

## Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies

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**Arabinogalactan-proteins (AGPs) are a diverse class of plant cell surface proteoglycans implicated in a range of fundamental processes associated with plant cell development. Anti-AGP monoclonal antibodies have been used extensively for the investigation of the developmental regulation of AGPs although virtually nothing is known about the structure of the carbohydrate epitopes recognised by these antibodies. In this report, a series of methyl glycosides of monosaccharides and a range of oligosaccharides that are elements of the carbohydrate component of AGPs have been investigated for recognition by previously derived anti-AGP monoclonal antibodies. No clear evidence was obtained for the involvement of terminal arabinofuranosides, nor of the galactan backbone, in the recognition of the glycan structure of AGPs by any of the antibodies used in this study. Interestingly, the most effective inhibitor of the binding of the monoclonal antibodies MAC207, JIM4 and JIM13 to exudate gum antigens was an acidic trisaccharide, isolated from a partial acid hydrolysate of gum karaya which has the structure: GlcA $\beta$ (1  $\rightarrow$  3) GalA $\alpha$ (1  $\rightarrow$  2)Rha, determined by a combination of FAB-MS, GC-MS and NMR spectroscopy.**

**Key words:** arabinogalactan proteins (AGPs)/exudate gums/monoclonal antibodies/oligosaccharides

### Introduction

Arabinogalactan-proteins (AGPs) are a class of abundant proteoglycans found at the plant cell surface and are predominantly associated with the plasma membrane and extracellular secretions. Although the functions of AGPs are unknown it has been speculated that they may have roles as lubricants, in nutrition or in some aspect of plant cell recognition (Clarke *et al.*, 1979; Fincher *et al.*, 1983; Chasan, 1994). The addition of isolated AGPs to carrot cell cultures has been reported to influence development (Kreuger and van Holst, 1993), and AGPs have been implicated in the control of plant cell proliferation (Basile and Basile, 1993; Serpe and Nothnagel, 1994). The generation and use of monoclonal antibodies

recognizing arabinogalactan-proteins has demonstrated the extensive regulation of AGP carbohydrate epitopes in relation to early stages of cell development in roots, shoots and embryos further implicating this class of plant cell surface molecule in aspects of plant cell development (Knox *et al.*, 1989; Pennell and Roberts, 1990; Stacey *et al.*, 1990; Knox *et al.*, 1991; Pennell *et al.*, 1991; Schindler *et al.*, 1995).

The arabinogalactan component of AGPs is known to contain a range of complex oligosaccharide structures involving not only arabinose and galactose but also other sugars. The structure of these arabinogalactans, known as type II, generally consists of a  $\beta$ (1  $\rightarrow$  3)-linked galactan backbone with  $\beta$ (1  $\rightarrow$  6)-linked branches consisting of  $\beta$ (1  $\rightarrow$  6)-galactan chains which can be heavily 3-substituted with arabinose residues and also substituted with other sugars including xylose, rhamnose, fucose, glucuronic acid, galacturonic acid and uronic acid 4-O-methyl derivatives (Clarke *et al.*, 1979; Fincher *et al.*, 1983; Saulnier *et al.*, 1992). Certain anti-AGP monoclonal antibodies are known to recognize carbohydrate containing epitopes as demonstrated by their sensitivity to periodate oxidation and by the ability of certain monosaccharides to act as hapten inhibitors (Pennell *et al.*, 1989). In most cases, however, the characterization of epitope structures has progressed no further than the identification of these monosaccharide haptens.

Recently, we have characterized the occurrence of epitopes for a panel of anti-AGP monoclonal antibodies in samples of commercially available exudate gums—arabic, karaya, ghatti and tragacanth (Yates and Knox, 1994), and these gums are therefore a possible abundant source of oligosaccharide fragments for the elucidation of epitope structure (Stephen *et al.*, 1990). In addition, we have embarked on a programme aimed at the synthesis of potential oligosaccharide components of the backbone, branch regions and terminal structures of AGPs to aid epitope characterization and for the preparation of novel immunogens. Here, we report details of some of these compounds and their efficacy as inhibitors of anti-AGP monoclonal antibody binding. Although arabinose is capable of inhibiting the binding of monoclonal antibodies MAC207, JIM14 and JIM15, none of the corresponding methyl glycosides of arabinose are, and an  $\alpha$ -L-arabinofuranosidase had no effect on the binding of these antibodies to gum arabic. We also report the isolation and structural characterisation of an acidic trisaccharide from gum karaya which acts as the most effective inhibitor of binding of the monoclonal antibodies MAC207 (Pennell *et al.*, 1989), JIM4 (Knox *et al.*, 1989) and JIM13 (Knox *et al.*, 1991) so far characterized. These results represent the first step towards a full elucidation of the complex oligosaccharide structures recognized by these antibodies.

**Table 1.** Concentrations (mg/ml) of haptens required for a 50% reduction in monoclonal antibody binding as determined in a competitive inhibition ELISA assay with gum arabic as immobilized antigen

Hapten	Concentration for 50% inhibition of binding of McAb					
	MC207	JIM4	JIM13	JIM14	JIM15	LM2
(1) L-Ara	0.6	—	—	>5	>5	—
(2) L-Araf- $\alpha$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(3) L-Araf- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(4) L-Arap- $\alpha$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(5) L-Arap- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(6) D-GlcA	1.7	—	—	—	1.0	1.1
(7) D-GlcA- $\beta$ (1- <i>O</i> -Me)	0.3	—	—	—	0.08	0.07
(8) D-Galp- $\beta$ (1-6)-D-Galp- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(9) D-Galp- $\beta$ (1-3)-D-Galp- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(10) L-Araf- $\alpha$ (1-3)-D-Galp- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(11) L-Arap- $\alpha$ (1-3)-D-Galp- $\beta$ (1- <i>O</i> -Me)	—	—	—	—	—	—
(X) D-GlcA- $\beta$ (1-3)-D-GalpA- $\alpha$ (1-2)-L-Rha	0.002	0.003	0.002	—	—	—

A dash in the above table signifies that the compound exhibited no inhibition of antibody binding at concentrations up to 10 mg/ml; >5 indicates that there was some inhibition of antibody binding by the compound at concentrations greater than 5 mg/ml. The binding of monoclonal antibodies JIM8 and JIM16 was not inhibited by the presence of any of the compounds listed in this table.

## Results

### *Mono- and disaccharide methyl glycosides as haptens for anti-AGP monoclonal antibodies*

A series of anti-AGP monoclonal antibodies has previously been characterized. These are MAC207 (Pennell *et al.*, 1989); JIM4 (Knox *et al.*, 1989), JIM13, JIM14, JIM15 and JIM16 (Knox *et al.*, 1991); and LM2 (Smallwood *et al.*, 1996). The binding of these antibodies to their diverse antigens in plant tissues and exudate gums is sensitive to periodate oxidation indicating the recognition of carbohydrate epitopes.

L-Ara and D-GlcA are effective haptens for the anti-AGP monoclonal antibody MAC207 (Pennell *et al.*, 1989), and at higher levels Ara is also capable of the inhibition of binding of JIM14 and JIM15 (Table I). D-GlcA is a more effective hapten for JIM15 than L-Ara and D-GlcA alone is an effective hapten for LM2 (Table I). The binding of the anti-AGP monoclonal antibodies JIM4, JIM13 and JIM16 cannot be inhibited by any monosaccharide haptens so far tested. In an attempt to determine further details of the epitope structures recognized by these AGPs methyl glycosides of L-Ara and D-GlcA were obtained or synthesized and their effectiveness to act as competitive inhibitors in ELISA assays determined. The results, shown in Table I, indicated that, surprisingly, none of the methyl-L-arabinosides were recognized by MAC207, JIM14, or JIM15 even though Ara could inhibit binding of these antibodies when present at high levels. In contrast, the  $\beta$ -methyl-D-glucuronoside was more effective than GlcA in the inhibition of binding of MAC207, JIM15 and LM2.

Disaccharide elements of the  $\beta$ (1  $\rightarrow$  3)-galactan backbone and the  $\beta$ (1  $\rightarrow$  6)-galactan side chains, characteristic of the glycan structure of AGPs were also synthesised chemically and found to be ineffective haptens for the antibodies (Table I). Furthermore, disaccharides containing L-arabinopyranoside and L-arabinofuranoside  $\alpha$ (1  $\rightarrow$  3) coupled to methyl galactoside were also ineffective haptens (Table I).

To explore further the apparent lack of involvement of the methyl-arabinosides in antibody recognition an  $\alpha$ -L-arabinofuranosidase was used to treat the immobilized antigens of gum arabic in an ELISA assay prior to incubation with anti-AGP monoclonal antibodies. No decrease in binding of anti-AGP monoclonal antibodies to gum arabic was observed, while an anti-hydroxyproline-rich-glycoprotein antibody, JIM11 (Smallwood *et al.*, 1994) showed a strong reduction in binding (data not shown).

These observations indicate that terminal arabino-L-furanosides are not a major component of the epitopes recognized by the anti-AGP monoclonal antibodies MAC207, JIM4, JIM13, JIM14, JIM15 and JIM16. The significance of the inhibition of binding of three of the antibodies by Ara is uncertain.

### *An oligosaccharide (X), isolated from a partial acid hydrolysate of gum karaya, is recognized by MAC207, JIM4 and JIM13*

The monoclonal antibodies are reactive with samples of the exudate gums (Yates and Knox, 1994). Partial acid hydrolysates of gum arabic and gum karaya were also active in competitive inhibition assays (data not shown). A preparation obtained by partial acid hydrolysis of gum karaya (Aspinall and Nasir-ud-din, 1965; Aspinall and Sanderson, 1970) was found to contain a component that was an effective inhibitor of three of the antibodies. This component, which was designated oligosaccharide X, was purified to one peak by HPAEC-PAD chromatography and was found to be an effective inhibitor of the binding of the monoclonal antibodies MAC207, JIM4 and JIM13, as determined by competitive inhibition ELISA assays as indicated in Table I. Total hydrolysis of a sample of oligosaccharide X followed by HPAEC-PAD chromatography indicated the presence of Rha, GalA and GlcA as determined by co-chromatography with authentic samples of the three monosaccharides (data not shown). The structure

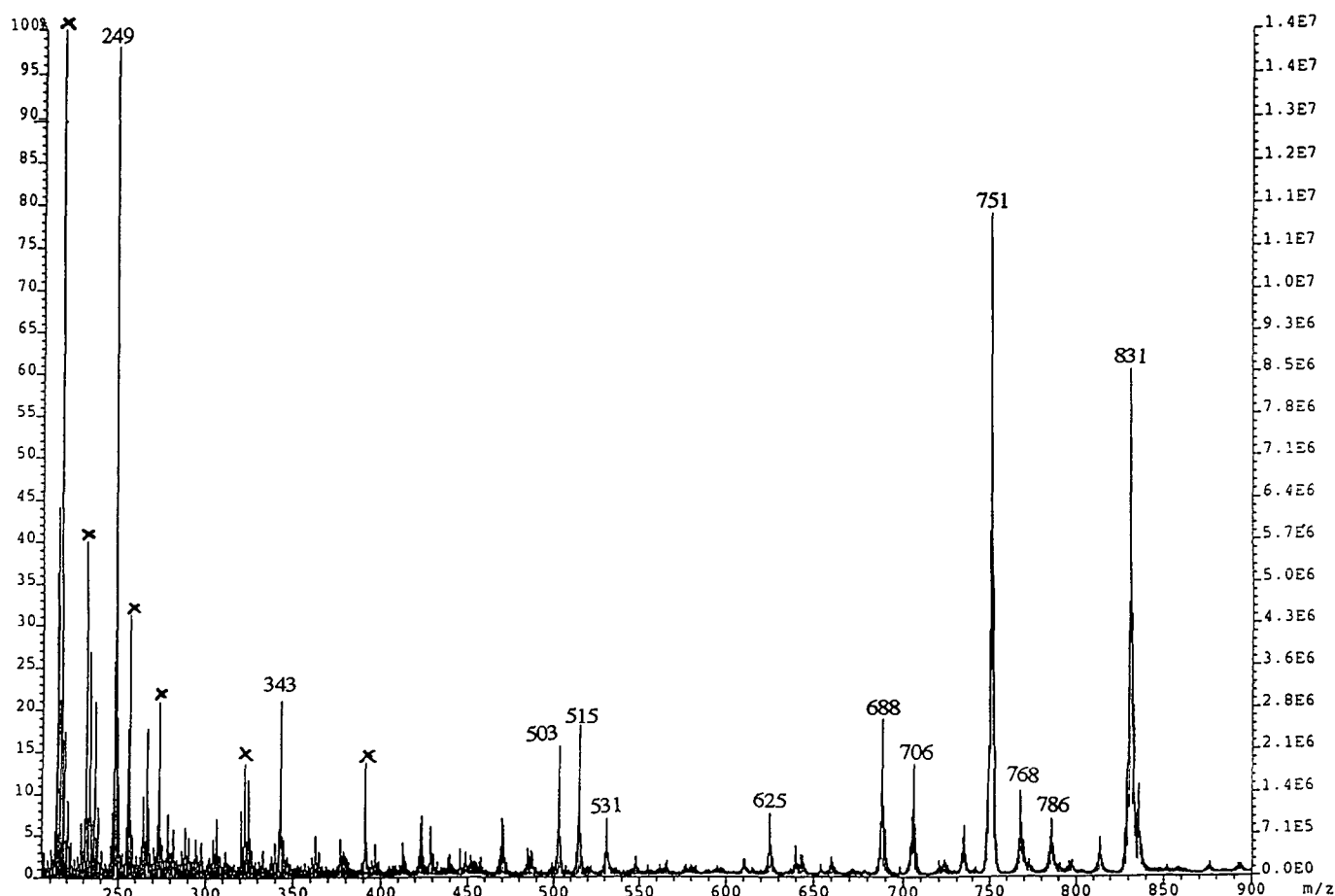


Fig. 1. Positive FAB spectrum of perdeuteroacetylated derivative of oligosaccharide X. Matrix derived peaks are marked with a cross. The peak at  $m/z$  343 is consistent with an A-type fragment ion  $\text{Hex}^+$ , probably derived from a minor contaminant.

of the purified oligosaccharide was determined by a variety of techniques.

#### FAB-MS of oligosaccharide X

Oligosaccharide X was perdeuteroacetylated and subjected to positive fast atom bombardment mass spectrometry (FAB-MS) (Figure 1). Major signals at  $m/z$  831 [ $\text{M} + \text{NH}_4$ ] $^+$  and  $m/z$  751 (A-type fragment ion) are consistent with an oligosaccharide of composition  $(\text{HexA})_2\text{Deoxy-Hex}$  in which one HexA is lactonised after derivatisation. Lactonisation of acidic sugars is commonly observed during the derivatisation procedure (Khoo et al., 1993). The signals at  $m/z$  249 and 515 are assigned to A-type fragment ions of composition  $\text{HexA}_L^+$  and  $\text{HexA}_L\text{HexA}^+$  respectively (where the subscript L designates the lactone). Additional fragment ions at  $m/z$  503 and 531 correspond to beta-cleavage and ring cleavage ions, respectively, arising from loss of the lactonised HexA from  $m/z$  751.

The perdeuteroacetylated sample was also analysed by FAB-MS in the negative mode (data not shown). A major signal was observed at  $m/z$  812 consistent with the  $[\text{M}-\text{H}]^-$  molecular ion of composition  $\text{HexA}_L\text{HexA}\text{DeoxyHex}$ . A major peak was present at  $m/z$  564 consistent with loss of  $\text{HexA}_L$  by beta cleavage suggesting that the lactonised HexA occupies a terminal position in the derivatised oligo-

saccharide. The strong negative spectrum and the observation of  $[\text{M}-\text{H}]^-$  rather than  $[\text{M}+\text{anion}]^-$  indicates that the deuterioacetylated sample still retains a functional group which can lose a proton readily, thus providing further evidence for the presence of HexA.

Oligosaccharide X was perdeuteromethylated and run in the positive FAB-MS mode. A major signal at  $m/z$  709 was present consistent with  $[\text{M}+\text{Na}]^+$  of  $(\text{HexA})_2\text{Deoxy-Hex}$  (data not shown).

#### GC-MS analysis of oligosaccharide X

The monosaccharide composition of oligosaccharide X was established by gas chromatography-mass spectrometry of trimethylsilyl (TMS), methyl glycoside derivatives. By comparison of both retention time and EI-mass spectra the DeoxyHexose component of the acidic trisaccharide was identified as Rha and the two Hexuronic acids were identified as GlcA and GalA (Table II).

Taken together the FAB-MS and GC-MS data suggest the sequence (GlcA, GalA)-Rha for oligosaccharide X.

#### Determination of rhamnose linkage by periodate oxidation/FAB-MS

Oligosaccharide X was deuteroreduced and subjected to mild periodate oxidation followed by reduction and permethylation. The FAB spectrum of the products gave a

strong signal at  $m/z$  589 which is consistent with the ammoniated molecular ion of  $(\text{HexA})_2$  attached to a three carbon fragment derived from rhamnitol (Figure 2). This is the result expected when periodate cleaves between the vicinal hydroxyls of carbon 3 and carbon 4 of 2-linked rhamnitol. A-type fragment ions for  $(\text{HexA})_2^+$  and  $\text{HexA}^+$  are also present at  $m/z$  451 and 233, respectively.

**Table II.** Monosaccharide composition of oligosaccharide X. Major peaks recorded in the total ion chromatogram were identified by retention time and the diagnostic fragment ions of their EI spectra

Retention time (min)	Diagnostic fragment ions	Monosaccharide
10.42	204, 290, 305	Rhamnose
15.92*	159, 204, 217, 331	Galacturonic acid
16.12	159, 204, 217, 331	Galacturonic acid
16.27	159, 204, 217, 331	Galacturonic acid
16.63	159, 204, 217, 331	Glucuronic acid
16.70	159, 204, 217, 331	Glucuronic acid
16.87*	159, 204, 217, 331	Glucuronic acid
17.00	159, 204, 217, 331	Glucuronic acid

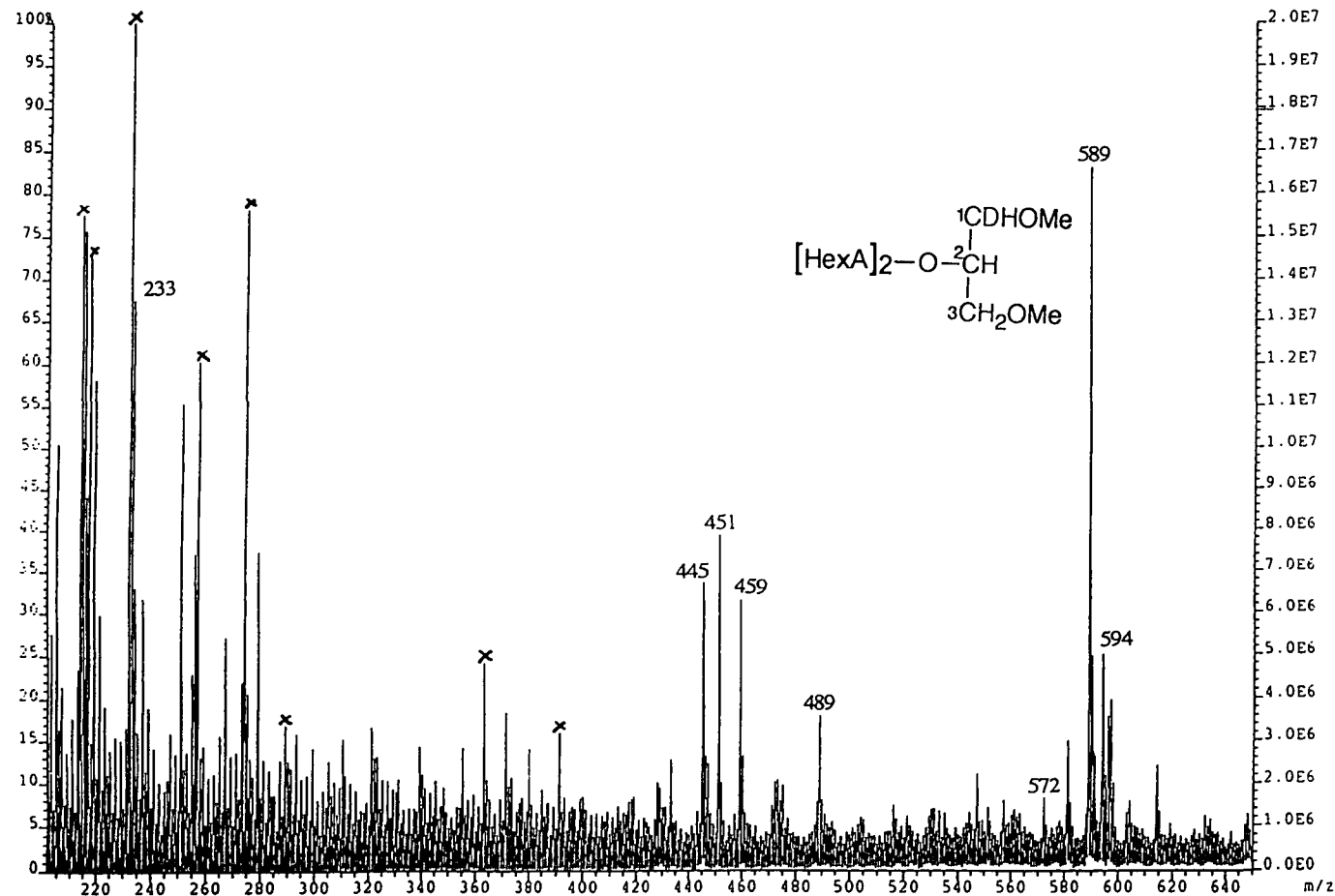
\*Major peak.

*FAB-MS study of anomeric configurations*

Anomeric configurations were investigated using chromium trioxide oxidation monitored by FAB-MS (Khoo and Dell, 1990) after protection of the carboxyl groups by esterification. To facilitate spectral interpretation esterification was carried out using a 1:1 (v/v) mixture of methanol/deuteromethanol. The spectrum of the oxidised product was dominated by a cluster of A-type fragment ions at  $m/z$  620,  $m/z$  623, and  $m/z$  626 (1:2:1) which corresponds to  $(\text{HexA})_2^+$  which has been oxidised at a single site only (data not shown). No signals were observed for the A-type for non-reducing HexA, either oxidised or unoxidised. These data suggest that oxidation had taken place at the non-reducing HexA but not at the penultimate residue, a result that would be expected if the terminal HexA has the configuration  $\beta$  and the internal HexA has the configuration  $\alpha$ . The FAB results are consistent with the results from the NMR experiments.

*NMR spectroscopy of oligosaccharide X*

The  $^1\text{H}$  NMR spectrum of oligosaccharide X, shown in Figure 3, was assigned by a combination of TOCSY and DQF COSY spectra using the anomeric and methyl signals (from Rha) as points of entry into the spectra (Figure 3).



**Fig. 2.** Positive FAB spectrum of oligosaccharide X after mild periodate cleavage. The sample was deuteroreduced prior to periodate treatment to introduce a D atom at C1 of rhamnitol. Matrix derived peaks are marked with a cross. The peaks at  $m/z$  445, 459 and 489 are unassigned and probably arise from minor contaminants.

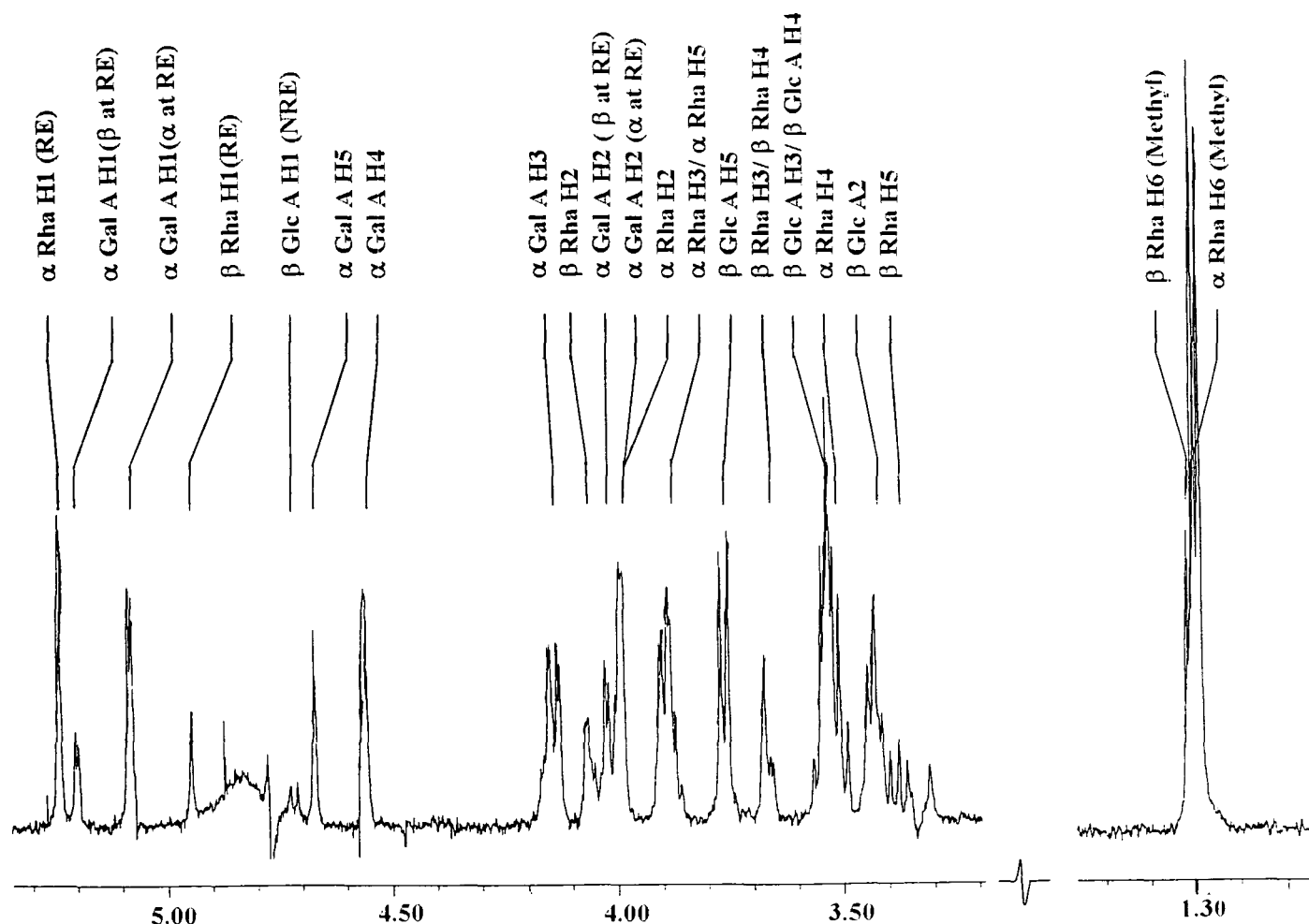


Fig. 3. One-dimensional  $^1\text{H}$  NMR spectrum of oligosaccharide X with assignments.

The TOCSY spectrum revealed the spin systems belonging to the constituent monosaccharides and the connectivity within each spin system was then traced through the DQF COSY spectrum to allow the  $^1\text{H}$  assignment to be completed (Table III). The  $^{13}\text{C}$  spectrum was assigned by an inverse detected  $^1\text{H}/^{13}\text{C}$  shift correlation method, once the full  $^1\text{H}$  spectrum had been assigned, employing heteronuclear multiple quantum coherence spectroscopy (HMQC) (van Halbeek, 1990). Anomeric configurations were determined by measurement of  $^3J_{\text{H1H2}}$  coupling constants. Measurement of  $^3J_{\text{H1H2}}$  (Table III) indicated the  $\alpha$  and  $\beta$  configurations for the H1 signals of Rha, the  $\alpha$  configuration for GalA and  $\beta$  configuration for GlcA. The two signals for H1 and H2 of  $\alpha$ -GalA corresponding to the  $\alpha$  and  $\beta$  anomers of the reducing end residue were assigned on the basis of their relative intensities. In the case of the H1 signal from GlcA, which lay under the HOD peak at  $30^\circ\text{C}$  the temperature was raised to  $40^\circ\text{C}$ , separating the signal from the HOD peak and allowing the coupling constant to be measured (Table III). As expected this signal integrated for one proton.

Analysis of the  $^{13}\text{C}$  spectrum (Table IV) revealed three highly shifted resonances which are unambiguously assigned as C2 of Rha (for both the  $\alpha$  and  $\beta$  anomers at the reducing end) and C3 of GalA indicating the positions of

the glycosidic linkages. In addition, the  $^1J_{\text{C,H}}$  coupling constant for the  $\alpha$  rhamnose anomer was 171 Hz in agreement with reported value of 170 Hz (Colquhoun *et al.*, 1990).

Two sets of signals, for H1 to H6( $\text{CH}_3$ ) in the  $^1\text{H}$  spectrum and for C1 to C6 in the  $^{13}\text{C}$  spectrum, are observed for Rha indicating that this is the reducing end residue (corresponding to  $\alpha$  and  $\beta$  anomers) and two sets of signals are observed for H1 and H2, and C1 and C2 of GalA indicating that this is the residue adjacent to the reducing end residue. Only one set of signals is observed for the non-reducing terminal GlcA. The pattern of chemical shifts for C1 signals is consistent with this sequence.

The positions of coincident signals (H3, H5 from  $\alpha$ -Rha, H3, H4 from  $\beta$ -Rha, and H3, H4 from  $\beta$ -GlcA) are all clearly indicated in the TOCSY spectrum by an apparent lack of signals and in the DQF COSY spectrum. The connectivity was traced from H1 to H2 to H3 and from  $\text{CH}_3$  to H5 to H4 in  $\alpha$ -Rha to reveal H3 and H5 as coincident and the integration of these signals is consistent with this. As expected, H4 showed connectivity to the H3/H5 position but showed no connectivity with either H1, H2 or  $\text{CH}_3$ . Furthermore, the  $^{13}\text{C}$  signals for these positions (C3, C5) could not be resolved but corresponded to a broad, intense signal in the  $^1\text{H}/^{13}\text{C}$  shift correlation spectrum. In

**Table III.**  $^1\text{H}$  Chemical shift assignments (ppm) and selected  $^3J_{\text{HH}}$  coupling constants (Hz) of oligosaccharide X

	H1	H2	H3	H4	H5	H6, 6', 6''
$\alpha$ -Rha (RE)	5.248 (1.53)	3.999	3.891*	3.523	3.891*	1.332
$\beta$ -rha (RE)	4.951 (<1.0)	4.069	3.670*	3.670*	3.377	1.302
$\alpha$ -GalA ( $\alpha$ at RE)	5.088 (3.97)	4.011	4.145	4.564	4.676	—
$\alpha$ -GalA ( $\beta$ at RE)	5.208 (3.66)	4.027	4.145	4.564	4.676	—
$\beta$ -GlcA (NRE)	4.724 (7.90)**	3.440	3.537*	3.537*	3.738	—

\*, Indicates that resonances are coincident.

RE and NRE signify reducing end residue and non-reducing end residue, respectively.

\*\*, This coupling constant was measured at 40°C and the two reducing end forms ( $\alpha$  and  $\beta$  Rha) for the H1 signal of GlcA could be resolved.

the case of  $\beta$ -Rha, the connectivity was traced from H1 to H2 to H3 to H4 and from CH<sub>3</sub> to H5 to reveal H3 and H4 as coincident. In this case, the  $^1\text{H}/^{13}\text{C}$  correlated spectrum showed two cross peaks (C3, C4) which could not therefore be unambiguously assigned. For  $\beta$ -GlcA, the connectivity was traced from H1 to H2 to H3 and from H5 (a doublet) to H4 to reveal H3 and H4 as coincident, and therefore the two cross peaks in the  $^1\text{H}/^{13}\text{C}$  shift correlation are also ambiguously assigned.

These results are also consistent with spectra of other rhamnose and uronic acid containing compounds and with the spectrum of Rha (King and Bishop, 1974; de Bruyn *et al.* 1976; McNeil *et al.* 1984; Cassels *et al.* 1990; Colquhoun *et al.* 1990; Livant *et al.*, 1992).

In summary, the analyses indicated that oligosaccharide X is a trisaccharide with the structure GlcA $\beta$ (1  $\rightarrow$  3)GalA $\alpha$ (1  $\rightarrow$  2)Rha.

## Discussion

While Ara at high levels leads to a reduction in binding of MAC207, JIM14 and JIM15 to gum arabic, none of the corresponding methyl arabinosides, which may reasonably be expected to serve as more realistic models of the ring forms of Ara present in AGPs, showed any inhibition of antibody binding at up to 10 mg/ml. Furthermore, gum arabic which is known to contain terminal  $\alpha$ -L-arabinofuranose residues (Stephen *et al.*, 1990) showed no reduction in its affinity for the anti-AGP monoclonal antibodies when treated with an  $\alpha$ -L-arabinofuranosidase and maintained considerable capacity to interact with the antibodies after partial acid hydrolysis (data not shown). These observations indicate that terminal Araf residues appear to play little role in the binding of the panel of antibodies to AGPs. The significance of the ability of Ara to inhibit the binding of three of the antibodies, and the role for Ara in epitopes recognized by the anti-AGP antibodies used in this report, therefore remains uncertain.

In contrast, the capacity of GlcA to inhibit the binding of antibodies MAC207, JIM15 and LM2 was substantiated by the relative increased effectiveness of methyl- $\beta$ -glucuronoside to act as a hapten inhibitor (Table I).

The involvement of uronic acid in the epitope recognized by MAC207 was further implicated by the structural elucidation of an effective hapten oligosaccharide isolated from

**Table IV.**  $^{13}\text{C}$  Chemical shift assignments/ppm for oligosaccharide X.

	C1	C2	C3	C4	C5	C6
$\alpha$ -Rha (RE)	94.6	79.2	71.7	76.6	71.7	19.9
$\beta$ -Rha (RE)	97.2	82.9	60.5*	75.4*	74.8	19.9
$\alpha$ -GalA ( $\alpha$ at RE)	100.7	71.7	82.5	73.3	74.9	n.m.
$\alpha$ -GalA ( $\beta$ at RE)	104.2	70.1	82.5	73.3	74.9	n.m.
$\beta$ -GlcA (NRE)	106.7	76.3	74.9*	78.0*	80.3	n.m.

\*, Indicates that the assignment may need to be reversed because the proton signals are coincident.

n.m. indicates that these resonances were not measured.

gum karaya that has been determined to have the structure GlcA $\beta$ (1  $\rightarrow$  3)GalA $\alpha$ (1  $\rightarrow$  2)Rha. This acidic trisaccharide is the most effective hapten inhibitor of MAC207 so far characterized and, furthermore, is the first identified hapten inhibitor of binding of the monoclonal antibodies JIM4 and JIM13. This acidic trisaccharide has previously been identified as a component of gum karaya (Aspinall and Nasar-ud-din, 1965; Aspinall and Sanderson, 1970). In addition, the structure of the acidic trisaccharide contains similar structural features to the repeating unit of a mucilage from *Abroma augusta* which has recently been reported (Nakar *et al.*, 1994). It is not yet known which structural features of this trisaccharide are responsible for interaction with the antibodies. As the GalA $\alpha$ (1  $\rightarrow$  2)Rha linkage is characteristic of the pectic polysaccharides (McNeil *et al.*, 1984) and the AGP antibodies do not recognize pectic polysaccharides the GlcA $\beta$ (1  $\rightarrow$  3)GalA component may be important for recognition. This is likely in the case of MAC207 which is also inhibited by GlcA and Me- $\beta$ -GlcA. It is however, of interest that JIM15 and LM2 which are both inhibited by GlcA and Me- $\beta$ -GlcA are not inhibited by this trisaccharide, perhaps indicating that in the epitope recognised by these antibodies the putative GlcA is not attached to a further uronic acid.

The antibodies MAC207, JIM4 and JIM13 recognize distinct epitopes at the plant cell plasma membrane. This is most clearly demonstrated by their recognition of different patterns of cells at the carrot root apex. MAC207 binds to the plasma membrane of all cells, JIM4 binds to only two sets of double-layered pericycle cells and JIM13 binds to cells in the region of the developing xylem (Knox *et al.*, 1989, 1991). The work reported here suggests that these

distinct epitopes may have a common structural feature and from this more complex epitopes are developed. The possibility of a common structure in the recognition of gum arabic is supported by an epitope mapping technique using biotinylated antibodies (Yates and Knox, 1994). Future investigations will include the synthesis of the disaccharide portions of the acidic trisaccharide together with a search for more oligosaccharides capable of recognising the anti-AGP monoclonal antibodies.

## Materials and Methods

### Chemical synthesis of monosaccharides and disaccharides

**Compounds (1) to (5).** Methyl-L-arabinosides:  $\alpha$ - and  $\beta$ -methyl-arabinofuranosides (compounds (2) and (3)) were prepared by the addition of L-Ara to a methanolic solution of hydrogen chloride (Fletcher, 1963). The  $\alpha$ - and  $\beta$ -methyl glycosides were separated by chromatography on silica plates (Merck 70-230) EtOAc/MeOH, 15/4 (v/v); Rf ( $\alpha$  methyl) = 0.79, Rf ( $\beta$  methyl) = 0.70, 3 ascents). Detection was by charring in an ethanolic solution of sulphuric acid (15% v/v).  $\alpha$ -L-methylarabinopyranoside (compound (5)) was prepared as follows: L-Ara was peracetylated in pyridine and acetic anhydride (Wolfrom and Thompson, 1963). The peracetate was then reacted with HBr/AcOH according to the method described (Barczai-Martos and Korosy, 1950). The brominated compound was recrystallised from ether/hexane (3 $\times$ ) and was employed as the glycosyl donor in a reaction with methanol in dichloromethane with silver triflate as promotor in the presence of 4A molecular sieves according to the procedure described (Hanessian and Banoub, 1977; Rashid and Mackie, 1992) and deprotected by standard methods (Mashimo and Sato, 1970). The product was purified in the above solvent system on silica plates; Rf = 0.57 (3 ascents).  $\beta$ -methyl-L-arabinopyranoside (97% pure, compound (6)) was obtained from Sigma and was purified on silica gel in the above solvent system Rf = 0.60 (3 ascents). The  $^1\text{H}$  NMR spectra ( $^3J_{1,2}$  data shown below) were in agreement with the literature values (Joseleu *et al.*, 1977); (observed; ( $\text{CD}_3\text{OD}$ ) Me- $\alpha$ -L-Araf  $^3J_{1,2}$  = 1.5 Hz, Me- $\beta$ -L-Araf  $^3J_{1,2}$  = 4.5 Hz, Me- $\alpha$ -L-Arap  $^3J_{1,2}$  = 7.6 Hz and Me- $\beta$ -L-Arap  $^3J_{1,2}$  = 3.0 Hz).

**Compounds (6, 7).** D-GlcA (compound (7)) and methyl- $\beta$ -D-glucuronoside (compound (9)) were both obtained from Sigma and were used unpurified.

**Compounds (8) to (11).** Apart from the  $\beta$ -(1  $\rightarrow$  6) linked disaccharide (8) which requires the synthesis of the methyl-2,3,4-tri-O-acetyl- $\beta$ -D-galactopyranoside (Kovac *et al.*, 1984) as a suitable nucleophile, all synthesised disaccharides (9–11) were prepared from a single glycosyl acceptor, the methyl-2,4,6-tri-O-benzyl- $\beta$ -D-galactopyranoside. The 3-position of the free hydroxyl group was obtained after a regioselective alkylation with dibutyltin oxide-crotyl bromide (Anisuzzaman *et al.*, 1988) and two subsequent steps. Glycosyl donors involved in glycosidation reactions with this single nucleophile were prepared from the appropriate free sugar (D-Gal or L-Ara) or from methyl- $\alpha$ -L-arabinofuranoside (see above) after peracetylation or perbenzylation in pyridine followed by activation of the anomeric centre with HBr/AcOH (Barczai-Martos and Korosy, 1950). The optimum conditions for the coupling of the glycosyl donor and the glycosyl acceptor involved silver triflate as promotor, dichloromethane as solvent and took place in the presence of 4A molecular sieves according to the procedure described (Hanessian and Banoub, 1977; Rashid and Mackie, 1992). The fully substituted disaccharides were then purified on silica gel and the expected configurations were verified by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. A deacylation step followed by debenzoylation (where appropriate) afforded the totally deprotected target oligosaccharides (8)–(11).

Sufficient quantities of each mono- or disaccharide for NMR spectroscopy and testing in competitive ELISA assays were obtained either by extraction from a number of silica plates or by column chromatography in identical solvent systems on silica (silica gel Merck 60).

### Spectroscopic and analytical data for compounds (8) to (11)

#### Compound 8

$\alpha_D^{25}$  =  $-28^\circ$  (c 0.5,  $\text{H}_2\text{O}$ )

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 4.26 (d,  $\text{H}_{1'}$ ,  $^3J_{1,2}$  = 7.7 Hz); 4.13 (d,  $\text{H}_1$ ,  $^3J_{1,2}$  = 7.9 Hz)

$^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 103.80 ( $\text{C}_1$ ); 103.27 ( $\text{C}_{1'}$ ); 75.14 ( $\text{C}_5$ ); 73.79 ( $\text{C}_5$ ); 72.67, 72.61 ( $\text{C}_3$ ,  $\text{C}_3'$ ); 70.72, 70.62 ( $\text{C}_2$ ,  $\text{C}_2'$ ); 68.95 ( $\text{C}_6$ ); 68.67, 68.60 ( $\text{C}_4$ ,  $\text{C}_4'$ ); 60.96 ( $\text{C}_6$ ); 57.32 ( $\text{OCH}_3$ )

#### Compound 9

$\alpha_D^{25}$  =  $+20^\circ$  (c 0.5,  $\text{H}_2\text{O}$ )

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 4.39 (d,  $\text{H}_{1'}$ ,  $^3J_{1,2}$  = 7.5 Hz); 4.17 (d,  $\text{H}_1$ ,  $^3J_{1,2}$  = 7.9 Hz)

$^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 104.34 ( $\text{C}_1$ ); 103.44 ( $\text{C}_{1'}$ ); 82.43 ( $\text{C}_3$ ); 75.04 ( $\text{C}_5$ ); 74.75 ( $\text{C}_5$ ); 72.49 ( $\text{C}_3$ ); 71.03 ( $\text{C}_2$ ); 69.82 ( $\text{C}_2$ ); 68.55 ( $\text{C}_4$ ); 68.44 ( $\text{C}_4$ ); 60.93 ( $\text{C}_6$  and  $\text{C}_6'$ ); 57.11 ( $\text{OCH}_3$ )

#### Compound 10

$\alpha_D^{25}$  =  $-60^\circ$  (c 0.5,  $\text{H}_2\text{O}$ )

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 5.00 (d,  $\text{H}_{1'}$ ,  $^3J_{1,2}$  = 1.3 Hz); 4.15 (d,  $\text{H}_1$ ,  $^3J_{1,2}$  = 7.9 Hz)

$^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 109.15 ( $\text{C}_1$ ); 103.45 ( $\text{C}_{1'}$ ); 83.72 ( $\text{C}_4$ ); 81.19 ( $\text{C}_2$ ); 80.26 ( $\text{C}_3$ ); 76.44 ( $\text{C}_3$ ); 74.86 ( $\text{C}_5$ ); 69.75 ( $\text{C}_2$ ); 68.43 ( $\text{C}_4$ ); 61.10 ( $\text{C}_5$ ); 60.83 ( $\text{C}_6$ ); 57.02 ( $\text{OCH}_3$ )

#### Compound 11

$\alpha_D^{25}$  =  $+26^\circ$  (c 0.5,  $\text{H}_2\text{O}$ )

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 4.41 (d,  $\text{H}_{1'}$ ,  $^3J_{1,2}$  = 7.6 Hz); 4.24 (d,  $\text{H}_1$ ,  $^3J_{1,2}$  = 8 Hz)

$^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm) : 104.49 ( $\text{C}_1$ ); 103.34 ( $\text{C}_{1'}$ ); 82.21 ( $\text{C}_3$ ); 74.69 ( $\text{C}_5$ ); 71.96 ( $\text{C}_3$ ); 70.85 ( $\text{C}_2$ ); 69.72 ( $\text{C}_2$ ); 68.27, 68.14 ( $\text{C}_4$ ,  $\text{C}_4'$ ); 65.97 ( $\text{C}_5$ ); 60.84 ( $\text{C}_6$ ); 57.01 ( $\text{OCH}_3$ )

### Isolation of an acidic trisaccharide from a partial acid hydrolysate of gum karaya

A sample derived by partial acid hydrolysis from gum karaya (a kind gift from Dr. E. Percival, Royal Holloway and Bedford New College, University of London) was found to contain an antibody-reactive component. The oligosaccharide X was purified from this mixture by HPAEC-PAD employing a DIONEX 500 ion-exchange chromatography system equipped with a PA100 column. In all cases the flow rate was 1 ml/min and the operating temperature was  $30^\circ\text{C}$ . The column was cleaned (200 mM NaOH, 10 min) and then re-equilibrated (10 min) after every five runs. Oligosaccharide X had a retention time of 17.25 min in gradient system 1 (constant 40 mM NaOH and 0–0.85 M NaOAc over 20 min [curve 7]) and 6.25 min in gradient system 2 (constant 40 mM NaOH and 0 to 0.39 M NaOAc over 1 min [linear] and then to 0.41 M over 20 min [linear]). A total of 75 runs in gradient system 2 were made and oligosaccharide (12) was collected manually and immediately neutralised with acetic acid. The samples were then combined and salts were removed by filtration through a nominal 1000 Da cut-off filter (Amicon) and were washed extensively with water. The solution was then lyophilised to yield approximately 1 mg for use in the structural analyses and in competitive inhibition ELISA assays.

### Derivatization of oligosaccharide X

Permethyl and perdeuteromethyl derivatives of oligosaccharide X were prepared using the sodium hydroxide permethylation procedure, whilst peracetyl and perdeuteroacetyl derivatives were prepared using acetic anhydride/deuteroacetic anhydride pyridine (1:1, v/v, 100  $\mu\text{l}$ ), at  $80^\circ\text{C}$  for 2 h, both procedures as previously described (Dell, 1990).

### FAB-MS

FAB mass spectra were obtained using a VG Analytical ZAB-2SE FPD mass spectrometer fitted with a Caesium ion gun operated at 20–25 KV. Data were acquired and processed using the VG Analytical Opus software. Monothioglycerol was used as the matrix.

### Methanolysis

Methanolysis of oligosaccharide X was performed as described previously (Dell, 1990). Briefly, methanolic HCl was prepared by bubbling HCl gas in to either methanol or methanol/deuteromethanol (1:1, v/v); 100  $\mu\text{l}$  of the reagent was added to the sample at room temperature for 30 min before evaporation of the reagent under a stream of nitrogen.

\*Indistinguishable

### Periodate oxidation

Oligosaccharide X was treated with 400  $\mu$ l of 10 mg/ml NaBD<sub>4</sub>, 2 M aq NH<sub>3</sub> for 2 h at room temperature. The sample was neutralised with glacial acetic acid and desalted on a Dowex (50W-X8(H)) column, and the borates removed by co-evaporation with 10% acetic acid in methanol under nitrogen. The reduced acidic trisaccharide was treated with 20  $\mu$ l of 10 mM sodium metaperiodate (NaIO<sub>4</sub>) in acetate buffer (0.1 M, pH 5.5), at room temperature for 4 h in the dark. The reaction was quenched with 2  $\mu$ l of ethylene glycol and left to stand for 30 min. The sample was re-reduced with NaBH<sub>4</sub> as described above before permethylation for FAB-MS analysis.

### Chromium trioxide oxidation

Chromium trioxide oxidation of oligosaccharide X was performed as described previously (Khoo and Dell, 1990). Products were dissolved in methanol for aliquoting into the FAB matrix.

### Derivatization for sugar analysis

Oligosaccharide X was hydrolysed to its monosaccharide components by incubation with 200  $\mu$ l of 1.0 M methanolic-HCl at 80°C for 16 h. The products of the hydrolysis were re-N-acetylated using 500  $\mu$ l of methanol. Ten microliters of pyridine and 50  $\mu$ l of acetic anhydride, and the reactants were incubated at 25°C for 15 min. Trimethylsilylation was carried out using 200  $\mu$ l of Tri-Sil 'Z' derivatizing agent (Pierce), at 25°C for 30 min. The products were dried under nitrogen and dissolved in hexane prior to GC-MS analysis.

### GC-MS analysis

GC-MS analysis was carried out on a Fisons Instruments MD800 instrument. Samples for analysis were dissolved in hexane prior to on-column injection on a DB-5 (30 m  $\times$  0.32 mm i.d., 0.25  $\mu$ m film thickness; J&W Scientific) column at 65°C. The oven was held at 65°C before increasing to 140°C at 25°C/min and then to 200°C at 10°C/min and finally to 300°C at 25°C/min.

### NMR experimental conditions

All spectra were recorded on a Varian 500 MHz spectrometer in D<sub>2</sub>O at 30°C, except a one-dimensional <sup>1</sup>H spectrum at 40°C to measure <sup>3</sup>J<sub>H1H2</sub> for D-GlcA. The chemical shift data were measured relative to internal acetone at 2.235 ppm at 30°C in D<sub>2</sub>O with reference to external TSP. Mixing times for TOCSY were 100, 150, and 200 ms. When all NMR experiments had been completed, it was demonstrated by HPAEC-PAD chromatography that hydrolysis or base-catalysed degradation which has been observed in reducing oligosaccharides had not occurred in the oligosaccharide (12) (Lee 1990; An et al., 1994). In the case of <sup>13</sup>C spectra the chemical shift values were measured relative to internal acetone at 30°C in D<sub>2</sub>O (33.08 ppm) with TSP as external standard.

### Monoclonal antibodies and competitive inhibition enzyme-linked immuno-sorbent assays (ELISAs)

The generation of the monoclonal antibodies MAC207, JIM4, JIM13, JIM14, JIM15, JIM16 and LM2 have been described previously (Pennell et al., 1989; Knox et al., 1989, 1991; Smallwood et al., 1996). The antibodies were used unpurified from hybridoma supernatants in all cases. The procedure for the determination of the efficacy of a particular compound to inhibit antibody binding to the antigen was as described previously (Yates and Knox, 1994). Essentially the concentration of a compound (mg/ml) that is required to effect a 50% reduction in antibody binding at 90% of maximal binding is determined. Gum arabic (Sigma) was used as the immobilised antigen throughout. ELISA plates were incubated with gum arabic (1 mg/ml, 24 h, 4°C) and then blocked (3% w/v BSA in PBS, 1 h). All potential haptens under test were single species as determined by t.l.c., <sup>1</sup>H-NMR and/or HPAEC-PAD chromatography.

The ability of an  $\alpha$ -L-arabinofuranosidase (Megazyme Pty. Ltd., Australia) to alter antibody binding characteristics to immobilized gum arabic was also determined using ELISAs. The enzyme was incubated with immobilized antigen in the microtitre plate (1 U activity per well in 100  $\mu$ l 50 mM NaOAc buffer, pH 4.0) for 2 h at 30°C. After incubation the plates were washed extensively in water, and re-blocked (3% w/v BSA in PBS, 1 h) prior to incubation with the anti-AGP monoclonal antibodies

and the subsequent determination of binding. JIM11, an anti-HRGP monoclonal antibody (Smallwood et al., 1994), was also used in these assays.

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### Abbreviations

AGP, arabinogalactan-protein; Ara, arabinose; DeoxyHex, deoxyhexose; DQF-COSY, double-quantum filtered correlated spectroscopy; ELISA, enzyme-linked immunosorbent assay; FAB, fast atom bombardment; GC, gas chromatography; GalA, galacturonic acid; GlcA, glucuronic acid; Hex, hexose; HexA, hexuronic acid; HexA<sub>L</sub>, lactone of hexuronic acid; HMQC, heteronuclear multiple quantum coherence spectroscopy; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HRGP, hydroxyproline-rich glycoprotein; MS, mass spectrometry; *m/z*, mass to charge ratio; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Rha, rhamnose; TOCSY, total correlation spectroscopy.

### References

- An, J., Zhang, L., O'Neill, M.A., Albersheim, P. and Darvill, A.G. (1994) Isolation and structural characterization of endo-rhamnogalacturonase generated fragments of the rhamnogalacturonan I. *Carbohydr. Res.*, **264**, 83–96.
- Anisuzzamen, K.M., Anderson, L. and Navia, J.L. (1988) Synthesis of a close analogue of the repeating unit of the antifreeze glycoproteins of polar fish. *Carbohydr. Res.*, **174**, 265–278.
- Aspinall, G.O. and Nasar-ud-din (1965) Plant gums of the genus *Sterculia*. Part I. The main structural features of *Sterculia urens* gum. *J. Chem. Soc.*, 2710–2720.
- Aspinall, G.O. and Sanderson, G.R. (1970) Plant gums of the genus *Sterculia*. Part IV. Acidic oligosaccharides from *Sterculia urens* gum. *J. Chem. Soc.*, 2256–2258.
- Barczai-Martos, M. and Korosy, F. (1950) Preparation of acetobromesugars. *Nature*, **165**, 369.
- Basile, D.V. and Basile, M.R. (1993) The role and control of the place-dependent suppression of cell division in plant morphogenesis and phylogeny. *Mem. Torrey Bot. Club*, **25**, 63–84.
- Cassels, F.J., Fales, H.F., London, J., Carlson, R.W. and van Halbeek, H. (1990) Structure of streptococcal adhesion carbohydrate receptor. *J. Biol. Chem.*, **265**, 14127–14135.
- Chasan, R. (1994) Arabinogalactan-proteins: getting to the core. *Plant Cell*, **6**, 1519–1521.
- Clarke, A.E., Anderson, R.L. and Stone, B.A. (1979) Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, **18**, 521–540.
- Colquhoun, I.J., de Ruiter, G.A., Schols, H.A. and Voragen, A.G.J. (1990) Identification by NMR spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectin with rhamnogalacturonase. *Carbohydr. Res.*, **206**, 131–144.
- de Bruyn, A., Anteanis, M., de Gussem, R. and Dutton, G.G.S. (1976) <sup>1</sup>H-NMR study of L-rhamnose, methyl  $\alpha$ -L-rhamnoside and 4-O- $\beta$ -D-galactopyranosyl-L-rhamnose in deuterium oxide. *Carbohydr. Res.*, **47**, 158–163.
- Dell, A. (1990) Preparation and desorption mass spectrometry of permethyl and peracetyl derivatives of oligosaccharides. *Methods Enzymol.*, **193**, 647–660.
- Fincher, G.B., Stone, B.A. and Clarke, A.E. (1983) Arabinogalactan-proteins: structure, biosynthesis and function. *Annu. Rev. Plant Physiol.*, **34**, 47–70.
- Fletcher, H.G. (1963) The anomeric tri-O-benzoyl-D-arabinofuranosyl bromides. In Whistler, R.L. and Wolfrom, M.L. (eds.), *Methods in Carbohydrate Chemistry*, Vol. II. Academic Press, New York, pp. 229–230.



- Hanessian, S. and Banoub, J. (1977) Chemistry of the glycosidic linkage. An efficient synthesis of 1,2 trans-disaccharides. *Carbohydr. Res.*, **53**, c13-c16.
- Joseleau, J.-P., Chambat, G., Vignon, M. and Barnoud, F. (1977) Chemical and  $^{13}\text{C}$  NMR studies on two arabinans from the inner bark of young stems of *Rosa glauca*. *Carbohydr. Res.*, **58**, 165–175.
- Khoo, K.-H. and Dell, A. (1990) Assignment of anomeric configuration of pyranose sugars in oligosaccharides using a sensitive FAB-MS strategy. *Glycobiology*, **1**, 83–91.
- Khoo, K.-H., Morris, H.R., McDowell, R.A., Dell, A., Maccarana, M. and Lindahl, U. (1993) FABMS/derivatisation strategies for the analysis of heparin-derived oligosaccharides. *Carbohydr. Res.*, **244**, 205–223.
- King, R.R. and Bishop, C.T. (1974) Synthesis of 2-O-, 3-O-, and 4-O- $\beta$ -D-glucopyranosyl-L-rhamnose, and of 2-O- $\beta$ -D-galactopyranosyl-L-rhamnose. *Can. J. Chem.*, **52**, 3913–3917.
- Knox, J.P., Day, S. and Roberts, K. (1989) A set of cell surface glycoproteins forms a marker of cell position, but not cell type, in the root apical meristem of *Daucus carota* L. *Development*, **106**, 47–56.
- Knox, J.P., Linstead, P.J., Peart, J., Cooper, C. and Roberts, K. (1991) Developmentally regulated epitopes of cell surface arabinogalactan-proteins and their relation to root tissue pattern formation. *Plant J.*, **1**, 317–326.
- Kovac, P., Sokolowski, E.A. and Glaudemans, C.P.J. (1984) Synthesis and characterization of methyl 6-O- $\alpha$  and  $\beta$ -D-galactopyranosyl- $\beta$ -D-galactopyranoside. *Carbohydr. Res.*, **128**, 101–109.
- Kreuger, M. and van Holst, G.-J. (1993) Arabinogalactan-proteins are essential in somatic embryogenesis of *Daucus carota* L. *Planta*, **189**, 243–248.
- Lee, Y.C. (1990) High performance anion-exchange chromatography for carbohydrate analysis. *Anal. Biochem.*, **189**, 151–162.
- Livant, P., Roden, L. and Rama Krishna, N. (1992) NMR studies of a tetrasaccharide from hyaluronic acid. *Carbohydr. Res.*, **237**, 271–281.
- Mashimo, K. and Sato, Y. (1970) Synthesis of isojmaline. *Tetrahedron*, **26**, 803–812.
- McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. (1984) Structure and Function of the primary cell walls of plants. *Annu. Rev. Biochem.*, **53**, 625–633.
- Nakar, N., Hazza, B.K., Mosihuzzaman, M., Rahman, M.M. and Anderson, R. (1994) Structural studies of a mucilage from *Abroma augusta* root bark. *Carbohydr. Polymers*, **24**, 277–280.
- Pennell, R.I., Knox, J.P., Scofield, G.N., Selvendran, R.R. and Roberts, K. (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. *J. Cell Biol.*, **108**, 1967–1977.
- Pennell, R.I., Janniche, L., Peart, J., Scofield, G.N., Kjellbom, P. and Roberts, K. (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *Plant Cell*, **3**, 1317–1326.
- Rashid, A. and Mackie, W. (1992) Efficient and stereo-selective synthesis of methyl 3-O-(3,6-anhydro- $\beta$ -D-galactopyranosyl- $\alpha$ -D-galactopyranoside and methyl 3,6 anhydro-4-O- $\beta$ -D-galactopyranosyl- $\alpha$ -D-galactopyranoside. *Carbohydr. Res.*, **223**, 147–155.
- Saulnier, L., Brillouet, J.-M., Moutounet, M., Herve du Penhoat, C. and Michon, V. (1992) New investigations of the structure of grape arabinogalactan-protein. *Carbohydr. Res.*, **224**, 219–235.
- Schindler, T., Bergfeld, R. and Schopfer, P. (1995) Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. *Plant J.*, **7**, 25–36.
- Serpe, M.D. and Nothnagel, E.A. (1994) Effects of Yariv phenyl glycosides on *Rosa* cell suspensions: evidence for the involvement of arabinogalactan-proteins in cell proliferation. *Planta*, **193**, 542–550.
- Siciliano, R.A., Morris, H.R., Bennett, H.P.J. and Dell, A. (1994) O-Glycosylation mimics N-glycosylation in the 16-KDa fragment of bovine pro-opiomelanocortin. *J. Biol. Chem.*, **269**, 910–920.
- Smallwood, M., Beven, A., Donovan, N., Neill, S.J., Peart, J., Roberts, K. and Knox, J.P. (1994) Localization of cell wall proteins in relation to the developmental anatomy of the carrot root apex. *Plant J.*, **5**, 237–246.
- Stacey, N.J., Roberts, K. and Knox, J.P. (1990) Patterns of expression of the JIM4 arabinogalactan-protein epitope in cell cultures and during somatic embryogenesis in *Daucus carota* L. *Planta*, **180**, 285–292.
- Stephen, A.M., Churms, S.C. and Vogt, D.C. (1990) Exudate gums. In Dey, M. (ed.), *Methods in Plant Biochemistry*, Vol 2, Carbohydrates, Academic Press, New York, pp 483–522.
- Smallwood, M., Yates, G.A., Willats, W.G.T., Martin, H. and Knox, J.P. (1996) Immunochemical comparison of membrane-associated and secreted arabinogalactan-proteins in rice and carrot. *Planta*, **198**, 452–459.
- van Halbeek, H. (1990) NMR of complex carbohydrates. In Live, D., Armitage, I.M. and Patel, D. (eds.), *Frontiers of NMR in Molecular Biology*. UCLA Symposium Series, Vol. 109. Alan R. Liss, New York, pp. 195–213.
- Wolfson, M.L. and Thompson, A. (1963) Acetylation. In Whistler, R.L. and Wolfson, M.L. (eds.), *Methods in Carbohydrate Chemistry*, Vol II. Academic Press, New York, pp. 211–215.
- Yates, E.A. and Knox, J.P. (1994) Investigation into the occurrence of plant cell surface epitopes in exudate gums. *Carbohydr. Polymers*, **24**, 281–286.

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