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Note

Location of a second *O*-acetyl group in xanthan gum by the reductive-cleavage method

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Xanthan gum is the extracellular polysaccharide produced by the bacterium *Xanthomonas campestris*. Its structure has been shown[1,2] to consist of a (1 → 4)-linked β-D-glucan backbone with β-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 2)-α-D-Man*p*-(1 → side chains substituted at O-3 of alternating glucose residues. In addition, the side chains may contain *O*-acetyl and pyruvic acid acetal groups, which are located at the 6-position of the internal mannose residue and at the 4,6-position of the terminal mannose group, respectively. The amount of non-carbohydrate substituents vary depending on the strain of bacteria, the fermentation conditions and the recovery procedure[3–5].

Samples of xanthan gum with high acetyl content have been observed[6]. The proportion of acetyl groups was shown to exceed the theoretical value of one residue per pentasaccharide repeat unit. Although the location of additional acetyl groups is uncertain, it has been speculated that multiple acetylation of the internal mannosyl residue is possible in some xanthan gum samples, as is acetylation of the terminal mannosyl group[7]. In *X. campestris*, three genes have been identified[8,9] that encode enzymes which catalyze modifications of mannose residues. It was later shown[10] that one of these genes encodes an acetylase activity, termed acetylase II, directing acetylation of the terminal mannose group; however, the position of substitution was not determined. We now report the location of a second *O*-acetyl group in xanthan gum using the reductive-cleavage technique[11].

A sample of xanthan gum was hydrolyzed using trifluoracetic acid and analyzed for the presence of organic acids by high-performance liquid chromatography (HPLC). The results of this analysis indicated the presence of acetic acid (6.8%) and pyruvic acid (2.7%). Assuming a sodium form of xanthan gum containing an average of 1.0 acetyl groups and 0.5 pyruvic acid acetal substituents per repeat unit, the theoretical content for acetic acid and pyruvic acid is 6.3 and 4.4%,

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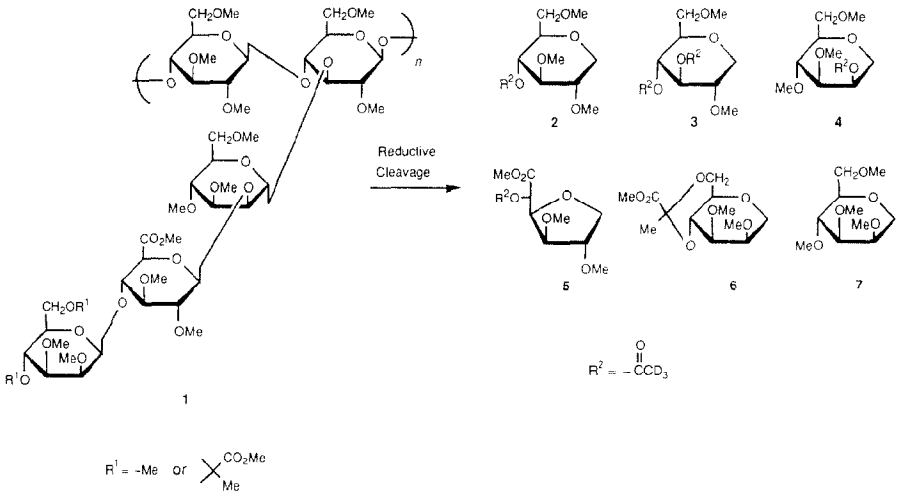


Scheme 1.

respectively. Therefore, the sample contains approximately 1.08 acetate groups and 0.31 pyruvic acid acetal substituents per repeat unit.

Reductive-cleavage of the per-*O*-methylated sample of xanthan gum (**1**, Scheme 1) was carried out in the presence of 5 equiv of triethylsilane (Et_3SiH), 1 equiv of boron trifluoride etherate ($BF_3 \cdot Et_2O$), and 5 equiv of trimethylsilyl methanesulfonate (Me_3SiOMs) as previously described[12]. The mixture of products was acetylated using perdeuterated acetic anhydride (Ac_2O-d_6) to differentiate acetate groups arising from a linkage position and acetate groups native to the polysaccharide (*vide infra*). The resulting anhydroalditol deuterioacetates were analyzed by gas–liquid chromatography (GLC) and the numbered peaks (Fig. 1, upper trace) were identified based on their GLC retention times and chemical-ionization (CI) and electron-ionization (EI) mass spectra. Peak 1 was identified as 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol (**7**) and Peak 6 was determined to be 1,5-anhydro-4,6-*O*-[(*S*)-1-methoxycarbonylethylidine]-2,3-di-*O*-methyl-D-mannitol (**6**). These components arise from the terminal, nonreducing mannose end group. Peaks 2, 3, and 5 were identified as the three singly linked residues, 2-*O*-($^2H_3$)acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol (**4**), 4-*O*-($^2H_3$)acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**2**), and methyl 2-*O*-($^2H_3$)acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**5**), respectively. Peak 4 was identified as 3,4-di-*O*-($^2H_3$)acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**3**), arising from the doubly linked glucose residue.

Integration of all peaks and correction for molar response[13,14] gave the molar fraction for each of the products (**2**–**7**) which are displayed in Table I. As is evident, the experimental values were in good agreement with those expected[15].

A second reductive-cleavage experiment was performed on the sample of neutral methylated xanthan gum to identify sugar residues bearing acetyl groups

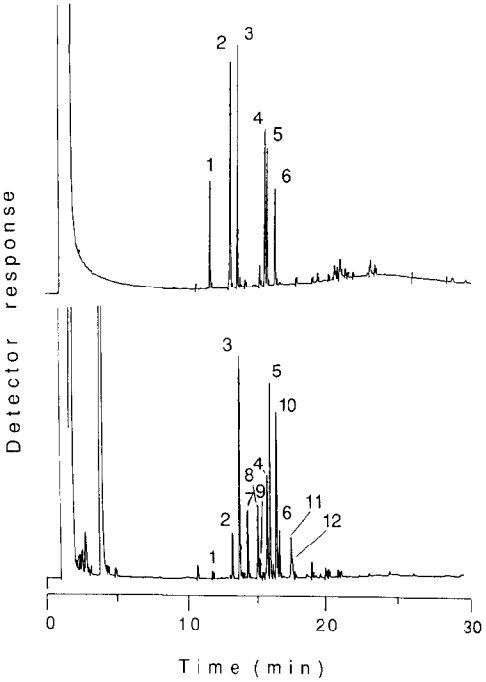


Fig. 1. Gas–liquid chromatograms of the anhydroalditol acetates derived by reductive-cleavage of per-*O*-methylated (base-catalyzed) xanthan gum (upper) and per-*O*-methylated (neutral conditions) xanthan gum (lower). The numbered peaks were identified as follows: (1) **7**; (2) **4**; (3) **2**; (4) **3**; (5) **5**; (6) **6**; (7) **10**; (8) **8**; (9) **11**; (10) **9**; (11) **12**; (12) **13**.

and to locate their positions of substitution. In this experiment, xanthan gum was methylated using the conditions of Prehm[16]. The *O*-acetyl groups remained intact (as judged by NMR spectroscopy), and the methylated polysaccharide containing native acetyl groups was subjected to reductive-cleavage with Et_3SiH and trimethylsilyl trifluoromethanesulfonate ($CF_3SO_3SiMe_3$) and acetylation with Ac_2O-d_6 in

TABLE I

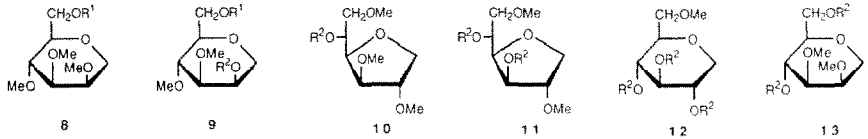
Molar ratios of products (compounds **2–13**) derived by reductive-cleavage of per-*O*-methylated xanthan gum [a]

Polysaccharide	Molar ratio											
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A	1.00	0.56	0.71	0.53	0.28	0.72						
B	0.82	0.33	0.20	0.91	0.17	0.08	0.24	0.67	0.18	0.05	0.34	0.06

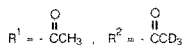
[a] Key: A, methylated (base-catalyzed) xanthan gum reductively cleaved using Et_3SiH, Me_3SiOMs and $BF_3 \cdot Et_2O$; B, methylated (neutral conditions) xanthan gum reductively cleaved using Et_3SiH and $CF_3SO_3SiMe_3$.

situ[17]. Model studies[18] indicated the stability of *O*-acetyl groups to $CF_3SO_3SiMe_3$-catalyzed reductive-cleavage and indeed, we have previously demonstrated[19] the direct analysis of polysaccharides containing such a group.

The gas–liquid chromatogram of the products is shown in Fig. 1, lower trace. As is evident, Peaks 1–6, in addition to several other peaks, were present. Peak 8 was identified as 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**8**) and Peak 10 was identified as 6-*O*-acetyl-2-*O*-($^2H_3$)acetyl-1,5-anhydro-3,4-di-*O*-methyl-D-mannitol (**9**). The presence of these two components demonstrate that one acetyl group is located at the 6-position of the 2-linked mannosyl residue and that a second acetyl group is located at the 6-position of the terminal mannose group.



8 9 10 11 12 13



Peaks 7 and 9 were identified as 5-*O*-($^2H_3$)acetyl-1,4-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**10**) and 3,5-di-*O*-($^2H_3$)acetyl-1,4-anhydro-2,6-di-*O*-methyl-D-glucitol (**11**), respectively. These products arise from ring contraction of 4-linked glucose residues during $CF_3SO_3SiMe_3$-catalyzed reductive-cleavage when water is present in the mixture[17]. Peak 11 was identified as 2,3,4-tri-*O*-($^2H_3$)acetyl-1,5-anhydro-6-*O*-methyl-D-glucitol (**12**) arising from incomplete methylation of the 3,4-linked glucose residue. Peak 12 was identified as 4,6-di-*O*-($^2H_3$)acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-mannitol (**13**). This component is most likely the result of hydrolysis of the pyruvic acid acetal during reductive-cleavage; model studies[20] indicated the possibility of slow reduction of 4,6-*O*-(1-methoxycarbonylethylidine) substituents.

Integration and correction for molar response[13,14] gave the mole fraction for each of the products which are listed in Table 1. The mole fractions for 6-*O*-acetyl-2-*O*-($^2H_3$)acetyl-1,5-anhydro-3,4-di-*O*-methyl-D-mannitol (**9**) and 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**8**) were 0.67 and 0.24, respectively. This experiment suggests 6-*O*-acetylation at approximately 67% of the 2-linked mannosyl residues and 24% of the terminal mannose groups. These numbers may be slight underestimations as is evident by the combined molar ratios of 2-linked mannosyl groups **4** and **9** (0.87 combined) and terminal mannose groups, **6**, **7**, **8**, and **13** (0.55 combined). The reason for the lower than expected recovery of terminal mannose components is not evident. Future experiments are designed to examine conditions which will allow for quantitative recovery of all components.

In this report we have described a two-step procedure that unequivocally establishes both the identity of acetylated sugar residues and the position of

substitution of their acetate groups. Perdeuterated acetic anhydride was used as an acetylating agent to differentiate between acetyl groups native to the polysaccharide and acetyl groups indicating linkage position. In addition, minor components arising from undermethylation of the polysaccharide and reaction byproducts are clearly identified as such. Only two components were observed by GLC–EIMS to contain fragment ions at m/z 43 ($CH_3C{\equiv}O^+$, acylium ion), which indicates native acetyl groups. These two components were identified as 6-*O*-acetyl-2-*O*-($^2H_3$)acetyl-1,5-anhydro-3,4-di-*O*-methyl-D-mannitol (**9**) and 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**8**). The presence of these two components demonstrates that one acetyl group is located at the 6-position of the 2-linked mannosyl residue and that a second acetyl group is located at the 6-position of the terminal mannose group.

EXPERIMENTAL

General.—High-performance liquid chromatography (HPLC) was performed using a Dionex BioLC system. Chromatography was performed on a Dionex HPICE-ASI ion-exclusion column, eluted with 10 mM octanesulfonic acid at a flow rate of 0.8 mL/min. Organic acids were detected by a Dionex Model CDM-1 conductivity detector following chemical suppression using a Model AMMS-ICE anion micromembrane suppressor. The sample was prepared by hydrolysis using 0.5 M trifluoroacetic acid. Gas–liquid chromatography (GLC) was performed on a Hewlett–Packard Model 5830A gas–liquid chromatograph equipped with a Hewlett–Packard Model 18850A integrator, a flame-ionization detector, and a J&W Scientific DB-5 fused silica capillary column (30 m × 0.53 mm, i.d.). The temperature of the column was held for 2 min at 110°C and then programmed to 250°C at 5°C/min. GLC–MS analyses were performed on a Finnigan MAT 90 high-resolution, double-focusing mass spectrometer. Column effluents were analyzed by CIMS with ammonia as the reagent gas and by EIMS. The EI mass spectra for all the acetylated anhydroalditols in the mannitol and glucitol series have been previously reported[21]. Anhydroalditols containing *O*-($^2H_3$)acetyl groups display similar fragmentation patterns as their *O*-($^1H_3$)acetylated counterparts. ^1H NMR spectroscopy of the permethylated polysaccharide ($CDCl_3$) was performed on a Varian Gemini 300 NMR spectrometer.

The xanthan gum sample used in this analysis was isolated from a culture of a strain of *X. campestris* derived from the wild-type *X. campestris* strain NRRL B1459. Modified Hakomori[22] methylation was carried out as described by Blakeny and Stone[23] using lithium methylsulfinyl carbanion[24] as the base. Neutral methylation was performed as described by Prehm[16] on a sample of xanthan gum that had been converted to its tetrabutylammonium salt. The conversion was accomplished by passing an aq 0.25% solution of xanthan gum through a column (30 × 200 mm) of Bio-Rad AG 50W-X4 analytical grade cation-exchange resin, tetrabutylammo-

nium form. Fully methylated polysaccharides were extracted into CH_2Cl_2, then purified by chromatography on a column (15 × 350 mm) of Sephadex LH-20 in 2:1 (v/v) CH_2Cl_2–MeOH. Fractions testing positive in the phenol–H_2SO_4 assay[25] were combined and used for further analysis. Reductive-cleavage using a combination of Me_3SiOMs and $BF_3 \cdot Et_2O$ as the catalyst was performed as described by Jun and Gray[12]. The reaction was allowed to proceed to 2.5 h, and the products were isolated and acetylated[12] using Ac_2O-d_6 (Aldrich). Reductive-cleavage using $CF_3SO_3SiMe_3$ as the catalyst was performed, as described by Rolf and Gray[11] for 3.5 h, followed by acetylation (Ac_2O-d_6) in situ.

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