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Assignment of the ^{1}H and ^{13}C NMR spectra of 2-aminobenzamide-labeled xylo-oligosaccharides

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ABSTRACT

Xylose, xylo-oligosaccharides with a degree of polymerization (DP) from 2 to 6, xylo-oligosaccharides carrying a single arabinofuranosyl, glucosyluronic acid, or 4-*O*-methyl glucosyluronic acid residues and 4-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p* were labeled at their reducing ends with 2-aminobenzamide (2AB) in the presence of sodium cyanoborohydride ($NaBH_3CN$). These derivatives were then analyzed by high-performance anion-exchange chromatography (HPAEC) and structurally characterized by electrospray-ionization mass spectrometry (ESI-MS) and by ^{1}H and ^{13}C NMR spectroscopy. Reacting Xyl_3-Xylitol-2AB with UDP-Xyl in the presence of rice microsomes resulted in the formation of small amounts of Xyl_{4-6}-Xylitol-2AB showing that the 2AB-labeled compound is an acceptor for xylosyltransferase. The 2AB-labeled xylo-oligosaccharides and the 2AB-labeled xylo-oligosaccharides carrying 4-*O*-methyl glucuronic acid are fragmented by xylanase and α-glucuronidase present in the culture filtrate of *Fomitopsis palustris* FFPRI 0507. Thus, the 2AB-labeled xylo-oligosaccharides are useful for studying enzymes involved in xylan degradation and biosynthesis.

1. Introduction

Glucuronoxylans (GXs) are quantitatively major components of the secondary cell walls of woody plant tissues. GX has a linear backbone composed of 1 → 4-linked β-D-xylosyl (Xyl) residues, some of which are substituted at *O*-2 with a single α-D-glucosyluronic acid (GlcA) or 4-*O*-methyl-α-D-glucosyluronic acid (MeGlcA) residue. The Xyl residues may also be substituted with α-L-arabinofuranosyl (Ara*f*) and *O*-acetyl residues (O'Neill and York, 2003; Shimizu, 1991). The presence and distribution of these substituents in xylans is dependent on the plant source and may affect their physico-chemical properties (Ebringerová, Hromádková, & Heinze, 2005; Izydorczyk & Biliaderis, 1995). Xylo-oligosaccharides have been reported to enhance the growth of bifidobacteria (Pepper & Olinger, 1988) and are defined as prebiotics (Fooks, Fuller, & Gibson, 1999; Modler, 1994). Recently, there has been increased interest in the structure and biosynthesis of xylans in hardwoods and grasses as these polysaccharides are major component of plant biomass, a renewable energy source that has potential for use in the large scale production of biofuels (Ragauskas et al., 2006; Somerville, 2007).

A number of glycosyltransferases (GTs) are required for biosynthesis of the xylan backbone, together with enzymes that add and modify side chains (Peña et al., 2007). Several genes have been identified that are believed to encode some of these enzymes. For example, analysis of *Arabidopsis* mutants has revealed that *FRAGILE FIBER8 (FRA8), IRREGULAR XYLEM8 (IRX8)*, and *IRX9*, *PARVUS*, *and IRX14* are required for the synthesis of normal amounts of xylan and cellulose in secondary cell walls and for the formation of normal vascular tissue morphology (Brown, Zeef, Ellis, Goodacre, & Turner, 2005; Brown et al., 2007; Persson, Wei, Milne, Page, & Somerville, 2005; Persson et al., 2007; Peña et al., 2007; Zhong et al., 2005). However, none of these genes have been functionally characterized nor have any of the enzymes involved in xylan biosynthesis been purified to homogeneity and biochemically characterized.

Fluorescent-labeled oligosaccharides are useful acceptor substrates for *in vitro* determination of GTs and glycosyl hydrolase activities. We previously reported the NMR assignment of 2-aminobenzaminated (2AB) pectic oligosaccharides (Ishii, Ichita, Matsue, Ono, & Maeda, 2002) and galacto- and arabino-oligosaccharides (Ishii, Ono, & Maeda, 2005). In this study, we will provide a complete assignment of signals in ^{1}H and ^{13}C NMR spectra of 2AB-labeled xylo-oligosaccharides and show usefulness of the oligosaccharides for detection of xylosyltransferase (XylT) activity, xylanase and α-glucuronidase activity.

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1 Xylitol-2AB
R

2 β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T R

3 β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T A R

4 β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T E A R

5 β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T E B A R

6 β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T E I B A R

7 α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T E R

8 4-*O*-Me-α-D-Glc*p*A-(1→2)- D-Xylitol-2AB
T R

9 α-D-Glc*p*A-(1→2)- D-Xylitol-2AB
T R

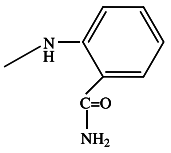
10 4-*O*-Me-α-D-Glc*p*A-(1→2)-β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T A R

11 α-D-Glc*p*A-(1→2)-β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T A R

12 4-*O*-Me-α-D-Glc*p*A-(1→2)-β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T B A R

13 α-D-Glc*p*A-(1→2)-β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→4)-D-Xylitol-2AB
T B A R

14 β-D-Xyl*p*-(1→4)- β-D-Xyl*p*-(1→3)- α-L-Rha*p*-(1→2)- α-D-Gal*p*A-(1→4)-D-Xylitol-2AB
T E B A R



2-aminobenzamide (2AB)

Fig. 1. Structures of compounds **1**–**14**. **1**–**3**, 2-AB-labeled D-xylitol to xylotriose (DP 1–3); **4**, xylotetraose consisting of a D-xylitol R, two internal residues (A and E), and a non-reducing terminal residue T; **5**, xylopentaose consisting of a D-xylitol R, three internal residues (A, B, and E), and a non-reducing terminal residue T; **6**, xylohexaose consisting of a D-xylitol R, four internal residues (A, B, I, and E), and a non-reducing terminal residue T; **7**, trisaccharide consisting of a D-xylitol R, one internal xylosyl residue A and a non-reducing terminal Ara*f* residue T; **8**, disaccharide consisting of a D-xylitol R, and a terminal 4-*O*-Me-Glc*p*A T; **9**, disaccharide consisting of a D-xylitol R, and a terminal Glc*p*A T; **10**, trisaccharide consisting of a D-xylitol R, an internal Xyl residue A, and a terminal 4-*O*-Me-Glc*p*A T; **11**, trisaccharide consisting of a D-xylitol R, an internal Xyl residue A, and a terminal Glc*p*A T; **12**, tetrasaccharide consisting of a D-xylitol R, internal Xyl residues (A and B), and a terminal 4-*O*-Me-Glc*p*A T; **13**, tetrasaccharide consisting of a D-xylitol R, internal Xyl residues (A and B), and a terminal Glc*p*A T; **14**, pentasaccharide consisting of a D-xylitol R, internal Gal*p*A, Rha, and Xyl residues (A, B, and E, respectively) and a non-reducing terminal xylosyl residue T.

2. Experimental

2.1. Material

2AB and $NaBH_3CN$ were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). 1,4-Linked β-D-xylo-oligosaccharides with a degree of polymerization (DP) of 2–6 and xylanase were obtained from Megazyme (Wicklow, Ireland). UDP-Xyl was obtained from Carbosource Services (Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA). 1,4-Linked β-D-xylo-oligosaccharides (DP 1–3) with a single GlcA or MeGlcA residues were generated from kenaf (*Hibiscus cannabinus*) xylan (Komiyama, Kato, Aimi, Ogihara, & Shimizu, 2008). Briefly, the xylan was extracted with aq. 10% KOH from bast and cores of kenaf and then hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. The hydrolyzates were kept

Table 1
Electrospray-ionization mass spectrometry data for compounds **1**–**14**

Nominal mass	Molecular ion	Compound[a]	Molecular weight
293	$(M+Na)^+$	**1**	270
425	$(M+Na)^+$	**2**	402
557	$(M+Na)^+$	**3**	534
689	$(M+Na)^+$	**4**	666
821	$(M+Na)^+$	**5**	798
953	$(M+Na)^+$	**6**	930
557	$(M+Na)^+$	**7**	534
483	$(M+Na)^+$	**8**	460
469	$(M+Na)^+$	**9**	446
615	$(M+Na)^+$	**10**	592
601	$(M+Na)^+$	**11**	578
747	$(M+Na)^+$	**12**	724
733	$(M+Na)^+$	**13**	710
879	$(M+Na)^+$	**14**	856

[a] Structures shown in Fig. 1.

at pH 8 for 4 h at room temperature to hydrolyze the lactone, and then applied to a column of Dowex 1×8 (acetate form 5×650 mm). The neutral sugars were eluted with water until the anthrone reaction was negative. Acidic sugars were then eluted with 5 M acetic acid. The acidic sugars were fractionated by chromatography on a column of the strong anion exchange re-

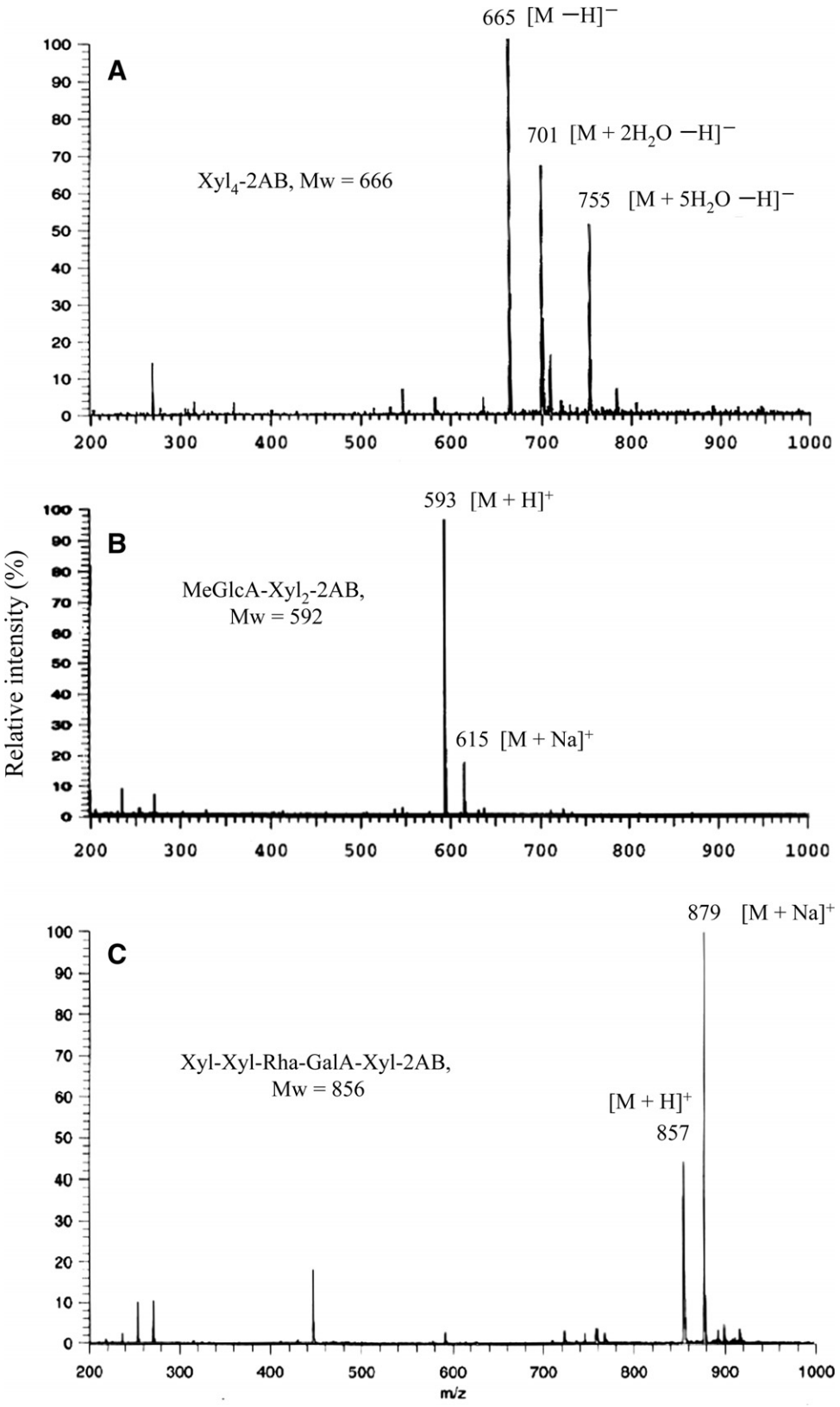


Fig. 2. ESI-MS spectra of compounds **4**, **12**, and **14**. (A) Negative-ion mode mass spectrum of compound **4**; (B) positive-ion mode mass spectrum of compound **12**; (C) positive-ion mode mass spectrum of compound **14**.

sin Diaion (acetate form, 15 × 930 mm, Mitsubishi Chemical) by elution with A, 80 mM sodium acetate (pH 5.9) giving 9 fractions. Fractions 1–9 were rechromatographed on a column of Aminex A-27 (10 × 830 mm) by step-wise elution with B, 250 mM acetic acid, C, 500 mM acetic acid, and D, 1.0 M acetic acid. Each acid was identified by the volume distribution coefficients (D_v) calculated in the usual way (Samuelson, 1963), acid hydrolysis, and subsequent identification of the glycoses products by GLC-MS of the derivatives (Komiyama et al., 2008). β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p* was purified from birch (*Betula verrucosa*) (Shimizu, Ishihara, & Ishihara, 1976). α-L-Ara*f*-(1 → 2)-β-D-Xyl*p*-(1 → 4)-D-xylose was prepared by saponification with sodium hydroxide of *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-xylose (Ishii & Hiroi, 1990).

2.2. 2AB labeling of oligosaccharides

The oligosaccharides were labeled with 2AB and purified as described previously (Ishii et al., 2002).

2.3. Analytical methods

High-performance anion-exchange chromatography (HPAEC) was performed using a CarboPac PA-1 column (4 × 250 mm) and a metal-free Dionex Bio LC interfaced to an Auto Ion series 400 data station (Dionex, Sunnyvale, CA, USA) with a fluorescence detector (Shimadzu RF-10A$_{XL}$, Kyoto, Japan) at λ_{ex} = 330 nm and λ_{em} = 420 nm (Ishii, Ohnishi-Kameyama, & Ono, 2004). HPAEC conditions were as follows; a linear gradient of sodium acetate (0 mM) for 2 min, to 250 mM for 60 min in 100 mM NaOH at flow rate of 1.0 mL min^{-1}. Normal phase liquid chromatography (LC) was performed using a Shimadzu LC system (Shimazdu LC-10AD, Kyoto, Japan), a fluorescence detector and an Amide-80 column (4.6 × 250 mm, TOSOH, Tokyo, Japan) eluted at 1.0 mL min^{-1} at 30 °C. The eluent and sample were eluted as follows: eluent A, 50 mM ammonium formate (pH 4.4) and eluent B, CH_3CN, and at initial conditions of 75% B and then a linear gradient of eluent B from 75% (V/V) to 50% (V/V) in 50 min. Electrospray-ionization mass spectroscopy (ESI-MS) analysis was performed with a Thermo-Quest LCQ DUO mass spectrometer (Thermoelectron Waltham, MA, USA) operated in the positive- or negative-ion modes with a spray voltage of 4.55 kV, a capillary voltage of 3.1 V and a capillary temperature of 180 °C. Mass spectra were obtained between *m/z* 150 and 2000 (Ishii et al., 2002). 1D, 2D-double quantum filtered correlation spectroscopy (DQFCOSY), 2D-total correlation spectroscopy (TOCSY) with 100 ms mixing time, 2D {^{1}H–^{13}C} ^{1}H-detected heteronuclear single quantum coherence (HSQC), and ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectroscopy (HMBC) were performed at 303 K and 800 MHz with a Brucker Avance 800 NMR spectrometer (Brucker Biospin, Karlsruhe, Germany) as previously described (Ishii et al., 2002). The 2AB-labeled oligosaccharides (2–3 mg) were dissolved in 99.96% isotopically enriched D_2O and then freeze-dried. The derivatives were then dissolved in 99.96% enriched D_2O (0.4 mL) prior to

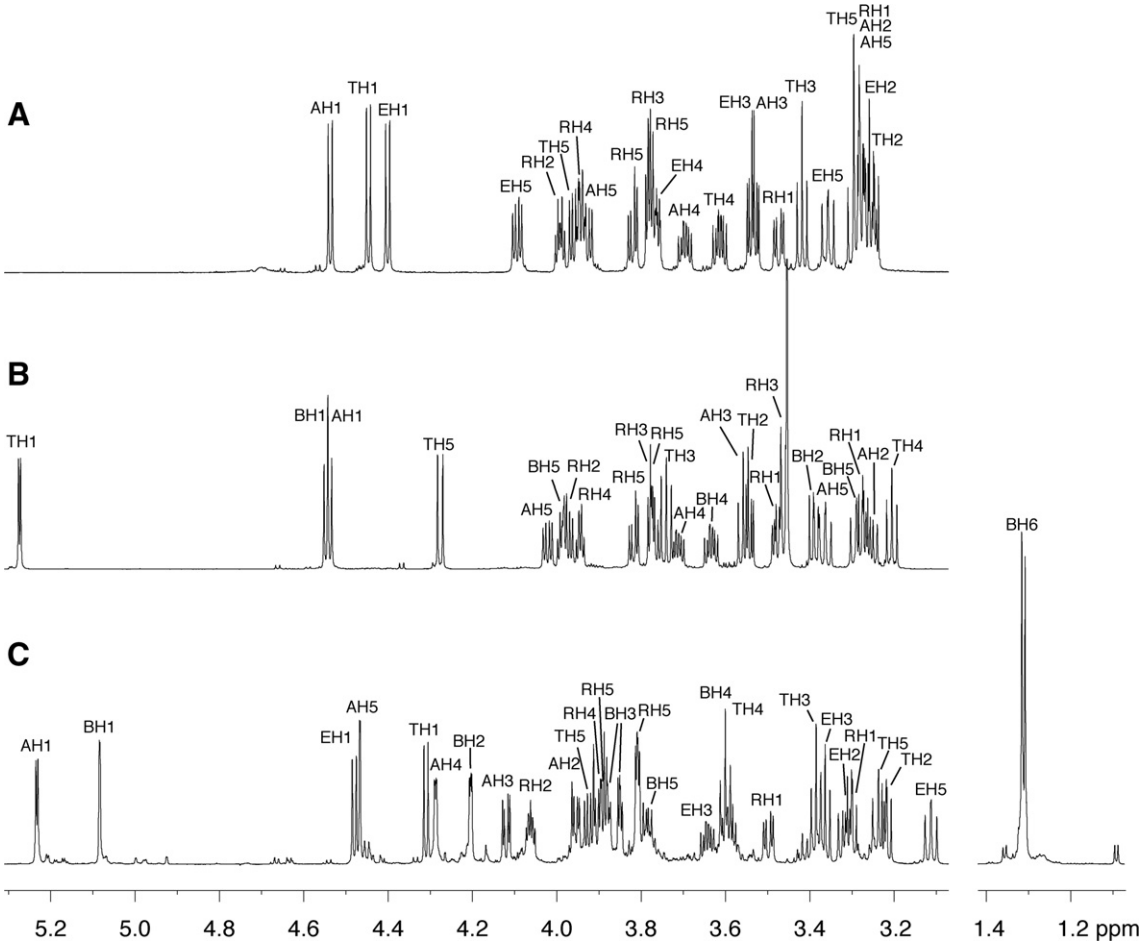


Fig. 3. ^{1}H NMR spectra of compounds **4**, **12**, and **14**. (A) compound **4**; (B) compound **12**; (C) compound **14**.

NMR spectroscopic analysis. In a typical two-dimensional (^{1}H–^{1}H) 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. ^{1}H and ^{13}C chemical shifts were measured relative to internal 2-methyl 2-propanol at δ 1.230 and 31.30, respectively.

2.4. Enzymic degradation of 2AB-labeled oligosaccharides

2AB-labeled xylo-tetrasaccharide (compound **4**, Fig. 1) and 2AB-labeled 4-*O*-Me glucurono-xylo-trisaccharide (compound **12**, Fig. 1) were dissolved in 100 mM acetate buffer (pH 4.0, 20 μL) and treated for 30 min at 35 °C with the culture filtrate (10 μL, about 1 μg protein) obtained from *Fomitopsis palustris* FFPRI 0507. *F. palustris* was grown for 10 days in the dark (Ishihara & Shimizu, 1984). The medium contained ~124 μg mL^{-1} protein and the proteins had xylanases and glucuronidases activities (Ishihara & Shimizu, 1984). Enzymic digests were analyzed by normal phase LC as described above.

2.5. Xylosyltransferase assay

Microsomal membrane fractions were prepared from etiolated rice seedlings according to the procedure described by Konishi

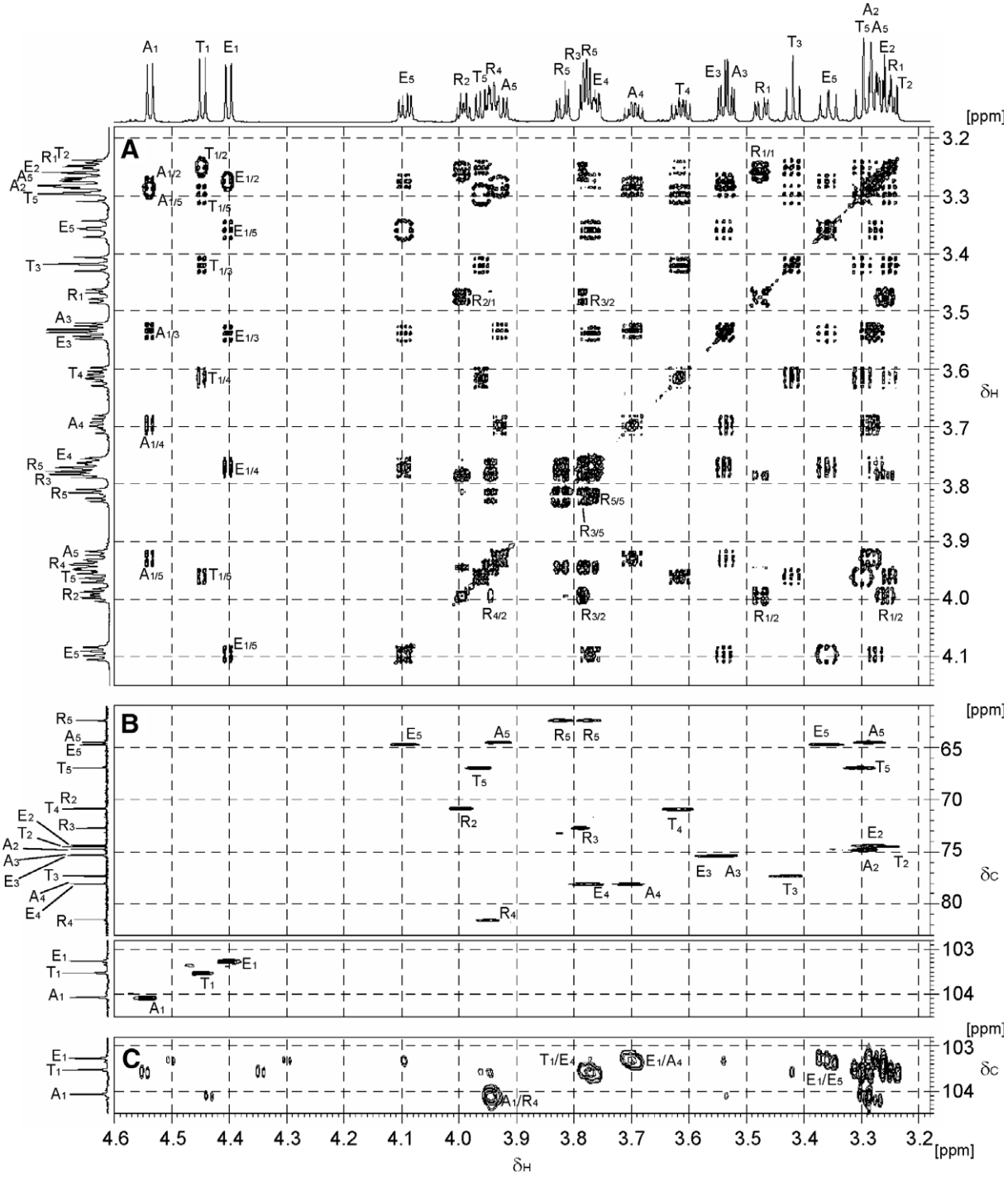


Fig. 4. TOCSY (A), HSQC (B), and HMBC (C) spectra of compound **4**.

Table 2
^{1}H and ^{13}C chemical shifts and first-order coupling constants (Hz) for compounds **1–7**

Compound	Residue	^{1}H chemical shifts[a] (ppm)							First-order coupling constants[b] (Hz)								^{13}C chemical shifts[c] (ppm)				
		H-1_a	H-1_b	H-2	H-3	H-4	H-5_a	H-5_b	$^{3}J_{1a,2}$	$^{3}J_{1b,2}$	$^{2}J_{1a,1b}$	$^{3}J_{2,3}$	$^{3}J_{3,4}$	$^{3}J_{4,5a}$	$^{3}J_{4,5b}$	$^{2}J_{5a,5b}$	C-1	C-2	C-3	C-4	C-5
1	R	3.432	3.271	3.962	3.642	3.821	3.696	3.619	4.3	5.0	13.3	8.5	9.0	6.8	12.0	10.0	47.26	71.45	73.77	74.55	64.32
2	R	3.476	3.266	3.983	3.772	3.939	3.816	3.767	4.6	5.5	13.0	8.0	8.5	5.3	12.0	10.0	46.97	70.87	72.75	81.53	62.35
	T	4.520	–	3.253	3.415	3.570	3.853	3.239	7.6	–	–	9.2	9.0	5.5	10.0	10.5	104.22	74.82	77.29	70.95	66.82
3	R	3.473	3.250	3.993	3.784	3.943	3.820	3.768	4.7	5.5	13.5	8.0	8.9	5.3	12.2	10.2	46.43	70.87	72.68	81.48	62.36
	A	4.538	–	3.291	3.532	3.694	3.929	3.278	7.7	–	–	9.2	9.0	5.3	10.1	10.1	104.04	74.75	75.30	78.06	64.48
	T	4.380	–	3.237	3.411	3.610	3.961	3.290	7.8	–	–	9.2	9.1	5.5	10.2	10.6	103.44	74.40	77.28	70.85	66.90
4	R	3.474	3.251	3.992	3.783	3.943	3.820	3.772	4.7	5.2	13.5	8.0	8.5	4.5	12.2	10.0	46.92	70.70	72.69	81.50	62.37
	A	4.537	–	3.279	3.529	3.696	3.927	3.283	7.7	–	–	9.0	9.4	5.3	12.0	10.5	104.07	74.76	75.34	78.03	64.47
	E	4.401	–	3.272	3.540	3.761	4.094	3.357	7.7	–	–	9.1	9.3	5.3	11.8	10.4	103.27	74.32	75.30	78.06	64.68
	T	4.447	–	3.259	3.418	3.613	3.959	3.296	7.8	–	–	9.3	9.2	5.5	11.6	10.8	103.53	74.45	77.29	70.86	66.89
5	R	3.475	3.251	3.992	3.775	3.947	3.820	3.768	4.7	5.2	13.5	8.0	8.5	4.7	12.2	10.0	46.92	70.79	72.69	81.50	62.37
	A	4.538	–	3.283	3.532	3.699	3.927	3.295	7.7	–	–	9.0	9.4	5.3	12.0	10.5	104.07	74.76	75.33	78.06	64.47
	B	4.469	–	3.283	3.545	3.781	4.094	3.366	7.7	–	–	9.1	9.3	5.3	12.0	10.4	103.33	74.37[d]	75.35	78.02	64.66
	E	4.402	–	3.273	3.538	3.781	4.094	3.353	7.7	–	–	9.1	9.3	5.3	12.0	10.4	103.27	74.32[d]	75.35	78.02	64.66
	T	4.446	–	3.253	3.416	3.612	3.958	3.295	7.8	–	–	9.3	9.2	5.5	11.5	10.8	103.52	74.45	77.29	70.86	66.89
6	R	3.475	3.251	3.991	3.775	3.947	3.820	3.767	4.7	5.2	13.5	8.0	8.5	4.5	12.0	10.0	46.92	70.79	72.69	81.50	62.37
	A	4.538	–	3.283[d]	3.530	3.699	3.927	3.295	7.7	–	–	9.0	9.4	5.0	12.0	10.0	104.07	74.76	75.30	78.06	64.47
	B	4.469[d]	–	3.281[d]	3.538[d]	3.777[d]	4.094	3.366	7.7	–	–	9.0	9.3	5.0	12.0	10.0	103.34	74.37[d]	75.34	78.06	64.66
	I	4.468[d]	–	3.281[d]	3.538[d]	3.775[d]	4.094	3.366	7.7	–	–	9.0	9.3	5.0	12.0	10.0	103.34	74.37[d]	75.34	78.02	64.66
	E	4.403	–	3.273[d]	3.546[d]	3.770[d]	4.094	3.353	7.7	–	–	9.0	9.3	5.0	12.0	10.4	103.27	74.33[d]	75.34	78.02	64.66
	T	4.446	–	3.253	3.416	3.613	3.958	3.295	7.8	–	–	9.3	9.2	5.0	12.0	10.8	103.52	74.45	77.29	70.86	66.89
7	R	3.481	3.254	3.985	3.770	3.950	3.820	3.773	4.6	5.5	12.0	8.5	9.0	6.0	4.0	11.0	46.96	70.85	72.76	81.54	62.23
	A	4.559	–	3.408	3.575	3.635	3.882	3.269	7.8	–	–	9.1	8.0	5.5	12.0	10.0	104.09	74.85	83.28	69.58	66.60
	T	5.314	–	4.165	3.944	4.161	3.803	3.691	1.3	–	–	3.4	5.1	5.4	2.6	11.0	109.81	82.89	78.17	85.64	62.91

[a] ^{1}H and ^{13}C chemical shifts are quoted from methyl proton of internal 2-methyl-2-propanol (1.230 ppm) at 800 MHz and methyl carbon of 2-methyl-2-propanol (31.30 ppm) at 150 MHz, respectively, at 30 °C.
[b] ^{1}H chemical shift and coupling constant assignments are based on 1D ^{1}H, DQFCOSY, and TOCSY spectra.
[c] ^{13}C chemical shifts assignments are based on 1D ^{13}C, HSQC, and HMBC spectra.
[d] Interchangable, uncertain.

et al. (2007). The reaction was performed for 2 h at 20 °C in 50 mM MES–KOH buffer (pH 6.8) containing 0.3 mM UDP-Xyl, 0.5 mM 2AB-labeled xylo-tetrasaccharide (compound **4**, Fig. 1), 0.5% (W/V) Triton X-100, 5 mM $MnCl_2$, 6% (W/V) sucrose, 0.5% (W/V) BSA, and the microsomal membrane fractions (protein content, 30 μg) in a total volume of 30 μL. The reaction was stopped by addition of AcOH (1.5 M, 30 μL), and the mixture boiled for 1 min. The reaction mixture was centrifuged, and the supernatant analyzed by HPAEC (Ishii et al., 2004). The reaction products were digested with xylanase and the products were analyzed by HPAEC (Ishii et al., 2004).

3. Results and discussion

3.1. 2-Aminobenzamide labeling of oligosaccharides

Xylose and xylo-oligosaccharides with DP of 2–6 (compounds **1**–**6**, see Fig. 1), arabinoxylan trisaccharide (compound **7**, see Fig. 1), xylo-oligosaccharides with DP 1–3 carrying a single GlcA or MeGlcA residues (compounds **8**–**13**, Fig. 1), and β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p* (compound **14**, Fig. 1) were labeled with 2AB (Ishii et al., 2002). Each 2AB-oligosaccharide eluted as a single peak when analyzed by HPAEC (data not shown). The positive- or negative-ion modes ESI-MS spectra of **1**–**14** confirmed the molecular weight of each derivative (Table 1). The mass spectra of compounds **4**, **12**, and **14** are shown in Fig. 2.

3.2. Assignment of ^1H and ^{13}C NMR spectra of the 2AB-labeled oligosaccharides

The ^1H NMR spectra of 2AB-labeled oligosaccharides were recorded at 800 MHz. The ^1H and ^{13}C NMR spectra of the 2AB-xylo-oligosaccharides (compounds **1**–**6**, see Fig. 1), 2AB-arabinoxylan trisaccharide (compound **7**), 2AB-xylo-oligosaccharides with GlcA or MeGlcA (compounds **8**–**13**), and 2AB-$(Xyl)_2$-Rha-Gal*p*A-Xyl (compound **14**) were assigned using DQF COSY, TOCSY, HSQC, and HMBC experiments. All of the signals in the NMR spectra were assigned to the oligosaccharides or 2AB, thereby confirming that the derivatives were homogeneous. Herein we describe the complete assignment of the ^1H NMR spectrum (see Fig. 3A) of 2AB-labeled xylo-tetrasaccharide (compound **4**). The D-xylitol residue (the former reducing end of the oligosaccharide) is clearly no longer in the pyranose ring form because C-1 is substituted with two protons rather than one. These two protons gave quartets at δ 3.474 and 3.251 (RH1 in Fig. 3A), with coupling constants of 4.7 and 5.2 Hz. The doublet at δ 4.447 (J 7.8 Hz) is assigned to the resonance of the H-1 of the terminal non-reducing Xyl residue T, whereas the H-1 resonance of the residue next to the former reducing end (residue A) is at δ 4.537. The remaining doublet at δ 4.401 is the resonances of the H-1 of the internal sugar residue (residue E). The chemical shift values of H-1s of the non-reducing Xyl residues and the magnitude of the coupling constants (7.7–7.8 Hz) are consistent with a β-linkage (Utille, Kováč, Sauriol, & Perlin, 1986). The anomeric resonances are all well resolved from the non-anomeric sugar proton signals. The DQFCOSY and TOCSY spectra allowed the assignment of the proton signals from H-1 to H-5 (Fig. 4A). The proton signals of H-5 were assigned by HSQC (Fig. 4B). By comparing the spectra for compound **4** with those of compound 6, the signals in the spectra of all the xylo-oligosaccharide derivatives were assigned (Table 2). The ^{13}C NMR spectra of the 2AB-labeled oligosaccharides were analyzed by HSQC and HMBC spectroscopy. The HMBC spectra gave extensive intramolecular correlations between the proton and carbon

Table 3
^1H and ^{13}C chemical shifts and first-order coupling constants (Hz) for compounds **8**–**13**

Compound	Residue	^1H chemical shifts[a] (ppm)							First-order coupling constants[b] (Hz)							^{13}C chemical shifts[c] (ppm)						
		H-1_a	H-1_b	H-2	H-3	H-4	H-5_a	H-5_b	CH_3	$^3J_{1a,\ 2}$	$^3J_{1b,\ 2}$	$2J_{1a,\ 1b}$	$^3J_{2,3}$	$^3J_{3,4}$	$^3J_{4,\ 5a}$	$^3J_{4,5b}$	$^3J_{5a,5b}$	C-1	C-2	C-3	C-4	C-5	C-6	CH_3
8	R	3.4821	3.552	3.980	3.789	3.824	3.682	3.564	–	4.5	5.2	11.0	8.0	9.0	6.0	12.0	11.0	44.11	77.57	72.69	73.30	64.23	–	–
	T	5.091	–	3.573	3.747	3.226	3.977	–	3.458	4.0	–	–	9.5	9.7	10.0	–	–	99.05	73.09	74.02	83.98	74.46	177.96	61.62
9	R	3.561	3.490	4.012	3.802	3.837	3.680	3.566	–	4.5	5.2	11.0	8.0	9.0	6.0	12.0	11.0	44.17	77.77	72.68	73.23	64.24	–	–
	T	5.111	–	3.556	3.735	3.472	4.000	–	–	4.0	–	–	9.5	9.5	10.0	–	–	99.16	72.30	74.41	73.63	74.52	178.11	–
10	R	3.468	3.265	3.983	3.760	3.918	3.826	3.779	–	4.5	5.5	12.0	8.0	9.0	6.0	12.0	11.0	46.99	70.61	72.65	81.22	62.17	–	–
	A	4.651	–	3.368	3.450	3.573	3.845	3.235	–	7.7	–	–	9.0	9.0	6.0	10.0	10.0	104.15	78.77	76.10	71.19	66.57	–	–
	T	5.360	–	3.697	3.740	3.208	4.269	–	3.455	4.0	–	–	9.0	9.0	10.1	–	–	99.28	72.97	73.93	84.13	73.88	178.40	61.63
11	R	3.469	3.268	3.987	3.762	3.939	3.833	3.787	–	4.5	5.5	11.0	9.0	9.0	6.0	12.0	11.0	47.01	70.65	72.65	81.26	62.18	–	–
	A	4.667	–	3.426	3.467	3.594	3.853	3.243	–	7.6	–	–	9.0	9.0	6.0	10.0	10.0	104.17	78.86	73.66	71.20	66.59	–	–
	T	5.382	–	3.578	3.724	3.466	4.305	–	–	3.8	–	–	9.0	9.0	10.2	–	–	99.37	72.84	74.32	76.19	73.80	178.69	–
12	R	3.480	3.274	3.977	3.775	3.947	3.818	3.773	–	4.5	5.2	11.0	8.0	9.0	6.0	12.0	12.0	46.93	70.85	72.75	81.37	62.32	–	–
	A	4.539	–	3.253	3.558	3.717	4.022	3.364	–	7.4	–	–	9.0	9.0	6.0	11.0	11.0	104.01	74.71	75.36	77.75	64.40	–	–
	B	4.539	–	3.385	3.469	3.630	3.983	3.286	–	7.2	–	–	9.0	9.0	6.0	11.0	11.0	103.20	78.10	76.01	71.05	66.62	–	–
	T	5.274	–	3.546	3.740	3.205	4.277	–	3.454	3.9	–	–	9.0	9.0	10.1	–	–	99.14	72.94	74.06	84.17	72.94	178.45	61.61
13	R	3.482	3.253	3.989	3.776	3.945	3.819	3.769	–	4.5	5.2	11.0	8.0	9.0	6.0	12.0	12.0	46.93	70.86	72.76	81.37	62.33	–	–
	A	4.543	–	3.280	3.565	3.727	4.034	3.374	–	7.7	–	–	9.0	9.0	6.0	11.0	11.9	104.01	74.72	75.37	77.77	64.42	–	–
	B	4.560	–	3.440	3.475	3.643	3.983	3.294	–	7.6	–	–	9.0	9.0	6.0	11.0	11.0	103.24	78.18	76.01	71.07	66.65	–	–
	T	5.293	–	3.519	3.723	3.473	4.310	–	–	3.9	–	–	9.0	6.0	10.2	–	–	99.23	72.81	74.33	73.76	73.70	178.71	–

a, b, and c See Table 1.

atoms of each residue (Fig. 4C), confirming the connectivity of each glycosyl residue in the oligomers. All the ^{13}C assignments are shown in Table 2.

The signals in the spectra of xylo-oligosaccharides carrying a single GlcA or MeGlcA residues are summarized in Table 3. The ^{1}H, TOCSY, HSQC, and HMBC spectra of compound **12** are shown in Figs. 3 and 5A, B, and C, respectively.

Herein we describe the complete assignment of the ^{1}H NMR spectrum (Fig. 3C) of 2AB-labeled pentasaccharide (compound **14**). The D-xylitol residue (the former reducing end of the oligosaccharide) gave two protons having quartets at δ 3.503 and 3.111 (RH1 in Fig. 3C), with coupling constants of 4.5 and 5.5 Hz. The doublet at δ 4.307 (J 7.9 Hz) is assigned to the resonance of the H-1 of the terminal non-reducing Xyl residue T, whereas the H-1 resonance of the residue next to the former reducing end (residue A, Gal*p*A) is at δ 5.232 (J 3.8 Hz). The doublet at δ 5.083 (J 1.3 Hz) is the resonances of the H-1 of the internal rhamnosyl residue (residue B). The remaining doublet at δ 4.481 (J 7.9 Hz) is the resonance of the H-1 of the internal xylosyl residue (residue E). The chemical shift values of H-1s of the non-reducing Xyl residues (residue T and E) and the magnitude of the coupling constants (7.9 Hz) are consistent with a β-linkage (Utille et al., 1986). The non-anomeric proton

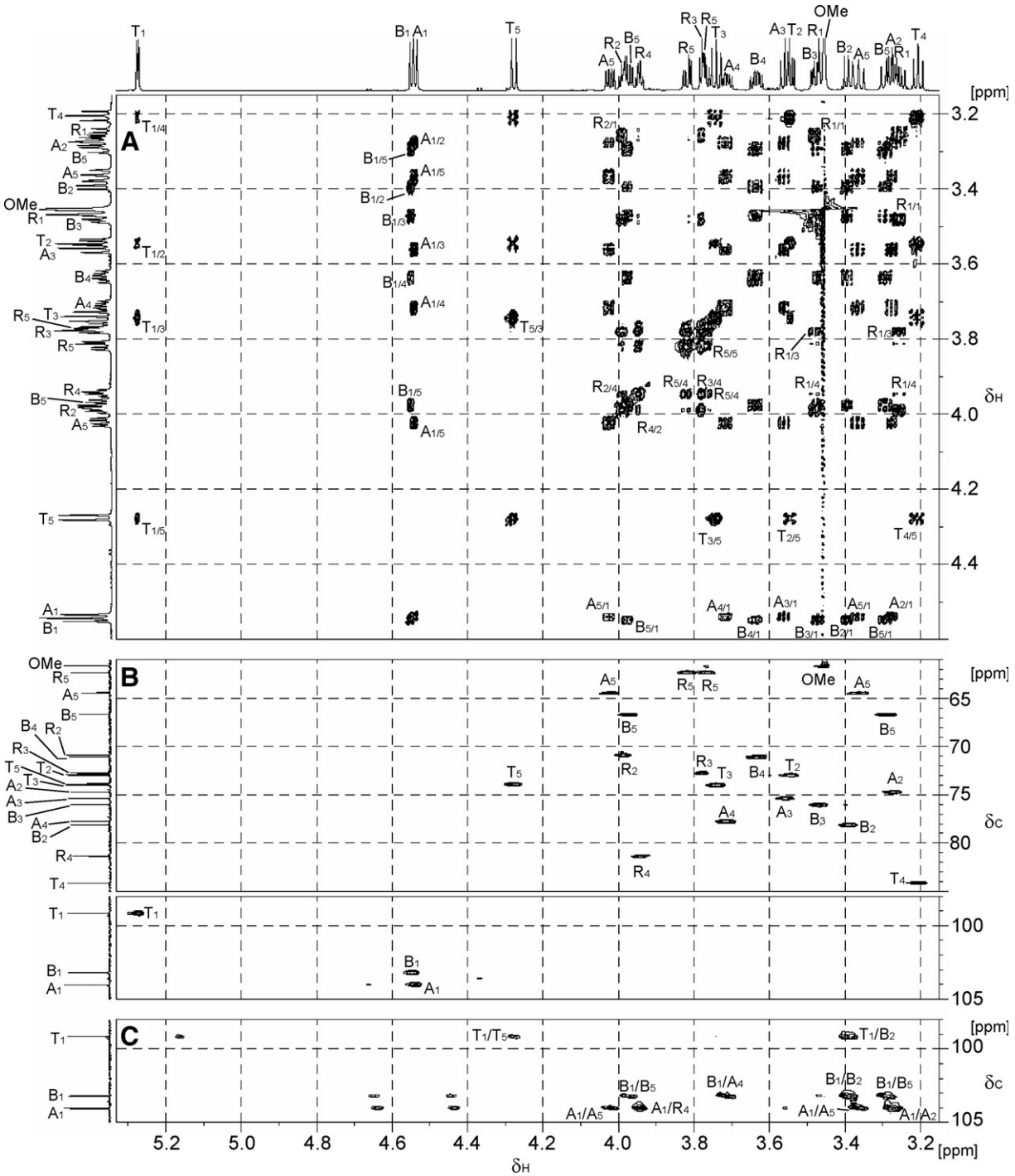


Fig. 5. TOCSY (A), HSQC (B), and HMBC (C) spectra of compound **12**.

Table 4
^{1}H and ^{13}C chemical shifts and first-order coupling constants (Hz) for compound **14**

Compound	Residue	^{1}H chemical shifts[a] (ppm)								First-order coupling constants[b] (Hz)									^{13}C chemical shifts[c] (ppm)					
		H-1$_a$	H-1$_b$	H-2	H-3	H-4	H-5$_a$	H-5$_b$	H-6	$^{3}J_{1a,2}$	$^{3}J_{1b,2}$	$^{2}J_{1a,1b}$	$^{3}J_{2,3}$	$^{3}J_{3,4}$	$^{3}J_{4,5a}$	$^{3}J_{4,5b}$	$^{2}J_{5a,5b}$	$^{3}J_{5,6}$	C-1	C-2	C-3	C-4	C-5	C-6
14	R	3.503	3.311	4.062	3.850	3.901	3.881	3.808	–	4.5	5.5	13.0	8.5	9.0	6.0	12.0	11.0	–	47.57	70.93	72.76	81.20	62.59	–
	A	5.232	–	3.957	4.120	4.287	4.467	–	–	3.8	–	–	10.4	3.4	1.3	–	–	–	100.56	76.17	71.14	72.80	73.91	177.23
	B	5.083	–	4.204	3.880	3.600	3.788	–	1.310	1.3	–	–	4.0	10.0	10.0	–	–	6.2	103.28	71.39	83.61	72.82	70.83	18.41
	E	4.481	–	3.301	3.364	3.646	3.897	3.111	–	7.9	–	–	9.0	9.0	4.5	12.0	10.0	–	105.91	74.86	75.22	77.94	64.42	–
	T	4.307	–	3.219	3.385	3.589	3.928	3.237	–	7.9	–	–	9.0	9.0	4.5	12.0	10.0	–	103.39	74.46	77.31	70.86	66.86	–

[a, b, and c] See Table 1.

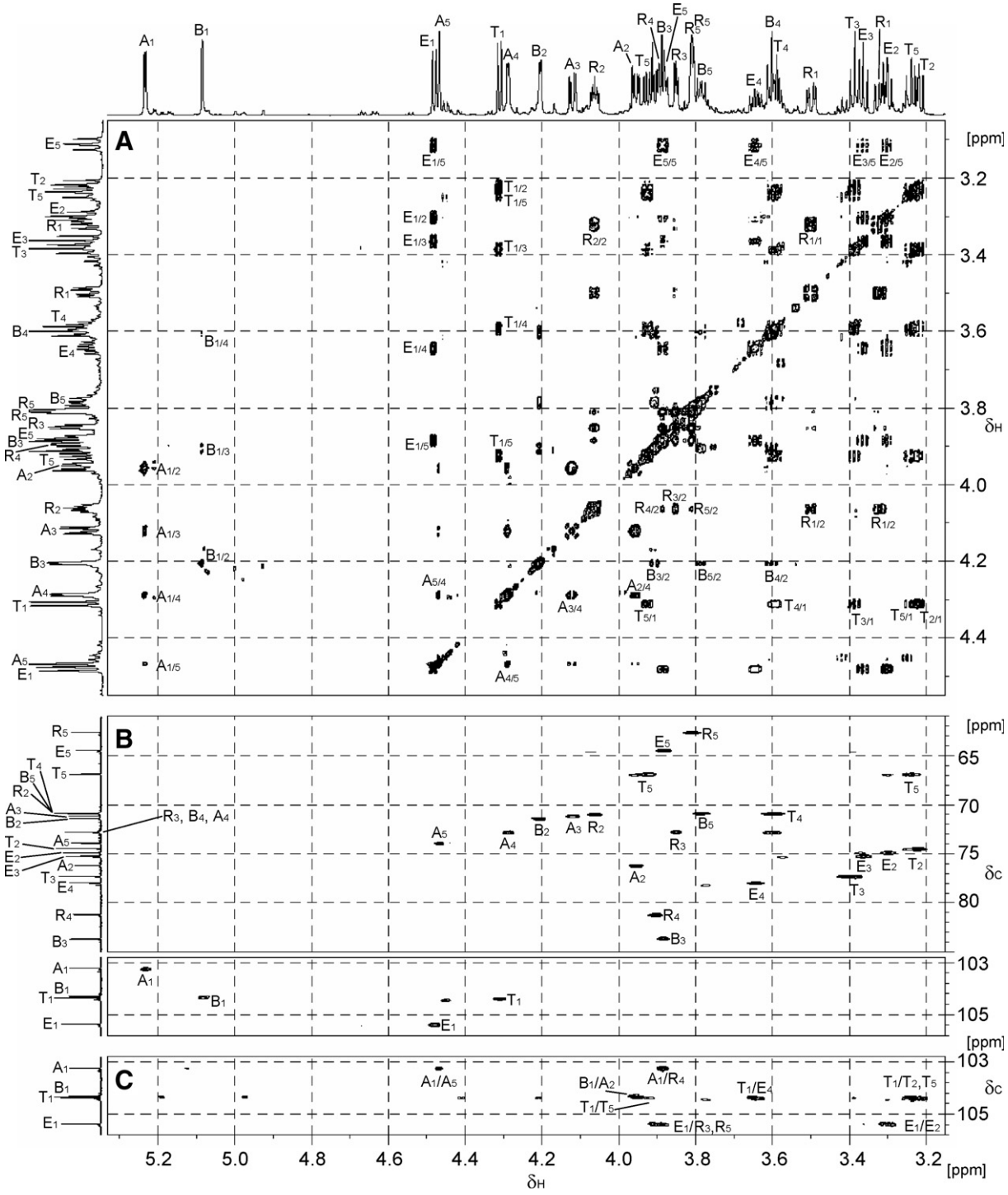


Fig. 6. TOCSY (A), HSQC (B), and HMBC (C) spectra of compound **14**.

signals of α-Gal*p*A, α-Rha*p*, and Xyl*p* residues were assigned on the basis of their scalar coupling patterns and chemical shifts in the DQFCOSY and TOCSY spectra (Table 4 and Fig. 6A). The linkages between each glycosyl residue were confirmed from HMBC spectrum (Fig. 6C). The ^{13}C NMR spectrum was assigned by HSQC (Fig. 6B). The HMBC spectrum gave extensive intramolecular correlations between proton and carbon atoms of each residue (Fig. 6C). The ^{1}H NMR spectrum of the reduced form of pentasaccharide **14** isolated from *Arabidopsis thaliana* stems was recently assigned (Peña et al., 2007). The chemical shift values of 2AB-labeled pentasaccharide **14** and the reduced form of **14** were almost same.

3.3. Enzymatic degradation

Treatment of 2AB-labeled xylo-tetraose (compound **4**) with culture filtrate of *F. palustris* generated 2AB-labeled xylobiose, and xylotriose (data not shown). Treatment of 2AB-labeled 4-*O*-MeGlcA-xylotriose (compound **12**) with the culture filtrate generated 2AB-labeled xylitol, xylobiose and xylotriose (Fig. 7B). These results show that 2AB-labeled 4-*O*-MeGlcA-xylotriose (compound **12**) was substrate for α-glucuronidase and xylanase. Fluorescent detection of 2AB-labeled oligosaccharides allows us to detect α-glucuronidase activity at picomol concentration.

3.4. Xylosyltransferase assay

Small amounts of 2AB-labeled xylo-oligosaccharides with DPs of 5, 6, and 7 were formed when 2AB-labeled xylotetraose (compound **4**) was reacted with UDP-Xyl and a rice microsomal fraction (Fig. 8B). 2AB-xylobiose was the major product detected when the transferase reaction products were treated with *endo*-xylanase (Fig. 8C). Xylosyltransferase activity was reported by using pyridylaminated β-(1 → 4)-xylotriose (Kuroyama & Tsumuraya, 2001; Urahara et al., 2004) and anthranilic acid (AA) labeled xylo-oligomers (Lee, O'Neill, Tsumuraya, Darvill, & Ye, 2007). A high XylT activity was reported when AA labeled xylo-oligomers was reacted with microsomes from *Arabidopsis* stems and UDP-Xyl. One reason for the low activity of XylT from rice microsomes was the amount of protein used for assay: 30 μg of rice microsomes was used, while 100 μg of *Arabidopsis* microsomes was used.

4. Conclusion

We have synthesized in high yield (about 90%) 2AB-labeled xylo-oligosaccharides and have assigned all of the signals in their ^{1}H and ^{13}C NMR spectra. Present data on 2AB-labeled xylo-oligosaccharides will be useful for detection of XylT, xylanase, and α-glucuronidase activities originated from plant and microorganisms.

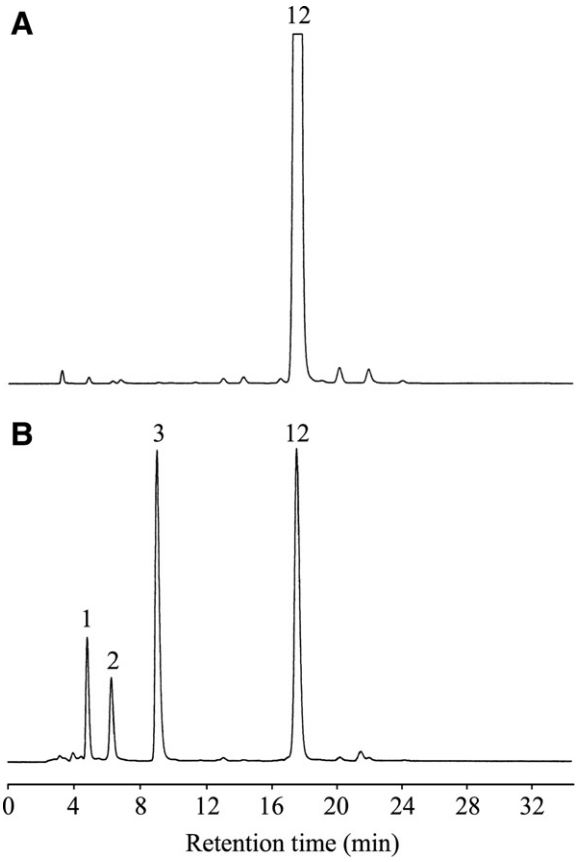


Fig. 7. Normal phase LC chromatograms of compound **12** (A) and its enzyme-digested products (B) monitored by fluorescent detection. The numbers above peaks (**1**–**3**) indicate the elution positions of xylitol-2AB. xylobiose-2AB and xylotriose-2AB (see Fig. 1).

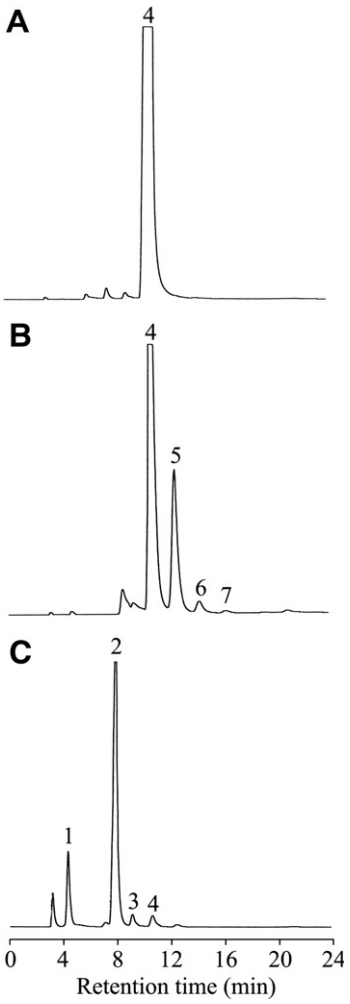


Fig. 8. HPAE chromatograms of compound **4** (A), the Xyl transfer products (B) and its xylanase-digested products (C) resolved on a CarboPac PA-1 column and monitored with a fluorescent detector. The numbers above peaks indicate DP of Xyl residues.

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