PERGAMON

Phytochemistry 60 (2002) 67–77

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Patterns of methyl and *O*-acetyl esterification in spinach pectins: new complexity

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Received 11 October 2001; received in revised form 18 January 2002

Abstract

Driselase-digestion of cell walls from suspension-cultures of spinach (*Spinacia oleracea* L.), followed by anion-exchange chromatography, gel-permeation chromatography, preparative paper chromatography and preparative paper electrophoresis, yielded ten uronic acid-containing products in addition to free galacturonic acid (GalA). These included 4-*O*-methylglucuronic acid, α-L-rhamnopyranosyl-(1→4)-D-glucuronic acid and several oligosaccharides containing GalA residues. The structures were unambiguously determined by a combination of 1- and 2-dimensional NMR spectroscopic techniques. Five of the six homogalacturonan-derived oligosaccharides purified contained 3-*O*-acetyl-GalA residues; however, methyl-esterified GalA residues occurred adjacent to both 2-*O*-acetyl-GalA and 3-*O*-acetyl-GalA residues. An acetylated, rhamnogalacturonan-I-derived oligosaccharide that was purified also contained 3-*O*-acetyl-GalA residues. Taken together with published data, our findings indicate considerable diversity in the patterns of pectin esterification. The implications for the action of pectin esterases are discussed.

Keywords: *Spinacia oleracea*; Chenopodiaceae; Spinach; Homogalacturonan; Rhamnogalacturonan-I; Pectic polysaccharides; Oligosaccharides; Oligogalacturonides; Driselase; Methyl esters; Acetyl esters; NMR spectroscopy; DQFCOSY; HMQC; HMBC

1. Introduction

Pectins, which are major components of the primary cell walls of dicotyledonous plants, are polysaccharides rich in α-D-galacturonic acid (GalA) residues. Four distinct pectic domains are generally recognised: homogalacturonan, rhamnogalacturonan I (RG-I), RG-II (O'Neill et al., 1990) and xylogalacturonan (Schols et al., 1995). Homogalacturonan is an unbranched chain of (1→4)-linked α-D-Gal*p*A residues which may (Powell et al., 1982) or may not (Zhan et al., 1998) be interrupted by occasional single Rha residues. The RGs and xylogalacturonan have also been described as 'hairy regions', isolated during enzymic degradation studies of extracted pectins (De Vries et al., 1982; Schols et al., 1995). RG-I has a backbone consisting of the repeating disaccharide unit, …-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-…, with a variety of neutral side-chains attached to *O*-4 of about 50% of the Rha residues; the side-chains contain α-L-Ara*f*, β-D-Gal*p* and small proportions of α-L-Fuc*p* residues (An et al., 1994). RG-II contains *O*-methyl etherified sugars and several other unusual sugar residues (Darvill et al., 1978; Vidal et al., 2000). It is likely that the backbones of the four pectic domains are glycosidically linked to each other to form a single, long backbone.

Pectic polysaccharides contain not only sugar residues but also ester-linked groups including *O*-acetyl esters (O'Neill et al., 1990) and *O*-feruloyl esters (Fry, 1982) as well as galacturonoyl esters (i.e. those with an alcohol group ester-linked at C-6 of a GalA residue). Galacturonoyl esters include both methyl (O'Neill et al., 1990) and unidentified non-methyl esters (Kim and Carpita, 1992; Brown and Fry, 1993b).

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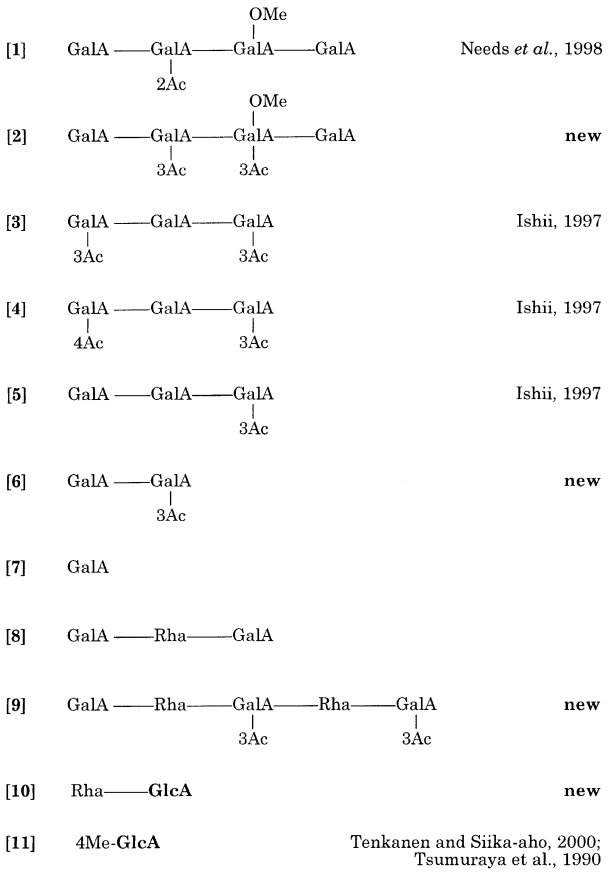
0031-9422/02/$ - see front matter
PII: S0031-9422(02)00039-0

The discovery that *Arabidopsis thaliana* has as many as 70 genes encoding putative pectinmethylesterases (Arabidopsis Genome Initiative, 2000) has focused new interest on the potential substrates and action patterns of these 70 enzymes, and on pectic esters in general.

Homogalacturonans are partially substituted with methyl and *O*-acetyl ester groups. To investigate the distribution of *O*-acetyl groups, Ishii (1997) digested potato-tuber pectin with 'Driselase', a mixture of fungal glycosylhydrolases that tends not to release ester-linked groups with the exception of GalA methyl esters (Brown and Fry, 1993a). Among the products, Ishii detected a population of molecules of the trisaccharide, α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→4)-D-GalA ($GalA_3$), carrying one or two *O*-acetyl groups (compounds **3**, **4** and **5** in Scheme 1). One of the acetyl esters was always located on *O*-3 of the reducing terminal GalA moiety; the other, if present, was on *O*-3 (in some molecules) or *O*-4 (in other molecules) of the non-reducing terminal GalA residue. Similarly, by Driselase-digestion of carrot root pectin, Needs et al. (1998) isolated $GalA_4$ with a methyl ester group on GalA residue B (lettering from the reducing end) and an acetyl group on *O*-2 of residue C (**1** in Scheme 1).



Scheme 1. Driselase digestion products reported in the present work and by previous authors. Compounds **1**–**7** arise from homogalacturonan; compounds **7**–**9** arise from RG-I; compound **10** is of unknown polymeric origin; compound **11** may arise from glucurono(arabino)xylan or an arabinogalactan-protein. 3Ac = acetyl group ester-linked to O-3 of a GalA residue; 4Me = methyl group attached to O-4 of a GlcA residue; OMe = methyl ester group attached at position 6 of a GalA residue.

Some of the GalA residues in RG-I have been reported to be *O*-acetylated but no methylesterification has been detected. In the case of RG-I from cell cultures and plants of cotton, carrot, tobacco and tomato, HF solvolysis yielded a cyclised disaccharide of GalA and Rha in which the GalA residue carried an acetyl group at *O*-3 (Komalavilas and Mort, 1989). In RG-I from sycamore cell-suspension cultures, enzymic dissection yielded oligosaccharide products that indicated the presence of an acetyl group at *O*-2 in some of the molecules and at *O*-3 in others (Lerouge et al., 1993). Driselase digestion of potato-tuber RG-I yielded a population of molecules based on the pentasaccharide, GalA–Rha–GalA–Rha–GalA, with *O*-acetyl groups on none, one, two or all three of the GalA residues (Ishii, 1997). In the mono- and di-acetylated pentasaccharides, it tended to be the non-reducing end that was acetylated. The *O*-acetylation was at position 2 of the GalA (Ishii, 1997). In addition, a population of heptasaccharide molecules was isolated (GalA–Rha–GalA–Rha–GalA–Rha–GalA) in which some of the GalA residues were 2,3-di-*O*-acetylated (Ishii, 1997). In Driselase digests of bamboo shoot cell walls, Ishii (1995) found a range of oligosaccharides with the general structure GalA–$(Rha–GalA)_n$–Rha–GalA, where $n = 0–3$. Some of the GalA residues were 2-*O*-acetylated; others were 2,3-di-*O*-acetylated.

Comparison of pectic fragments isolated after enzymic hydrolysis of the homogalacturonans and RG-Is of various tissues from various plant species suggests significant variation in patterns of esterification. In the present work we have isolated and characterised several major uronate-containing products obtained after Driselase digestion of the cell walls of cultured spinach cells. Since some of the structures are new, our results further support the existence of variation in the patterns of pectic esterification and may provide some clues as to the functions of the surprisingly large number of pectic esterases apparently encoded in the *Arabidopsis* genome.

2. Results

Spinach cell walls were digested with Driselase and the soluble products subjected to anion-exchange chromatography on QAE-Sephadex. The majority of the uronate-containing material was eluted with 0.4–2.5 M buffer (Fig. 1). Analytical paper chromatography (PC) of the QAE fractions showed that the major neutral mono- and disaccharide products were eluted in 0.01–0.05 M buffer, with a peak in 0.02 M buffer (Fig. 2); these were not analysed further. Free GalA was

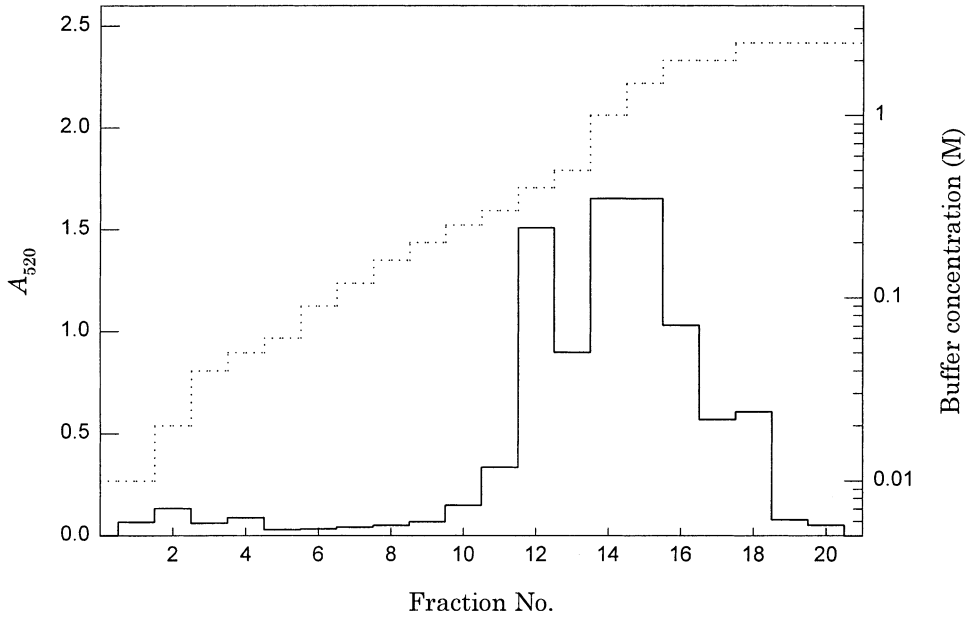


Fig. 1. Preparative anion-exchange chromatography of Driselase digestion-products. Spinach cell walls were digested with Driselase and the soluble products were fractionated on QAE-Sephadex. Material was eluted with a step-gradient of buffer (pH 4.7; pyridine–HOAc, 1:1 v/v, containing 0.5% w/v chlorobutanol) (·····), and each 200-ml fraction was assayed for uronic acids by the *m*-hydroxybiphenyl method (A_{520}; —).

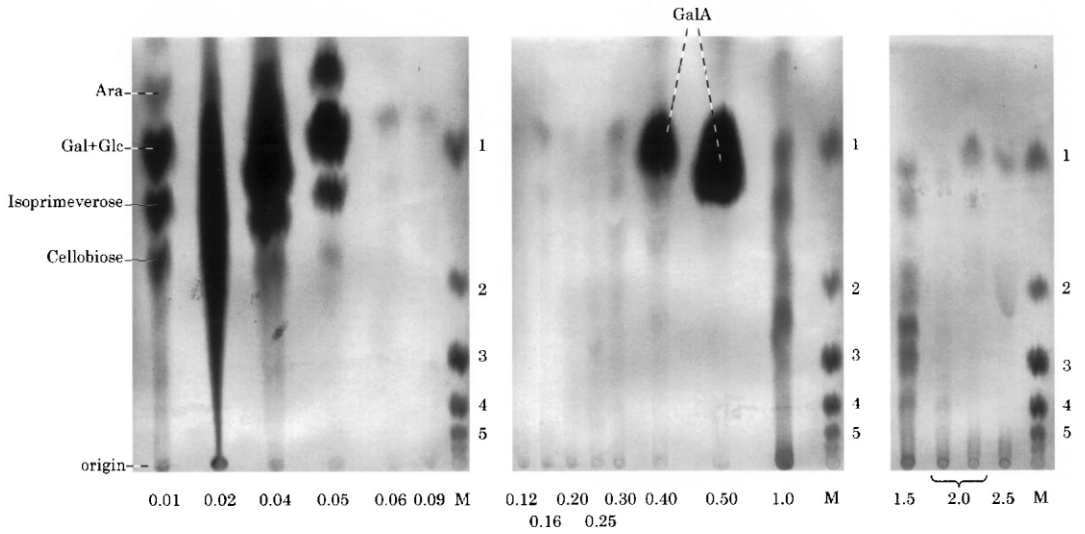


Fig. 2. Paper chromatography (in EtOAc–HOAc–H_2O, 10:5:6) of fractions eluted from QAE-Sephadex. Driselase-digestion products were eluted from QAE-Sephadex, as shown in Fig. 1. A portion (1% of the total) of each QAE fraction (0.01, 0.02, … 2.5 M buffer, as indicated) was analysed by analytical PC and stained with aniline hydrogen-phthalate to detect reducing sugars. In the case of the 0.02 M buffer fraction, this quantity of sample overloaded the PC; however, a diluted sample produced a pattern similar to that of the 0.01 M buffer fraction (results not shown). 'M' = marker mixture containing GalA to $GalA_5$ (1–5).

the main compound eluted by 0.4 and 0.5 M buffer; however, smaller amounts of additional reducing sugars were also detectable by PC in these fractions (Fig. 2), which were therefore further fractionated by preparative GPC. The 1.0 and 1.5 M buffer eluted several additional reducing oligosaccharides, which had a wide range of R_F values: these compounds were also fractionated by preparative GPC. The uronate-containing compounds eluted by 2.0 and 2.5 M buffer mainly had very low R_F values on PC (other than a small amount of free GalA) and were therefore likely to consist of larger, more complex oligosaccharides; these were not analysed further.

Preparative GPC on Bio-Gel P-2 indicated that the uronic acid-containing products had a wide range of molecular sizes (Fig. 3). Analytical PC and PE (results not shown) of the GPC fractions indicated that a large range of compounds were present, and suggested which

GPC fractions were similar enough to be pooled (pools A–G in Fig. 3). Based on the patterns of analytical PC and PE, preparative PC and/or PE were applied to each GPC pool.

Portions of each purified compound were analysed by GPC, PE, and PC; the data (Table 1) indicate optimised methods for purification of these compounds. Many of the purified compounds decreased in R_F after mild alkaline hydrolysis, suggesting that relatively hydrophobic ester group(s) had been present. In each case, alkaline hydrolysis gave a single compound, as resolved by PC. In some cases, the product of alkaline hydrolysis co-chromatographed with a simple α-(1→4)-linked oligogalacturonide marker (degree of polymerisation, DP = 2–4); in other cases, the product migrated between two of the oligogalacturonides, indicating the presence of residue(s) other than α-(1→4)-D-GalA (Table 1).

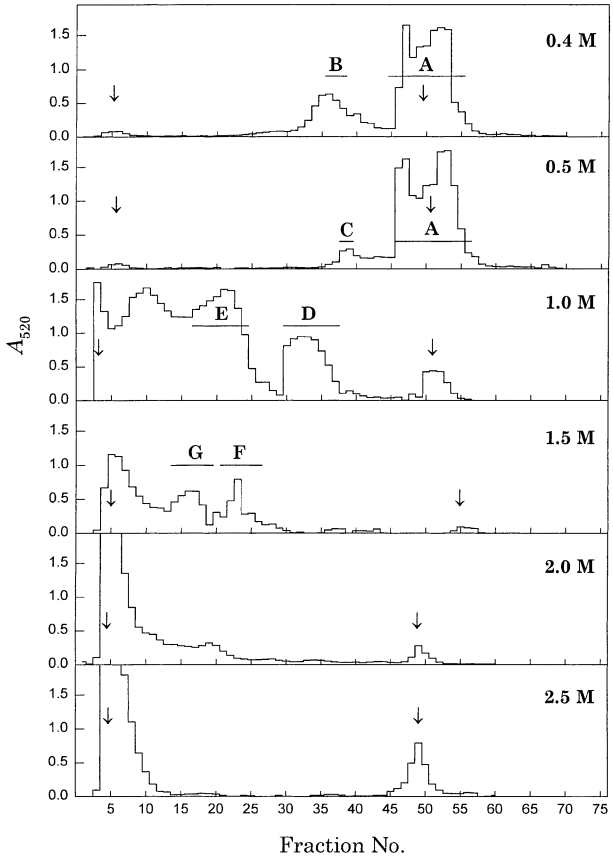


Fig. 3. Preparative gel-permeation chromatography of fractions eluted from QAE-Sephadex. Driselase-digestion products were eluted from QAE-Sephadex, as shown in Fig. 1. Selected QAE fractions (the 0.4–2.5 M buffer eluates as indicated) were subjected to preparative GPC on Bio-Gel P-2 and the eluates were assayed for uronic acid (A_{520}); some fractions prior to the void volume were discarded. The GPC fractions that were pooled and that eventually yielded identifiable products are indicated (A–G). The void volume (peak centre of blue dextran marker·) and the peak centre of free GalA are indicated on each histogram by the left and right arrow respectively.

Each sample was examined by NMR spectroscopy. Two of the samples (one from each of pools E and G) were shown by NMR to be complex mixtures whose components were not identified are not discussed further.

A third sample was shown by NMR spectroscopy to be an approximately equimolar mixture of compounds **3** and **4** (Scheme 1; Table 1), and the remaining samples were shown to be single compounds (Scheme 1, Table 1). Since all these compounds were reducing sugars the presence of both α and β forms rendered the NMR spectra of the larger oligosaccharides extremely complex. The procedure for solving the structures of all these compounds was essentially the same as described below for compound **9** [GalA-(1→2)-Rha-(1→4)-3-*O*-Ac-GalA-(1→2)-Rha-(1→4)-3-*O*-Ac-GalA].

The 600-MHz 1-D proton spectrum of **9** showed signals from 37 protons, all in the region 1.2–5.4 ppm. A doublet observed at 1.24 ppm of intensity corresponding to six protons showed the presence of two methyl groups and a singlet at 2.21 ppm also of intensity corresponding to six protons suggested the presence of two acetyl groups. The remaining signals lay in the range 3.3–5.6 ppm (Fig. 4a). Twelve clear signals were inverted in a series of 1-D TOCSY experiments and showed the presence of two separate 8-spin systems, each of which included the methyl doublet, and four separate 5-spin systems. These results were confirmed by a 2-D TOCSY experiment (Fig. 4b). The intensities of the individual proton signals from two of the 5-spin systems were approximately half those of the other proton signals, suggesting that these arose from the α and β anomers of a reducing terminal sugar and that the compound was a pentasaccharide. The proton connectivity for each spin system was identified from a 2-D DQFCOSY spectrum (not shown) and in every case was in accordance with observed couplings where these could be measured. The sub-spectra for the two 8-spin systems were as expected for Rha residues with H-2 equatorial and H-3, H-4 and H-5 axial. The sub-spectra for the 5-spin systems were those expected for GalA residues. Higher chemical shifts for H-3 in the sub-spectra of the reducing terminal moieties and of one of the other 5-spin systems suggested acetylation at the 3-position in these residues. The ^{13}C chemical shifts for each residue were determined from the 2-D HMQC one-bond proton–carbon chemical shift correlation spectrum (not shown). Long-range correlations obtained from the 2-D HMBC multiple-bond proton–carbon chemical shift correlation spectrum (not shown) (i) allowed the assignment of the carbonyl resonances of the GalA residues, (ii) confirmed the position of acetylation in the GalA residues and (iii) identified the glycosidic linkages and hence the sequence of residues. In particular, clear correlations were observed between GalA H-4 and Rha C-1 for residues 1 (reducing terminus) and 2, and residues

Table 1
Chromatographic and electrophoretic data for uronic acid-containing compounds released from spinach cell walls by Driselase digestion

Compound No.	Fraction (molarity of buffer and GPC pool; see Fig. 3)	m_{GlcA} on PE, pH 3.5	R_{GalA} before alkaline hydrolysis[a]	R_{GalA} after alkaline hydrolysis[a]	Apparent DP of alkaline hydrolysis product[b]	K_{av} on Bio-Gel P-2	Structure by NMR[c]
Homogalacturonan-derived							
1	1.0 (E) and 1.5 (F)	1.01	0.44	0.19	4	0.42	GalA–2AcGalA–GalA(OMe)–GalA
2	1.0 (E)	1.07	0.67	0.24	4	0.35	GalA–3AcGalA–3AcGalA(OMe)–GalA
3	1.5 (F)	1.22	0.94	0.37	3	0.39	3AcGalA–Gal–3AcGalA
4	1.5 (F)	1.38	0.99	0.37	3	0.40	4AcGalA–GalA–3AcGalA
5	1.5 (F)	1.25	0.54	0.37	3	0.51	GalA–GalA–3AcGalA
6	1.0 (D)	1.12	0.83	0.57	2	0.67	GalA–3AcGalA
7	0.4 and 0.5 (A)	0.77	1.00	1.00	1	1.00	GalA[d]
RG-I-derived							
8	1.0 (D)	0.72	0.53	0.52	2–3	0.60	GalA–Rha–GalA
9	1.0 (E)	0.81	0.53	0.23	3–4	0.26	GalA–Rha–3AcGalA–Rha–3AcGalA
–	1.0 (E)	1.02	0.40	0.27	3–4	0.30	Mixture
–	1.5 (G)	≈1.27	0.62	0.15	4–5	0.20	Mixture
Others							
10	0.4 (B)	0.77	0.92	0.92	1–2	0.69	Rha–GlcA
11	0.5 (C)	0.98[e]	1.25	1.25	n/a	0.73	4-*O*-Methylglucuronic acid

[a] Chromatographic mobility relative to that of GalA on PC in EAW.

[b] Judged by PC in EAW. For example, '3' indicates that the product of alkaline hydrolysis exactly co-migrated with authentic α-(1→4)-trigalacturonide (= $GalA_3$); '3–4' indicates that the product migrated on PC (EAW) between $GalA_3$ and $GalA_4$.

[c] Structures are written with the reducing terminus to the right; the GalA and Rha residues are all α-D-pyranose and α-L-pyranose respectively; the GalA–GalA bonds are (1→4), the GalA–Rha bonds are (1→2), the Rha–GalA bonds are (1→4), and the Rha–GlcA bond is (1→4); OMe = methyl ester; 3Ac = *O*-acetylated on the 3-position.

[d] Derived from both homogalacturonan and RG-I.

[e] Stained a characteristic reddish colour with aniline hydrogen-phthalate, in contrast to the orange colour given by the other compounds.

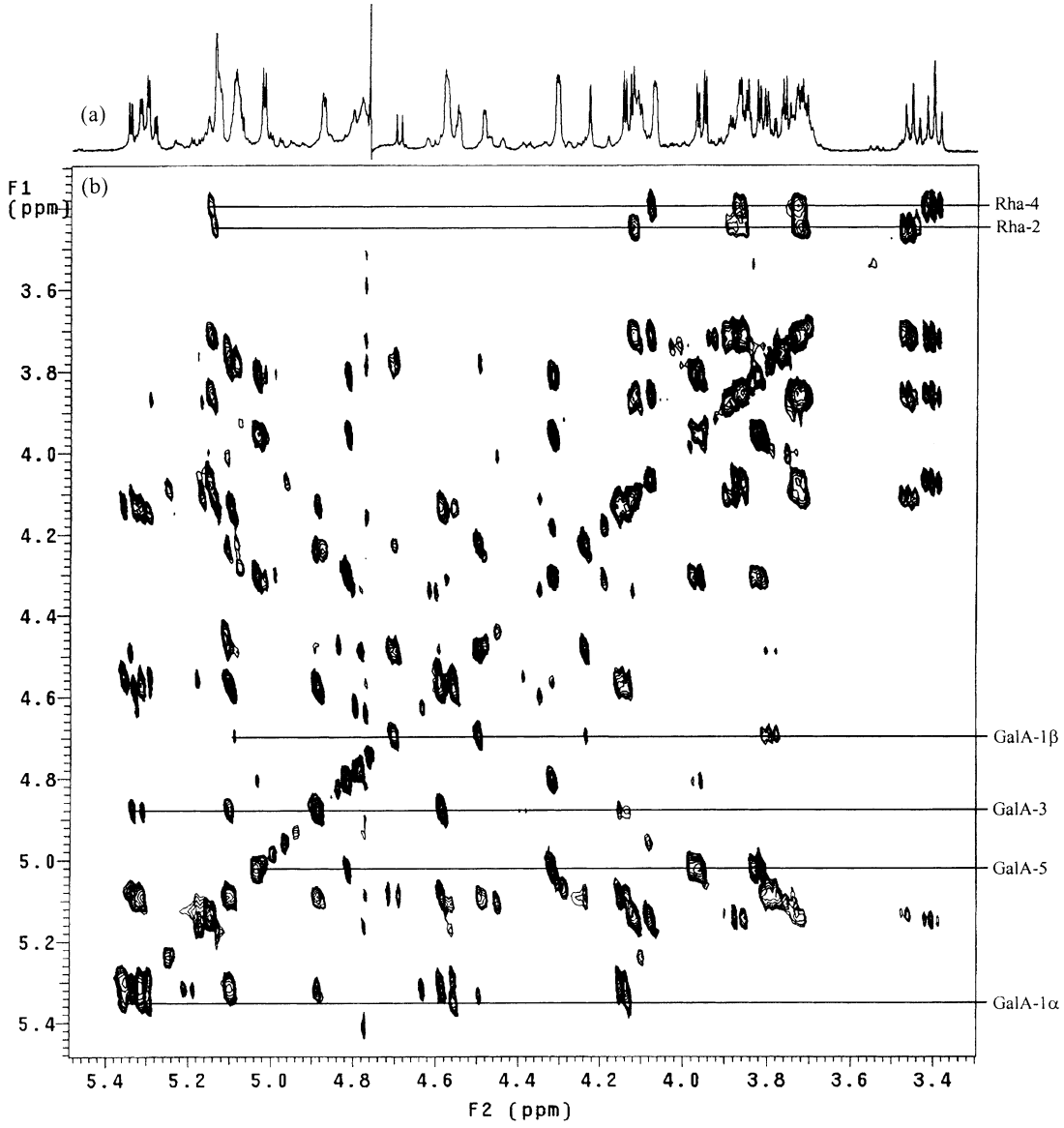


Fig. 4. NMR spectra of compound **9** [α-D-GalA-(1→2)-α-L-Rha-(1→4)-3-*O*-Ac-α-D-GalA-(1→2)-α-L-Rha-(1→4)-3-*O*-Ac-D-GalA]. (a) One-dimensional single-pulse 600-MHz proton NMR spectrum over the range 3.3δ–5.5δ, i.e. excluding all methyl resonances. (b) Two-dimensional TOCSY NMR spectrum over the same region as in (a); one horizontal connectivity pattern is marked for each residue, numbering the reducing terminus as 1.

3 and 4, and between Rha H-2 and GalA C-1 and between Rha C-2 and GalA H-1 for residues 2 and 3 and residues 4 and 5. The spectroscopic data for all compounds are given in Tables 2 and 3. The chemical shifts of some of the protons on the non-reducing residues are sometimes affected by the anomeric configuration of the reducing terminal GalA moiety. The presence of an *O*-acetyl group increases the chemical shift of the adjacent ring proton by 1.0–1.3 ppm.

After alkaline hydrolysis, each of compounds **1**–**6** and **9** was readily Driselase-digestible to its constituent monosaccharides (GalA or GalA + Rha; data not shown). This susceptibility to enzymic digestion supports the conclusion that the oligosaccharides contained sugar residues in the usual anomeric and enantiomeric forms (α-D-GalA and α-L-Rha), characteristic of known pectins.

In summary, the 10 uronic acid-containing compounds, in addition to free GalA, that were characterised by NMR spectroscopy fell into three categories (Scheme 1; Table 1): (a) esterified oligogalacturonides, thought to be derived from homogalacturonan; (b) esterified and non-esterified oligosaccharides containing alternating GalA and Rha residues, presumably derived from RG-I; and (c) two products based on GlcA rather than GalA.

Table 2
Proton NMR data for isolated products **1**–**6** and **8**–**11**

Compound[a]	^{1}H Chemical shift δ (ppm) [coupling constant $J_{n,n+1}$ (Hz)]							
Residue[b]	H-1	H-2	H-3	H-4	H-5	H-6	CH_3CO	CH_3O
1 (pH 3.6)								
GalA-1α	5.31 (3.8)	3.81 (10.7)	4.00 (3.5)	4.41 (1.1)	4.47			
GalA-1β	4.60 (7.9)	3.48 (10.2)	3.76 (3.4)	4.35 (0.9)	4.11			
[c]GalA-2	5.12 (3.9)	3.77 (10.7)	4.03 (3.2)	4.49	5.10			3.76
2-Ac-GalA-3	5.06 (3.8)	4.88 (10.9)	4.24 (3.2)	4.49 (0.6)	4.90		2.18	
GalA-4	5.09 (3.9)	3.74 (10.5)	3.93 (3.4)	4.29 (1.3)	4.79			
2 (pH 3.7)								
GalA-1α	5.32 (3.8)	3.88 (10.6)	4.01 (3.4)	4.45 (1.1)	4.53			
GalA-1β	4.61 (7.8)	3.55 (10.1)	3.77 (3.4)	4.40 (1.2)	4.17			
3-Ac-[c]GalA-2	5.17, 5.18 (4.1)	4.00 (11.0)	5.24, 5.26 (3.2)	4.65 (1.4)	5.12, 5.19		2.08	3.75
3-Ac-GalA-3	5.07 (3.7)	4.26 (11.1)	4.91 (3.5)	4.50 (1.5)	4.72		2.19	
3-Ac-GalA-4	5.11 (4.0)	3.74 (10.6)	3.94 (3.6)	4.30 (1.6)	4.87			
3 (pH 3.5)								
3-Ac-GalA-1α	5.40 (3.5)	4.08 (11.0)	5.26 (3.2)	4.60	4.67		2.08	
3-Ac-GalA-1β	4.76 (7.5)	3.72 (10.5)	5.04 (3.1)	4.55 (1.2)	4.32		2.08	
GalA-2	5.08 (3.8)	3.82, 3.83 (10.8)	4.07 (3.7)	4.46 (1.0)	4.72			
3-Ac-GalA-3	5.12 (3.7)	3.95 (10.8)	5.11 (3.3)	4.47 (1.4)	5.08		2.11	
4 (pH 4.0)								
3-Ac-GalA-la	5.38 (3.7)	4.07 (10.8)	5.25 (2.8)	4.57	4.58		2.09	
3-Ac-GalA-1α	4.74 (7.7)	3.72 (10.5)	5.02 (3.0)	4.52	4.23		2.10	
GalA-2	5.07 (3.8)	3.77, 3.78 (10.7)	4.05, 4.06 (3.0)	4.43, 4.44 (1.0)	4.65			
4-Ac-GalA-3	5.11 (3.8)	3.73 (10.5)	4.11 (3.5)	5.64 (1.6)	5.00		2.16	
5 (pH 3.7)								
3-Ac-GalA-1α	5.39 (3.9)	4.08 (10.8)	5.24 (2.9)	4.58	4.62		2.09	
3-Ac-GalA-1β	4.73 (7.6)	3.72(10.6)	5.02 (3.0)	4.53	4.27		2.09	
GalA-2	5.08 (4.0)	3.76 (11.6)	4.06, 4.07 (2.6)	4.42	4.67, 4.68			
GalA-3	5.08 (4.0)	3.73 (10.3)	3.92 (3.5)	4.31	4.97, 4.98			
6 (pH 3.7)								
3-Ac-GalA-1α	5.39 (3.5)	4.07 (10.7)	5.22 (2.5)	4.54	4.55		2.10	
3-Ac-GalA-1β	4.74 (7.7)	3.72 (10.3)	4.99 (2.8)	4.49	4.20		2.10	
GalA-2	5.04 (3.5)	3.72(10.1)	3.93, 3.95 (3.1)	4.28, 4.29	4.65, 4.66			
8 (pH 3.7)								
GalA-1α	5.30 (3.7)	3.89 (10.4)	4.07 (3.1)	4.42 (1.5)	4.61			
GalA-1β	4.59 (7.9)	3.56 (10.0)	3.85 (3.1)	4.35 (1.1)	4.24			
Rha-2	5.25	4.12 (3.4)	3.87, 3.90 (9.8)	3.43 (9.8)	3.70, 3.71 (6.2)	1.25		
GalA-3	5.03 (3.9)	3.82 (10.4)	3.98 (3.2)	4.32 (1.3)	4.83, 4.84			
9 (pH 4.1)								
3-Ac-GalA-1α	5.36 (3.9)	4.14 (10.5)	5.30 (2.9)	4.56	4.59		2.21	
3-Ac-GalA-1β	4.70 (8.0)	3.79 (10.2)	5.09 (3.3)	4.49 (0.8)	4.24		2.21	
Rha-2	5.14 (1.5)	4.11, 4.12 (3.8)	3.86, 3.89 (9.6)	3.46 (10.0)	3.72 (6.3)	1.24		
3-Ac-GalA-3	5.10 (3.8)	4.14 (10.7)	5.33 (3.0)	4.58 (2.9)	4.89		2.21	
Rha-4	5.15 (1.4)	4.08 (3.9)	3.86 (9.6)	3.40 (10.2)	3.73 (6.3)	1.24		
GalA-5	5.03 (3.8)	3.82 (10.5)	3.97 (3.5)	4.31 (1.0)	4.81			
10 (pH 6.2)								
GlcA-1α	5.24 (3.8)	3.61 (9.9)	3.75 (9.4)	3.55 (10.0)	4.14			
GlcA-1β	4.63 (8.1)	3.31 (9.2)	3.56 (9.5)	3.59 (9.5)	3.76			
Rha-2	4.73 (3.7)	3.94, 3.95 (3.3)	3.77, 3.78 (9.9)	3.43 (9.5)	4.03, 4.05 (6.3)	1.26		
11 (pH 3.9)								
4-Me-GlcA-α	5.21 (3.7)	3.58 (9.8)	3.75 (9.3)	3.26 (9.9)	4.11			3.48
4-Me-GlcA-β	4.60 (8.0)	3.29 (9.5)	3.53 (9.3)	3.28 (9.8)	3.74			3.48

[a] In parentheses: pH of solution used for NMR spectroscopy.
[b] Residues are numbered from the reducing terminus.
[c] Methyl ester at position 6.

Table 3
Carbon-13 NMR data for isolated products **1**–**6** and **8**–**11**

Compound	^{13}C Chemical shift δ (ppm)								
Residue	C-1	C-2	C-3	C-4	C-5	C-6	$\underline{C}H_3CO$	$\underline{C}H_3CO$	$\underline{C}H_3O$
1									
GalA-1α	94.9	70.5	71.0	81.3	73.0	177.2			
GalA-1β	98.8	73.9	74.7	80.5	76.6	176.3			
[a]GalA-2	102.2	70.6	70.9	79.8	72.7	173.5			55.3
2-Ac-GalA-3	98.8	73.3	68.9	80.9	73.8	177.1	23.0	176.7	
GalA-4	102.1	70.8	71.9	73.2	74.4	177.4			
2									
GalA-1α	95.2	70.5	70.9	81.4	72.8	176.7			
GalA-1β	99.2	73.9	74.6	80.8	76.5	175.9			
3-Ac-[a]GalA-2	102.3	69.0	73.5	78.8	72.8	172.8	22.8	175.6	55.5
3-Ac-GalA-3	99.6	68.8	73.1	81.4	74.2	176.7	23.4	176.7	
GalA-4	102.5	70.9	71.8	73.0	74.4	176.7			
3									
3-Ac-GalA-1α	94.9	68.8	73.9	79.8	72.8	175.6	22.8	175.9	
3-Ac-GalA-1β	98.7	72.1	76.8	79.0	76.4	175.0	22.8	175.9	
GalA-2	103.1	70.4	70.7	81.1	73.9	176.7			
3-Ac-GalA-3	102.2	68.7	75.0	70.8	73.2	176.7	22.8	175.9	
4									
3-Ac-GalA-1α	94.9	68.8	74.1	79.7	73.2	176.9	22.8	176.0	
3-Ac-GalA-1β	98.6	72.1	76.8	78.9	76.8	175.0	22.8	176.0	
GalA-2	103.0	70.5	71.1	80.9	74.1	177.5			
4-Ac-GalA-3	102.1	71.0	70.6	75.6	73.2	177.0	23.1	176.0	
5									
3-Ac-GalA-1α	95.0	68.8	84.2	80.0	73.2	–	22.9	–	
3-Ac-GalA-1α	98.3	72.3	77.2	79.1	76.8	–	22.9	–	
GalA-2	103.2	70.7	71.2	81.4	74.2	–			
GalA-3	102.5	80.9	81.6	73.1	74.4	–			
6									
3-Ac-GalA-1α	95.1	69.0	74.5	80.1	73.7	176.8	23.6	176.3	
3-Ac-GalA-1β	98.8	72.1	77.2	79.4	77.2	175.8	23.6	176.3	
Ga;A-2	103.3	70.9	72.4	73.6	75.3	178.0			
8									
GalA-1α	95.1	70.7	72.4	80.4	72.7	178.0			
GalA-1β	99.0	74.1	76.1	79.7	76.5	177.4			
Rha-2	101.6	79.0	72.0	74.5	72.1	22.9			
Gal-3	100.3	70.5	71.8	73.2	74.2	178.8			
9									
3-Ac-GalA-1α	95.0	68.9	75.6	76.9	73.0	176.4	23.3	175.8	
3-Ac-GalA-1β	98.7	72.4	78.5	76.3	76.9	176.4	23.3	175.8	
Rha-2	100.8	80.1	72.0	74.5	72.2	19.2			
3-Ac-GalA-3	101.4	69.1	75.6	76.9	73.6	176.5	23.3	175.8	
Rha-4	100.8	79.9	72.1	74.6	72.2	19.2			
GalA-5	101.5	70.6	72.0	73.3	74.5	177.6			
10									
GlcA-1α	94.8	74.2	73.9	82.3	74.5	178.7			
GlcA-1β	98.7	77.1	77.0	81.7	79.1	178.0			
Rha-2	103.0	72.8	72.6	74.5	71.4	18.9			
11									
4-Me-GlcA-1α	94.6	73.6	74.4	84.8	73.6	178.7			62.3
4-Me-GlcA-1β	98.5	76.5	77.5	84.8	78.4	178.1			62.3

Residues are numbered from the reducing terminus.
[a] Methyl ester at position 6.

3. Discussion

Driselase is readily able to hydrolyse α-D-Gal*p*A and α-L-Rha*p* linkages in homogalacturonan and RG-I after these polysaccharides have been stripped of ester groups by treatment with cold, dilute alkali. Driselase is also able to hydrolyse both partially and fully methylesterified oligogalacturonides, completely, to release free GalA and methanol (Brown and Fry, 1993b). However, most other ester (Fry, 1982; Brown and Fry, 1993a) and amide (Perrone et al., 1998) bonds that have been tested are resistant to Driselase digestion, and the presence of a resistant ester group appears to protect one or more neighbouring glycosidic bonds that would otherwise have been hydrolysable by Driselase; therefore, oligosaccharides can be isolated which carry *O*-acetyl and/or *O*-feruloyl ester groups (Fry, 1982; Ishii, 1991; Brown and Fry, 1993b). It appears likely that the presence of a resistant ester bond (e.g. *O*-acetyl) may also protect one or more neighbouring methyl ester groups from hydrolysis by Driselase. Thus, it is possible to begin to map the distribution of methyl and acetyl esters within pectins.

Komalavilas and Mort (1989) detected only 3-*O*-acetylated GalA residues in fragments of RG-I from several plant species. Ishii (1995, 1997) found predominantly 2-*O*-acetylation in the RG-I of bamboo and potato, and suggested that the apparent absence of 2-*O*-acetylation in Komalavilas and Mort's work was purely because they had fragmented their pectin using HF, generating a cyclic disaccharide, -Rha-(1→2)-GalA-(1→2)-, which would necessarily have lost any acetyl group that was originally present on *O*-2 of the GalA. Ishii (1995) also detected oligosaccharides containing 2,3-di-*O*-acetyl-GalA residues. Lerouge et al. (1993) found both 2-*O*- and 3-*O*-monoacetylated GalA residues in oligosaccharides isolated enzymically from sycamore RG-I. In our work on spinach pectins, the major RG-I-derived oligosaccharide contained two 3-*O*-acetylated GalA residues. This is a significant difference from Ishii's results and suggests the occurrence of variation in the acetylation pattern of RG-I.

Turning to homogalacturonan-derived oligosaccharides, Ishii (1997) found predominantly 3-*O*-acetylation in potato homogalacturonan. Our work with spinach pectin gave identical results in this respect. One oligogalacturonide had a 4-*O*-acetyl group on the non-reducing terminal GalA residue (Ishii, 1997 and present work): this could have been formed from the corresponding 3-*O*-acetylated compound by acyl migration during the digestion or purification steps. Such acyl migration has been noted with *O*-feruloyl sugar esters (Birkofer et al., 1966; Fry, 1982). Any natural 4-*O*-acetylation of homogalacturonan would only be possible on the non-reducing terminal GalA residue and would therefore be very rare.

Needs et al. (1998), on the other hand, working with carrot pectin, isolated a tetragalacturonide containing one 2-*O*-acetylated GalA residue, adjacent to which was a methyl-esterified GalA residue (compound **1**). We also isolated this oligosaccharide, as a major product from spinach pectin. The structure of compound **1** may indicate that methyl-esterified domains of homogalacturonan are 2-*O*-acetylated whereas the domains that lack methyl esters are 3-*O*-acetylated. However, in spinach, we found an additional, new, tetragalacturonide (compound **3**) which had one 3-*O*-acetylated GalA residues adjacent to a methyl-esterified 3-*O*-acetylated GalA residue. Therefore, it appears that a

diverse range of esterification patterns can occur, with possible taxonomic or ontological variation.

The other uronic acid-containing fragments isolated were based on glucuronic acid. It is of interest that Driselase evidently contains a glucuronidase activity capable of releasing free 4-*O*-methylglucuronic acid. It is unclear whether this product was released by hydrolysis of xylans or of arabinogalactan-proteins. Xylans often contain 4-*O*-methyl-α-D-glucuronic acid residues linked to a β-(1→4)-xylan backbone (Tenkanen and Siika-aho, 2000) and arabinogalactan-proteins are reported to contain 4-*O*-methyl-β-D-glucuronic acid residues linked to an oligomer of β-(1→6)-galactan (Tsumuraya et al., 1990). The disaccharide α-L-Rha*p*-(1→4)-D-GlcA was possibly derived from arabinogalactan-proteins, which are known to possess this dimeric unit (Nothnagel, 1997).

The main conclusion of our work is that the description of esterification patterns in pectic polysaccharides is likely to be far from complete. Future work, directly comparing diverse tissues from related and unrelated plant species, will establish whether the differently esterified fragments isolated to date represent taxonomic or developmental variation. The unfolding complexity in the distribution of ester groups within the pectin molecule may be connected with the existence of over 70 different putative pectinmethylesterase genes in *Arabidopsis* (Arabidopsis Genome Initiative, 2000). It remains to be established whether or not most of these genes really do encode pectinmethylesterases and, if so, whether the action of the encoded 'isoenzymes' on methyl esters is influenced by the siting of adjacent acetyl or other ester groups.

4. Experimental

4.1. Preparation of spinach cell walls

Suspension-cultured spinach cells (*Spinacia oleracea* L., cv. Monstrous Viroflay) were maintained in Murashige and Skoog medium (Sigma Chemical Co., Poole, UK) as described (Fry, 1982). Cells were harvested by filtration through muslin, washed with water and freeze-dried. Dried cells (25 g) were stirred in 2×350 ml phenol/acetic acid/water (2:1:1, w/v/v) at room temperature for 2×24 h. The cell wall-rich residue was collected by centrifugation (800×*g*, 10 min) and freed of phenol by repeated washing in buffer A (pyridine/acetic acid/water, 1:1:98 by vol.). The insoluble material (12.35 g) was then stirred in 250 ml of 0.5% Driselase [previously purified as described by Fry (1982)] in buffer A containing 0.5% 1,1,1-trichloro-2-methylpropan-2-ol (to prevent microbial growth), for 48 h at 25 °C. The digest was then centrifuged (2500×*g*, 15 min) and the supernatant freeze-dried.

4.2. Anion-exchange chromatography of pectins

QAE-Sephadex A-25 (Sigma Chemical Co) was washed in 2 M NaOAc, poured as a 5×25-cm bed, and equilibrated with 10 mM pyridine/HOAc buffer (pH ≅ 4.7; this and all buffers contained 0.5% 1,1,1-trichloro-2-methylpropan-2-ol). A portion (9.26 g) of the Driselase digestion products was dissolved in 500 ml of 10 mM buffer and applied to the QAE-Sephadex. Material was then eluted with a step-gradient of 0.01–2.5 M buffer (200 or 400 ml of each concentration). Aliquots (200 μl) of each fraction were assayed for the uronic acid content by the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). The range of oligosaccharides present in each fraction was investigated qualitatively by paper chromatography (PC) in EAW and BPW of 0.1 and 1% of the total in each fraction. Fractions of interest were freeze-dried and stored at −70 °C.

4.3. Gel-permeation chromatography

The uronate-rich fractions (eluted by 0.4–2.5 M buffer) from QAE-Sephadex were further fractionated by gel-permeation chromatography (GPC) on a column (3.5×87 cm) of Bio-Gel P-2, eluted with buffer C (pyridine/acetic acid/water, 1:1:23 v/v/v, containing 0.5% chlorobutanol). Fractions (6 ml) were assayed for uronic acids by the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) and investigated qualitatively by analytical PC. Fractions which appeared to contain oligosaccharides in common were pooled and freeze-dried; portions were then analysed by high-voltage paper electrophoresis (PE) at pH 3.5.

4.4. Purification of compounds

Fractions of interest were then further fractionated, as appropriate, by preparative PC and/or preparative PE, loading at 1 mg (cm paper)$^{-1}$. The compounds were eluted from paper by the method of Eshdat and Mirelman (1972) after staining of guide strips. The eluted compounds were finally passed through another column of Bio-Gel P-2 (1.4×90 cm) to remove traces of carbohydrate carried over from the chromatography paper; small portions of the collected fractions were re-analysed by PC so that K_{av} values of the purified compounds could be accurately determined. K_{av} values were defined with reference to the peak centres of Blue Dextran ($K_{av} = 0$) and GalA ($K_{av} = 1$).

4.5. Estimation of degree of polymerisation of esterified oligosaccharides

A portion (∼100 μg) of each purified compound was de-esterified in 40 μl 0.5 M NaOH at 25 °C for 1 h and

then acidified with 4 μl of 10 M acetic acid; a control portion was incubated in pre-mixed NaOH/HOAc. Both samples were then analysed by PC in EAW and stained with $AgNO_3$.

4.6. Paper chromatography and electrophoresis

PC was performed by the descending method on 57-cm lengths of Whatman 3MM paper in EtOAc/HOAc/H_2O (10:5:6; EAW) for 24 h or in BuOH/pyridine/H_2O (4:3:4; BPW) for 48 h. GalA was used as reference marker, and mobilities are reported as R_{GalA}, defined as [(distance moved by compound)/(distance moved by GalA)]. An oligogalacturonide mixture [$GalA_{1-6}$, prepared by pectinase digestion of homogalacturonan (Brown and Fry, 1993b)] was also used as an external marker.

High-voltage paper electrophoresis (PE) was performed on Whatman 3MM paper in pH 3.5 buffer (HOAc/pyridine/water, 10:1:189) at 3 kV for 70 min. Orange G, glucuronic acid and glucose were used as markers. Electrophoretic mobilities of compounds are reported as m_{GlcA} values, defined as [(distance from Glc to compound)/(distance from Glc to GlcA)], i.e. corrected for electro-endo-osmosis. PCs and PEs were stained for reducing sugars with aniline hydrogenphthalate (Partridge, 1949; modified by Fry, 2000) or with $AgNO_3$ (Trevelyan et al., 1950).

4.7. NMR spectroscopy

The NMR spectra were measured at 25 °C on D_2O solutions using a Varian INOVA 600-MHz spectrometer operating at 599.9 MHz for protons and 150.9 MHz for ^{13}C nuclei. Chemical shifts for both ^1H and ^{13}C nuclei are referenced to methyl signals in trimethylsilyltetradeuteriopropionate Na^+ salt (TSP) as zero ppm.

1-D TOCSY (Kessler et al., 1989) proton spectra were obtained using the sequence: D1–180°sel–90°–D2–90°–D3–90°–AQ. Typical parameters were D1 = 2 s (presaturation of residual HOD signal), 180°sel = 300 ms (= 20 Hz), D2 = 0.12 s (pulsed spin lock), D3 = 0.01 s (z-filter), AQ = 2.56 s, SW = 7000 Hz, 32 k data points with 128 transients per FID. A shaped (IBURP) (Green and Freeman, 1991) selective spin inversion pulse was applied on- and off-resonance on alternate scans and the FIDs alternately added and subtracted to give a 'difference' FID which, on transformation, gave spectra showing only TOCSY responses.

2-D TOCSY phase-sensitive proton spectra were obtained using the sequence (Bax and Davis, 1985): D1–90°-t1-D2-AQ. An 8-step phase cycle (hyper-complex acquisition) was used. Typical parameters were D1 = 1.5 s (pre-saturation), D2 = 0.2 s (spin lock), AQ = 0.205 s, SW = 5000 Hz, 2 k data points, 128 increments each with 24 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1-data to 512 before data transformation.

2-D DQFCOSY phase-sensitive proton spectra were obtained using the sequence (Pianti et al., 1982): D1–90°–t_1–90°–90°–AQ. An 8-step phase cycle (hyper-complex acquisition) was used. Typical parameters were D1 = 1.4 s, AQ = 0.205 s, SW = 5000 Hz, 2 k data points, 256 increments each with 16 transients per FID were used. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F1-data to 2 k before transformation.

The 2-D proton-detected one-bond ^1H–^{13}C correlation (HMQC) spectrum was obtained using the sequence (Summers et al., 1986): D1–90°(^1H)–D2–180°(^1H); 180°(^{13}C)–D2–90° (^1H)–D3–90° (^1H)–D2–90° (^{13}C)–t_1/2–180°(^1H)–t_1/2–90°(^{13}C)–D2–AQ. The experiment was preceded by 64 dummy scans to establish thermal equilibrium. A 4-step phase cycle (hyper-complex acquisition) was used with ^{13}C broad band decoupling during acquisition of the proton signals. Typical parameters were D1 = 1.5 s (pre-saturation), D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 600 ms (to minimise signals from protons bonded to ^{12}C nuclei), SW(^1H) 3000 Hz, 2 k data points, SW(^{13}C) = 15 000 Hz, AQ = 0.34 s, 128 increments each with 40 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 128 to 512 W before transformation.

The 2-D proton detected long-range ^1H–^3C correlation (HMBC) spectrum was obtained using the gradient selection sequence (Ruiz Cabello et al., 1992): D1–90° (^1H)–D2–90° (^{13}C)–D3–90° (^{13}C) - zgrd1–t_1/2–180°(^1H)–t_1/2-zgrd1–90°(^{13}C)–zgrd2–AQ. A 16-step phase cycle (hyper-complex acquisition) was used with no ^{13}C broad band decoupling during the acquisition of the proton signals. Typical parameters were D1 = 1.4 s, D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 55 ms (optimised for signals from protons with couplings to carbon of ca. 9 Hz), zgrad1 = 4 gauss/cm (0.002 s), zgrd2 = 2 gauss/cm (0.002 s), SW(^1H) = 2500 Hz, 2 k data points, SW(^{13}C) = 25 000 Hz, AQ = 0.410 s, 128 increments each with 256 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 128 W to 1024 W before transformation.

Acknowledgements

P.P. thanks the European Communities' Biotechnology Programme for the award of a research fellowship within the Project of Technological Priority, 1993–1996.

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