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Location of *O*-acetyl groups in the heteropolysaccharide of the cactus *Pereskia aculeata*

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ABSTRACT

The complex polysaccharide of the leaves of *Pereskia aculeata* is highly branched, containing units of arabinofuranose, arabinopyranose, galactopyranose, galactopyranosyluronic acid, and rhamnopyranose. Glucopyranose units are also present. The location of 25 molar % of acetate ester groups was determined using modified Bouveng procedures, one of the changes being the use of refluxing methanolic sodium methoxide for the removal of *N*-methyl-*N*-phenylcarbamoyl groups. With the aid of g.l.c.–m.s. on a capillary column of DB-210, which separated the various isomeric mono-, di-, and tri-*O*-methyl arabinitol acetates, and by examination of e.i. fragmentation patterns of various derivatives deuterated at C-1, the *O*-acetyl groups were located. They were found mainly as monosubstituents at O-2 and O-3 of nonreducing arabinofuranosyl end-groups and at O-2 of 4-*O*-substituted galacto- and gluco-pyranosyl units.

INTRODUCTION

A heteropolysaccharide isolated from the leaves of the cactus *Pereskia aculeata* has a chemical structure consisting principally of a main chain of (1→4)-linked β-D-galactopyranosyl units, partly substituted at O-3 by residues of α-L-arabinopyranose. These in turn are disubstituted, at O-2 and O-4, by nonreducing end-groups of α-L-arabinofuranose. Also present are β-D-galactopyranosyluronic acid units, which occur as nonreducing end-groups or internally, and are linked (1→2) to rhamnopyranosyl units. The polysaccharide contains 0.25 mol/unit of *O*-acetyl groups[1], and the objective of the present study was to determine their locations.

RESULTS AND DISCUSSION

In a preliminary experiment, *O*-acetyl locations were elucidated in terms of overall substitution pattern on each component monosaccharide. This was done using the method of Bouveng, in which the *O*-acetyl groups were replaced by *O*-methyl, furnishing a modified polysaccharide which was hydrolyzed to provide fragments that could be analyzed[2]. The original method, involving the steps partly acetylated polysaccharide → acetylated phenylcarbamate → acetylated *N*-methyl-*N*-phenylcarbamate → *O*-methylated *N*-methyl-*N*-phenylcarbamate → *O*-methylated polysaccharide →

O-methyl-aldoses, was improved by a number of modifications. In the phenylcarbamoylation step the polymer was solubilized in dimethyl sulfoxide (DMSO) + phenyl isocyanate at 50°, conditions that are known not to promote *O*-acetyl migration[3]. The solvent was thus superior to *N*,*N*-dimethylformamide (DMF) at 100° (ref. 2), in which the polysaccharide was only partly soluble, and to pyridine at 50°, in which the ester groups would be subject to base-catalyzed migration. The phenylcarbamate groups were then *N*-methylated with silver oxide–methyl iodide–DMF and the product *O*-deacetylated with methanolic sodium methoxide at 4°. This reagent is preferable to strong sulfuric acid[2], which would cause concomitant degradation. The *O*-deacetylated material was *O*-methylated with silver oxide–methyl iodide–DMF, and the *N*-methyl-*N*-phenylcarbamate groups were removed from the *O*-methyl derivative with sodium methoxide in refluxing methanol–tetrahydrofuran. This reagent is an improvement over lithium aluminum hydride in tetrahydrofuran[2], which forms a reaction mixture that is difficult to work up, and to methylsulfinyl carbanion in DMSO[4], which does not always effect complete removal of *N*-methyl-*N*-phenylcarbamate groups.

TABLE I

Relative retention times of *O*-methyl arabinitol acetates in g.l.c. on a DB-210 capillary column at 195°

Alditol acetate[a]	*Relative retention time*	*Alditol acetate*[a]	*Relative retention time*
2,3,5-Me_3Rib	1.00	2,3-Me_2Ara	1.68
2,3,5-Me_3Ara	1.03	2,5-Me_2Ara	1.75
2,3,4-Me_3Ara	1.08	5-MeAra	1.88
3,5-Me_2Ara	1.37	2-MeAra	2.24
2,4-Me_2Ara	1.60	3-MeAra	2.39
3,4-Me_2Ara	1.67	[b]	[b]

[a] 2,3,5-Me_3Rib = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylribitol, etc. [b] Under the conditions specified the relative retention times of 2-*O*-, 3-*O*-, and 2,3-di-*O*-methylxylitol acetates are 2.50, 2.47, and 1.47 respectively.

The partly *O*-methylated polysaccharide was converted into a mixture of aldose and partly *O*-methylated aldose derivatives. This was reduced with sodium borodeuteride, the product was acetylated, and the mixture examined by g.l.c.–m.s. on a capillary column of DB-210, which was found to separate the various isomeric mono-, di-, and tri-*O*-methyl arabinitol acetates present (Table I). The main fragments, identified by their typical e.i. breakdown patterns[5] and retention times, were acetates of (1-^{2}H) arabinitol (35%) and its 2-*O*- (6%) and 3-*O*-methyl derivatives (3%), rhamnitol (3%), galactitol (35%) and its 2- (3%), 2,3- (1%) and 2,6-di- (1%), and 2,3,6-tri- (2%) *O*-methyl derivatives, and glucitol (7%) and its 2-*O*-methyl derivative (3%). Consideration of these values, combined with those for peaks present in amounts of less than 1% (Table II), accounts for 0.29 mol of acetate per mol of sugar unit, close to 0.25 mol/mol found in the polysaccharide by direct determination[1]. The small difference might result from the nonappearance of galacturonic acid derivatives on g.l.c., and the removal of some units by β-elimination during the Bouveng experiment.

TABLE II

Indentity and percentage content of alditol acetates and *O*-methyl derivatives formed on Bouveng analysis of *P. aculeata* heteropolysaccharide[a]

Acetylated[b] *fragment*	*Mole %*	*Acetylated fragment*	*Mole %*	*Acetylated fragment*	*Mole %*
2,3,5-Me_3Ara	0.5	Rha	3	2-MeGal	3
3,5-Me_2Ara	0.6	3-MeAra	3	2-MeGlc	3
2,4,6-Me_3Gal	0.6	2,6-Me_2Gal	1	Gal	35
2,3,6-Me_3Gal	2	Ara	35	Glc	7
2-MeAra	6	2,3-Me_2Gal	1		

[a] Analyzed by g.l.c.–m.s. on a capillary column of DB-210. [b] 2,3,5-Me_3Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

The production of small quantities of *O*-methyl derivatives raises the question of their formation as artifacts. These might arise by incomplete phenylcarbamoylation of the polysaccharide or by loss of phenylcarbamoyl groups during the treatment with cold sodium methoxide in methanol–tetrahydrofuran, which removed the *O*-acetyl groups, or during the *O*-methylation process. To test for these possibilities, the *O*-acetyl phenylcarbamate derivative was deacetylated with sodium methoxide in methanol–tetrahydrofuran, then converted into the fully phenylcarbamoylated polysaccharide (the *O*-deacetylated polysaccharide was insoluble in the phenylcarbamoylation mixture). This on being subjected to the improved Bouveng procedure gave a final product containing, by g.l.c., only the acetates of rhamnitol, arabinitol, galactitol, and glucitol, without even traces of their *O*-methyl derivatives.

The exact locations of the *O*-acetyl substituents in the structurally complex polysaccharide were determined as follows. The previously mentioned acetylated *N*-methyl-*N*-phenylcarbamate derivative was treated with sodium methoxide in cold methanol–tetrahydrofuran, and the product was treated with silver oxide–*N,N*-dimethylformamide–trideuteriomethyl iodide. The resulting material was heated under reflux in methanolic sodium methoxide in order to remove *N*-methyl-*N*-phenylcarbamate groups, forming a partly *O*-(dcuteriomethyl)ated polysaccharide. This was further treated with unlabeled methyl iodide–silver oxide–*N,N*-dimethylformamide to give a fully methylated polysaccharide that was converted into a mixture of partly *O*-methylated aldoses. This was successively reduced with sodium borohydride and acetylated, and the mixture of partly *O*-methylated alditol acetates was then examined by g.l.c.–m.s. on a column of DB-210, which resolved more peaks than previously obtained[1] with OV-17 blended with OV-225. The profile was the same as that observed after conventional methylation analysis of the polysaccharide using the procedure of Haworth[6] followed by that of Kuhn *et al.*[7] (Table III). The only difference was a small peak of 3,4-di-*O*-methylrhamnitol acetate[1] that was not found because of β-elimination of the uronic acid residue at O-2 of rhamnopyranose, converting it into a nonreducing end-group, which gave rise to the 2,3,4-tri-*O*-methyl derivative.

TABLE III

Distribution of *O*-acetyl groups in each structural unit of heteropolysaccharide[a]

Acetylated methylation fragment[b]	*Mole %*	*O-Acetyl positions (approx. %)*	*Acetylated methylation fragment*[b]	*Mole %*	*O-Acetyl positions (approx. %)*
2,3,5-Me_3Ara	18	O-2 (7%) O-3 (7%) O-5 (2%)	2,3,6-Me_3Gal	(19)[c]	O-2 (6%) O-3 (~7%) O-6 (2%)
3,5-Me_2Ara	5	O-3 (4%)	2,3,6-Me_3Glc	(13)[c]	O-2 (12%) O-3 (~0%) O-6 (2%)
2,4-Me_2Ara	5	O-2 (1%) O-4 (2%)	3-MeAra	5	O-4 (4%)
2,3,4,6-Me_4Gal	7	O-2 (3%) O-3 (?) O-4 (4%) O-6 (2%)	2,6-Me_2Gal	18	O-2 (1%)
			2,4-Me_2Gal	1	O-2 (2%)
			2,3-Me_2Gal	2	O-2 (7%) O-3 (2%)
2,3,4-Me_3Rha	1	O-3 (12%)			
2,3-Me_2Ara	2	O-2 (3%) O-4 (4%)	2-MeGal	2	O-2 (1%)
2,4,6-Me_3Gal	2	O-2 (1%) O-4 (1%)			

[a] *O*-Acetyl groups were replaced with *O*-trideuteriomethyl groups by the Bouveng procedure and the resulting polysaccharide submitted to a conventional methylation analysis giving the described acetates of *O*-methyl alditols. [b] 2,3,5-Me_3Ara = 1,4-di-*O*-acetyl-2,3,5-di-*O*-methylarabinitol, etc. [c] 2,3,6-Me_3Gal and 2,3,6-Me_3Glc acetates were not separated by g.l.c. and gave a combined value of 32%. Values in parentheses are derived from the ratio (3:2) obtained in runs in which they were separated.

The e.i. spectrum of each partly *O*-methylated alditol acetate contained, in most cases, peaks due to recognizable fragments accompanied by satellite peaks at +3 daltons, corresponding to the introduction of *O*-C^2H_3 groups. The positions of these groups could be determined by employing the e.i.-fragmentation mechanisms outlined by Jansson *et al.*[5]. An approximate quantitation of *O*-acetyl at each substitution position on a given type of structural unit was provided by determination of the relative percentual height (r.p.h.) of the satellite peak (s.p.), compared with the sum of its height plus that of the parent peak. Selected were peaks of ion fragments that were relevant to the location of *O*-methyl groups, preference being given to larger fragments. Sometimes, the origin of the fragment was better demonstrated via examination of material in which deuterium had been introduced at C-1 by reduction of the *O*-methylaldose mixture with sodium borodeuteride. Interpretations of the e.i. spectra of *O*-methyl alditol acetates are presented below, in the order of elution of the compounds from the DB-210 column (see also Table III).

1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinitol. — The r.p.h.'s of the s.p.'s having *m/z* 45 ($CH_2{=}OMe^+$; C-5) and 129 ($CH_2{=}COAc{\cdot}CH{=}OMe^+$; C-5,4,3) were 2% and

7% respectively. The r.p.h. value of 7%, observed for the *m/z* 117 fragment ($CH_2OAc{\cdot}CH{=}OMe^+$; C-1,2) can be attributed to OMe-2, since cleavage should take place between C-2 and C-3. The values for acetyl substitution at O-2, O-3, and O-5 of the nonreducing end-groups of arabinofuranose were thus 7, 7, and 2%, respectively. In the series where C-1 is linked to deuterium, the values were 9%, 10%, and 2%, respectively. Cleavage between C-2 and C-3 was confirmed by the presence of fragments of *m/z* 118 and 121 (satellite). A peak at *m/z* 117 had only 7% of the height of *m/z* 118, indicating little cleavage between C-3 and C-4.

1,5-Di-O-*acetyl-2,3,4-tri*-O-*methylrhamnitol.* — The e.i. spectrum contained only one interpretable group of peaks. The main peak appeared at *m/z* 161 ($CH_2OAc{\cdot}CHOMe{\cdot}CH{=}OMe^+$; C-1,2,3) and the satellite at 164, with an r.p.h. of 12%, indicating this degree of acetylation at O-3 of the rhamnopyranosyl groups in the original polysaccharide.

1,2,4-Tri-O-*acetyl-3,5-di*-O-*methylarabinitol.* — The r.p.h. of the s.p. of a fragment having *m/z* 189 ($CH_2OAc{\cdot}CHOAc{\cdot}CH{=}OMe^+$; C-1,2,3) was 4% in spectra obtained from mixtures prepared via both sodium borohydride and borodeuteride treatments. The peaks with *m/z* 45 did not have satellites. Thus, acetylation occurs at O-3 of 2-*O*-substituted arabinofuranosyl units to the extent of 4%.

1,3,5-Tri-O-*acetyl-2,4-di*-O-*methylarabinitol.* — A peak at *m/z* 117 ($CH_2OAc{\cdot}CH{=}OMe^+$) was formed by cleavage between C-2 and C-3 and between C-3 and C-4. The r.p.h. of its s.p. at 120 was 2%. In the series deuterated at C-1, the r.p.h. of the s.p. related to *m/z* 118 was 1% and the r.p.h. of the s.p. related to *m/z* 117 was 2%. Thus, the 3-*O*-substituted arabinopyranosyl units are 1% acetylated at O-2 and 2% at O-4.

1,5-Di-O-*acetyl-2,3,4,6-tetra*-O-*methylgalactitol.* — Only a partial determination of *O*-acetyl distribution could be carried out. However, typical fragments were obtained having *m/z* 45 ($CH_2{=}OMe^+$; C-6), 161 ($CH_2OAc{\cdot}CHOMe{\cdot}CH{=}OMe^+$; C-1,2,3), and 87 ($CH_3{\cdot}CO{\cdot}CH{=}OMe^+$; C-6,5,4), and showing s.p.'s of r.p.h. 2, 9, and 4%, respectively. In the C-1-deuterated series, the values were 3, 11, and 6%, respectively. A fragment at *m/z* 118 having an s.p. at 121 with an r.p.h. of 3% likely arose *via* cleavage between C-2 and C-3 to form $CHDOAc{\cdot}CH{=}OMe^+$ (C-1,2). Thus, nonreducing end-groups of galactopyranose are substituted to the extent of 15% by acetyl groups distributed between O-2, O-3, O-4, and O-6.

1,4,5-Tri-O-*acetyl-2,3-di*-O-*methylarabinitol.* — Peaks were observed at *m/z* 117 ($CH_2OAc{\cdot}CH{=}OMe^+$; C-1,2) and 129 ($CH_2{=}COAc{\cdot}CH{=}OMe^+$; C-5,4,3) having s.p.'s of r.p.h. 3% and 4%, respectively. In the C-1-deuterated series the values were 4% and 5%, respectively, indicating an even distribution of ~8% acetyl between O-2 and O-3 of 4-*O*-substituted arabinopyranosyl and/or 5-*O*-substituted arabinofuranosyl units.

1,3,5-Tri-O-*acetyl-2,4,6-tri*-O-*methylgalactitol.* — The r.p.h.'s of the s.p.'s of the *m/z* 117 ($CH_2OAc{\cdot}CH{=}OMe^+$; C-1,2) and 129 peaks ($CH_2{=}COAc{\cdot}CH{=}OMe^+$; C-6,5,4) were 2 and 0.5%, respectively. In the C-1-deuterated series, the values were 1

and 1%. The peak at *m/z* 45 ($CH_2=OMe^+$; C-6) did not have a satellite. Thus, 3-*O*-substituted galactopyranosyl units have 1% of acetyl at each of O-2 and O-4 in the polysaccharide.

*1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol.* — Peaks were detected at *m/z* 45 ($CH_2=OMe^+$; C-6), 117 ($CH_2OAc{\cdot}CH=OMe^+$; C-1,2), and 233 ($CH_2OMe{\cdot}CH_2OAc{\cdot}CH_2OAc{\cdot}CH=OMe^+$; C-6,5,4,3) having s.p.'s of r.p.h. 2, 6, and 9%, respectively. These showed substitution with acetyl groups at O-2 (6%), O-3 (~7%), and O-6 (2%) of galactose.

*1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.* — Peaks were observed at *m/z* 45 ($CH_2=OMe^+$; C-6), 117 ($CH_2OAc{\cdot}CH=OMe^+$; C-1,2), and 233 ($CH_2OMe{\cdot}CH_2OAc{\cdot}CH_2OAc{\cdot}CH=OMe^+$; C-6,5,4,3) whose s.p.'s had r.p.h.'s of 2%, 12%, and 1%. The 4-*O*-substituted glucopyranosyl units in the polysaccharide are therefore acetylated predominantly at O-2.

*1,2,4,5-Tetra-*O*-acetyl-3-*O*-methylarabinitol.* — A fragment was detected at *m/z* 87 [$CH_3{\cdot}CO{\cdot}CH=OMe^+$; C-1(5), 2(4),3] having an s.p. at 90 with an r.p.h. of 4%, equal in relative size to the s.p. at *m/z* 91 in the C-1-deuterated series.

*1,3,4,6-Tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol.* — The only interpretable peaks in the mass spectrum were *m/z* 117 ($CH_2OAc{\cdot}CH=OMe^+$; C-1,2) and its s.p. at 120, r.p.h. 1%. The value for the corresponding signal in the C-1-deuterated series was 2%, thus indicating 1% of acetylation at O-2.

In summary, in a preliminary experiment an improved Bouveng procedure was used to determine the overall distribution of *O*-acetyl groups in the polysaccharide. The major substitutions were at O-2 and O-3 of arabinosyl units and at O-2 of galactose and glucose residues. Surprisingly, small proportions of galactosyl and arabinosyl units were tri-*O*-substituted with acetyl groups (Table II). A more accurate determination of the locations of the acetyl groups showed that they occur mainly as monosubstituents on O-2 and O-3 of nonreducing arabinofuranosyl end-groups and O-2 of (1→4)-linked galacto- and gluco-pyranosyl units, although a large number of other substitution types were present (Table III).

Of the 14 different species of aldosyl units detected, 8 showed the presence of acetyl groups distributed between two or more positions. Whether the observed distribution arose during biosynthesis, or whether the acetyl groups were laid down in only one position and thereafter underwent migration while still in the plant, is open to question.

EXPERIMENTAL

Preparation of O*-acetyl* N*-methyl-*N*-phenylcarbamate derivative from heteropolysaccharide.* — Dried heteropolysaccharide[1] (polysaccharide P; 350 mg) was dissolved in DMSO (12 mL) at 50°, phenylisocyanate (2 mL) added, and the mixture was maintained for 12 h at this temperature. It was then added to excess ethanol and the precipitated phenylcarbamate derivative (460 mg) was collected following centrifugation, 3 washes with ethanol, and drying. The derivatization proces was repeated twice.

The product was dissolved in DMF (6 mL), methyl iodide (1.5 mL) and Ag_2O (1.0 g) were added, and the mixture was stirred in the dark for 24 h. More Ag_2O (1.0 g) was then added, followed by another portion after 48 h. After 72 h reaction time the mixture was diluted with excess dichloromethane, filtered, and the filtrate was evaporated to a residue of the *N*-methyl-*N*-phenylcarbamate derivative.

O-Deacetylation of N-*methyl*-N-*phenylcarbamate derivative.* — To the derivative in 1:1 THF–MeOH (6 mL) at 4°, 0.2M NaOMe in MeOH (1 mL) was added. After 24 h at 4°, the solution was neutralized (AcOH) and evaporated to dryness, the residue was suspended in water, and the mixture was dialyzed. The residue, obtained on evaporation, did not contain *O*-acetyl groups[8].

O-Methylation and O-deuteriomethylation of O-deacetylated material. — The above product was divided into 2 equal parts. The first was *O*-methylated with DMF–MeI–Ag_2O (6 mL; 0.8 mL; 1.0 g; with further additions of Ag_2O after 24 h and 48 h), and the product was isolated as described above. The remainder was deuteriomethylated by the same method, MeI being replaced by MeI-d_3.

Removal of N-*methyl*-N-*phenylcarbamate groups from O-methylated,* N-*methylated phenylcarbamate derivatives.* — Each product was dissolved in 1:1 THF–MeOH (6 mL), 0.2M NaOMe in MeOH (1 mL) was added, and the mixture was heated for 1 h under reflux. It was then neutralized (AcOH) and evaporated to dryness, and the residue was dialyzed. Evaporation gave partly *O*-methylated polymers that still showed i.r. absorption for phenylcarbamate at 700, 770, and 1705 cm^{-1}. The decarbamoylation process was then repeated twice in order to completely remove these substituents.

Analysis of partly O-methylated polysaccharides by g.l.c.–m.s. of derived partly O-methylated alditol acetates. — A portion of partly *O*-methylated polysaccharide was hydrolyzed[9] with 72% w/w H_2SO_4 for 1 h at 22°, followed by 0.5M H_2SO_4 for 18 h at 98°, to form a mixture of partly *O*-methylated aldoses. This was then reduced with aqueous NaB^2H_4 and the product was acetylated to give partly *O*-methylated alditol acetates, which were submitted to g.l.c.–m.s. using a Model 4000 Finnigan unit interfaced with an Incos 2300 Data System. E.i. spectra were obtained every 2 s by repetitive scanning from *m/z* 40 to 420. A capillary column (0.25 mm i.d. × 30 m) of DB-210 was used, injections were made in the split mode at 50°, and a rapid temperature-increase program (40°.min^{-1} to 195°, then hold) was executed. The carrier gas was He (linear velocity, 22 $cm.s^{-1}$). Peaks were identified by their e.i.-breakdown patterns[5] and retention times.

Analysis of product formed by O-methylation of partly O-deuteriomethylated polysaccharide. — The *O*-deuteriomethylated polysaccharide was methylated by the method of Hakomori[10] until the product gave an i.r. spectrum lacking OH absorption at ~3200 cm^{-1}. The product was then converted, by the method just described, into a mixture of partly *O*-methylated alditol acetates, one portion of which was reduced with $NaBH_4$ and the other with NaB^2H_4. These products were acetylated and the acetates examined by g.l.c.–m.s. using the DB-210 column (Table III).

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