the fully methylated polysaccharide following reduction of its ester groups with either lithium aluminum hydride or lithium aluminum deuteride. The products of the latter reductive cleavage were the same as before, except for the absence of **6** and the presence of 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-glucitol, or its 6,6-dideuterio isomer. Although the reductive-cleavage technique is suitable for the direct analysis of polysaccharides containing 4-linked D-glucopyranosyluronic residues, it does not establish whether the uronic residue is a 4-linked pyranoside or a 5-linked furanoside. The expected product is, however, derived from the 4-linked D-glucopyranosyluronic residue after sequential methylation, reduction of its ester group and reductive cleavage.

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INTRODUCTION

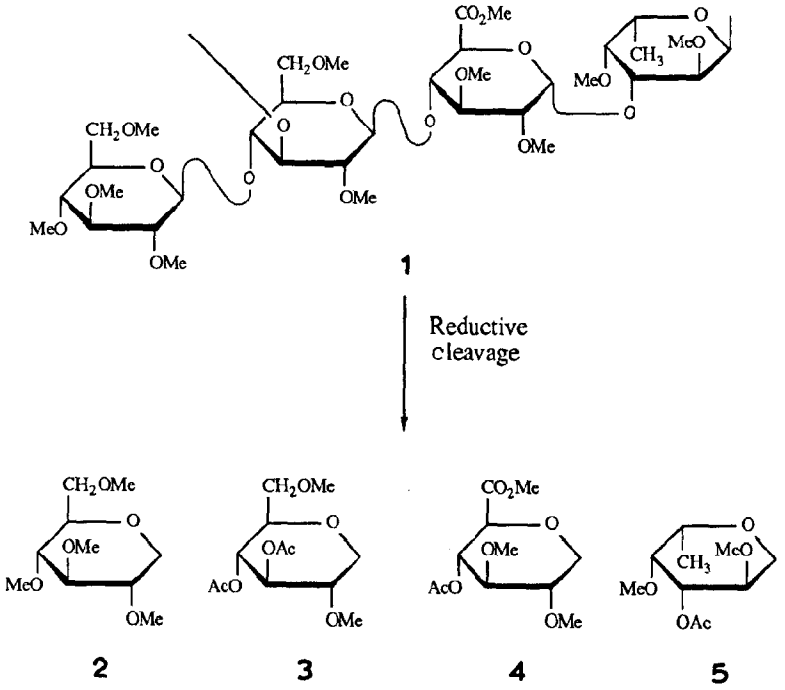
The development of the reductive-cleavage method[1] for determination of the structure of complex carbohydrates was prompted by both the laboriousness of "standard" methylation analysis and its inability to distinguish between certain combinations of ring form and position of linkage. In exploring the utility of this new method, we have chosen to examine a variety of structurally well-characterized polysaccharides as models. In previous reports, we have demonstrated the applicability of this technique to polysaccharides containing D-mannopyranosyl[2], D-fructofuranosyl[3], D-glucopyranosyl[4,5], and 2-acetamido-2-deoxy-D-glucopyranosyl[6] residues.

During the course of these studies, it became apparent[5] that carboxylic acid esters are stable under the conditions of the reductive-cleavage reaction, suggesting that it might be possible to analyze directly, polysaccharides containing uronic acid residues by sequential methylation and reductive cleavage. In this report, we describe our studies with a polysaccharide that contains 4-linked D-glucopyranosyluronic residues, namely, the acidic polysaccharide secreted by *Klebsiella aerogenes* type 54 strain A3. The K54 polysaccharide is comprised of the tetrasaccharide repeating-unit[7] shown, and, in addition, is acetylated at O-2 of the α-L-fucopyranosyl residue[8].

$$\begin{array}{l} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{-}(1\rightarrow \\ \qquad\qquad\quad 4 \\ \qquad\qquad\quad \uparrow \\ \qquad\qquad\quad 1 \\ \qquad\quad \beta\text{-D-Glc}p \end{array}$$

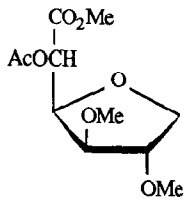
RESULTS

Reductive cleavage of the permethylated polysaccharide. — The structures of the permethylated K54 polysaccharide (**1**) and its expected reductive-cleavage products (**2–5**) are shown in Scheme 1. 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 extraction with aqueous sodium hydrogencarbonate[4,9]. 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 spectrometry (e.i.m.s.) revealed the presence of four anhydroalditol derivatives. The component eluted first (9.1 min) was identified as **2** through comparison to an independently synthesized standard. The component eluted second (9.4 min) was identified as **5** on the basis of its c.i. mass spectrum, which indicated that it possessed a molecular weight of 218, and its ^1H-n.m.r. spectrum, which was obtained on a sample isolated by preparative g.l.c. The component eluted third (20.1 min) was identified as **3** by comparison to an authentic standard.



Scheme 1

The remaining component from the reductive cleavage of **1** was expected to be methyl 3-*O*-acetyl-2,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**4**). Indeed, the c.i. mass spectrum of the component eluted last (20.4 min) established that it had a molecular weight of 262, as expected for compound **4**. However, the ^{1}H-n.m.r. spectrum of this component, obtained on a sample isolated by preparative g.l.c., demonstrated that it was methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**6**)! The ^{1}H-n.m.r. spectrum of this component displayed the expected resonances for acetoxyl (δ 2.10), methoxyl (δ 3.37, 3.38) and methyl ester (δ 3.77) substituents, and the expected number of ring-hydrogen atoms. The most downfield resonance (δ 5.10) was, however, a doublet (J 8.8 Hz), demonstrating that the acetyl group was present at O-2 (O-5 of the original D-glucopyranosyluronic



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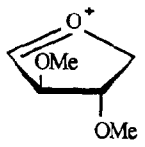


7, $m/z = 131$

residue). The multiplicity of the δ 5.10 resonance precluded all other isomeric structures of both compound **4** and compound **6**. The e.i. mass spectrum of this component was also in accord with structure **6**, displaying an intense ion (**7**) at *m/z* 131 which arises through fragmentation and loss of the exocyclic substituent.

Integration of the g.l.c. profile, and correction for molar response[2,10], gave the molar ratios listed in Table I. The molar ratios of compounds **2, 5,** and **6** are in reasonable agreement with those expected. The lower-than-expected ratio for **6** is probably due to degradation during Hakomori methylation[11,12], and not to the formation of **4**, as none of the latter product was detected by g.l.c.–c.i.m.s. analysis. The molar ratio (0.72) for compound **3** is substantially lower than expected, for reasons unknown.

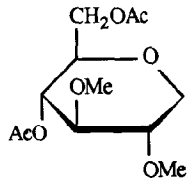
Reductive cleavage of the permethylated and reduced polysaccharide. — Reductive cleavage of the permethylated K54 polysaccharide was also performed after reduction of its ester groups with lithium aluminum hydride[17]. Reductive cleavage, acetylation and g.l.c.–c.i.m.s. and g.l.c.–e.i.m.s. analyses were performed as described previously for the permethylated polysaccharide. The gas–liquid chromatogram of the product was the same as in the previous experiment, except for the absence of **6** and the presence of a new component eluted at a slightly longer retention time (21.8 min). The c.i. mass spectrum of the new component demonstrated that it had a molecular weight of 276, as expected for 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-glucitol (**8**). Verification that this new component was indeed **8** was accomplished by independent synthesis. Integration of the peaks in the g.l.c. profile corresponding to compounds **2, 3, 5,** and **8**, and correction for molar response as described previously, gave the molar ratios listed in Table I. Within experimental error, compounds **2, 3,** and **5** were formed in proportions identical to those obtained by the direct reductive cleavage of **1**, and compound **8** was obtained in the same proportion as was compound **6** in the aforementioned reductive cleavage.

TABLE I

MOLAR RATIOS OF PRODUCTS DERIVED BY REDUCTIVE CLEAVAGE[a] OF PERMETHYLATED *K. aerogenes* TYPE 54, STRAIN A3 POLYSACCHARIDE (**1**) AND ITS REDUCED ($LiAlH_4$ OR $LiAlD_4$) DERIVATIVE

Reducing agent	*Molar ratio*				
	2	**3**	**5**	**6**	**8**
None[b]	1.00	0.72	0.90	0.86	—
$LiAlH_4$[b]	1.00	0.71	0.92	—	0.89
$LiAlD_4$[c]	0.93	0.66	0.86	—	1.00[d]

[a]Reductive cleavage was carried out with $Me_3SiOSO_2CF_3$ as the catalyst. [b]Methylated by the procedure of Hakomori[13,14]. [c]Methylated with methyl trifluoromethanesulfonate and trimethylphosphate in the presence of 2,6-di(*tert*-butyl)pyridine[15,16]. [d]6,6-Dideuterio isomer of compound **8**.



8

In an effort to establish whether the somewhat smaller than expected proportion of **8** was due to degradation of the glucuronic acid residue during Hakomori methylation[13], the K54 polysaccharide was methylated under neutral conditions with methyl trifluoromethanesulfonate and trimethyl phosphate in the presence of 2,6-di-(*tert*-butyl)pyridine[15,16]. After reduction of the ester groups with lithium aluminum deuteride, reductive cleavage and subsequent analyses were conducted as described previously. Compounds **2, 3, 5,** and **8** (as its 6,6-dideuterio isomer) were again produced, as expected, but compound **8** was indeed derived in a greater proportion than when methylation was performed by the procedure of Hakomori[13] (see Table I). Interestingly, and for reasons unknown, compound **3** was, however, again derived in a substantially lower proportion than expected.

Standard methylation analysis. — The positions of linkage in the K54 polysaccharide were checked by standard methylation analysis. The polysaccharide was methylated by using methyl trifluoromethanesulfonate[15,16], the product reduced with lithium aluminum deuteride and hydrolyzed, and the resulting monomers were reduced with sodium borodeuteride, and the alditols then 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 four partially methylated alditol acetates that were observed were those expected, based upon the previously reported[7] structure of the polysaccharide. The molar ratios obtained for these products were in good agreement with the respective values derived by reductive cleavage where the same preparation of methylated and ester-reduced ($LiAlD_4$) polysaccharide was analyzed. Products arising from the terminal (nonreducing) D-glucopyranosyl group and the branched D-glucopyranosyl residue were recovered in somewhat greated proportions by standard methylation analysis, but the proportions of products arising from the 4-linked D-glucopyranosyluronic residue and the 3-linked L-fucopyranosyl residue were identical in the two methods.

DISCUSSION

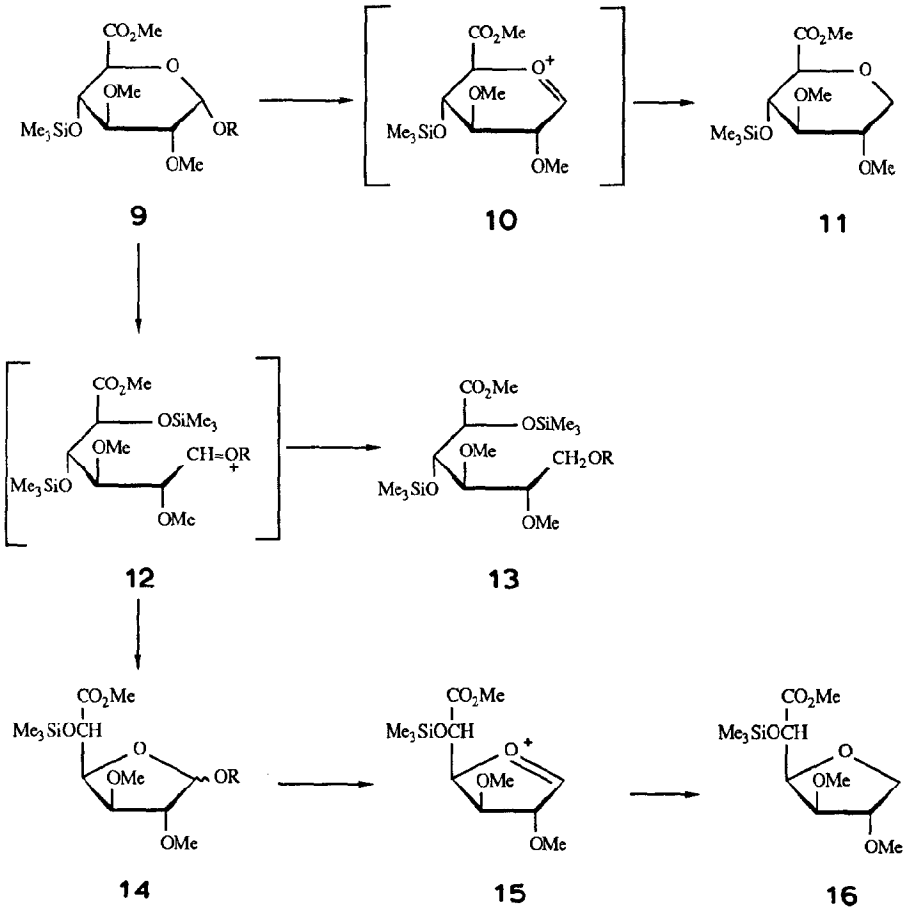
Our previous finding[5] that esters are stable to reductive-cleavage conditions suggested that simplified methodologies might be developed for the structural characterization of complex carbohydrates containing uronic acids, sialic acids, pyruvic acetals, lactic acid ethers, and esterified fatty acids. We have therefore begun to examine polysaccharides containing these residues and substituents by

the reductive-cleavage technique. The polysaccharide examined in the present study was chosen because of its content of 4-linked D-glucopyranosyluronic residues. Because of the relative stability of uronic acid glycosides to acid hydrolysis, it was suspected that reductive cleavage of the glycosidic linkage of the D-glucopyranosyluronic residue would occur slowly, if at all.

However, treatment of the fully methylated K54 polysaccharide under the usual conditions for total reductive cleavage[4] resulted in complete cleavage of all glycosidic linkages, and four anhydroalditol products were detected by subsequent g.l.c.–c.i.m.s. and g.l.c.–e.i.m.s. analysis. Three of the anhydroalditols produced (**2, 3,** and **5**) were those expected, based upon the structure of the polysaccharide as previously proposed[7], but the anhydroalditol arising from the 4-linked D-glucopyranosyluronic residue was not the expected product, **4**, but the isomeric anhydroalditol **6**. Because the latter product (**6**) would be that expected upon reductive cleavage of a 5-linked D-glucofuranosyluronic residue, it was not clear whether the structure that had been published was incorrect, or whether rearrangement of the methylated D-glucopyranosyluronic residue had occurred during reductive cleavage, to produce **6**. In order to distinguish between these possibilities, the fully methylated polysaccharide was subjected to reductive cleavage after reduction of its ester groups with either lithium aluminum hydride or lithium aluminum deuteride. These experiments unequivocally established that the K54 polysaccharide contains a 4-linked D-glucopyranosyluronic residue, as compound **8** and its 6,6-dideuterio isomer were obtained in the separate experiments.

These results therefore established that the formation of **6** during reductive cleavage of the fully methylated K54 polysaccharide was artifactual. Even this result was not very surprising. Because we had previously demonstrated[9] that reductive cleavage of glycosides proceeds *via* formation and reduction of cyclic oxonium ions, the formation of **4** by reductive cleavage of the fully methylated polysaccharide would require the intermediacy of oxonium ion **10** (see Scheme 2). That the pyranosyl residue **9** does not yield **11**, and hence **4** upon acetylation, is most probably attributable to destabilization of oxonium ion **10** by the electron-withdrawing methoxycarbonyl substituent. An energetically more-feasible route for cleavage of the glycosidic linkage of **9** is its isomerization to **14** *via* the more-stable *acyclic* oxonium ion **12**. Intermediate **14** would then be expected to undergo reductive cleavage readily, as the cyclic furan oxonium ion **15** would be more stable than the cyclic pyran oxonium ion **10**, and thus more readily formed. Reduction of intermediate **15**, followed by acetylation of the product (**16**) would yield the rearrangement product (**6**) actually observed. Interestingly, the product (**13**) arising from reduction of acyclic oxonium ion **12** was not formed, as judged by g.l.c.–c.i.m.s. analysis after acetylation.

The results obtained herein are likely to be general for other polysaccharides that contain 4-linked D-glucopyranosyluronic residues, *i.e.*, the furan anhydroalditol **6** will be observed as a consequence of rearrangement and reductive cleavage, at least when $Me_3SiOSO_2CF_3$ is used as the catalyst. Although the data are not re-



Scheme 2

ported herein, **6** was also formed when boron trifluoride etherate[1,4] was used to catalyze reductive cleavage. The latter catalyst is considerably more selective[2,4], however, and incomplete reductive cleavage was observed. In an analysis where **6** is observed as a product, the ring form and position of linkage of the D-glucuronic residue are not known with certainty, and the analysis must be repeated after reduction of the ester substituent. The stability of the glucuronic ester to reductive cleavage is still useful, however, in that it allows direct analysis of a polysaccharide of unknown structure that may or may not be known to contain uronic acid residues. From these results, it is also anticipated that direct analysis of polysaccharides containing terminal (nonreducing) D-glucuronic groups and 2- and 3-linked D-glucuronic residues would be feasible. In these cases, reductive cleavage, if it occurs, would be relatively slow, but ring contraction would not be possible. It is conceivable in these cases that products will arise as a result of the formation and

reduction of acyclic oxonium ions (related in structure to **12** and **13**; see Scheme 2), and so studies with polysaccharides that contain these structural features are essential if a strategy for the satisfactory analysis of glucuronic acid-containing polysaccharides by the reductive-cleavage technique is to be developed.

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. ^{1}H-2D-COSY spectra were obtained on the same instrument, as an aid to assigning individual proton resonances. Analytical g.l.c. was performed in a Hewlett–Packard Model 5890A gas–liquid chromatograph equipped with a Hewlett–Packard Model 3392A integrator, a flame-ionization detector, and a Hewlett–Packard cross-linked methylsilicone fused silica capillary column (0.2 mm × 25 m). The temperature of the column was held for 10 min at 120° and then programmed to 300° at 4°/min. Preparative g.l.c. was performed in 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 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.

Methylation was carried out by a modification[14] of the Hakomori[13] procedure. The fully methylated polysaccharide was purified by adsorption to, and elution from, a C-18 reverse-phase Sep-Pak cartridge, as described by Mort *et al.*[18]. Methylation was also carried out by using $MeOSO_2CF_3$ in trimethyl phosphate, with 2,6-di-(*tert*-butyl)pyridine added as a proton scavenger[15,16]. The fully methylated polysaccharide was purified by chromatography on a column (3 × 39 cm) of Sephadex LH-20, eluted with 2:1 (v/v) dichloromethane–methanol. Methylated samples were analyzed by ^{1}H-n.m.r. and i.r. spectroscopy, to determine whether methylation was complete[19]. Prior to methylation, polysaccharides were dissolved in water and the solutions lyophilized. Reduction of the methyl ester groups in the fully methylated polysaccharide was accomplished with either lithium aluminum hydride or lithium aluminum deuteride in freshly distilled tetrahydrofuran[17]. Reductive cleavage and *in situ* acetylation were conducted as described previously[4], except that reduction and acetylation were each performed for 8 h. Standard methylation analysis was performed by the method of Lindberg[17], and the resultant, partially methylated alditol acetates were analyzed by g.l.c.–c.i. and g.l.c.–e.i. mass spectrometry[20].

Samples of authentic 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**2**) and

3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**3**) were obtained as previously described[4,5].

3-O-Acetyl-1,5-anhydro-2,4-di-O-methyl-L-fucitol (**5**). — Compound **5** was isolated, from the reductive-cleavage products of the fully methylated K54 polysaccharide, by preparative g.l.c. The temperature of the column was held for 5 min at 160° and was increased to 300° at 5°/min. ^{1}H-N.m.r. ($CDCl_3$) data: δ 1.24 (d, 3 H, *J* 6.5 Hz, H-6), 2.15 (s, 3 H, AcO), 3.14 (dd, 1 H, *J* 9.9, 11.2 Hz, H-1a), 3.42, 3.52 (2 s, 6 H, 2 MeO), 3.45 (d, 1 H, *J* 3.1 Hz, H-4), 3.50 (complex, 1 H, H-5), 3.68 (dt, 1 H, *J* 5.4, 9.9 Hz, H-2), 4.11 (dd, 1 H, *J* 5.4, 11.2 Hz, H-1e), and 4.79 (dd, 1 H, *J* 3.1, 9.9 Hz, H-3); g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 219 (100) and 236 (6); g.l.c.–e.i.m.s.: *m/z* 43 (66), 45 (17), 58 (14), 59 (23), 74 (78), 75 (21), 101 (100), 114 (16), 128 (8), 154 (3), and 159 (1).

Methyl 2-O-acetyl-3,6-anhydro-4,5-di-O-methyl-L-gulonate (**6**). — Compound **6** was isolated by preparative g.l.c., as described for compound **5**. For **6**: ^{1}H-n.m.r. ($CDCl_3$): δ 2.13 (s, 3 H, AcO), 3.37, 3.38 (2 s, 6 H, 2 MeO), 3.77 (s, 3 H, MeO_2C), 3.78–3.81 (complex, 2 H, H-4,6), 3.85–3.92 (complex, 1 H, H-5), 4.10 (dd, 1 H, *J* 4.6, 9.9 Hz, H-6'), 4.27 (dd, 1 H, *J* 4.2, 8.8 Hz, H-3), and 5.11 (d, 1 H, *J* 8.8 Hz, H-2); g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 263 (47) and 280 (100); g.l.c.–e.i.m.s.: *m/z* 43 (71), 45 (31), 58 (65), 59 (23), 69 (17), 71 (97), 72 (15), 73 (27), 75 (20), 85 (19), 87 (52), 97 (15), 99 (43), 101 (52), 102 (20), 114 (20), 117 (14), 129 (32), 131 (100), 132 (12), 139 (10), 142 (37), 143 (30), 145 (10), 156 (25), 171 (24), 174 (5), 188 (5), 202 (24), 203 (16), 231 (9), 262 (0.5), and 263 (1.5).

4,6-Di-O-acetyl-1,5-anhydro-2,3-di-O-methyl-D-glucitol (**8**). — 1,5-Anhydro-D-glucitol[21] was converted into its 4,6-*O*-benzylidene derivative by treatment with α,α-dimethoxytoluene and *p*-toluenesulfonic acid in *N,N*-dimethylformamide as described by Evans[22]. The crude syrup so obtained was dissolved in CH_2Cl_2, and the solution was washed successively with saturated aqueous sodium hydrogencarbonate and water, and evaporated, to give crystals which were dried overnight under high vacuum. Recrystallization from ethyl acetate gave pure 1,5-anhydro-4,6-*O*-benzylidene-D-glucitol (11%; m.p. 169°). Treatment of the latter product by successive methylation[13,14], hydrolysis[23], and acetylation afforded **8** as an oil; ^{1}H-n.m.r. ($CDCl_3$): δ 2.07, 2.08 (2 s, 6 H, 2 AcO), 3.08–3.45 (complex, 4 H, H-1a,2,3,5), 4.01–4.20 (complex, 3 H, H-1e,6,6'), and 4.88 (t, 1 H, *J* 9.5 Hz, H-4); g.l.c.–c.i.m.s. (NH_3, positive): *m/z* 277 (65) and 295 (100); g.l.c.–e.i.m.s.: *m/z* 43 (100), 45 (11), 58 (59), 143 (4), 156 (5), 171 (1), 203 (0.4), and 216 (0.4).

Molar-response values (flame-ionization detection) of anhydroalditol derivatives **2**, **3**, **5**, **6**, *and* **8**. — The integral values of all g.l.c. peaks were corrected for molar response by the effective carbon-response (e.c.r.) method[10], which had been shown[2] to be applicable to anhydroalditols. The e.c.r. values of anhydroalditol derivatives were normalized to **2** set at unity and are: **2 (1.00)**, **3** (1.18), **5** (1.09), **6** (1.00), and **8** (1.20). Integrated areas were divided by the appropriate, normalized e.c.r. values, in order to correct for molar response.

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