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CONFLICTING EVIDENCE FOR NON-METHYL GALACTURONOYL ESTERS IN *DAUCUS CAROTA*

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Key Word Index—*Daucus carota*; Umbelliferae; carrot root; cell wall; pectic polysaccharides; non-methyl galacturonoyl esters; Driselase resistance; acetylation.

Abstract—An alcohol-insoluble residue from carrot root, analysed by an established method, appeared to contain partially non-methyl esterified pectic polysaccharides. Digestion with Driselase, to which such esters are thought to be resistant, gave small amounts of acidic oligosaccharides. Treatment with alkali at room temperature hydrolysed some of these components and gave more acidic 1,4-linked oligogalacturonic acids as products. Since Driselase is known to cleave pectic methyl esters, these components appeared to contain non-methyl galacturonoyl esters. However, the predominant component of this type was shown to be a monomethyl esterified, singly-acetylated tetragalacturonic acid. It was converted to galacturonic acid when re-treated with Driselase and, thus, was a product of incomplete digestion. Other, more minor components with similar properties were either other methyl esterified oligomers or were present in such small amounts that their characterization was not possible.

INTRODUCTION

The concept that covalent cross-links might exist between the polymeric components of the plant cell wall is well established and, indeed, certain types, such as phenolic cross-links, have received much attention [1]. In plant cell walls, the galacturonic acid residues of pectic polysaccharides are often methyl-esterified [2]. The idea that non-methyl galacturonoyl esters (NMGEs) might also occur and function as cross-links between pectic polysaccharides and other wall polymers is not new [3], but a method to detect them has only recently been developed. Kim and Carpita [4] detected NMGEs in maize coleoptiles and studied changes in their contents during growth. McCann *et al.* [5] have used the approach to detect NMGEs in suspension culture cells of tobacco. Brown and Fry demonstrated [6] that a synthetic galacturonoyl-glucose ester was not cleaved by Driselase (a mixture of *exo*- and *endo*-hydrolases, which also contains pectin methyl esterase, and which is known to degrade cell wall material to, principally, monomeric and dimeric material [1]). Driselase digestion generated ^{14}C-labelled oligogalacturonides from suspension-cultured spinach cells, which seemed to contain NMGEs [7]. Nevertheless, none of these approaches established the nature of the esterified alcohol(s).

The concept of a cross-link which is made and cleaved during developmental changes [4] in the wall is particularly attractive, as it would present an opportunity for enzymic control of wall properties. Isolation of such linkages might be expected to be difficult, however, because only a few cross-links would be necessary to cause a dramatic effect on the structural properties of the wall [8].

As part of our ongoing study of the structure and function of cell wall polymers, we decided to further investigate the nature of NMGEs. Our aims were two-fold. First, to establish the presence of NMGEs in the walls of dicotyledonous, free-growing plants; Kim and Carpita [4] studied the primary walls of wheat internodes. Pectic polysaccharides of graminaceous monocots comprise only a minor proportion of the wall [9] and, thus, may not be typical of the more abundant pectic polysaccharides of dicotyledons. Brown and Fry [7] and McCann *et al.* [5] used suspension cultures of the dicots spinach and tobacco; there is increasing evidence that cultures may not always accurately reflect the nature of the walls of free-grown plants [10]. Secondly, we hoped to identify the nature of the esterified alcohols and, perhaps, to infer the role of NMGEs in the wall.

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RESULTS AND DISCUSSION

Estimation of NMGE content

We chose to study an alcohol-insoluble residue (AIR) of carrot root for two reasons. First, Koch and Nevins [11] have demonstrated that the degree of methyl-esterification, of an AIR of tomato pericarp, was higher than that in walls prepared by other methods. We thus expected to minimize degradation of other galacturonoyl esters by using an AIR. Secondly, Brown and Fry [7] reported that the walls of a carrot suspension culture contained NMGEs. Consequently, they might also occur in mature carrot root and, indeed, analysis by us, of carrot root AIR as described by Kim and Carpita [4], suggested that 8% of its galacturonic acid residues were present as NMGEs. Table 1 summarizes the carbohydrate composition and degree of esterification of carrot AIR. Table 2 gives the data, with error limits, from which the degree of methyl and total esterification and, hence, the degree of non-methyl esterification, were calculated [4]. Although the apparent NMGE content was smaller than the ranges measured during maize coleoptile [4] and tobacco suspension culture [5] growth, it was comparable with that of spinach cultures [7].

Driselase digestion and high-performance anion-exchange chromatography of products

We digested Carrot AIR with Driselase (purified from the crude commercial material) for 5 days. Although solubilization appeared rapid—the majority of material dissolved within 30 min—treatment was extended to maximize degradation. A residue (8%) was removed by centrifugation. The added enzymes and any residual solubilized high M_r material were removed by ultrafiltration, through a membrane with a nominal 10 kD cutoff. A sample of this ultrafiltrate was concentrated by evaporation and examined by high-performance anion-exchange-chromatography (HPAEC) at pH 6. This pH ensured that only acidic components were retained and separated by the column, and that any esterified residues were not hydrolysed. Post-column addition of base ensured that the pulsed electrochemical detector (PED) operated at high sensitivity. A sample was treated with NaOH at pH 12.8 for 1 h for comparison (Fig. 1). The complexity of the traces made interpretation difficult but certain components diminished after hydrolysis; others appeared at longer retention times—in particular tri- and tetra-galacturonic acid (OG3 and OG4). This strongly suggested the presence of NMGEs in the digest. (Incubation of the pre-purified

Table 1. Sugar composition and degree of galacturonic acid esterification of an alcohol-insoluble residue of carrot root

Anhydro sugars (μg mg^{-1} dry weight)									Ester (mol%)		
Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA*	Total	dm†	de‡	NMGE§
15	2	50	21	22	63	303	292	768	49	57	8

* Total uronic acid (GalA + GlcA) as measured by colorimetric assay [20]. GlcA content was, however, found to be negligible by carboxyl reduction and GC-MS.

† Degree of methyl-esterification of GalA. This was calculated from the ratio of the MeOH, liberated by hydrolysis, to the GalA content (both expressed in μmol mg^{-1} – see Table 2).

‡ Total degree of esterification of GalA as determined by selective reduction of GalA residues to 6,6-dideuterogalactosyl residues and GC-MS [4] (see Table 2).

§ NMGE (%) = de − dm.

Table 2. Analysis of carrot alcohol-insoluble residue by the method of Kim and Carpita [4]

GalA/μmol mg^{-1}*†	MeOH/μmol mg^{-1}‡	Total GalA as a percentage of final Gal§	Esterified GalA as a percentage of final Gal¶
1.66 ± 0.10	0.82 ± 0.02	80.9 ± 1.1	46.0 ± 2.1

* Total uronic acid as measured by colorimetric assay [20]. GlcA content was, however, found to be negligible by carboxyl reduction and GC-MS.

† All data error limits are 95% certainty limits calculated from the standard error of the mean of quadruplicate determinations [4].

‡ Measured by GC after base hydrolysis [21].

§ All GalA was reduced to 6,6-dideuterogalactosyl residues. The ratio of these residues to the resultant total Gal content was determined by carbohydrate analysis and GC-MS [4].

¶ All esterified GalA residues were selectively reduced to 6,6-dideuterogalactosyl residues. After reduction of unesterified GalA residues to unlabelled galactosyl residues, the ratio of the labelled residues to the resultant total Gal content was determined by carbohydrate analysis and GC-MS [4]. The ratio of this figure to the total GalA as a percentage of final Gal (column 3) gave the de (see Table 1).

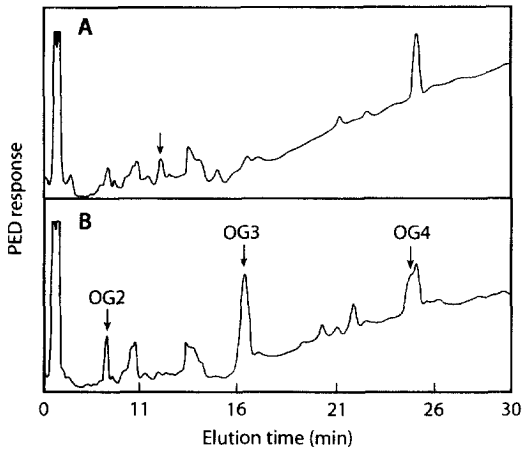


Fig. 1. High-performance anion-exchange chromatography (semipreparative Dionex PA-1 column) of the diafiltered Driselase digest. **A**, unhydrolysed digest (the fraction subsequently studied is arrowed); **B**, digest after base hydrolysis at pH 12.8 (the oligogalacturonic acid product peaks are marked).

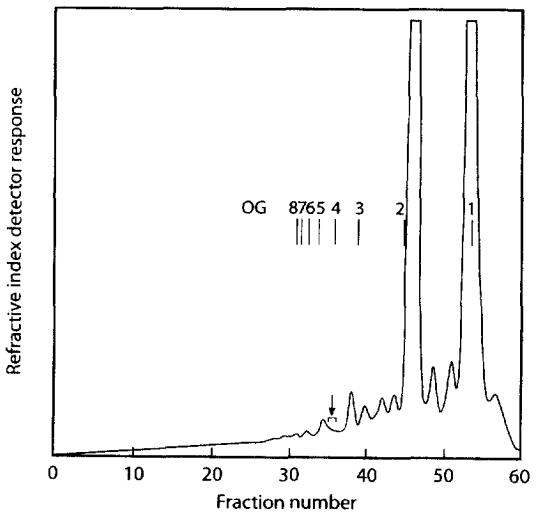


Fig. 2. Size-exclusion chromatography (Fractogel 40S, pH 6) of the diafiltered Driselase digest. Elution times of oligogalacturonic acid standards are shown for comparison. The fraction subsequently studied is arrowed.

Driselase alone, in buffer, as a control, for the same period produced no autolysis products detectable by HPAEC under the conditions used).

We concentrated in our study on components of the digest which were: (a) hydrolysed by base, and (b) replaced by an OG of higher charge than the component removed. (Any β-elimination of the oligomers was expected to be slower than ester hydrolysis. It would lead in part to smaller OGs which would be recognized together with the primary non-eliminated product.) The sole arbiter of these properties during screening was R_t during HPAEC. The components of interest might also contain substituents on ring hydroxyl groups; these would go undetected if they were base-labile (e.g. acetyl) or did not noticeably affect the R_t of the OG produced by hydrolysis. These possibilities would only be distinguished by additional experiments (see below).

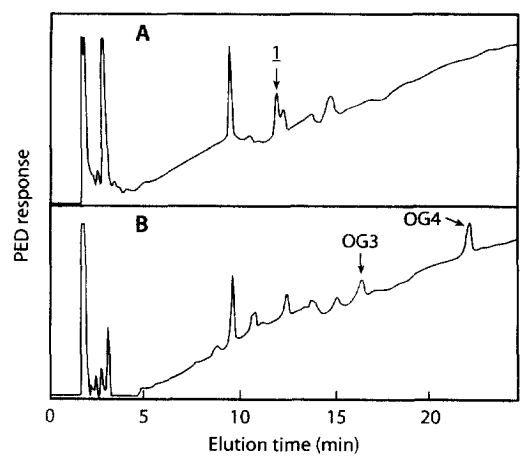
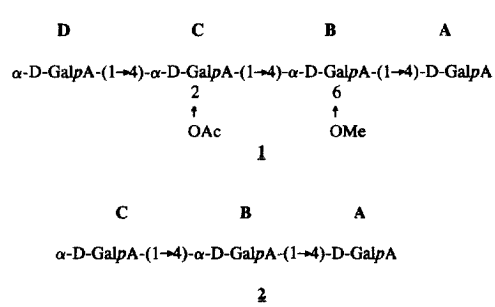


Fig. 3. High-performance anion-exchange chromatography (semipreparative Dionex PA-1 column) of a fraction from size-exclusion chromatography which contained compound **1**. **A**, unhydrolysed; **B**, after base hydrolysis at pH 12.8.

Size-exclusion chromatography of products of Driselase digestion

A sample of the digest was subjected to size-exclusion chromatography (SEC) on Fractogel 40S at pH 6 (Fig. 2). Fractions of the eluate were examined by HPAEC (4 × 250 mm CarboPac PA-1 column) before and after hydrolysis, as described above, and compared with a run of oligogalacturonide standards. The traces thus obtained for a fraction, which eluted on SEC after OG5 but before OG4, are shown in Fig. 3. Hydrolysis of this fraction produced OG4 and OG3 and removed a component, **1**. Sequential SEC, on Biogel P2 at pH 3.3 and on Fractogel 40S, gave a fraction which contained **1** as the only hydrolysable component, and OG4 as the only acidic hydrolysis product. The latter suggested that the esterified alcohol was of low M_r and uncharged (when it would coelute with the early eluting salt peaks) or was undetectable by the PED program used. Methylation or acetylation of the products gave no detectable com-



ponents when examined by GC. This suggested that the esterified alcohol gave either highly volatile or involatile products when derivatized and, thus, was unlikely to be a polyol (i.e. a low M_r sugar or diol which might cross-link the polymers).

Examination of **1** *by fast atom bombardment mass spectrometry*

A sample of a fraction containing **1** alone, prepared by preparative HPAEC, was desalted and examined by negative ion FAB mass spectrometry which showed $[M\text{-}H]^-$ *m/z* 777. A sample of OG4 gave, as expected, $[M\text{-}H]^-$ *m/z* 721. This suggested that **1** was either a diethyl galacturonoyl ester, or a monomethyl galacturonoyl, monoacetyl ester of OG4. **1** Eluted at 13.7 min on HPAEC (4 × 250 mm CarboPac PA-1 column), after OG2 (10.4 min) but before OG3 (17.7 min) and OG4 (23.1 min). These data were felt to be consistent, in principle, with a triply-charged (monoesterified) or a doubly-charged (diesterified) structure for **1**. A fragment ion of *m/z* 601 was also observed; this corresponded to loss of a terminal unsubstituted residue from either the non-reducing end or reducing end of **1** by Schemes B and C of Dell [12], respectively.

Acetic acid analysis of carrot AIR

Hydrolysis of carrot AIR and analysis of acetic acid by GC [13] indicated a degree of acetylation of 23%, if the GalA residues were exclusively substituted. Although xyloglucans can also be acetylated [14], this result was consistent with an acetylated structure for **1**.

Partial hydrolysis of **1**

Partial hydrolysis of **1**, at pH 9 for 15 s and 2 min (Fig. 4), supported a singly methyl-esterified and acetylated structure. A peak first appeared just before **1**, suggesting an initial product of the same charge as **1**, consistent with deacetylation. After 2 min hydrolysis, another peak appeared just before OG4; this was consistent with initial methyl ester cleavage to give monoacetylated OG4.

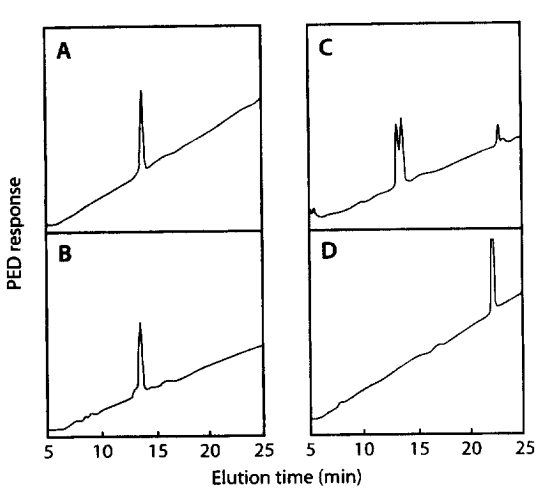


Fig. 4. High-performance anion-exchange chromatography (analytical Dionex PA-1 column) of compound **1**. **A**, unhydrolysed; **B**, **C**, after partial base hydrolysis at pH 9 for 15 s and 2 min, respectively; **D**, after hydrolysis at pH 12.8 for 60 min.

Location of substituents by ^1H *NMR*

A sample of **1** was isolated by repeated fractionation of the digest by SEC and preparative anion-exchange chromatography. The location of the *O*-acetate and methyl ester substituents was determined by a combination of one- and two-dimensional (COSY and ROESY) ^1H NMR experiments. The same methods provided confirmation that **1** was a linear tetramer of α-D-galacturonic acid. The ^1H assignments (carbohydrate region only) of **1** are shown in Table 3. Singlet resonances were observed for the methyl groups of the two substituents (OMe at δ 3.76; OAc at δ 2.18). Integration of these singlets relative to the assigned carbohydrate resonances showed that there was one substituent of each type present in the tetrasaccharide. Apart from one or two ambiguities (resolved by consideration of the ROESY spectrum), the COSY spectrum allowed all the ^1H chemical shifts in the four sugar rings to be obtained. The sequence of the sugar units and, hence, the location of substituents, was determined from the ROESY spectrum and by comparison of the chemical shifts of **1** with those of a reference compound, **2** (Table 3). Although chemical shifts of **2** have been published previously [15], we re-recorded the spectrum to facilitate comparison with **1** under comparable conditions.

The chemical shifts for the protons of the reducing and non-reducing termini of **2** were almost identical with the values for the units labelled **A** and **D** in **1**. This shows not only that **A** and **D** in **1** correspond to reducing and non-reducing ends, respectively, but also that both residues are very likely unsubstituted, as in **2**. The occurrence of ROESY cross-peaks (indicating proximity of protons H1 and H4′ across the glycosidic linkage) at δ 5.12/4.39 (**A**4α) and δ 5.12/4.34 (**A**4β) showed that the anomeric signal at δ 5.12 arose from **B**1. The assignment was confirmed by observation (COSY spectrum) of a small splitting of the signals for **B**3 (proximity to α/β reducing end), a phenomenon also noted for the corresponding **B**3 in **2**. The remaining anomeric signal (δ 5.06) is therefore associated with **C**1. From the COSY spectrum, the chemical shift of **C**2 was found at δ 4.87, i.e. considerably downfield of the expected position (δ 3.77, cf **B**2 in **2**) for an unsubstituted GalA residue. Thus, the acetyl group is linked to *O*-2 of unit **C**.

As the chemical shifts of **B**4 and **C**4 are identical (δ 4.49), an ambiguity arises in the COSY spectrum assignment of the H5 signals (δ 5.10 and 4.85) for rings **B** and **C**. The situation is further complicated by the near overlap of two signals (one of them from **D**1) at δ 5.10. Assignment of the H5 signals is important

Table 3. ^{1}H Chemical shifts for compound **1** and reference compound **2**

Compound	Unit	Chemical shift (δ) H-1	H-2	H-3	H-4	H-5
1	Aα	5.30	3.80	3.98	4.39	4.41
	Aβ	4.59	3.47	3.74	4.33	4.05
	B	5.12	3.76	4.01, 4.03*	4.49	5.10
	C	5.06	4.87	4.23	4.49	4.85
	D	5.09	3.73	3.92	4.26	4.66†
2	Aα	5.30	3.85	3.99	4.42	4.40
	Aβ	4.59	3.49	3.75	4.37	4.04
	B	5.10	3.77	4.00, 4.02*	4.41	4.75
	C	5.05	3.71	3.91	4.26	4.74

* The two values are for unit B linked to α- and β-forms of the reducing end unit.
† Measured at 40° as obscured by residual water signal at 27°.

because it provides the clearest indication (at the pH considered here) of the presence or absence of a methyl ester group. If the unit is esterified, the associated H5 signal is shifted downfield from its usual position ($\approx\delta$ 4.75) towards the region of the anomeric signals [15, 16], so that for **1** the esterified sugar ring can be identified as the one with H5 at δ 5.10. The ROESY cross-peak at δ 5.10/4.49 could indicate an interaction between protons in the same sugar ring (i.e. **B5**/**B4** or **C5**/**C4**) or across a glycosidic linkage (i.e. **D1**/**C4**). However, there is a cross-peak at the same position in the COSY spectrum, which must arise from **B5**/**B4** or **C5**/**C4** spin-coupling ($^3J_{45}$ is 1.5 Hz for α-D-GalA residues but $^4J_{14}$, between neighbouring rings, is zero). The problem is resolved by observation of a further ROESY cross-peak at δ 5.10/4.02. Consideration of the relevant chemical shifts (Table 1) and of which protons are in proximity suggests that this cross-peak can only be assigned as **B5**/**B3** (cross-ring interaction), indicating that δ 5.10 is the **B5**, not the **C5**, chemical shift. It is concluded that the tetrasaccharide is methyl-esterified on ring **B** and that the acetyl group is located at *O*-2 of ring **C**.

Komalavilas and Mort [17] report that the galacturonosyl residues of the hairy regions of a variety of tissues, including carrot, are acetylated at *O*-3. **1** originates from smooth regions of homogalacturonan, where the pattern of acetylation may differ.

Re-digestion of **1** *with Driselase*

Our findings suggested that **1** was a product of incomplete digestion. This was confirmed when **1** was converted entirely to monomeric products when re-treated with Driselase. Its initial survival was presumably due to product inhibition by the large amounts of galacturonic acid produced and/or steric hinderance of each ester, by the other, of the action of esterase(s) in Driselase. Driselase is reported to contain a pectin methyl esterase [6]; the latter also catalyses deacetylations [18]. We found that Driselase released acetic acid from *p*-nitrophenyl acetate, confirming this activity.

Finally, we looked for other NMGEs in the digest. Fractions over the entire M_r weight range afforded by SEC of the digest were screened, hydrolysed and unhydrolysed, by HPAEC. (The final concentration of eluent and the run time of HPAEC were both increased, to detect any larger, more highly charged components—up to OG15—in appropriate fractions.) Although components of the required properties were found, they were much less abundant than **1** (although in the absence of standards their levels could not be quantified). Also, most had the retention time of one of a mixture of the synthetic partially methyl esterified OGs (prepared by the method of Brown and Fry [7], data not shown), and/or were as highly base labile (see below). This suggested that they were compounds of the same type as **1**; they were not further studied. To examine the possibility that NMGEs were contained in fragments of higher M_r, the original retentate from ultrafiltration of the digest was suspended in buffer and ultrafiltered through a 30 kD filter. Alkaline hydrolysis of the filtrate did not produce significant amounts of OGs, so this approach was abandoned.

Brown and Fry [7] detected compounds which apparently contained NMGEs in, amongst others, a carrot suspension culture, but did not examine them in detail. It is thus possible that some of these were of the same type as **1**. They characterized oligomers from spinach in greater detail. They did not, however, consider the possible role of acetyl groups or report the effect of redigestion on their products. Nonetheless, but crucially, the compounds they obtained were considerably more resistant to hydrolysis than **1** and were thus likely to contain authentic NMGEs.

Compound **1** was only isolated in low yield (*ca* 0.3 mg g^{-1} AIR, equivalent to 1 in 4000 GalA methyl esterified as part of **1**), which is consistent with its production by incomplete digestion. Although the carrot AIR apparently contained NMGEs, we were unable to isolate them by digestion with Driselase.

The reasons for this remain undetermined. Perhaps NMGEs did not survive Driselase digestion or they (partially) survived, but were diverse in structure and too small in the amount to characterize.

EXPERIMENTAL

Preparation of AIR. Carrot root (cv. Autumn King) was chopped into 5 mm thick discs, frozen in liquid N_2 and stored at $-30°$. AIR of carrot was prepd as described in Ref. [19].

Quantification of NMGE content of AIR. This was achieved by established methods [4, 20], except that the methanol produced by hydrolysis was determined directly by GC [21].

Purification of Driselase. Driselase was purified as described in Ref. [22], except that, after $(NH_4)_2SO_4$ ppn, the product was dissolved in NaOAc buffer (25 mM + 0.01% NaN_3, pH 5) and diafiltered through a hollow fibre cartridge (cut-off 10 kD) to remove residual salts and any other low M_r contaminants. A sample of the final filtrate was examined by HPAEC-PED (see below) and was shown to be free of detectable contaminants. An aliquot was desalted in a diafiltration cell (Amicon PM10 membrane, 10 kD cut-off), lyophilized and weighed, indicating a total yield of 1.9 g.

Driselase digestion of AIR and isolation of oligomeric products. AIR (15 g) was suspended in a soln of Driselase (1.66 g, purified as above) in NaOAc buffer (25 mM + 0.01% NaN_3, pH 5). The mixt. was placed on an orbital shaker (140 rpm) at room temp. for 90 h. The digest was centrifuged (2500 *g*) and the supernatant ultrafiltered through a hollow fibre cartridge (10 kD cut-off) to a final vol. of 100 ml. The retentate was then cell-ultrafiltered to near dryness through a PM10 membrane. (This circumvented extended diafiltration against buffer and subsequent concn of the dild filtrate by evapn, otherwise needed to avoid exposing the hollow fibre cartridge to inappropriately high protein concn.) Samples of the two filtrates were examined by HPAEC-PED (see below) and found to be essentially identical; they were combined and stored at $-20°$.

Preparative SEC of products of Driselase digestion. Biogel P2/100 mM HCO_2Na, pH 3.3 + 0.1% $PhCO_2Na$ and Fractogel HW 40S/100 mM NaOAc, pH 6 + 0.01% NaN_3 (packing/eluent) were used, in 1 m × 25 mm id columns, at a flow rate of 25 ml hr^{-1}. RI detection was used. The maximum loading of digest was equivalent to 500 mg of AIR in 2.5 ml. When the digest was pre-fractionated by prep. anion-exchange chromatography, material from 2 g of digest was applied. Frs (180) of 150 drops (2.28 ml) were collected. (Prep. anion-exchange chromatography removed the majority of digestion products before SEC on Biogel P2, allowing **1** to be isolated more quickly and in larger quantities. This procedure was not adopted initially, so as to limit the number of frs to be screened and to facilitate detection of components with the required properties, such as **1**.) Frs containing **1** were purified by semi-prep. HPAEC.

Preparative anion-exchange chromatography of products of Driselase digestion. Ultrafiltered Driselase-digest (equivalent to 2 g of AIR) in 25 mM NH_4OAc, pH 5.5, was loaded onto a DEAE Trisacryl M column (2.6 × 8.5 cm). The column was eluted with a gradient of NH_4OAc (25–500 mM, 300 ml, pH 5.5) at a flow-rate of 2 ml min^{-1}; 13 ml frs were collected.

HPAEC of products of Driselase digestion. CarboPac PA-1 analytical and semi-prep. columns were used (4 × 250 and 9 × 250 mm, respectively, Dionex Corporation); the analytical column was equipped with a CarboPac PA-1 (4 × 50 mm) guard column. A pulsed electrometric detector (PED) was used in conjunction with a 3 mm Au working electrode and an Ag/AgCl ref. electrode in integrated amperometric mode (0.1 V, 0 s; 0.1 V, 0.5 s; 0.6 V, 0.51 s; 0.6 V 0.59 s; −0.6 V, 0.60 s; −0.6 V, 0.65 s; integration period 0.3–0.5 s) at a sensitivity of 200 nC. The columns were eluted at 1 and 4 ml min^{-1} over 30 min with a linear gradient (120–600 mM) of NaOAc, pH 6. Samples (25, 100–250 μl, respectively) were injected. NaOH (200 mM) was added post-column at 0.5 and 2 ml min^{-1}, respectively. Later we devised a more sensitive electrode program (0.2 V, 0 s; 0.2 V, 0.2 s; 0.2 V, 0.40 s; 0.75 V, 0.41 s; 0.75 V, 0.60 s; −0.15 V, 0.61 s; −0.15 V, 1.0 s; integration period 0.2–0.4 s).

Generation of oligogalacturonide standards for HPAEC. A soln of polygalacturonic acid (30 g) in NaOAc (20 mM, 1.5 l, pH 4.5) was treated for 24 hr at 21° with α-(1-4)-endopolygalacturonase (15 units: 1 unit releases 1 μmol reducing sugar min^{-1} at 40°). The enzyme had been purified to homogeneity (unpublished results). The reaction was terminated by heating (100°, 15 min). A fr. enriched in lower M_r oligogalacturonides (dp < 10–12) was prepd using an EtOH–NaOAc pptn method [23].

Hydrolysis of digestion products. NaOH (46–48% by wt, 3 μl) was added to a sample of oligomers in 100 mM HCO_2Na (100 μl, pH 3.3 + 0.1% $PhCO_2Na$) and vortexed to give a pH of 12.8. After 1 h, HOAc (2 μl) was added to give a final pH of 5.75. Addition of greater quantities of HOAc resulted in a large, negative, broad, early running peak when the sample was run on HPAEC and was thus avoided. Subsequently, much shorter reaction times (e.g. 5 min) were found to completely hydrolyse **1**.

Fast atom bombardment mass spectroscopy of **1** was performed in a glycerol matrix as described in Ref. [24]. Acetic acid analysis of AIR was performed as described in Ref. [13].

Partial hydrolysis of **1**. Na_2CO_3 (60 μl, 0.5 M) was added to a soln of **1** in HCO_2Na buffer (60 μl, 100 mM, pH 3.3) and mixed to give a final pH of 9. After the designated reaction time, the reaction was quenched with HOAc (15 μl, 1 M) and examined by HPAEC.

Redigestion of **1** *with Driselase.* A sample of **1** purified by SEC and in 300 mM NaOAc buffer (1 ml) was

adjusted to pH 5 with HOAc (1 M). Purified Driselase (10 mg) was added. After 18 h, the mixt. was centrifuged (20,000 *g*, 30 min). The supernatant was ultrafiltered through a PM10 membrane and the ultrafiltrate examined by HPAEC.

NMR analysis of **1**. Spectra of **1** and **2** at pH 5 in D_2O were obtained at 400 MHz. Sample temp. was 27° and chemical shifts were determined using Me_2CO (δ 2.22 with respect to TMS) as int. reference. 2D NMR experiments (COSY and ROESY) were carried out as described previously [25].

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