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Note

Preparation, purification, and structural characterization of linear oligogalacturonides. An FAB-mass spectrometric and NMR spectroscopic study †

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Many plants respond to microbial attack by accumulating small lipophilic molecules, called phytoalexins, that have antibiotic activity[1–3]. Phytoalexins are absent in healthy plants. Molecules (and other stimuli) that signal plants to begin the process of phytoalexin synthesis are called elicitors[4,5]. A number of different biotic elicitors (e.g., complex carbohydrates isolated from fungal and plant cell walls) and abiotic elicitors (e.g., heavy metal salts and UV light) are known. We are interested specifically in carbohydrate elicitors that originate either from the cell wall of the host plant ("endogenous" elicitors) or the wall of the invading microbe[6–9]. The endogenous carbohydrate elicitors are released from the plant cell wall by pectic-degrading enzymes secreted by the invading microorganism[10–12]. It has been demonstrated with in vitro bio-assays that oligogalacturonides [(1 → 4) linked oligomers of α-D-galacturonic acid] of dp 9 to 15 derived by partial acid hydrolysis of plant cell walls and citrus pectin have high elicitor activity[8,9,13]. Complete structural and conformational characterization of these elicitors has not been reported; in particular, the correlation between the size of the oligosaccharides and their biological activity is poorly understood.

We report the preparation and purification of milligram quantities of oligogalacturonides of dp 6 to 16 (see Fig. 1). As previously described[9,13], partial acid hydrolysis of citrus pectin produces oligogalacturonides in a dp range from monomer to, at least, a hexadecamer. The crude hydrolysis mixtures have been sub-divided by low-resolution anion-exchange chromatography using successive gradient steps into fractions (A, B, and C) with increasing average dps. Fraction C

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1 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *A* *R*

2 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *E* *A* *R*

3 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *E* *B* *A* *R*

4 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *E* *I* *B* *A* *R*

5 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → [4)-α-D-Gal*p*A-(1 →]$_2$4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *E* I_2 *B* *A* *R*

10 α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → [4)-α-D-Gal*p*A-(1 →]$_7$4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 4)-D-Gal*p*A
T *E* I_7 *B* *A* *R*

Fig. 1. Structures of oligogalacturonides purified and characterized in this study. **1–4**, Tri- to hexa-galacturonides (dp 3 to 6); **5**, heptagalacturonide, consisting of a reducing-end residue ***R***, five internal residues, designated ***A***, ***B***, ***I*** (two internal residues that have identical NMR signals), and ***E***, and a nonreducing terminal residue ***T***; ***10***, dodecagalacturonide, composed of residues ***R***, ***A***, ***B***, seven ***I***'s, ***E***, and ***T***.

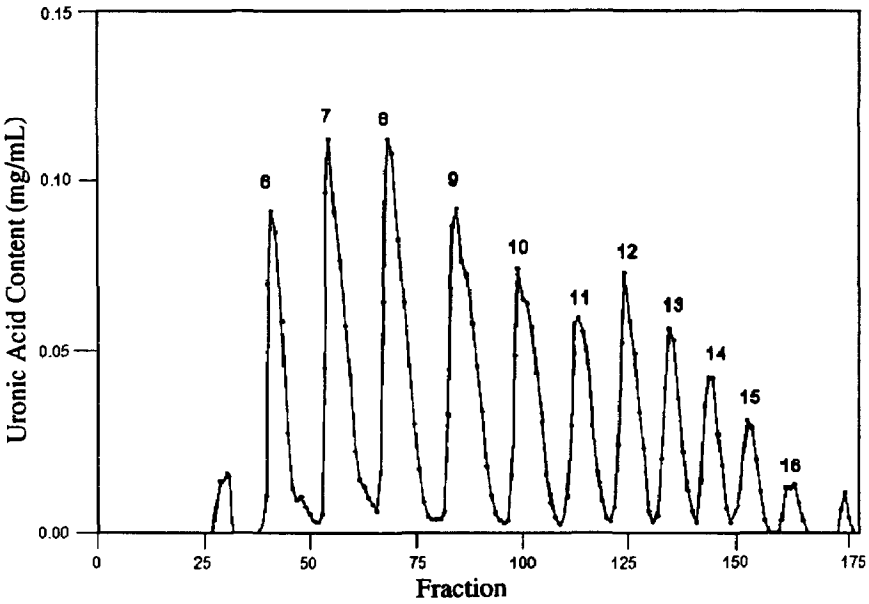

Fig. 2. High-resolution anion-exchange chromatography of fraction C on Q-Sepharose. Eleven major uronic acid containing components were obtained (4–14; dp 6 to 16). The number on top of each peak indicates the degree of polymerization of the oligogalacturonide, as determined by FABMS.

contains oligogalacturonides large enough to elicit phytoalexins, i.e., with dp > 8, as determined by testing them on the soybean cotyledon bioassay[8,9,14,15] (data not shown). Fraction C was resolved into at least eleven separate peaks of uronic acid-rich material using a Q-Sepharose high-resolution anion-exchange column. We obtained baseline separation of peaks when fractionating up to 21 mg of material on a 1.4 × 40 cm column (see Fig. 2). Fractionation of 160 mg of material on a 3.5 × 40 cm column resulted in the resolution of the eleven major peaks with approximate yields of 11 to 14 mg of material per peak. This traditional ambient-pressure strong-anion-exchange chromatography provides a convenient method to purify substantial amounts of larger oligogalacturonides (dp 6–16) and compares favorably with a more sophisticated preparative HPLC method[16] that can isolate smaller oligogalacturonides (dp ≤ 7) in gram amounts. The purity of the oligogalacturonides with dp ≤ 12 was found to be ~90%, as determined by high-pH anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD) of the individual components of fraction C. In an attempt to further purify oligogalacturonides of size dp ≥ 10, material from fraction C peaks enriched in these oligogalacturonides was passed through a gel filtration column containing Sephadex G-25 in 1% acetic acid. For each of the oligogalacturonides, uronic acid-positive material eluted in a single narrow peak with small shoulders. HPAE analysis of the centers of these peaks showed an increased purification of the major peak from 85 to 95%. Further purification (up to 99%) of oligogalacturonides (dp 6–16) was obtained by using semi-preparative HPAE[17].

We determined the dp of the partially purified and purified oligogalacturonides by fast-atom bombardment mass spectrometry (FABMS). According to FABMS analysis, the consecutive peaks from both the small and large anion-exchange columns are enriched in material with molecular weights that correspond exactly to the $[M - H]^-$ molecular ions of oligogalacturonides of consecutive size from dp 6 to 14 (compounds **4–12**). To obtain good quality FAB-mass spectra of larger underivatized oligogalacturonides, we have found it critical to remove salts and to exchange all cationic counterions for a single cationic species by the use of cation-exchange resins. The effectiveness of this procedure was demonstrated by Jin and West[13] who, using cation-exchange chromatography with Dowex 50 (H^+), were able to obtain the molecular ion for the (underivatized) galacturonic acid oligomer with dp 12. The solubility of the oligogalacturonides in the FAB matrix and the abundance of molecular ions are both enhanced by the use of an appropriate counterion and ionization matrix. We have obtained FAB-mass spectra of oligogalacturonides with up to 14 residues (m/z 2481 for $[M - H]^-$) by using ammonium as the counterion and thioglycerol as the matrix. This report is the first account of the successful characterization of underivatized oligogalacturonides of up to dp 14 by FABMS.

The oligogalacturonides were further characterized by NMR spectroscopy. The ^1H NMR spectra of mono- through penta-galacturonides, measured for solutions in D_2O at 220 MHz at a pH of 1 and 6, had been described previously[18]. In order to improve the resolution, we measured the NMR spectra at 500 MHz (and at pH 5.5–6.5). Figs. 3 and 4 show the one dimensional ^1H and ^{13}C NMR spectra of oligogalacturonides **1**, **5**, and **10** (with dp 3, 7, and 12, respectively). The ^1H and ^{13}C NMR assignments of oligogalacturonides **1–14** (dp 3–16) were made based on comparison of the 1D spectra supported by 2D $\{^1H–^1H\}$ long-range COSY (COSYLR, i.e., COSY optimized for small couplings)[19] and $\{^1H–^{13}C\}$ HETCOR[20] of all fourteen carbohydrates, assisted by 2D heteronuclear multiple quantum coherence (HMBC)[21] spectra of trigalacturonide **1** and heptagalacturonide **5** (see Table I).

We describe the complete assignment of the ^1H spectrum of heptagalacturonide **5** (see Fig. 3b) as a typical example. The doublets at δ 5.320 and 4.610, with coupling constants of 3.9 and 7.8 Hz, are assigned to the α and β H-1s of the reducing-end sugar residue ***R***. The doublet at relatively high field (δ 5.041, J 4.0 Hz) is the resonance of the H-1 of the nonreducing end sugar residue ***T***, whereas the H-1 resonance of the residue next to the reducing end, ***A*** H-1, is found at relatively low field (δ 5.10). The remaining partly overlapping doublets (δ 5.05–5.07) are the resonances of H-1s of the internal sugar residues ***I***. From the chemical shift values of H-1s of the nonreducing sugar residues and the magnitude of their coupling constants (~ 4.0 Hz), we conclude that the D-galacturonate residues are α-linked. The assembly of the anomeric resonances is well resolved from the nonanomeric sugar proton signals. Subsequent 2D NMR techniques were employed to assign ring proton signals that were not well resolved. The long-range

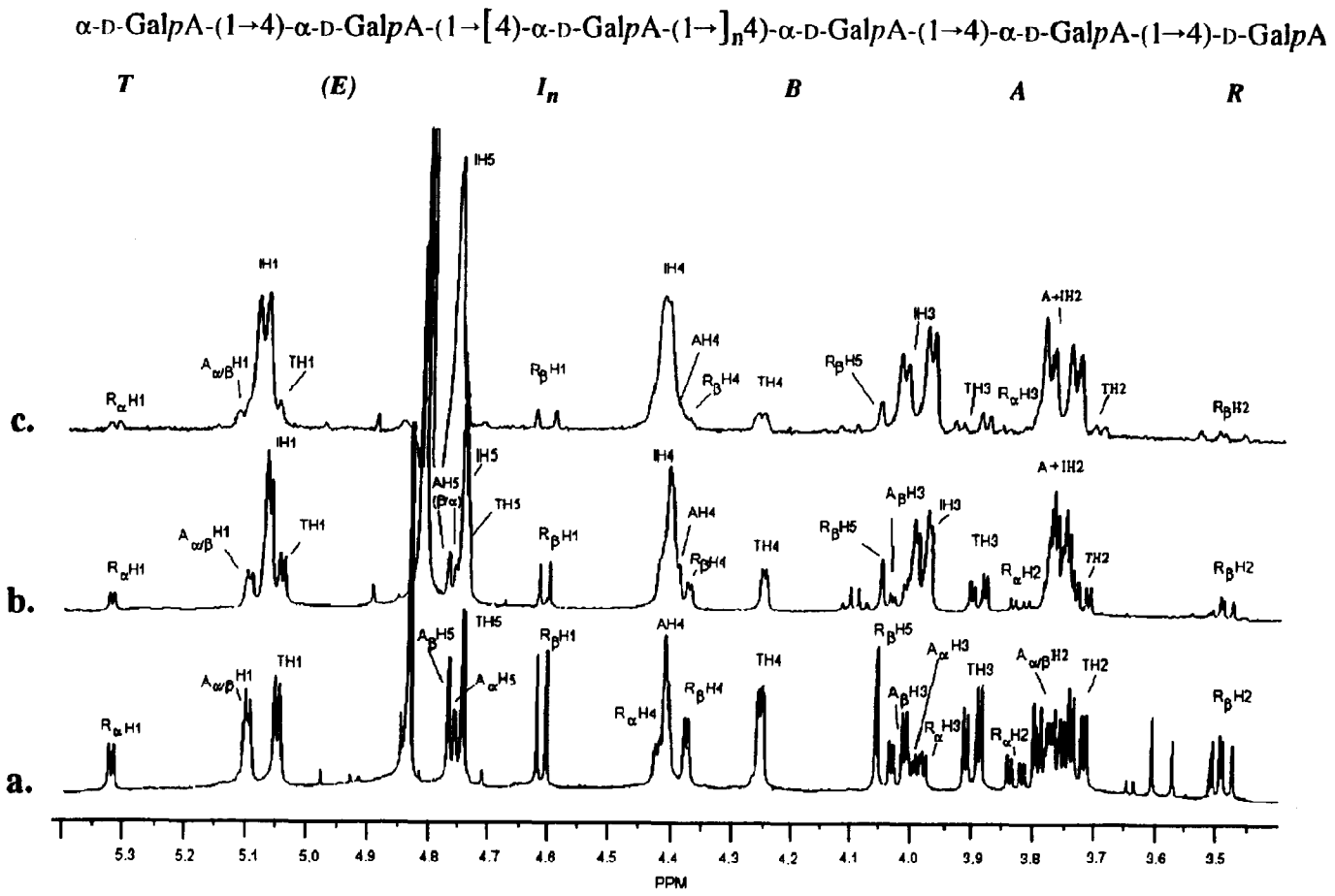

Fig. 3. ^{1}H NMR spectra of: a, trigalacturonide **1**; b, heptagalacturonide **5**; c, dodecagalacturonide **10**. Note that the spectra of **5** and **10** are very similar; only the signals of the residues **I** are much stronger in the spectrum of **10** in comparison to signals of any other residues.

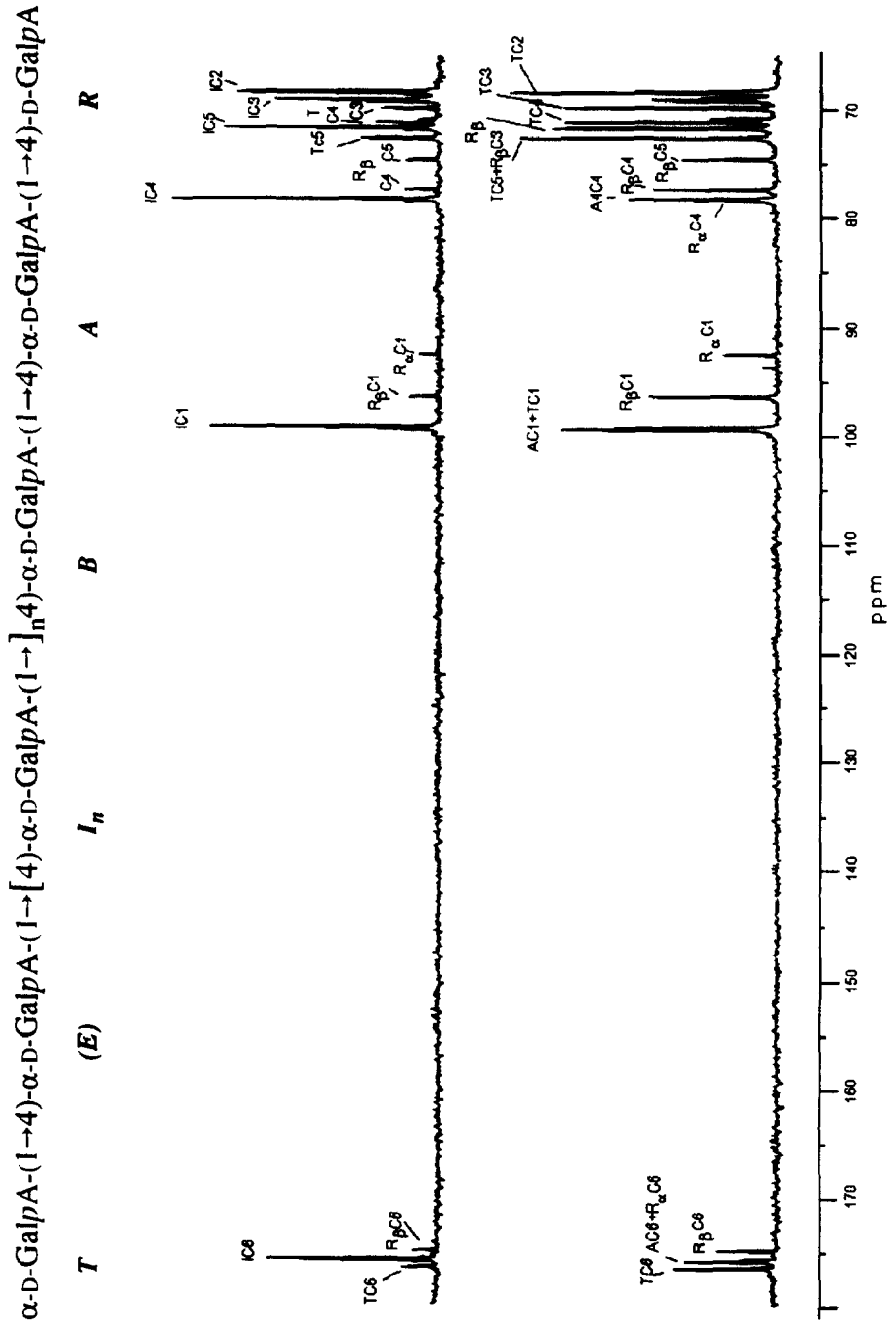

Fig. 4. ^{13}C NMR spectra of: a, trigalacturonide **1**; b, heptagalacturonide **5**.

COSY spectrum of heptagalacturonide **5** is shown in Fig. 5. Off-diagonal cross peaks in the COSY spectra arise via scalar coupling between a pair of vicinal or geminal protons, allowing the connectivity of adjacent protons within a given glycosyl residue to be determined. In order to visualize connectivities through the small coupling constants between the GalA H-4 and H-5 resonances (~ 1.0–1.5 Hz), the delay period of the COSYLR experiment was carefully optimized. The

5. α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→[4)-α-D-Gal*p*A-(1→]$_2$4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→4)-D-Gal*p*A(α/β)

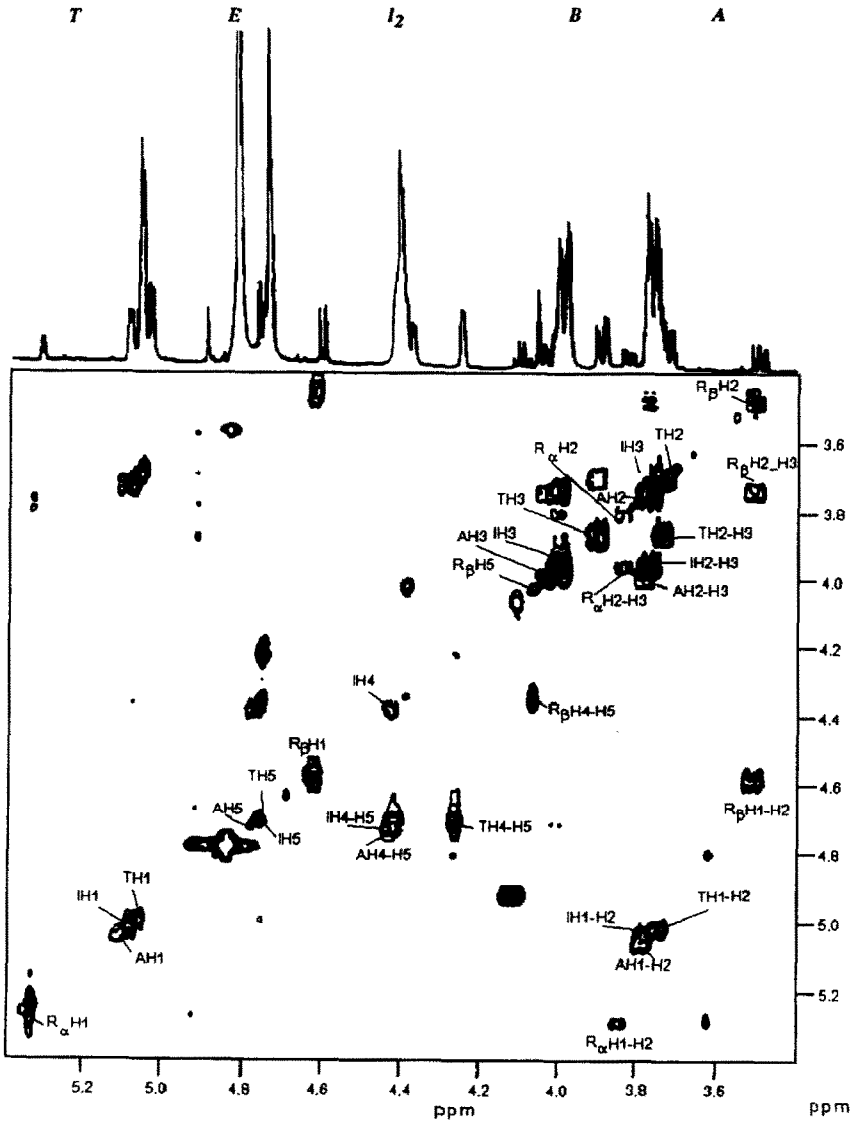

Fig. 5. 2D {^{1}H–^{1}H} COSY spectrum (optimized for small coupling constants) of heptagalacturonide **5**. The 1D projection of the COSY spectrum is shown at the top.

TABLE I

^{1}H and ^{13}C chemical shifts and $^{1}H-^{1}H$ coupling constants for oligogalacturonides

Oligo-galact-uronide	Residue	^{1}H Chemical shifts (ppm) [a]					$^{1}H-^{1}H$ Coupling constants (Hz)				^{13}C Chemical shifts (ppm) [a]					
		H-1	H-2	H-3	H-4	H-5	$J_{H-1,H-2}$	$J_{H-2,H-3}$	$J_{H-3,H-4}$	$J_{H-4,H-5}$	C-1	C-2	C-3	C-4	C-5	C-6
Trigalacturonide 1 [b,c]																
	Rα	5.320	3.830	3.990	4.423	4.410	3.8	10.6	3.1	1.1	92.49	68.31	69.21	78.43	70.88	175.63
	Rβ	4.613	3.494	3.765	4.376	4.059	7.8	10.2	3.3	1.1	96.42	71.70	72.60	77.35	74.57	174.83
	Aα	5.099	3.775	4.001	4.405	4.756	3.7	10.7	3.2	1.1	99.27	68.41	68.93	77.35	71.70	175.84
	Aβ	5.095	3.783	4.022	4.410	4.767	3.8	10.8	3.1	1.1	99.27	68.41	69.14	78.29	71.70	175.84
	T	5.048	3.730	3.901	4.255	4.743	3.9	10.5	3.4	1.5	99.41	68.50	69.83	71.14	72.60	176.49
Tetragalacturonide 2 [b,d]																
	Rα	5.320	3.824	3.987	4.429	4.405	3.9	10.6	n.d. [f]	1.1	92.47	68.28	69.30	78.39	70.81	175.63
	Rβ	4.608	3.495	3.760	4.370	4.051	7.9	10.2	3.3	1.1	96.43	71.70	72.57	77.36	74.52	174.83
	Aα	5.097	3.764	3.996	4.412	4.754	3.8	10.7	3.4	1.2	99.30	68.46	69.93	78.39	71.63	175.84
	Aβ	5.092	3.772	4.016	4.416	4.766	3.8	10.7	3.2	1.2	99.30	68.46	69.00	78.18	71.63	175.84
	B	5.065	3.769	3.984	3.984	4.741	3.8	10.7	3.2	1.2	99.30	68.46	69.17	78.26	71.63	175.84
	T	5.035	3.726	3.891	4.249	4.734	3.9	10.5	3.4	1.5	99.41	68.57	69.84	71.15	72.57	176.49
Pentagalacturonide 3 [b]																
	Rα	5.320	3.826	3.99	4.43	4.41	3.7	10.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Rβ	4.609	3.493	3.762	4.371	4.054	7.8	10.2	3.5	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Aα	5.091	3.765	3.996	4.42	4.754	3.8	10.7	3.4	0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Aβ	5.088	3.773	4.022	4.418	4.766	3.8	10.7	3.4	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	B	5.065	3.768	3.988	4.40	3.745	3.9	10.7	3.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	E	5.061	3.764	3.984	4.392	3.739	4.1	10.8	3.2	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	T	5.037	3.726	3.892	4.249	4.735	4.0	10.5	3.4	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Hexagalacturonide **4** [b,d]															
Rα	5.320	3.827	3.99	4.44	4.41	3.8	10.5	n.d.	n.d.	92.52	68.48	69.17	78.32	70.91	175.81
Rβ	4.613	3.494	3.765	4.376	4.059	7.8	10.2	3.3	1.1	96.49	71.71	72.64	77.34	74.61	174.88
Aα	5.096	3.768	4.000	4.43	4.757	3.9	10.6	3.3	1.1	99.38	68.48	69.17	78.32	71.71	175.82
Aβ	5.095	3.783	4.022	4.410	4.767	3.8	10.8	3.1	1.1	99.38	68.48	69.17	78.32	71.71	175.82
B	5.067	3.771	3.993	4.408	4.747	4.0	10.7	3.3	n.d.	99.38	68.48	69.17	78.32	71.71	175.82
I	5.063	3.763	3.988	4.408	4.740	4.0	10.7	3.3	n.d.	99.38	68.48	69.17	78.32	71.71	175.82
E	5.063	3.762	3.986	4.395	4.740	4.0	10.7	3.2	n.d.	99.38	68.48	69.20	78.32	71.71	175.82
T	5.040	3.729	3.894	4.251	4.736	4.0	10.5	3.4	1.5	99.38	68.86	69.88	71.19	72.64	176.51
Heptagalacturonide **5** [b,c,e]															
Rα	5.320	3.828	3.99	4.44	4.41	3.9	10.5	n.d.	n.d.	92.52	68.80	69.18	78.30	70.91	175.60
Rβ	4.610	3.497	3.765	4.376	4.052	7.8	10.2	3.3	1.0	96.49	71.71	72.64	77.35	74.60	174.88
Aα	5.098	3.769	3.988	4.42	4.756	4.0	10.6	3.3	1.2	99.37	68.86	69.18	78.31	71.71	175.82
Aβ	5.094	3.776	4.025	4.423	4.769	4.0	10.6	3.3	1.2	99.37	68.20	69.18	78.31	71.71	175.82
B	5.069	3.770	3.991	4.409	4.747	4.0	10.6	3.2	1.2	99.37	68.49	69.18	78.31	71.71	175.82
I	5.063	3.763	3.988	4.409	4.744	4.0	10.7	3.2	1.3	99.37	68.49	69.18	78.31	71.71	175.82
E	5.063	3.762	3.988	4.396	4.742	4.0	10.6	3.5	1.2	99.37	68.40	69.18	78.31	71.71	175.82
T	5.041	3.728	3.896	4.253	4.734	4.0	10.5	3.4	1.5	99.37	68.40	69.89	71.19	72.64	176.51

[a] ^{1}H and ^{13}C chemical shifts are quoted with reference to DSS. They were measured from internal acetone-d_5 (2.167 ppm) and acetone-d_6 (30.88 ppm), respectively, at 500 and 125 MHz, at 21°C in solutions buffered at pH 5.5–6.5. [b] ^{1}H chemical shift and coupling constant assignments are based on 1D ^{1}H and 2D {^{1}H–^{1}H} long-range COSY spectra. [c] ^{13}C chemical shift assignments are based on 1D ^{13}C and 2D {^{1}H–^{13}C} HETCOR and HMBC spectra. [d] ^{13}C chemical shift assignments are based on 1D ^{13}C spectra. [e] Assignments for compounds **6**–**14** are identical to those for compound **5**. [f] n.d., Not determined.

COSYLR spectra allowed the assignment of nearly all of the proton signals of $(GalA)_7$. By comparing the spectra of $(GalA)_3$ to $(GalA)_7$ and $(GalA)_{12}$ (see Fig. 3), the signals in the spectra of all the other compounds were assigned. The H-5 resonances of all the nonreducing residues were at δ 4.73–4.76 and the H-5 resonance of the β reducing sugar residue is found at δ 4.052. The assignment of the resonances at δ 4.4, where the H-4s of α-linked residues are expected, was proved by 2D $\{^1H-^1H\}$ COSYLR spectra (see Fig. 5). All the H-4 resonances except the H-4 of the nonreducing end sugar GalA ***T*** are overlapping at δ 4.35–4.44, whereas the H-4 of the nonreducing end sugar residue ***T*** is found at δ 4.253. The position of the H-4 resonances of the internal residues showed a downfield shift of 0.1–0.2 ppm compared to that of nonreducing terminal residue, reflecting that the D-galacturonides are $(1 \rightarrow 4)$-linked. The H-3 resonance of the residue next to the reducing end was found at δ 3.98–4.02 and the H-3 of the nonreducing end residue is at δ 3.896. The H-3 signals of all other residues except those of the β reducing end residue are overlapping at δ 3.98–4.00. The resonances of the H-2 atoms of all sugar residues (except those of the nonreducing end and the β reducing end residues) overlap with those of the H-3 atoms. The resonances at δ 3.728 and the well-resolved doublet-of-doublets at δ 3.497 are assigned to H-2s of the nonreducing end and β reducing end residues, respectively. The detailed assignments of the ^1H NMR spectra of these oligogalacturonides (compounds **1**–**10**) are compiled in Table I.

Once the ^1H NMR spectra of the oligogalacturonides had been assigned, their ^{13}C NMR spectra (Fig. 4) were completely analyzed by $\{^1H-^{13}C\}$ HETCOR spectroscopy. The resulting spectra allowed ^{13}C signals to be assigned by virtue of the one-bond $^1J_{CH}$ scalar coupling to assigned protons. The C-6 (C=O carbonyl) signals of the GalA residues were assigned by the $^3J_{CH}$ coupling to the assigned H-5 of the same residues by HMBC experiments. Detailed ^{13}C assignments are listed in Table I.

Ultimately, we wish to determine the physicochemical basis behind the size requirement of an oligogalacturonide for elicitor activity. The ^1H and ^{13}C NMR parameters for oligogalacturonides of dp 3 to 16 established in this study are the first step towards that goal. The NMR parameters for internal residues in the oligomers are identical to each other, and they are clearly different from those of the terminal and penultimate residues (see Table I). These features are reminiscent of those reported for microbial polysaccharides, consisting of polymers of sialic acid residues. For example, it has been established that, for oligomers of α-$(2 \rightarrow 8)$-linked sialic acid residues, a dp > 7 is required to bind to antibodies specific to a group B meningococcal polysaccharide[22,23]. It has been shown recently[23] that the oligosialyl epitope is not conformationally stable until at least 8 sialic acid residues are present. Therefore, our current hypothesis is that oligogalacturonides of dp > 8 can achieve a functionally significant solution conformation that smaller oligogalacturonides cannot. The details of the preferred solution conformations of the oligogalacturonides remain to be established.

EXPERIMENTAL

Preparation of oligogalacturonides by partial acid hydrolysis of citrus pectin.—Oligogalacturonides were prepared by partial acid hydrolysis of citrus pectin using a protocol based on a previously developed procedure[9]. Citrus pectin was purchased from Sunkist growers (Corona, CA), suspended in 2 M CF_3CO_2H in ten bottles (1 g in 100 mL of 2 M CF_3CO_2H per bottle), and heated for 4 h at 85°C with brief shaking every 30 min. After cooling to room temperature, the suspensions were filtered (Whatman GF/A), the filtrates combined and dried to a paste by rotary evaporation under reduced pressure at 35°C. The hydrolysate was suspended in 10 mL of MeOH and dried four times, suspended in 100 mL of deionized H_2O, and adjusted to pH 7 with 5 M imidazole. The hydrolysate was stirred overnight at 4°C, and filtered progressively through Whatman GF/A, Millipore Type HA 0.45-μm, and Millipore Type GS 0.22-μm filters. The final filtrate was lyophilized and contained 3.5 g hydrolyzed material. This was called the crude pectin hydrolysate (CPH).

Low-resolution anion-exchange chromatography of CPH.—An anion-exchange column (3 × 12 cm) containing QAE-Sephadex (A-25-120, Sigma) was equilibrated with 125 mM imidazole–HCl, pH 7 (buffer A). CPH (1 g) in 100 mL of H_2O, adjusted to a conductivity of 6.7 mΩ^{-1}, slightly less than that of buffer A, with 5 M imidazole–HCl, pH 7, was loaded onto the column. The column was washed stepwise with 400 mL of buffer A, 400 mL of buffer B (550 mM imidazole–HCl, pH 7), and 200 mL of buffer C (750 mM imidazole–HCl, pH 7). The eluates from each wash were collected separately, coincident with the start of the washes, and designated as fractions A, B, and C. Fractions A, B, and C were concentrated by rotary evaporation to ca. 5 mL each and desalted using a column (3 × 18 cm) of Sephadex G-10 (Sigma) in deionized H_2O. The uronic acid content of the eluate was determined colorimetrically using the *m*-hydroxybiphenyl assay[24], and the salt concentration was determined by conductivity measurement.

Preparative high-resolution anion-exchange chromatography of fraction C.—A desalted preparation of fraction C was further fractionated using high-resolution anion-exchange chromatography. Samples containing ca. 21 mg of uronic acid equivalents as determined colorimetrically were adjusted to pH 7 and a conductivity slightly less than that of the 450 mM buffer (imidazole–HCl, pH 7, 22.8 mΩ^{-1}), loaded in a total volume of 5 mL onto a fast-flow anion-exchange column (1.4 × 40 cm) containing Q-Sepharose (Pharmacia) equilibrated with this 450 mM buffer, and the column was washed with a linear gradient of imidazole–HCl, pH 7, from 450 mM (1 L) to 750 mM (buffer C) (1 L). Fractions (10 mL) were collected and their uronic acid content determined colorimetrically. Peaks enriched in uronic acid were each desalted using either a Sephadex G-10 column or dialysis against deionized H_2O with 1000 mol wt cutoff dialysis membranes (Spectropor-7).

For large-scale fractionation, desalted preparations of fraction C containing ca. 160 mg uronic acid equivalents were adjusted to pH 7 and a conductivity slightly

less than that of 450 mM buffer (imidazole–HCl, pH 7), and loaded in a total volume of 40 mL onto a fast-flow anion-exchange column (3.5 × 40 cm) containing Q-Sepharose equilibrated with this 450 mM buffer. The column was eluted with a linear gradient of imidazole–HCl, pH 7, from 450 mM (2.6 L) to 750 mM (2.6 L). Fractions (10 mL) were collected and their uronic acid content determined colorimetrically. Peaks enriched in uronic-acid were each desalted as above.

Further purification of the dodecagalacturonide by gel filtration.—A column (1.6 cm × 95 cm × 2) of Sephadex G-25 (Sigma) was equilibrated with 1% acetic acid and calibrated with Dextran T-40 (mol wt 40 000) and glucose at a flow rate of 4.5 mL/cm^2/h. Elution of standards was analyzed colorimetrically using the anthrone assay[25]. Material (2.1 mg) from the peak corresponding to the dodecagalacturonide was loaded in a total volume of 0.5 mL and eluted in 1% acetic acid. Fractions (0.45 mL) were collected and assayed colorimetrically for uronic acids.

High-pH anion-exchange chromatography of oligogalacturonides with pulsed amperometric detection (HPAE-PAD).—Oligogalacturonide mixtures (CPH or fraction C) were filtered (0.2-μm Nylon 66) and then separated on a CarboPac I column (4 × 250 mm) using a Dionex BioLC system. The oligogalacturonides (ca. 500 μg total uronic acid in deionized water, 100 μL) were loaded onto the column using a polymeric micro-injection valve (Dionex) and eluted at 1 mL/min with 400 mM sodium acetate containing 150 mM sodium hydroxide (0–2 min), followed by a gradient of 400 mM to 900 mM sodium acetate in 150 mM sodium hydroxide (2.1–45 min). The column was reconditioned between runs by washing with 1 M sodium acetate in 150 mM NaOH (5 min; flow 1 mL/min) and then re-equilibrated with the starting buffer solution for at least 15 min. Carbohydrate was detected using a Dionex pulsed amperometric detector (PAD), at 10 K sensitivity, interfaced to a Bio-Rad model 700 data station. The HPAE column was calibrated using the oligogalacturonides that had been isolated by high-resolution Q-Sepharose anion-exchange chromatography and characterized by FABMS.

Fast-atom-bombardment mass spectrometry (FABMS).—Small aliquots of the partially purified oligogalacturonides were converted to their ammonium salt form by elution through a 1 mL column of Dowex 50X2-200 (NH_4^+) (Aldrich). Aliquots (ca. 10 μg in 1 μL) of the ammonium salts of the underivatized oligogalacturonides were applied to the FAB probe tip along with 1 μL 1 M HCl and 2 μL thioglycerol. FAB-mass spectra of oligogalacturonides were recorded with a VG ZAB-SE mass spectrometer operating at low resolution (1:1000) in the negative ion mode with an accelerating voltage of 8 kV. The mass spectrometer was calibrated with CsI. Isotopomeric ions of high-mass ion clusters were not resolved under the low-resolution conditions used. Signals corresponding to the average mass of the ion cluster were detected. The m/z values were converted into the nominal masses of the isotopomers containing only ^{12}C, ^{1}H, and ^{6}O isotopes (as reported throughout this article) using the CARBOMASS mass spectrometry software[26].

NMR analysis of oligogalacturonides.—The oligogalacturonides (dp 3–5) were

converted into their H^+ form by elution from a 1 mL column of Dowex 50X2-200 (H^+) (Aldrich) and then dissolved in 0.5 mL 0.3 M KH_2PO_4–0.6 M K_2HPO_4 buffer system. The oligogalacturonides (dp 6–14) were first dissolved into 100 mL 0.7 M KOAc–0.04 M HOAc and dialysed against deionized water, then dissolved in 0.5 mL D_2O buffered with 0.2 M KH_2PO_4–0.028 M K_2HPO_4 buffer solution. The protons of the hydroxyl groups of the oligogalacturonides and the phosphate salts were exchanged against deuterium by fivefold lyophilization from D_2O. All spectra were recorded at 21°C with a Bruker AM 500 (500 MHz for ^1H) instrument interfaced with an Aspect 3000 computer using the DISR88 software package.

A {^1H–^1H} long-range COSY spectrum[19] was recorded with N-type peak selection. The introduction of a relatively long delay before the beginning of data acquisition enhances the relative intensity of cross-peaks arising from small couplings. The delay was set to 0.25 s. The spectral width was 1250 Hz in the F_2 domain, and 625 Hz in the F_1 domain. The relaxation delay was 4.8 s. FIDs (512) each having 2 K data points were acquired with a t_1 increment of 800 μs. An unshifted sine-bell window function was used in processing the data both in the t_1 and t_2 time domains. The data matrix was expanded to a 2 K × 1 K data file before Fourier transformation.

2D {^1H–^{13}C} shift-correlated (HETCOR) spectra[20] were recorded with ^1H composite pulse decoupling during acquisition. The spectral width was 556 Hz in the F_1 domain, and 13889 Hz in the F_2 domain. The relaxation delay was 1 s. Incrementing t_1 in steps of 450 μs, 48 FIDs with 2 K data points were obtained. An unshifted sine-bell window function was used in processing the data in the t_1 domain, and a Gaussian window function was applied in the t_2 domain. The data matrix was expanded to a 2 K × 48 data file before Fourier transformation.

2D ^1H detected {^1H–^{13}C} multiple-bond shift correlation (HMBC) spectra were recorded as described by Bax and Summers[21]. The introduction of a WEFT pulse[27] sequence during the relaxation delay suppressed the solvent signal. The spectral widths in the F_2 and F_1 domains were 7353 Hz and 1250 Hz, respectively. Incrementing t_1 in steps of 34 μs, 128 FIDs with 2 K data points were obtained. An unshifted sine window function was applied in processing the data in t_2, no window function was applied in t_1.

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