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Carbohydrate Research 331 (2001) 265–270

CARBOHYDRATE RESEARCH

Location of *O*-acetyl groups in S-657 using the reductive-cleavage method

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Received 30 October 2000; accepted 31 January 2001

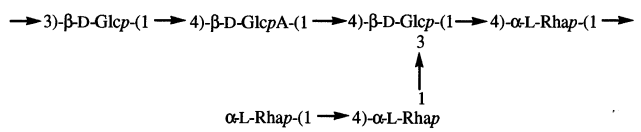
Abstract

A two-step procedure is described in this paper to identify the position of *O*-acetyl groups in S-657 polysaccharide. Reductive-cleavage experiments performed on the fully methylated (base-catalyzed) polysaccharide, followed by acetylation of anhydroalditols, identified individual sugar residues and their position of linkage. In a second experiment, the polysaccharide was methylated under neutral conditions leaving native acetate groups intact. Reductive cleavage of the neutral methylated polysaccharide using $CF_3SO_3SiMe_3$ as a catalyst, followed by acetylation in situ, identified sugar residues containing native acetate groups and established their position of substitution. Using this two-step procedure of analysis, S-657 polysaccharide is shown to contain *O*-acetyl groups on the 2-position and the 2,6-positions of 3-linked glucopyranosyl residues.

Keywords: S-657; Polysaccharide; Structural analysis; *O*-Acetyl groups

1. Introduction

The extracellular polysaccharide, S-657, is a developing commercial product produced by fermentation of a strain of *Sphingomonas* sp.[1] (previously identified as heteropolysaccharide S-657 prepared by fermentation of *Xanthomonas* ATCC 53159 strain[2]). The strain was isolated in November 1980 from an algal sample taken from a marsh located near Eureka, CA. The structure of the polysaccharide, as originally determined by Chowdhury et al.,[3] was shown to be comprised of hexasaccharide repeating-units having the structure



O-Acetyl groups were also shown to be present on the polysaccharide, although the position of substitution was not determined.

Several structurally related polysaccharides produced by members of the genus *Sphingomonas* have been commercialized, or are currently in various stages of commercial development, for use in controlling rheological properties of aqueous solutions. Some of the polysaccharides in this group include S-60 (gellan gum), S-130 (welan gum), S-194 (rhamsan gum), S-657, S-198, and S-88. Although produced from different organisms, the polysaccharides all contain the same tetrasaccharide repeating-unit, either without a side chain or with mono- or disaccharide side-chain substitution linked to one of the two

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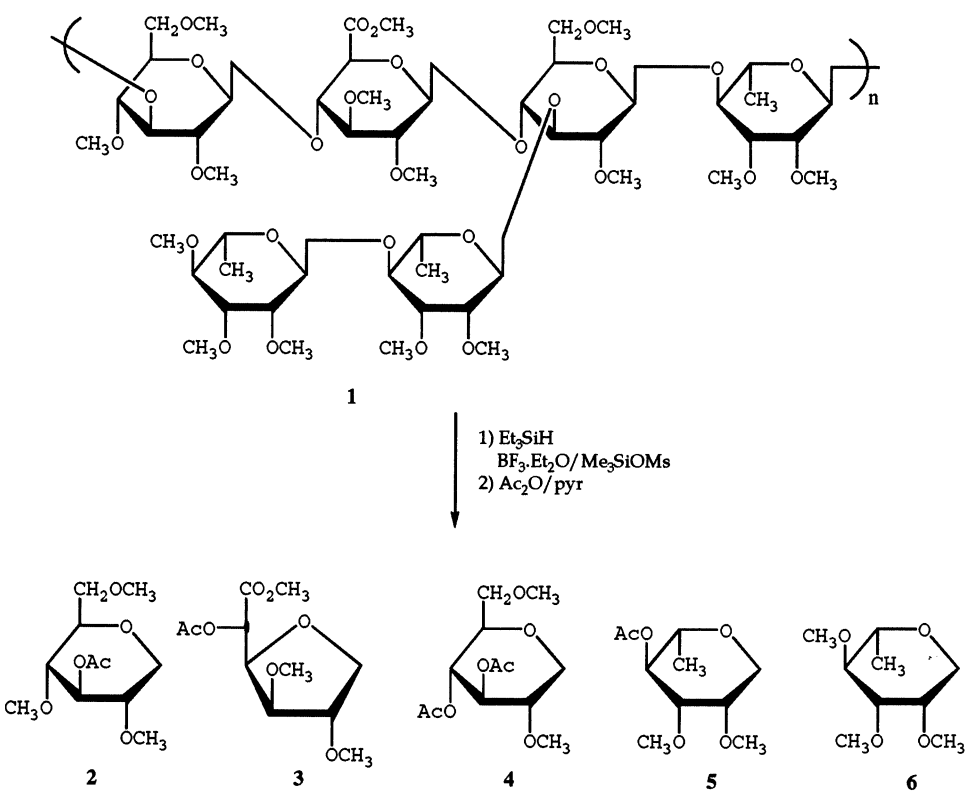
0008-6215/01/$ - see front matter
PII: S0008-6215(01)00045-3

glucopyranosyl residues.[4–8] In some of the polysaccharides L-rhamnose is partially replaced by L-mannose. All of the polysaccharides contain acetyl or glyceryl non-carbohydrate substituents. Gellan gum contains L-glycerate and acetate esterified to the 2-, and 6-positions, respectively, of the 3-linked glucopyranosyl residue.[9] Welan gum contains an acetate group esterified to the 2-position of the 3-linked glucopyranosyl residue.[10] These polysaccharides exhibit a wide range of functional properties.[11] Solutions of gellan gum form stable aqueous gels under appropriate ionic conditions, whereas solutions of welan, rhamsan, and S-657 do not gel but give very viscous solutions over a wide range of thermal, pH and salt conditions. Solutions of S-657 are more pseudoplastic than welan gum and exhibit superior suspending power, thermal stability over a temperature range from 5 to 150 °C, shear stability and salt tolerance. As a result, this polysaccharide has potential utility in a wide range of thickening, stabilization, cementitous and high-temperature applications. The molecular structure of S-657 as related to behavior in aqueous solution including thermal stability[12] and intrinsic viscosity as a function of ionic strength[13–15] have been discussed. As the degree and position of side-chain modification may be important in determining conformation,[16] and consequently properties in aqueous solution, and to further assist those studying this influence, we report herein the location of the *O*-acetyl groups in S-657 using the reductive-cleavage technique.[17]

We have observed variable acetate content in samples of S-657 polysaccharide produced during the course of fermentation development. These variations were attributed to the fermentation and recovery conditions employed. As such, a sample with a relatively high acetate content was selected for analysis. The sample was hydrolyzed using trifluoroacetic acid and analyzed for the presence of organic acids by high-performance liquid chromatography. The results of this analysis indicated the presence of 8.7% acetic acid. Based on the molecular weight of S-657 polysaccharide, assuming a potassium form, this sample contained approximately 1.5 acetate groups per repeat unit.

Reductive cleavage of Hakomori-methylated S-657.—Fully methylated (base-catalyzed) S-



Scheme 1.

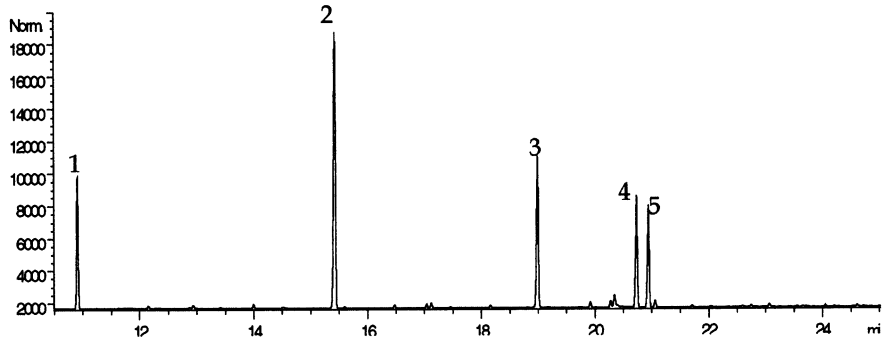


Fig. 1. Gas–liquid chromatogram of the acetylated anhydroalditols derived by reductive cleavage of base-catalyzed, per-O-methylated S-657 polysaccharide. Peaks were identified as follows: Peak 1 as 1,5-anhydro-2,3,4-tri-*O*-methyl-L-rhamnitol (**6**); Peak 2 as 4-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-rhamnitol (**5**); Peak 3 as 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**2**); Peak 4 as 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**4**); and Peak 5 as methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**3**).

657 (**1**, Scheme 1) was subjected to reductive cleavage in the presence of 5 equiv of triethylsilane (Et_3SiH) as the reducing agent and a mixture of 5 equiv of trimethylsilyl methanesulfonate (Me_3SiOMs) and 1 equiv of boron trifluoride etherate ($BF_3{\cdot}Et_2O$) as the catalyst.[18] The mixture of products was acetylated, and the resulting anhydroalditol acetates were analyzed by gas–liquid chromatography (GLC), the results of which are shown in Fig. 1. The numbered peaks were identified by comparison of their GLC retention times and electron-ionization (EI) mass spectra to those of published standards.[19,20] Peak 1 was identified as 1,5-anhydro-2,3,4-tri-*O*-methyl-L-rhamnitol (**6**) arising from the terminal, non-reducing end group. Peak 2 was identified as 4-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-rhamnitol (**5**). Peak 3 was identified as 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**2**) and Peak 5 was identified as methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**3**), all arising from singly linked residues. Peak 4 was identified as 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**4**), arising from the doubly linked hexose residue.

Integration of all peaks and correction for molar response[21,22] gave the mole fraction for each of the products (**2**–**6**), which are displayed in Table 1. The experimental values were in close agreement with those expected.[3] Slightly lower than expected recovery of the per-O-methylated rhamnitol component was attributed to volatility and subsequent loss during work-up of the reductive cleavage mixture. Less than quantitative recovery was also noted for the ring contracted methyl gulonate component **3**, most likely resulting from degradation during methylation and reductive depolymerization.

Reductive cleavage of neutral methylated S-657.—A second experiment was performed to determine the identity of the sugar residue(s) bearing acetate groups and to locate their position of substitution. In this experiment, methylation of S-657 was carried out under

Table 1
Mole fractions of products derived by reductive cleavage of per-O-methylated S-657 [a]

Compound	Mole%	
	A	B
1,5-Anhydro-2,3,4-tri-*O*-methyl-L-rhamnitol (**6**)	0.72	0.64
4-*O*-Acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-rhamnitol (**5**)	2.00	1.89
3-*O*-Acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**2**)	0.86	trace
3,4-Di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**4**)	0.80	0.60
Methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**3**)	0.55	1.00
2,3-Di-*O*-acetyl-1,5-anhydro-4,6-di-*O*-methyl-D-glucitol (**7**)		0.30
2,3,6-Tri-*O*-acetyl-1,5-anhydro-4-*O*-methyl-D-glucitol (**8**)		0.20

[a] Key: A, methylated (base-catalyzed) S-657 reductively cleaved using Et_3SiH, Me_3SiOMs and $BF_3{\cdot}Et_2O$; B, methylated (neutral conditions) S-657 reductively cleaved using Et_3SiH and $CF_3SO_3SiMe_3$.

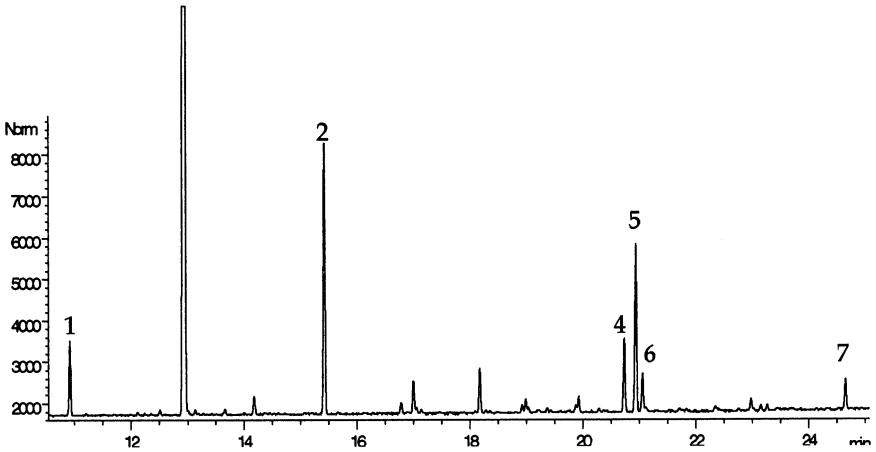


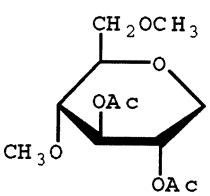
Fig. 2. Gas–liquid chromatogram of the acetylated anhydroalditols derived by reductive cleavage of the neutral methylated S-657 polysaccharide. Peaks were identified as follows: Peak 1 as 1,5-anhydro-2,3,4-tri-*O*-methyl-L-rhamnitol (**6**); Peak 2 as 4-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-L-rhamnitol (**5**); Peak 4 as 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-glucitol (**4**); Peak 5 as methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate (**3**); Peak 6 as 2,3-di-*O*-acetyl-1,5-anhydro-4,6-di-*O*-methyl-D-glucitol (**7**); and Peak 7 as 2,3,6-tri-*O*-acetyl-1,5-anhydro-4-*O*-methyl-D-glucitol (**8**).

neutral conditions as described by Prehm.[23] The *O*-acetyl group remained intact (as judged by NMR spectroscopy), and the permethylated polysaccharide containing native acetate groups was subjected to reductive cleavage with Et_3SiH and trimethylsilyl trifluoromethanesulfonate ($CF_3SO_3SiMe_3$) and acetylation in situ.[24] Model studies[25] indicated the stability of *O*-acetyl groups to $CF_3SO_3SiMe_3$-catalyzed reductive cleavage, and we have successfully utilized this approach to determine position of acetylation in other polysaccharides including welan gum[10] and xanthan gum.[26]

The gas–liquid chromatogram of the products (Fig. 2) was similar to the chromatogram from the previous experiment (Fig. 1), except Peak 3 disappeared and two new peaks were evident. Peak 6 was identified as 2,3-di-*O*-acetyl-1,5-anhydro-4,6-di-*O*-methyl-D-glucitol (**7**). The presence of this anhydroalditol establishes that an *O*-acetyl group is present on the 2-position of the 3-linked glucopyranosyl residue. Peak 7 was identified as 2,3,6-tri-*O*-acetyl-1,5-anhydro-4-*O*-methyl-D-glucitol (**8**). The presence of this anhydroalditol establishes that *O*-acetyl groups are present on the 2,6-positions of the 3-linked glucopyranosyl residue. No evidence of 3,6-di-*O*-acetyl-1,5-anhydro-2,4-di-*O*-methyl-D-glucitol was found in the chromatogram; therefore, acetylation of the 3-linked glucopyranosyl residue occurs on the 2-position and the 2,6-position exclusively. A few small peaks were observed in the chromatogram resulting from reductive cleavage of neutral methylated S-657. These peaks were attributed to artifacts of reductive cleavage with one exception. A small peak identified as 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol was observed in the chromatogram. This component was believed to be the result of partial loss of the rhamnosyl side chain during methylation. Several experiments were conducted in an attempt to reduce the loss of rhamnosyl side chain during methylation by altering the ratio of 2,6-di-*tert*-butylpyridine to methyl trifluoromethanesulfonate. Our attempts were unsuccessful in preventing the loss of the side chain leading to the methylation of resultant 4-linked glucopyranosyl residues and the formation of 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol following reductive cleavage and in situ acetylation. To demonstrate that this component was, in fact, generated due to side chain loss during methylation, reductive-cleavage experiments were performed followed by in situ acetylation using deuterated acetic anhydride. The use of deuterated acetic anhydride differentiates non-deuterated native *O*-acetyl groups from those deuterated *O*-acetyl esters marking linkage position. Using this technique, 4-*O*-($^2H_3$)acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol was identified in re-

ductive-cleavage experiments performed on neutral methylated polysaccharide, demonstrating the artifact of methylation.

Integration of all peaks and correction for molar response[21,22] gave the mole fraction for each of the products, which are listed in Table 1. The mole fraction for 2,3-di-*O*-acetyl-1,5-anhydro-4,6-di-*O*-methyl-D-glucitol (**7**) was 0.30, while the mole fraction for 2,3,6-tri-*O*-acetyl-1,5-anhydro-4-*O*-methyl-D-glucitol (**8**) was 0.20, for a combined mole fraction recovery of 0.50 of substituted 3-linked glucopyranosyl residues. Only a trace amount of 3-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol was present in the reaction mixture. HPLC analysis indicated approximately 1.5 acetate groups per repeating-unit for S-657 polysaccharide; therefore, recoveries of **7** and **8** were lower than anticipated. Based on the fact that only trace amounts of 3-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol were observed in the chromatogram, all 3-linked glucopyranosyl residues appear to contain acetate substitution. Acetylation may be directed to the 2-position of the 3-linked glucopyranosyl residue first, as no 6-*O*-acetyl groups were observed, followed by directed acetylation at the 6-position of the 2-O-acetylated, 3-linked glucopyranosyl residue.



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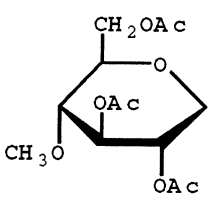

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The two-step procedure described in this paper establishes the position of O-acetylation in S-657 polysaccharide. Reductive-cleavage experiments performed on the fully methylated (base-catalyzed) polysaccharide, followed by acetylation of anhydroalditols, identified individual sugar residues and their position of linkage. A second experiment, performed on the neutral methylated polysaccharide, using reductive cleavage with $CF_3SO_3SiMe_3$ as a catalyst and acetylation in situ, served to identify sugar residues containing native acetate groups and established their position of esterification. In S-657, it is shown that the polysaccharide contains *O*-acetyl groups on the 2-position and the 2,6-positions of 3-linked glucopyranosyl residues.

2. Experimental

General.—Heteropolysaccharide S-657 was produced during the aerobic fermentation of suitable aqueous nutrient media under controlled conditions via inoculation with a culture of the organism ATCC 53159.[2] The polysaccharide was isolated from viscous fermentation broth by precipitation using 2.5 vols of 2-propanol and purified by exhaustive dialysis of a dilute solution against running deionized water. After 1 week of dialysis, the contents of the dialysis bag were lyophilized to afford a white, fluffy solid. This material was used directly for methylation. Modified Hakomori methylation[27] was carried out as described by Blakeny and Stone[28] using lithium methylsulfinyl carbanion as the base.[29] Neutral methylation was performed as described by Prehm,[23] with the exception that the ratio of 2,6-di-*tert*-butylpyridine to methyl triflate was doubled. Fully methylated polysaccharide was extracted into CH_2Cl_2, then purified by chromatography on a column of Sephadex LH-20 in 2:1 (v/v) CH_2Cl_2–methanol. Fractions testing positive in the phenol–H_2SO_4 assay[30] 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.[18] The reaction was allowed to proceed for 2 h, and the products were isolated and acetylated. Reductive cleavage using $CF_3SO_3SiMe_3$ as the catalyst was performed, as described by Rolf and Gray[17] for 3 h, followed by acetylation in situ. Gas-liquid chromatography was performed on a Hewlett–Packard model 5890 gas-liquid chromatograph equipped with a Hewlett–Packard ChemStation software package, a flame-ionization detector, and a Hewlett–Packard HP5 fused-silica capillary column (50 m × 0.20 mm i.d., 0.33 μm film thickness). The temperature of the column was held for 2 min at 110 °C and then programmed to 300 °C at 5 °C/min. GLC–MS analyses were performed on a

Hewlett–Packard model 6890 gas–liquid chromatograph equipped with a Hewlett–Packard model 5973 mass-selective detector, Hewlett–Packard ChemStation software, and a Hewlett–Packard HP5-MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.50 μm film thickness). The EI mass spectra for all of the acetylated anhydroalditols in the glucitol and rhamnitol series have been previously reported.[19,20] ^{1}H NMR spectroscopy of the permethylated polysaccharide ($CDCl_3$) was performed on a Varian Gemini 300 NMR spectrometer. 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.

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