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SELECTIVE FRAGMENTATION OF METHYLATED TOBACCO EXTRACELLULAR ARABINOGLUCURONOMANNAN BY CLEAVAGE OF β-D-GLUCOPYRANOSIDURONIC ACID UNITS*

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ABSTRACT

Permethylated extracellular arabinoglucuronomannan from suspension-cultured tobacco cells has been subjected to the following sequence of reactions: (*i*) saponification, (*ii*) decarboxylation–acetoxylation with lead tetraacetate, and (*iii*) reductive cleavage of the derived 5-acetoxypentopyranosidic linkages with sodium borohydride to give a series of 4-*O*-α-D-mannopyranosyl-D-xylitol derivatives with and without L-arabinofuranosyl side-chain substituents. The oligosaccharide-alditols have been separated, and characterized on the basis of n.m.r.- and mass-spectral data and of compositional analysis of (trideuteriomethyl)ated derivatives. The results provided evidence for alternating sequences of 4-linked β-D-glucosyluronic acid and 2-linked α-D-mannosyl residues in the main chain, with α-L-arabinofuranosyl groups attached to O-3 of some of the former, and β-L-arabinofuranosyl groups attached to O-3 of some of the latter residues.

INTRODUCTION

Until recently, polysaccharides based on internal chains of 4-linked β-D-glucuronic acid and 2-linked α-D-mannopyranose residues were known only amongst the exudate gums[1]. The discovery of arabinoglucuronomannans (AGMs) in the extracellular and intracellular polysaccharides of suspension-cultured cells of *Nicotinia tabacum* was of considerable interest, because other exudate gums of the arabinogalactan, arabinoxylan, and rhamnogalacturonan families bear close resemblances to the corresponding glycans from plant cell-walls. The main structural features of the tobacco polysaccharides were established by Kato *et al.*[2,3], who provided evidence for the glucuronomannan backbone, with attachment of L-arabinofuranosyl groups as single-unit side-chains to most residues of each type. In the case of the intracellular[2] AGM, ^{13}C-n.m.r. spectral data confirmed the anomeric configurations of the backbone residues, and a characteristic signal at $\delta \sim 110$ was

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assigned to α-L-arabinofuranosyl substituents. Similar evidence was reported for the extracellular[3] AGM, which carried arabinofuranosyl groups at a rather smaller proportion of the backbone residues, but also contained, as minor constituents, some terminal D-galactopyranose and some 5-linked L-arabinofuranose (or some 4-linked L-arabinopyranose) residues.

We have recently developed a selective fragmentation procedure[4] for the cleavage of internal glucosiduronic acid linkages in methylated glycans, with the generation of partially methylated xylitol-terminated oligosaccharides. This procedure has provided information on the detailed structures of glucuronomannans from *Anogeissus leiocarpus* gum[5] and the mucins of *Drosera* species[6]. Dr. K. Kato kindly provided us with a sample of the extracellular AGM, and a detailed examination was undertaken with the primary intention of seeking evidence for the distribution of L-arabinofuranosyl groups along the backbone of the glycan.

RESULTS AND DISCUSSION

The extracellular AGM was methylated, and analysis of the sugars formed on hydrolysis of the carboxyl-reduced, methylated glycan (see Table I) gave results in close agreement with those reported earlier[3]. Permethylated AGM was saponified with dilute alkali, and the resulting polysaccharide acid was treated with lead tetraacetate until no ionizable carboxyl groups could be detected. The methylated tobacco AGM derivative, containing 5-acetoxypentopyranosyl residues, was then treated with sodium borohydride to effect reductive cleavage at the modified sugar residues, and it furnished a mixture of partially methylated oligosaccharide-alditols.

A small portion of the oligosaccharide-alditol mixture was (trideuteriomethyl)ated, and g.l.c.–m.s. analysis (see Table II) revealed the presence of five separable oligosaccharides having retention times in the range for di-, tri-, and tetra-saccharides. Each individual component showed fragment-ions having uniform incorporation of isotopic label, and, with knowledge of the sugar residues

TABLE I

METHYLATION ANALYSIS OF CARBOXYL-REDUCED METHYLATED TOBACCO ARABINOGLUCURONOMANNAN

Alditol acetate	T^a	*Relative proportion (%)*
2,3,5-Me_3Ara	0.46	26.3
2,3-Me_2Ara	1.08	2.2
2,3,4,6-Me_4Gal	1.18	3.1
3,4,6-Me_3Man	1.83	17.4
4,6-Me_2Man	2.94	16.2
2,3-Me_2Glc	4.46	17.8
2-MeGlc	6.61	17.0

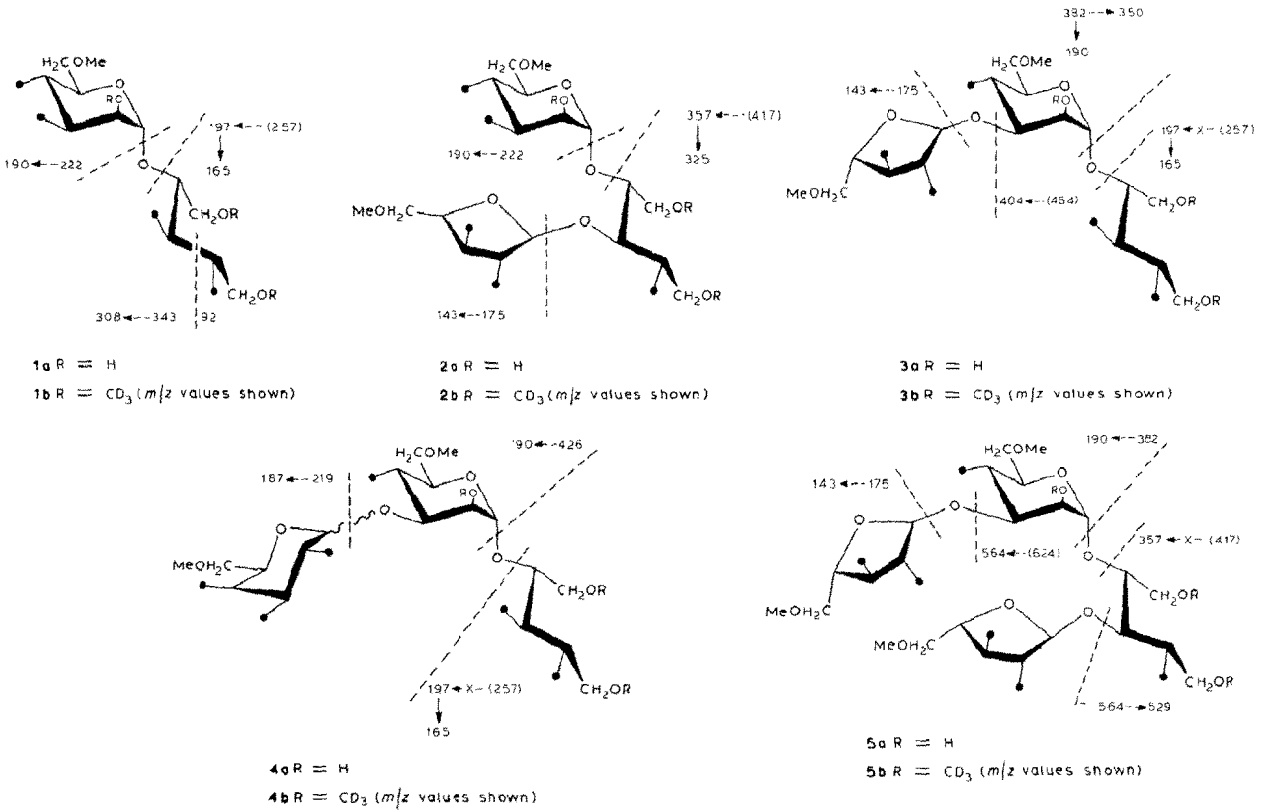
[a]Retention times relative to 2,3,4,6-Me_4Glc (5.95 min); column A, isothermal at 190°.

in the parent methylated polysaccharide, provisional assignments of structure were made for the peralkylated oligosaccharides **1b** to **5b**, whose structures are shown in Scheme 1, together with the more important fragment-ions.

Fractionation of the major portion of the mixture of oligosaccharide-alditols by preparative liquid chromatography (l.c.) followed by h.p.l.c. furnished samples of the major oligosaccharides **1a, 2a, 3a,** and **5a**. The configurations of glycosidic linkages were assigned from their ^{1}H-n.m.r. spectra, and the oligosaccharides were each converted into their (trideuteriomethyl)ated derivatives, which were checked for homogeneity and structural identity by g.l.c.–m.s. The fully alkylated derivatives were hydrolyzed, and the resulting sugars were converted into partially methylated alditol acetates for examination by g.l.c.–m.s. The results showed that the oligosaccharides **1a, 2a, 3a,** and **5a** contained a 4-*O*-α-D-mannopyranosyl-D-xylitol unit with free hydroxyl groups at C-2 of the D-mannosyl group and at C-1 and C-5 of the D-xylitol residue.

Disaccharide **1a** showed a single anomeric signal, at δ 4.97 (d, $J_{1,2}$ 2.0 Hz), and hydrolysis of the alkylated derivative **1b** gave 2*,3,4,6-tetra-*O*-methylmannose† and 1*,2,3,5*-tetra-*O*-methylxylitol. The trisaccharide **2a** showed two anomeric protons, at δ 4.94 (d, $J_{1,2}$ 2.0 Hz) and 5.23 (br. s), assignable to α-D-mannopyranosyl and α-L-arabinofuranosyl[7] residues respectively, and hydrolysis of the alkylated derivative **2b** furnished 2,3,5-tri-*O*-methylarabinose, 2*,3,4,6-tetra-*O*-methylmannose, and 1*,2,5*-tri-*O*-methylxylitol. The fragment-ions, shown together with the presence in the mass spectrum of an ion of high abundance at *m/z* 211 (V ion[8]), supported a branched structure with vicinal substituents in this derivative **2a** of the trisaccharide 3-*O*-α-L-arabinofuranosyl-4-*O*-α-D-mannopyranosyl-D-xylitol. The structure for the trisaccharide **3a** as a partially methylated derivative of *O*-β-L-arabinofuranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→4)-D-xylitol was established on the basis of two anomeric protons in the ^{1}H-n.m.r. spectrum, at δ 4.94 (d, $J_{1,2}$ 1.6 Hz) and 5.10 (d, $J_{1',2'}$ 4.7 Hz), respectively, assignable to α-D-mannopyranosyl and β-L-arabinofuranosyl[7] residues, and the formation of 2,3,5-tri-*O*-methylarabinose, 2*,4,6-tri-*O*-methylmannose, and 1*,2,3,5*-tetra-*O*-methylxylitol on hydrolysis of the peralkylated derivative **3b**. The following evidence supported the structure proposed for **5a** as being a partially methylated derivative of the branched tetrasaccharide 3-*O*-α-L-arabinofuranosyl-4-*O*-[*O*-β-L-arabinofuranosyl-(1→3)-α-D-mannopyranosyl]-D-xylitol. Hydrolysis of the peralkylated derivative **5b** gave 2,3,5-tri-*O*-methylarabinose, 2*,4,6-tri-*O*-methylmannose, and 1*,2,5*-tri-*O*-methylxylitol. The branched structure with vicinal glycosyl substituents on the xylitol residues was also indicated in the mass spectrum by the ion of high abundance at *m/z* 211 (V ions[8]), as shown previously for the same trisaccharide from methylated *Drosera* mucins[6]. Of the three anomeric protons, at δ 4.91 (d, $J_{1,2}$ 1.2 Hz), 5.12 (d, $J_{1',2'}$ 4.6 Hz), and 5.23 (d, $J_{1'',2''}$ 0.9 Hz), the last two were respectively assigned to β- and α-L-arabinofuranosyl[7] groups attached in an analogous manner to those in the two trisaccharides **2a** and **3a**. Because preparative separation failed to yield appreciable quantities of partially methylated trisaccharide **4a**, the struc-

†The asterisk designates a trideuteriomethyl substituent.



Scheme 1. The salient features of the mass-spectral fragmentations are shown for compounds **1b** to **5b**, with the convention that (257) → 197, or (257) -X → 197, indicates that an ion of the J_1 series is, or is not, observed [—● = –OMe].

TABLE II

RELATIVE PROPORTIONS AND PROVISIONALLY ASSIGNED STRUCTURES FOR PERALKYLATED OLIGOSACCHARIDE-ALDITOLS GENERATED FROM DEGRADATION OF TOBACCO AGM

Parent oligosaccharide, with placing of CD_3 groups in peralkylated derivative[a]	*Retention time (min)*[b]	*Relative proportion (%)*
*Man*p*-(1→4)-**Xylitol (**1b**)	2.75	23.5
*Man*p*-(1→4)-**Xylitol-(3←1)-Ara*f* (**2b**)	8.43	32.0
Ara*f*-(1→3)-*Man*p*-(1→4)-**Xylitol (**3b**)	9.45	23.0
Gal*p*-(1→3)-*Man*p*-(1→4)-**Xylitol (**4b**)	10.51	7.0
Ara*f*-(1→3)-*Man*p*-(1→4)-**Xylitol-(3←1)-Ara*f* (**5b**)	15.07	14.5

[a]* Indicates the number of CD_3 groups attached to designated residues. [b]On column B; 150° (2 min) then to 280° (8°/min, hold).

tural assignment as a derivative of *O*-D-galactopyranosyl-(1→3)-*O*-D-mannopyranosyl-(1→4)-D-xylitol is based on the mass-spectral data for the fully alkylated trisaccharide **4b**.

The results of the selective fragmentation provided confirmatory evidence for the backbone structure of the AGM as containing alternating glucuronic acid and mannose residues, through the isolation of the series of 4-*O*-α-D-mannopyranosyl-D-xylitol derivatives. An apparently non-regular distribution of arabinofuranosyl side-chains was indicated by the generation of oligosaccharides carrying (*a*) no side-chains, (*b*) two differently attached single side-chains, and (*c*) two side-chains. The occurrence of both α- and β-L-arabinofuranosyl side-chains attached at specific sites is of particular interest. Other glucuronomannans recently examined in this laboratory contain only one type each of arabinofuranosyl units but the sites of attachment are the same, namely, β-L-arabinofuranosyl groups attached to O-3 of mannose residues[5] in leiocarpan A, and α-L-arabinofuranosyl groups attached to O-3 of glucuronic acid residues in the *Drosera* mucins[6]. Although the anomeric configuration of the D-galactopyranosyl side-chains was not established, the site of attachment to O-3 of mannose residues is the same as in the *Drosera* mucins[6,9].

Earlier studies by Kato *et al.*[2,3] suggested that, in addition to residues of β-D-glucuronic acid and α-D-mannopyranose, only those of α-L-arabinofuranose were present in the tobacco arabinoglucuronomannans. Although insufficient quantities of the extracellular AGM remained for re-examination of the ^{13}C-n.m.r. spectrum in the light of the evidence for both α- and β-L-arabinofuranosyl residues, it was possible to obtain ^{13}C-n.m.r. data for leiocarpan A, in which β-L-arabinofuranose residues have been identified. In addition to assignments of anomeric carbon atoms for the three aforementioned types of sugar residues in the intracellular AGM, and by implication for the extracellular AGM also, signals at δ_C ~94, 98, and 103 were assigned to reducing L-arabinose inadvertently liberated by autohydrolysis. Of the latter signals, only the first two have been reported[10] for the anomeric carbon atoms

of α- and β-L(or D)-arabinopyranose, and we have confirmed this observation. The ^{13}C-n.m.r. spectrum for leiocarpan A showed incompletely resolved signals at δ_C 104.0, 103.1, and 102.7, which were assigned to β-D-xylopyranose, β-L-arabinofuranose, and β-D-glucuronic acid, and another, at δ_C 99.1, assigned to α-D-mannopyranose residues. The signal assigned to the β-L-arabinofuranosyl residues was no longer present in the spectrum of the arabinose-free, degraded glycan[11], which showed signals for anomeric carbon atoms at δ_C 104.1, 102.3, and 99.0. It is probable that the signal at δ_C ~103, which was mistakenly assigned by Kato *et al.*[2], was that of β-L-arabinofuranose residues. It is noteworthy that the intensity of the signal at δ_C ~109, which was correctly assigned to α-L-arabinofuranose residues, was of considerably lower intensity than might be expected if all of the arabinose residues had had this configuration.

EXPERIMENTAL

General methods. — The sample of arabinoglucuronomannan from extracellular polysaccharides of suspension-cultured tobacco cells was kindly provided by Dr. K. Kato[3]. Samples of leiocarpan A and the arabinose-free degraded glycan were prepared as described previously[11]. Methylations of glycan and oligosaccharides were performed by the Hakomori procedure as described by Jansson *et al.*[12]. The methylated derivatives were hydrolyzed with 2M trifluoroacetic acid[13] for 1 h at 120°, and the resulting sugars were determined as partially methylated alditol acetates by g.l.c.–m.s.[12]. N.m.r. spectra (^{1}H- and ^{13}C-) were recorded with a Bruker AM 300 spectrometer for solutions in $CDCl_3$ or D_2O, with tetramethylsilane or methanol as the internal standard. G.l.c. was performed with a Perkin–Elmer Sigma 3B chromatograph, using fused-silica columns (Chromatographic Specialities Limited): *A*, a 15-m wide-bore capillary of DB-225, or *B*, a 5-m, narrow-bore capillary of DB-5. For g.l.c.–m.s., columns were attached by a jet separator to a VG Micromass 16F mass spectrometer, which was operated with an inlet temperature of 250°, an ionization potential of 70 eV, and an ion-source temperature of ~250°.

Degradation of methylated tobacco arabinoglucuronomannan. — A sample of methylated tobacco AGM was reduced by boiling with M lithium triethylborohydride in oxolane for 4 h, the carboxyl-reduced glycan was isolated as described[14], and the hydrolysis products were converted into partially methylated alditol acetates for analysis by g.l.c.–m.s. as shown in Table I. Methylated AGM (55 mg) in water (12 mL) was saponified at 0° by dropwise addition of M sodium hydroxide to maintain a pH of 12. After 2 h, the solution was warmed to room temperature and kept for a further 15 h at pH 12. Sodium ions were removed by passage of the solution through a column of Amberlite resin IR-120(H^+), and the eluate was freeze-dried to give methylated polysaccharide acid (50 mg); $\nu_{max}^{NaCl\ disc}$ 1740 cm^{-1} (shifted completely to 1600 cm^{-1} on addition of triethylamine).

The methylated polysaccharide acid (47 mg) in dry benzene (8 mL) containing pyridine (0.2 mL) and freshly recrystallized lead tetraacetate was boiled for 2 h. A further portion (50 mg) of lead tetraacetate was added, and the mixture was heated for another 15 h. The mixture was cooled and filtered, the precipitate was washed with benzene, the filtrate and washings were combined and evaporated, and a solution of the residue in dichloromethane was washed with water, dried, and evaporated. The product was chromatographed on Sephadex LH-20 using 2:1 dichloromethane–methanol, to give modified methylated AGM (42 mg); ν_{max} 1750 cm^{-1}, but no absorption at 1600 cm^{-1} on addition of triethylamine, indicating, within the limits of detection, that all glucuronic acid residues had been modified. The modified methylated AGM (40 mg) in 1:1 oxolane–water (4 mL) was treated with sodium borohydride (100 mg) for 15 h. The excess of hydride was decomposed, and sodium ions were removed by addition of Amberlite resin IR-120(H^+), the suspension filtered, the filtrate concentrated, and methanol repeatedly added to and evaporated from the residue, to remove boric acid and furnish a mixture (35 mg) of partially methylated oligosaccharide-alditols. A portion of the mixture was (trideuteriomethyl)ated, and examination of the fully alkylated derivatives by g.l.c.–m.s. (see Table II) showed the presence of 5 components for which structures **1b** to **5b** were provisionally assigned.

The remainder of the mixture was separated initially on a 1-mm silica gel plate and then in batches (2 mg each) by h.p.l.c. using a Waters Associates analytical Partisil column (4.6 × 250 mm, 10-μm silica), with 97:3 benzene–methanol as eluant, to give four oligosaccharide fractions (**1a, 2a, 3a,** and **5a**). The fractions were examined by ^{1}H-n.m.r. spectroscopy and, after (trideuteriomethyl)ation, by g.l.c.–m.s. (*i*) directly (on column *B*) and (*ii*) after hydrolysis and conversion into partially methylated alditol acetates, on column *A*.

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