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Fluorescent labeling of pectic oligosaccharides with 2-aminobenzamide and enzyme assay for pectin

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Abstract

Oligogalacturonides [oligomers composed of (1→4)-linked α-D-galactosyluronic acid residues] with degrees of polymerization (DP) from 1 to 10, and a tri-, penta-, and heptasaccharide generated from the backbone of rhamnogalacturonan I (RG-I) were labeled at their reducing ends using aqueous 2-aminobenzamide (2AB) in the presence of sodium cyanoborohydride in over 90% yield. These derivatives were analyzed by high-performance anion-exchange chromatography (HPAEC) and structurally characterized by electrospray-ionization mass spectrometry (ESIMS) and by ^{1}H and ^{13}C NMR spectroscopy. The 2AB-labeled oligogalacturonides and RG-I oligomers are fragmented by *endo*- and *exo*-polygalacturonase and by Driselase, respectively. 2AB-labeled oligogalacturonide is an exogenous acceptor for galacturonosyltransferase of transferring galacturonic acid from UDP-GalA. Thus, the 2AB-labeled oligogalacturonides and RG-I oligomers are useful for studying enzymes involved in pectin degradation and biosynthesis and may be of value in determining the biological functions of pectic fragments in plants.

Keywords: 2-Aminobenzamide; Electrospray-ionization mass spectrometry; Fluorescent labeling; Galacturonosyltransferase; NMR; Pectin; Polygalacturonase

1. Introduction

Reductive amination of the reducing end of carbohydrates with UV and fluorescent tags is a widely used technique for generating derivatives that can be detected at picomol levels.[1–3] The use of numerous fluorophores including monosubstituted aminobenzene derivatives (2-aminobenzamide,[4–6] 2-aminobenzoic acid,[7,8] 4-aminobenzoic acid esters,[9,10] 4-aminobenzonitrile,[11,12] 3-aminobenzoic acid,[13] 4-aminobenzoic acid[14]) to 8-aminonapthalene-1,3,6-trisulfonate (ANTS)[15] have been described in the literature (for reviews, see Refs. 1–3). Such derivatives can be separated and quantified by HPLC, capillary electrophoresis, and gel electrophoresis, and characterized by NMR spectroscopy and mass spectrometry.

Several oligosaccharides derived from plants, fungi, and bacteria have been shown to elicit complex processes in plants, including the alteration of morphogenesis and the initiation of plant defense responses. Among these biologically active oligosaccharides oligogalacturonides (oligomers composed of (1→4)-linked α-D-galactosyluronic acid residues) have been demonstrated with in vitro bioassays to have various biological activities.[16,17] 2-Aminopyridine-derivatized oligogalacturonides from DP 3 to over 25 were separated by anion-exchange HPLC and used to analyze the endopolygalacturonase digestion of a labeled DP 20 oligomer.[18] Tyramine-derivatized[19] and biotin-labeled oligogalacturonides[20] were synthesized to study the biological functions of oligogalacturonides.

The labeling of carbohydrates with fluorescent tags has been reported to have some limitations. For example, the yield of the labeled derivatives is low when acidic oligosaccharides are reductively aminated with tyramine.[19]

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Here we report a procedure for the derivatization using aqueous 2-aminobenzamide (2AB) on acidic oligosaccharides derived from homogalacturonan and rhamnogalacturonan I (RG-I). The extent of derivatization and the structures of the derivatives were determined using HPLC, ESIMS, and NMR spectroscopy. 2AB-labeled oligogalacturonides were shown to be degraded by *endo*- and *exo*-polygalacturonase and to be an exogenous acceptor for galacturonosyltransferase (GalA T).

2. Results and discussion

Derivatization of oligosaccharides with aqueous 2AB.—Organic solvents have been used for labeling with fluorescent tags in previous methods.[1–3] Under our experimental conditions, acidic oligosaccharides were only partially soluble in methanol and dimethyl sulfoxide, and consequently the yield of labeled oligosaccharides was low. In contrast, a high yield of fluorescent derivatives was obtained by treating oligogalacturonides (DP 1–10) derived from homogalacturonan and oligosaccharides derived from RG-I (trimer, pentamer and heptamer) for 2 h at 65 °C with aqueous 0.2 M 2AB in the presence of sodium cyanoborohydride. The reaction mixture was applied to a Toyopearl HW-40 column, and the derivatized oligosaccharides that eluted at the column void volume were collected. The products were then analyzed by HPAEC with pulsed amperometric (PAD) and fluorescence detection (Fig. 1). 2AB-derivatized oligosaccharides are detected by both PAD and fluorescence ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 420$ nm), whereas underivatized oligosaccharides are only detected by PAD. For example, the decagalacturonide when reacted with AB gave one major 2AB-labeled peak (Fig. 1(A and B)), indicating that 2AB derivatization of decagalacturonide gave a yield of at least 90% and that no significant degradation occurred at the temperature (65 °C) used for the derivatization reaction. Fluorescent derivatives of the trisaccharide (RG_3), the pentasaccharide (RG_5) and the heptasaccharide (RG_7) generated from the RG-I backbone were also obtained in high yield when reacted with 2AB.

Characterization of the 2AB-labeled oligosaccharides.—The negative-ion electrospray-ionization mass spectra (ESIMSs) of the 13 2AB-derivatized oligosaccharides were dominated by a singly or doubly charged pseudomolecular ion. For example, the 2AB-labeled oligogalacturonides and RG-I oligomers typically gave an intensive singly charged ion $[M-1H]^-$, thereby confirming their molecular weights. 2AB-labeled nona- and decagalacturonides also gave doubly charged ions $[M-2H]^-/2$, in addition to the singly charged ion.

^1H NMR spectra of 2AB-labeled oligosaccharides.—The ^1H NMR spectra of 2AB-labeled oligogalacturonides and 2AB-labeled RG-I oligosaccharides were measured at 800 MHz at pH 7.0–7.5 (Fig. 3). The signals in the ^1H and ^{13}C NMR spectra of **1**–**13** (Fig. 2) were assigned using 2D $\{^1H-^1H\}$ double quantum filtered correlation spectroscopy (DQFCOSY), 2D total correlation spectroscopy (TOCSY), and 2D $\{^1H-^{13}C\}$ ^1H-detected heteronuclear single quantum coherence (HSQC), and ^1H-detected multiple-bond heteronuclear multiple quantum coherence spectroscopy (HMBC). The absence of signals not attributed to the oligogalacturonides, and 2AB confirmed that the derivatives were homogeneous (Fig. 3).

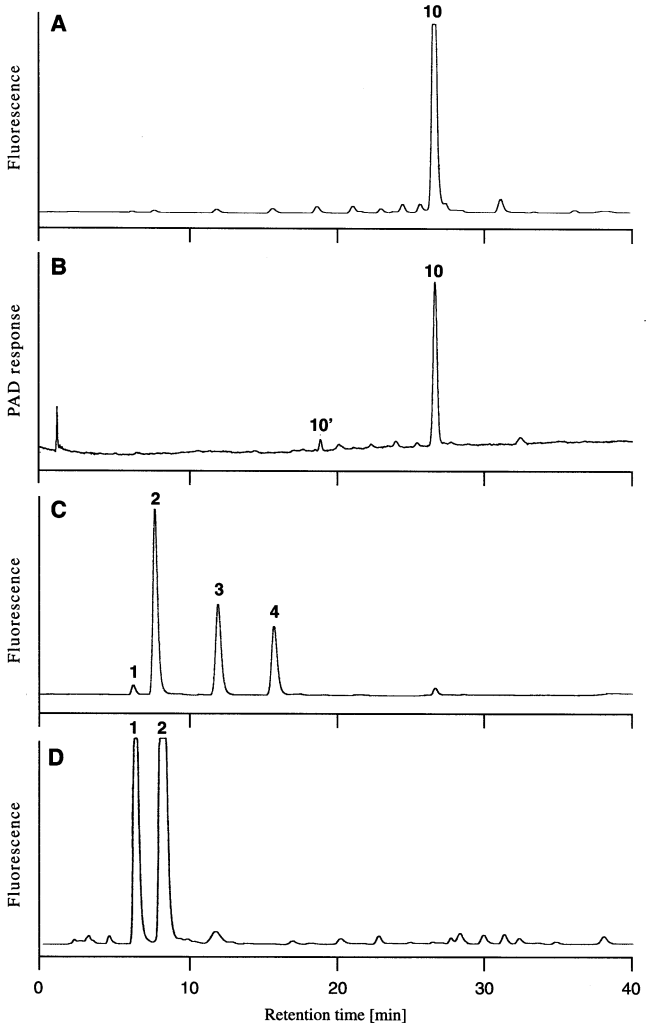

Fig. 1. HPAE chromatograms of the decagalacturonide derivatization mixture (A and B) resolved on a CarboPac PA-1 column with a linear gradient of NaOAc in 0.1 M NaOH and monitored by a fluorescent detector (A) ($\lambda_{em} =$ 420 nm) and by PAD (B). The decagalacturonide derivatization mixture contained 2AB-labeled decagalacturonide **10** and a small amount of underivatized decagalacturonide **10′**. (C) The EPG digest of the 2AB-labeled decagalacturonide monitored by the fluorescent detector. (D) The *exo*-polygalacturonase digest of the 2AB-labeled decagalacturonide monitored by the fluorescent detector.

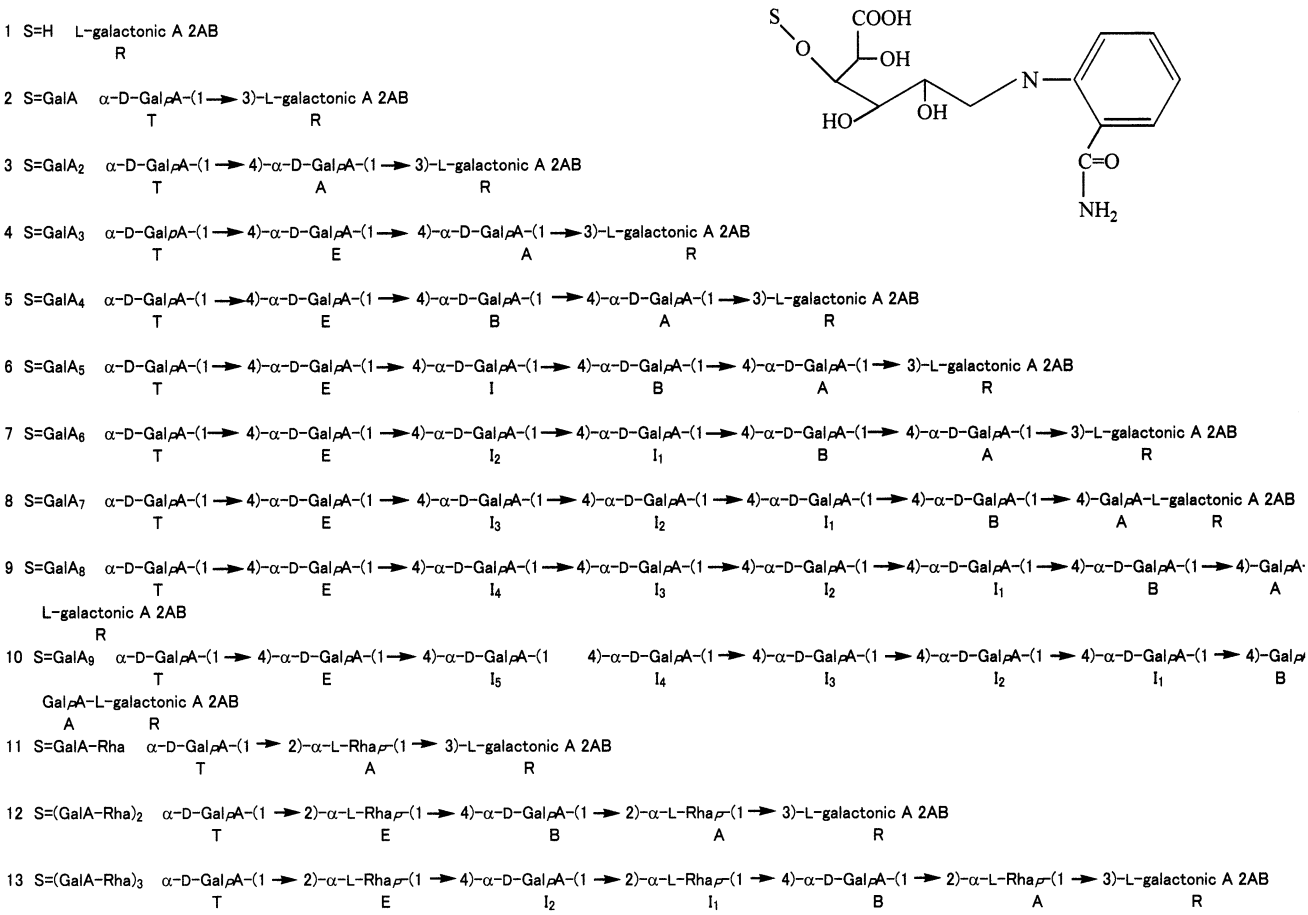

Fig. 2. Structures of compounds **1**–**13**. **1**–**3**, 2-AB labeled L-galactonic acid to trigalacturonides (DP 1–3); **4**, tetragalacturonide, consisting of a L-galactonic acid R, two internal residues, designated A and E, and a nonreducing terminal residue T; **5**, pentagalacturonide, consisting of an L-galactonic acid R, three internal residues, designated A, B, and E, and a nonreducing terminal residue T; **6**, hexagalacturonide, composed of R, four internal residues, designated A, B, I, E, and T; **7**, heptagalacturonide, composed of R, five internal residues, A, B, I_1, I_2, and E, and T; **8**, octagalacturonide, composed of R, six internal residues, A, B, I_1, I_2, I_3, and E and T; **9**, nonagalacturonide, composed of R, seven internal residues, A, B, I_1, I_2, I_3, I_4, and E and T; **10**, decagalacturonide, composed of R, eight internal residues, A, B, I_1, I_2, I_3, I_4, I_5, and E and T; **11**, RG_3, trisaccharide, composed of L-galactonic acid R, Rha residue A and a nonreducing terminal galacturonic acid residue T; **12**, RG_5, pentasaccharide, composed of R, three internal Rha and GalA residues, A, B, E, and T; **13**, RG_7, heptasaccharide, composed of R, five internal Rha and GalA residues, A, B, I_1, I_2, and E and T.

The complete assignment of the ^{1}H spectrum of **7** (2AB-labeled heptagalacturonide) is described as a typical example. The L-galactonic acid residue (the former reducing end of galacturonic acid residue) is clearly no longer a pyranose since there are two protons on carbon-6 instead of one, and their quartet signals are at δ 3.397 and 3.372, with coupling constants of 5.2 and 7.2 Hz. The doublet at δ 5.043 (J 3.5 Hz) is the resonance of the H-1 of the terminal nonreducing end Gal*p*A residue T, whereas the H-1 resonance of the residue next to the reducing end, (residue A), is found at δ 5.088. The remaining partly overlapping doublets (δ 5.060–5.065) are the resonances of H-1s of the internal sugar residues I. The chemical shift values of H-1s of the nonreducing Gal*p*A residues and the magnitude of their coupling constants ($\sim$3.0–3.5 Hz), are consistent with an α linkage. The anomeric resonances are well resolved from the non-anomeric sugar proton signals. The TOCSY and DQFCOSY spectra allowed the assignment of nearly all of the proton signals of (2AB $GalA_7$). By comparing the spectra of (2AB $GalA_3$) to (2AB $GalA_7$), the signals in the spectra of all the oligogalacturonide derivatives were assigned (Table 1). The signals in the ^{1}H spectra of 2AB-labeled oligogalacturonides of DP >8 are broader than those of DP <7 (Fig. 3). The anomeric proton of the Gal*p*A residue B in the 2AB-labeled heptagalacturonide is clearly distinguished from those of the internal residues. In contrast, the anomeric protons of the Gal*p*A residue B in 2AB-labeled oligomers of DP >8 are partly overlapped with those of the internal residues. These results suggest that oligogalacturonides of DP >8 have a different solution conformation from those of DP <7. Lo et al.[21] reported that the NMR parameters for

Table 1
^{1}H and ^{13}C chemical shifts (δ, ppm) and first-order coupling constants (Hz) for compounds **1**–**10**

Compound	Residue	^{1}H chemical shifts (ppm) [a]							First-order coupling constants (Hz) [b]							^{13}C chemical shifts (ppm) [c]					
		H-1	H-2	H-3	H-4	H-5	H-6_a	H-6_b	$^{3}J_{1,2}$	$^{3}J_{2,3}$	$^{3}J_{3,4}$	$^{3}J_{4,5}$	$^{3}J_{5,6a}$	$^{3}J_{5,6b}$	$^{2}J_{6a,6b}$	C-1	C-2	C-3	C-4	C-5	C-6
1	R		4.2587	3.970	3.648	4.133	3.414	3.351		<1	9.8	<1	4.9	8.5	13.5	181.09	73.17	72.40	71.41	69.49	47.75
2	R		4.282	4.183	3.690	3.975	3.387	3.354		4.5	8.4	1.1	6.4	7.2	13.7	180.08	71.05	78.02	69.93	68.42	46.01
	T	5.041	3.695	3.533	4.101	4.019			4.1	10.4	3.3	1.3				98.57	67.40	70.81	70.98	72.95	175.58
3	R		4.296	4.210	3.685	3.965	3.392	3.366		<1	10.4	<1	6.7	7.1	13.6	180.03	72.21	79.20	70.01	68.45	47.22
	A	5.074	3.745	3.611	4.253	3.995			2.9	10.5	2.6	<1				99.65	68.26	70.56	79.38	73.35	174.70
	T	5.028	3.675	3.864	4.239	4.663			3.9	10.5	3.3	1.3				100.73	71.78	71.21	72.50	73.93	176.49
4	R		4.305	4.215	3.691	3.965	3.402	3.382		<1	8.5	<1	5.0	5.5	13.0	177.72	72.20	79.27	70.05	68.41	47.26
	A	5.081	3.743	3.607	4.271	4.008			3.1	10.3	3.1	2.3	<1			99.74	69.47	70.48	79.65	73.31	175.91
	E	5.067	3.729	3.954	4.386	4.681			3.7	10.6	3.1	4.2	<1			100.67	69.85	70.57	79.65	73.04	177.06
	T	5.043	3.700	3.895	4.250	4.719			3.3	10.6	3.7	4.2	<1			100.67	71.79	71.23	72.51	73.88	177.60
5	R		4.277	4.192	3.675	3.949	3.384	3.360		1.4	8.5	<1	5.2	5.7	13.5	181.27	72.22	79.24	70.00	68.44	47.20
	A	5.061	3.733	3.594	4.252	3.987			4.1	10.4	3.1	<1				99.70	69.47	70.62	79.31	73.30	175.94
	B	5.045	3.728	3.940	4.379	4.662			3.9	10.7	3.7	<1				100.60	69.89	71.25	79.51	73.02	177.18 [e]
	E	5.045	3.741	3.970	4.374	4.662			3.9	10.7	3.7	<1				100.60	69.85	70.61	79.68	73.30	177.08 [e]
	T	5.023	3.690	3.881	4.235	4.7			3.9	10.7	3.4	<1				100.70	71.80	71.41	72.53	73.93	177.74
6	R		4.291	4.204	3.701	3.950	3.396	3.373		1.4	8.5	<1	5.2	7.2	13.5	181.25	72.22	79.22	70.02	68.42	47.20
	A	5.073	3.754	3.601	4.264	4.004			4.1	10.4	3.1	<1				99.68	69.47	70.02	79.30	73.30	175.97
	B	5.058	3.732 [e]	3.990 [e]	4.400 [e]	4.658			3.9	10.7	3.2	<1				100.60	69.90 [e]	70.50 [e]	79.55 [e]	73.02 [e]	177.07
	I	5.058	3.741 [e]	3.977 [e]	4.393 [e]	4.734 [e]			3.9	10.7	3.7	<1				100.60	69.86 [e]	70.55 [e]	79.55 [e]	72.98 [e]	177.10 [e]
	E	5.058	3.741 [e]	3.960 [e]	4.390 [e]	4.723 [e]			3.9	10.7	3.7	<1				100.60	69.86 [e]	70.62 [e]	79.69 [e]	72.98 [e]	177.18 [e]
	T	5.039	3.682	3.895	4.247	4.723 [e]			3.9	10.7	3.4	<1				100.69	71.78	71.25	72.53	73.92	177.75
7	R		4.302	4.212	3.699	3.951	3.397	3.372		<1	8.5	<1	5.2	7.2	13.5	181.25	72.21	70.02	79.15	68.46	47.23
	A	5.088	3.756	3.614	4.271	4.020			3.7	10.2	3.5	<1				100.63	69.47	70.02	79.32	73.32	175.93
	B	5.060	3.733	3.971	4.391	4.658			3.5	11	2.5	<1				99.58	69.88	70.54 [e]	79.58	73.00	176.09
	I_1	5.063 [e]	3.715 [e]	3.981	4.397 [e]	4.732 [e]			3.0	11	2.5	[d]				100.69	69.88	70.54 [e]	79.58	73.00	177.10
	I_2	5.065 [e]	3.745 [e]	3.985 [e]	4.405 [e]	4.720 [e]			3.0	11	2.5	[d]				100.69	69.88	70.54 [e]	79.58	73.00	177.10
	E	5.065 [e]	3.745 [e]	3.985 [e]	4.405 [e]	4.720 [e]			3.0	11	2.5	[d]				100.63	69.88	70.61 [e]	79.68	73.00	177.10
	T	5.043	3.704	3.894 [e]	4.247	4.721			3.5	10.2	3.4	[d]				100.63	71.74	71.24	72.53	73.93	177.17
8	R		4.288	4.206	3.693	3.947	3.397	3.375		<1	6.0	<1	6.2	6.9	13.0	177.76	72.19	79.20	70.04	68.43	47.20
	A	5.082	3.762	3.597	4.265	3.999			[d]	10.0	3.0	<1				99.68	70.04	69.48	79.31	73.33	181.02
	B	5.062	3.723	3.977	4.393	4.671			[d]	10.0	3.0	<1				100.56 [e]	69.93	70.52 [e]	79.57 [e]	73.03	175.94
	I_1	5.062	3.743	3.985	4.403	4.720			[d]	10.0	3.0	[d]				100.61 [e]	69.90	70.56 [e]	79.56 [e]	73.03	177.11
	I_2	5.062	3.743	3.985	4.403	4.720			[d]	10.0	3.0	[d]				100.61 [e]	69.90	70.63 [e]	79.55 [e]	73.03	177.11
	I_3	5.062	3.743	3.985	4.403	4.720			[d]	10.0	3.0	[d]				100.61 [e]	69.90	70.56 [e]	79.57 [e]	73.03	177.11
	E	5.062	3.743	3.985	4.403	4.720			[d]	10.0	3.0	[d]				100.61 [e]	69.90	70.56 [e]	79.68	73.03	177.11
	T	5.041	3.670	3.889	4.247	4.720			3.8	11.0	2.4	[d]				100.67	71.81	71.25	72.54	73.94	177.21
9	R		4.290	4.205	3.685	3.954	3.397	3.378		<1	6.0	<1	7.4	7.6	12.0	177.75	72.26	79.20	70.05	68.43	47.20
	A	5.072	3.759	3.597	4.263	4.002			[d]	10.5	2.2	<1				99.67	70.05	69.49	79.31	73.51	181.20
	B	5.062	3.721	3.967	4.393	4.670			[d]	10.5	3.0	<1				100.56 [e]	69.82 [e]	70.47 [e]	79.48 [e]	73.31	175.94
	I_1	5.062	3.739	3.984	4.404	4.720			[d]	10.5	3.0	[d]				100.62 [e]	69.91 [e]	70.56 [e]	79.57 [e]	73.31	177.10
	I_2	5.062	3.739	3.984	4.404	4.720			[d]	10.5	3.0	[d]				100.62 [e]	69.91 [e]	70.63 [e]	79.57 [e]	73.31	177.10
	I_3	5.062	3.739	3.984	4.404	4.720			[d]	10.5	3.0	[d]				100.62 [e]	69.91 [e]	70.56 [e]	79.57 [e]	73.31	177.10
	I_4	5.062	3.739	3.984	4.404	4.720			[d]	10.5	3.0	[d]				100.62 [e]	69.91 [e]	70.56 [e]	79.57 [e]	73.31	177.10
	E	5.062	3.739	3.984	4.404	4.720			[d]	10.5	3.0	[d]				100.62 [e]	69.91 [e]	70.56 [e]	79.67	73.31	177.10
	T	5.042	3.699	3.895	4.249	4.720			3.9	10.0	3.4	[d]				100.67	71.80	71.25	72.54	73.93	177.21

Table 1 (*Continued*)

Compound	Residue	^{1}H chemical shifts (ppm) [a]							First-order coupling constants (Hz) [b]							^{13}C chemical shifts (ppm) [c]					
		H-1	H-2	H-3	H-4	H-5	H-6$_a$	H-6$_b$	$^3J_{1,2}$	$^3J_{2,3}$	$^3J_{3,4}$	$^3J_{4,5}$	$^3J_{5,6a}$	$^3J_{5,6b}$	$^2J_{6a,6b}$	C-1	C-2	C-3	C-4	C-5	C-6
10	R		4.287	4.203	3.683	3.944	3.396	3.374		<1	5.7	<1	6.4	7.0	13.0	177.77	72.26	79.21	70.04	68.43	47.19
	A	5.051	3.755	3.596	4.263	3.997			[d]	9.8		<1				99.68	70.04	69.48	79.31	73.2	181.20
	B	5.061	3.723	3.965	4.392	4.670			[d]	10.0	3.7	<1				100.67	69.90	70.56 [e]	79.57	73.02	175.94
	I_1	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.62 [e]	79.57	73.02	177.12
	I_2	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.56 [e]	79.57	73.02	177.12
	I_3	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.56 [e]	79.57	73.02	177.12
	I_4	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.56 [e]	79.57	73.02	177.12
	I_5	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.56 [e]	79.57	73.02	177.12
	E	5.061	3.742	3.983	4.402	4.720			[d]	10.0	3.7	[d]				100.67	69.90	70.56 [e]	79.68	73.02	177.12
	T	5.040	3.699	3.895	4.247	4.720			3.9	11.0	3.4	[d]				100.67	71.81	71.25	72.54	73.93	177.12

[a] ^{1}H and ^{13}C chemical shifts are quoted from methyl proton of internal *tert*-butanol (1.230 ppm) at 800 MHz and methyl carbon of *tert*-butanol (31.30 ppm) at 150 MHz, respectively, at 30 °C, in solutions buffered at pD 7.0–7.5.

[b] ^{1}H chemical shift and coupling constant assignments are based on 1D ^{1}H, DQF COSY, and TOCSY spectra.

[c] ^{13}C chemical shift assignments are based on 1D ^{13}C, HSQC and HMBC spectra.

[d] Overlapped.

[e] Interchangeable, uncertain.

internal residues in oligogalacturonides of DP >8 are identical to each other and that they are different from those of the terminal and penultimate residues.

The ^{13}C NMR spectra of the 2AB-labeled oligogalacturonides were completely analyzed by HSQC spectroscopy. The C-6 (C=O carbonyl) signals of the GalA residues were assigned by DQFCOSY and TOCSY to the assigned H-5 of the same residue by the HMBC experiment. Detailed ^{13}C assignments are shown in Table 1.

The signals in the ^{1}H and ^{13}C spectra of the 2AB-labeled RG-I backbone-derived oligosaccharides (compounds **11**–**13**) were assigned using 1D and 2D NMR techniques and are summarized in Table 2.

The ^{13}C NMR spectra of RG-I oligomers were completely analyzed by HSQC spectroscopy. The C-6 (C=O carbonyl) signals of the GalA residues were assigned by the 2D TOCSY to the assigned H-5 of the same residues by HMBC experiments. The HMBC spectra for the RG-I oligomers confirmed the connectivity of each glycosyl residue in the oligomers. The ^{13}C signal assignments are summarized in Table 2. The chemical shifts of 2AB residue are summarized in Table 3.

Enzymatic degradation.—*endo*-Polygalacturonase (EPG) treatment of the 2AB-labeled decagalacturonide generated 2AB-labeled di-, tri-, and tetragalacturonides (Fig. 1(C)), whereas *exo*-polygalacturonase treatment of the same derivative resulted in the formation of

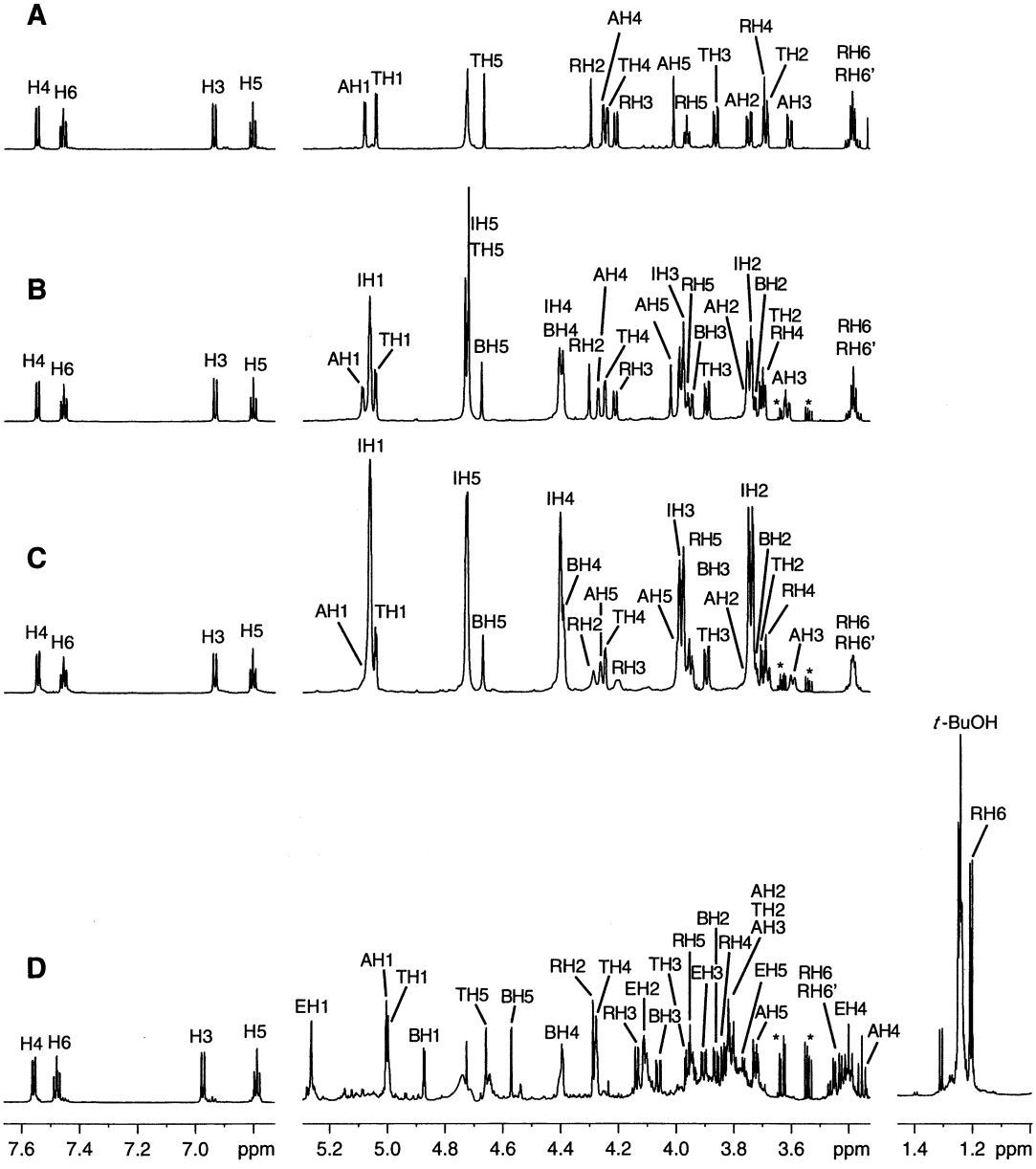

Fig. 3. ^{1}H NMR spectra of compounds **3**, **7**, **10**, and **12**. (A) Compound **3**; (B) compound **7**; (C) compound **10**; (D) compound **12**. The (*) indicates containment in the buffer solution.

Table 2
^{1}H and ^{13}C Chemical shifts (δ, ppm) and first-order coupling constants (Hz) for compounds **11**–**13**

Compound	Residue	^{1}H chemical shifts (ppm) [a]							First-order coupling constants (Hz) [b]							^{13}C chemical shifts (ppm) [c]					
		H-1	H-2	H-3	H-4	H-5	H-6_a	H-6_b	$^{3}J_{1,2}$	$^{3}J_{2,3}$	$^{3}J_{3,4}$	$^{3}J_{4,5}$	$^{3}J_{5,6a}$	$^{3}J_{5,6b}$	$^{2}J_{6a,6b}$	C-1	C-2	C-3	C-4	C-5	C-6
11	R		4.288	4.136	3.848	3.956	3.408	3.454		1.3	7.9	1.3	5.9	7.3	13.5	179.76	73.16	80.19	71.58	69.43	47.50
	A	5.005	3.845	3.738	3.363	3.823	1.204		1.3	5.8	10.0	9.8	6.3			99.06	78.38	70.98	73.60	69.67	18.23
	T	4.910	3.785	3.927	4.266	4.592			3.9	10.5	3.5	1.3				99.50	69.67	71.04	72.49	73.86	177.45
12	R		4.288	4.136	3.837	3.946	3.459	3.422		1.0	9.0	1.4	6.0	7.5	13.5	179.79	72.52	80.09	71.60	69.32	47.43
	A	5.004	3.819	3.821	3.353	3.723	1.203		1.3	4.2	10.0	9.8	6.5			99.16 [d]	78.46	71.09	73.62	71.09	18.24
	B	4.873	3.862	4.062	4.396	4.571			3.8	10.4	3.0	1.4				99.60	69.59	71.97	78.82	73.16	176.44
	E	5.263	4.112	3.905	3.399	3.780	1.244		1.3	3.6	10.1	9.8	6.2			100.07	77.82	71.13	73.68	70.97	18.27
	T	4.999	3.810	3.959	4.278	4.659			4.4	11.5	3.5	1.2				99.06 [d]	69.68	71.09	73.16	73.70	177.58
13	R		4.290	4.136	3.829	3.940	3.456	3.418		1.1	8.8	2.6	6.0	7.5	14.0	179.80	73.18	80.07	69.66	69.35	47.43
	A	5.009	3.797	3.815	3.357	3.721	1.202		1.0	6.0	10.0	10.0	6.0			99.01	78.41	71.58	73.74	70.97	18.26
	B	4.883	3.858	4.064	4.403	4.571			3.9	11.0	4.1	1.1				99.57	71.07	71.94	78.87	73.05	176.43
	I_1	5.264	4.109	3.879	3.402 [d]	3.792 [d]	1.236 [d]		1.0	4.0	10.0	10.0	6.0			100.07	77.77	71.07	73.74	70.65	18.26
	I_2	4.995	3.898	4.098	4.397	4.650			3.7	10.0	3.5	<1.0				99.13	69.56	71.94	78.87	72.99	176.57
	E	5.255	4.103	3.907	3.396 [d]	3.785 [d]	1.241 [d]		1.0	4.0	11.0	10.0	6.2			100.07	77.77	71.07	73.64	70.65	18.26
	T	4.995	3.763	3.957	4.274	4.642			3.7	11.0	3.5	<1.0				99.13	69.56	71.07	72.50	73.74	177.59

[a] ^{1}H and ^{13}C chemical shifts are quoted from methyl proton of internal *tert*-butanol (1.230 ppm) at 800 MHz and methyl carbon of *tert*-butanol (31.30 ppm) at 150 MHz, respectively, at 30 °C, in solutions buffered at pD 7.0–7.5.

[b] ^{1}H chemical shift and coupling constant assignments are based on 1D ^{1}H, DQF COSY and TOCSY spectra.

[c] ^{13}C chemical shift assignments are based on 1D ^{13}C and HSQC and HMBC spectra.

[d] Interchangeable and uncertain.

Table 3
^{1}H and ^{13}C chemical shifts (δ, ppm) and first-order coupling constants (Hz) for 2-aminobenzamide group

			H-3	H-4	H-5	H-6	
2-Aminobenzamide			6.927 $^{3}J_{3,4}$ 8.4	7.451 $^{3}J_{4,5}$ 7.1	6.794 $^{3}J_{5,6}$ 7.8	7.541	
	C-1	C-2	C-3	C-4	C-5	C-6	C=O
2-Aminobenzamide	117.06	148.44	113.73	134.1	117.3	129.67	176.01

2AB-labeled galactonic acid and digalacturonide (Fig. 1(D)). Driselase treatment of the 2AB-labeled RG_7 generated a series of 2AB-labeled RG-I oligosaccharides (Fig. 4(A and B)).

Galacturonosyltransferase assay.—Oligogalacturonides with DP >10 were effective exogenous acceptors for GalA T.[22] When 2AB-labeled decagalacturonide, as an exogenous acceptor for GalA T, was reacted with UDP-GalA and microsomal fractions, 2AB-labeled oligogalacturonides with DP 11, 12 and 13 formed (Fig. 5(A)). 2-AB-labeled oligogalacturonides with DP 2–9 formed at the same time. This is due to polygalacturonase present in the microsomal fraction. Polygalacturonase activity in the microsomes was previously reported for GalA T preparation from mung bean[23] and azuki bean.[24] When the reaction mixture was digested with EPG, di- and trigalacturonides were detected (Fig. 5B)). These results show that 2AB-labeled decagalacturonide was an exogenous acceptor for GalA T. Fluorescent detection of 2AB-labeled oligogalacturonides allows us to detect GaA T activity at picomol concentration.

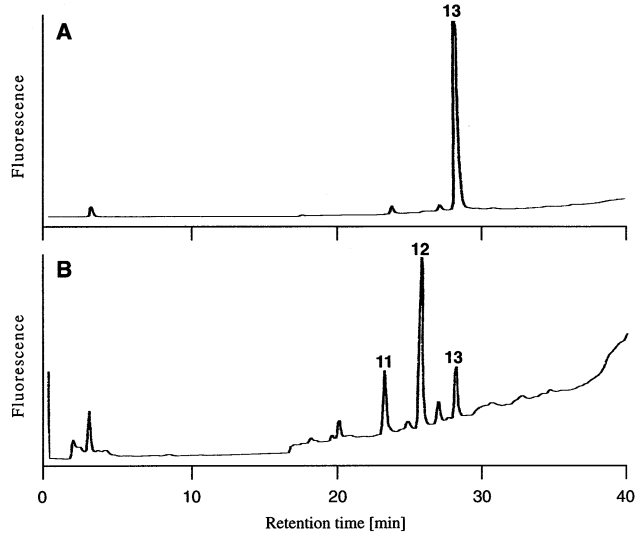

Fig. 4. HPAE chromatograms of the 2AB-labeled heptasaccharide RG_7 **13** monitored by the fluorescent detector. (B) The Driselase digest of 2AB-labeled RG_7 **13** monitored by the fluorescent detector. **11**, RG_3; **12**, RG_5.

We have synthesized in high yield 2AB-labeled oligogalacturonides and RG-I oligomers and have completely assigned the signals in their ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR data of oligogalacturonides up to DP 13[21] and RG-I oligomers having galactosyl residues[25] were reported. Present NMR data on 2AB labeled pectin oligosacchaides and the native oligomers will be useful for structural elucidation of pectic polysaccharides. Sensitive analysis with 2AB-labeling pectin helps to determine the activity of pectin synthesizing and degrading enzymes

3. Experimental

Materials.—2AB and $NaBH_3CN$ were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Digalacturonic and trigalacturonic acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Katayama Chemical Co. (Osaka, Japan) unless otherwise stated.

Oligosaccharide preparation.—RG-I oligosaccharides were generated by Driselase (from Basidiomycetes) treatment of bamboo shoots and destarched potato cell walls.[26,27] Acetyl and methyl esters in RG-I oligomers were saponified with 1 N NH_4 for 4 h at 4 °C. Oligogalacturonides were prepared by partial enzymic hydrolysis of apple pectin with immobilized polygalacturonase, followed by anion-exchange chromatography on a Q-Sepharose column and size-exclusion chromatography on a Bio-Gel P-2 column.[28] The purified oligosaccharides and 2AB-labeled oligosaccharides were then analyzed by HPAEC-PAD (Dionex BioLC) to determine their purity and by ESIMS to determine their DP.

2AB labeling of oligosaccharides.—Oligosaccharide labeling with 2AB was performed according to Sato et al.[7] and Huang et al.[8]. 2AB (0.2 M) was prepared in 1 M $NaBH_3CN$ by heating at 65 °C. The pH of the solution was then adjusted to pH 5.5–6.0 with 10% AcOH. The mixture (150–200 μL) was then added to the oligosaccharide (1 mg) in a glass tube with a Teflon-lined cap and kept for 2 h at 65 °C. The cooled

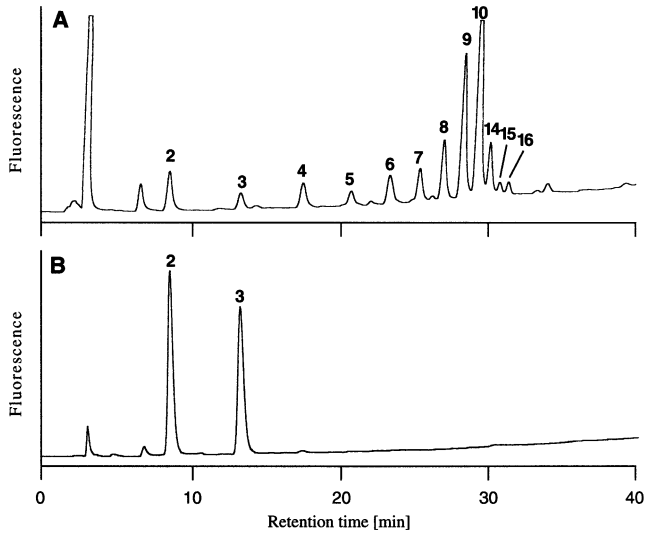

Fig. 5. HPAE chromatogram of the GalA transfer products (A) and its EPG digested product (B) monitored by the fluorescent detector. The numbers above peaks from **2** to **10** and one from **14** to **16** indicate DP of GalA to be 2–10 (see Fig. 2), and 11–13, respectively.

solution was mixed with 0.3 mL of CH_3COONH_4 buffer (10 mM, pH 7.0) and applied to a Toyopearl HW-40F column (1.5 × 8 cm). The column was eluted with the same buffer, and the $A_{254\ nm}$ of the eluant was monitored. The UV-positive fractions were pooled and freeze-dried.

ESIMS analysis of the 2AB-labeled oligosaccharides.—ESIMS analysis was performed with a ThermoQuest LCQ DUO mass spectrometer (Thermo Quest, Tokyo, Japan) operated in the negative-ion mode with a spray voltage of 4.55 kV, a capillary voltage of 3.1 V and a capillary temperature 180 °C. Solutions of oligosaccharides (1 mg) in 50% aq MeOH (100 μL) were adjusted to pH 10 by the addition of 1 M NH_4OH, diluted to ~50 ng/mL and then infused into the electrospray source at 5 μL/min with a syringe pump. Spectra were obtained between m/z 150 and 2000 with a step size of 0.1 amu and a dwell time of 25 ms.

NMR analysis of 2AB-labeled oligosaccharides.—The purified 2AB-labeled oligosaccharides were dissolved in 99.96% isotopically enriched D_2O and then freeze-dried. The residue was dissolved in 99.96% enriched D_2O and ND_4 (1.0 N), then added to adjust the pD to 7.0–7.5. 1D, 2D-DQFCOSY, 2D-TOCSY, HSQC, and HMBC spectra were recorded at 303 K and 800 MHz with a Bruker Avance 800 NMR spectrometer. The ^{13}C NMR spectra were recorded at 303 K with a Bruker Avance 600 NMR spectrometer. The TOCSY mixing time was 100 ms. HSQC, and HMBC were recorded using pulsed-field gradients for coherence selection. In a typical two-dimensional (^1H–^1H) spectrum, 4096 transients of 2048 data points were recorded with a spectral width of 3600 Hz in both dimensions, and the data were processed with zero filling to obtain a 4096 × 4096 matrix. ^1H and ^{13}C chemical shifts were measured relative to internal *tert*-butanol at δ 1.230 and 31.30, respectively.

High-performance anion-exchange liquid chromatography.—The 2AB-labeled oligosaccharides were analyzed by HPAEC using a CarboPac PA-1 column (4.5 × 250 mm) with a metal-free Dionex BioLC interfaced to an Auto Ion series 400 data station (Dionex, Sunnyvale, CA). The 2AB-labeled oligogalacturonides (0.1 mg/mL water) were eluted at 1 mL/min over 40 min with a linear gradient of 400–800 mM NaOAc in 100 mM NaOH.[26] 2AB-labeled RG-I derived oligosaccharides were eluted with 100 mM NaOH for 10 min, followed by a linear gradient from 0 to 1 M NaOAC in 100 mM NaOH (10–40 min).[27] The eluting compounds were detected with PAD or with a fluorescence spectrophotometer at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 420$ nm.

Enzymic degradation of the 2AB-labeled oligosaccharides.—2AB-labeled decagalacturonide dissolved in 50 mM acetate buffer (pH 5.0, 0.1 mg/mL) was digested with five units of EPG or one unit of *exo*-EPG for 3 h at 25 °C. 2AB-labeled RG_7 was digested with partially purified Driselase in the same way as EPG digest. Enzymic digests were analyzed with HPAEC as described above.

Assay of GalA T.—Microsomal fractions were prepared from etiolated pumpkin and adzuki according to a procedure described by Takeuchi and Tsumuraya.[24] Seedlings were grown in moist rock fiber for 7 days at 25 °C in the dark. Etiolated epicotyl segments (from the cotyledon, 5 cm in length, 15 g total fresh weight) were excised. UDP-GalA was synthesized by oxidation of UDP-Gal with galactose oxidase.[29] GalA T activity was measured as described by Takeuchi and Tsumuraya[24] with some modifications. The reaction mixture was incubated for 2 h at 30 °C in 50 mM Mes–KOH buffer (pH 6.8) containing 0.3 mM UDP-GalA, 0.5 mM 2AB-labeled decagalacturonide, 0.5% (w/v) Tritox X-100, 5 mM $MnCl_2$, 6% (w/v) sucrose, 0.5% (w/v) BSA, and the microsomal fraction (protein content, 30–35 μg) in a total volume of 30 μL. The reaction was stopped by addition of 0.3 M AcOH (80 μL), and portions (3 μL) were analyzed by HPAEC. Elongated oligogalacturonides with DP 11, 12, and 13 were identified by comparison of the retention time with those of authentic 2AB-labeled oligogalacturonides. Protein was determined by the Bradford assay using bovine serum albumin as a standard.[30]

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