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ISOLATION AND IDENTIFICATION OF *O*-(5-*O*-FERULOYL-α-L-ARABINOFURANOSYL)-(1→3)-*O*-β-D-XYLOPYRANOSYL-(1→4)-D-XYLOPYRANOSE AS A COMPONENT OF *Zea* SHOOT CELL-WALLS*

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ABSTRACT

Zea shoot cell-walls were hydrolyzed with 30mM oxalic acid followed by treatment with "Driselase" (a *Basidiomycetes* enzyme preparation) to obtain carbohydrate fragments containing ferulic acid. The structure of the major feruloyl compound was identified as *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose on the basis of ^{13}C-n.m.r., methylation analysis, and partial acid-hydrolysis, alkali hydrolysis, or esterase hydrolysis followed by analyses of the hydrolyzate.

INTRODUCTION

Ferulic acid is one of the phenolic acids considered to be esterified to carbohydrate in cell-walls derived from members of several plant families[1–11]. Such phenols are released from plant cell-walls upon treatment with alkali solutions and the phenolic acid–carbohydrate complex is liberated by a mixture of carbohydrate hydrolases. It has recently been suggested that phenolic acids may play an important structural role by cross-linking polymers to which the phenolic acids are bound[6,12–18]. Such coupling may be mediated by phenolic oxidation in the presence of peroxidases and H_2O_2, and may contribute to the control of wall extensibility and cell growth.

The linkage between ferulic acid and carbohydrate is based on analysis of low-molecular-weight carbohydrate esters of ferulic acid obtained from the enzymic hydrolyzate of cell walls or the lignin–carbohydrate complex. Most recently, Fry has isolated two feruloylated disaccharides, 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3-*O*-(3-*O*-feruloyl-α-L-arabinofuranosyl)-L-arabinose, from cell walls of suspension-cultured spinach cells[19]. He also has suggested that

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such oligosaccharides are derived from pectin[20]. Kato *et al.* have isolated *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose from sugar cane bagasse as the lignin–carbohydrate complex[21]. In contrast, Smith and Hartley[22] have recently identified a major feruloyl compound derived upon enzymic treatment of wheat bran cell-walls as 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose. There is therefore a significant difference between monocots and of dicots in terms of the carbohydrate to which ferulic acids are esterified: in monocots it is arabinoglucuronoxylan whereas the arabinan and galactan portions of pectin contain ferulic acid in dicots. Furthermore, it appears that ferulic acid occurs at 2-*O*-arabinosyl branch-points of linear backbones of (1→4)-linked β-D-xylopyranosyl residues in wheat bran, whereas in bagasse a 3-*O*-arabinosyl branch point occurs on the xylan backbone.

In the course of structural studies of carbohydrates in cell walls of growing *Zea* shoots, a monocot, we have found that arabinoglucuronoxylan, which is liberated from *Zea* shoot cell-walls by treatment with a purified endo-(1→4)-β-D-xylanase, contains ferulic acid (about 3 μg/100 μg of carbohydrate)[23]. The quantity of this feruloylated arabinoglucuronoxylan was not sufficient to investigate the linkage between arabinoglucuronoxylan and ferulic acid. Therefore we isolated feruloylated arabinoxylan-oligosaccharides on a large scale from *Zea* shoot cell-walls and have characterized the major fraction. This paper describes the results.

RESULTS AND DISCUSSION

Isolation of feruloylated oligosaccharides from Zea *shoot cell-walls.* — The isolation procedures for obtaining feruloylated oligosaccharides from LiCl-treated *Zea* shoot cell-walls are summarized in Fig. 1. LiCl was used as a pretreatment in these experiments to remove a fraction of wall-associated protein.

When *Zea* shoot cell-walls were hydrolyzed with 30mM oxalic acid followed by separation of the hydrolyzate into soluble (fraction I) and insoluble (fraction II) fractions, ~67% of the feruloyl groups present in the cell walls were found in fraction I and the remainder were found in fraction II. Fraction I was resolved into eight additional components on the basis of solubility in 50% methanol and subsequent chromatography of the 50% methanol-soluble materials on Sephadex LH-20 (Fig. 2). Table I records the yields and several properties of fractions I-1–I-8.

Fraction I-7, which accounted for 15.6% of the total feruloyl derivatives recovered, was subjected to rechromatography on the LH-20 column followed by preparative paper-chromatography. A major component having R_F 0.69 was obtained in a yield of 17.7 mg and designated substance A.

Fractions I-1 and I-2 were combined and hydrolyzed with the *Basidiomycetes* hydrolases "Driselase". Chromatography on Sephadex LH-20 of the hydrolyzate yielded five peaks containing feruloyl groups (Fig. 3). The last peak eluted from the LH-20 column was collected and subjected to rechromatography followed by preparative paper-chromatography. A major component having R_F 0.38 was ob-

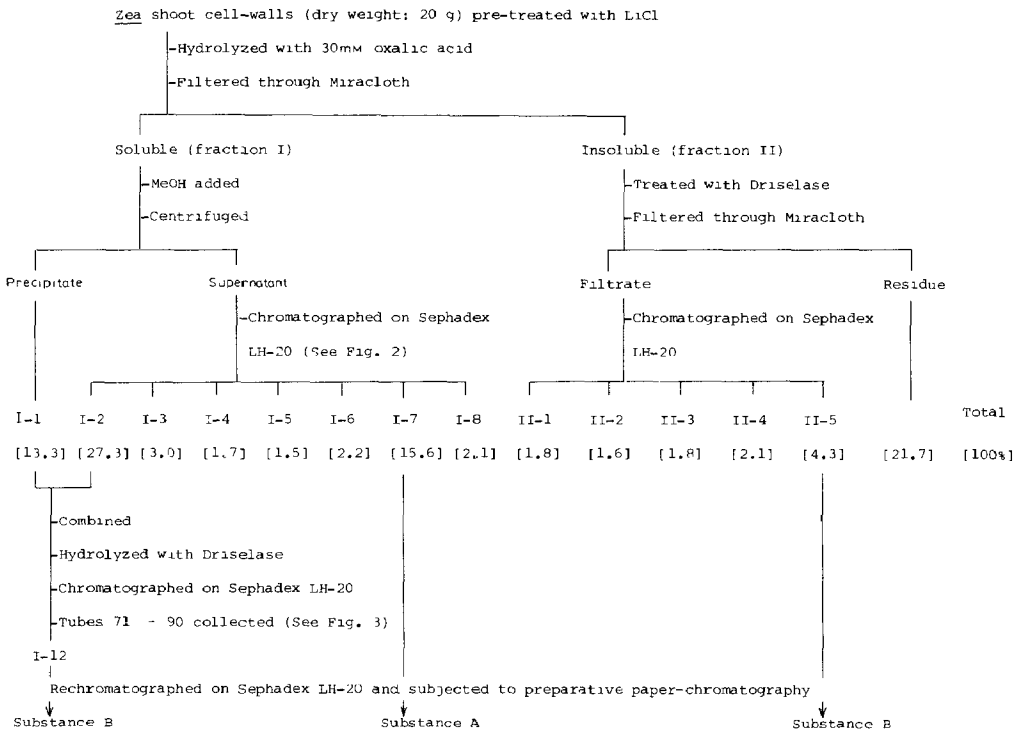

Fig. 1. A summary of isolation procedures of feruloylated oligosaccharides from *Zea* shoot cell-walls. Each step was performed as described in the text. The values in brackets give the percentage of total recovered feruloyl groups, which were determined by u.v. absorption with ferulic acid as a standard.

tained in a yield of 16.2 mg and designated substance B.

Fraction II, material insoluble after hydrolysis with 30mM oxalic acid, was treated with Driselase, and the solubilized portion was also resolved into five fractions (II-1–II-5) by chromatography on Sephadex LH-20. After rechromatography of fraction II-5 followed by preparative paper-chromatography, a major feruloylated carbohydrate (substance C) was obtained in a yield of 11.0 mg. Substance C was combined and analyzed with substance B because they exhibited similar properties.

Properties of substance A. — Substance A had an R_F value of 0.87 in solvent *A* and 0.69 in solvent *B*. The absorption spectrum between 220 and 400 nm of substance A showed λ_{max} at 325 nm in 2.5% methanol and also at 347 nm in M NaOH. Substance A, when treated with M NaOH, gave ferulic acid and arabinose. The molar ratio of ferulic acid and arabinose was 1:0.6, as determined by u.v. absorption of sodium ferulate at λ_{max} 347 nm and by the phenol–sulfuric acid method[24]. This substance was not studied further.

Properties of substance B. — Substance B was soluble in water and 50% methanol. Its R_F values in solvents *A* and *B* were 0.77 and 0.38, respectively. It had

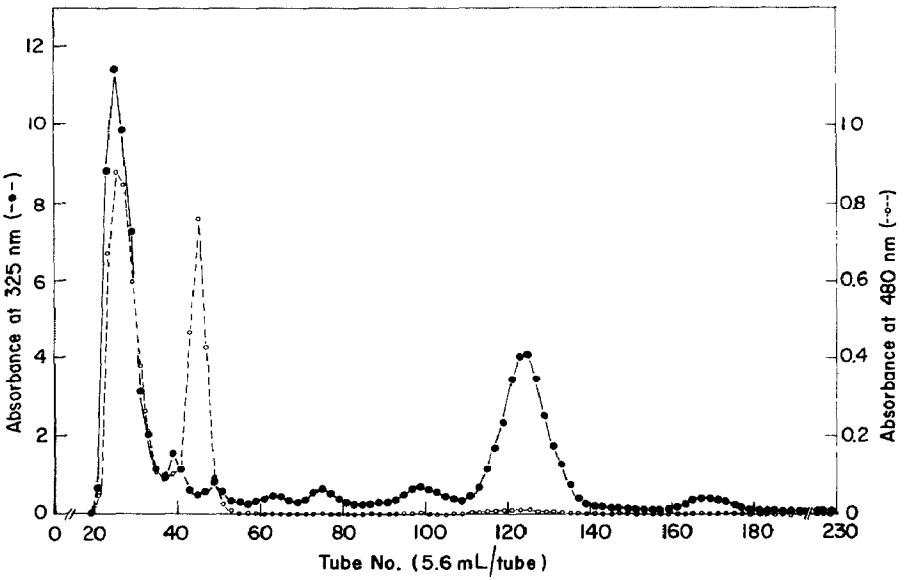

Fig. 2. Sephadex LH-20 chromatography of the 50% methanol-soluble fraction obtained from materials solubilized from *Zea* shoot cell-walls by treatment with oxalic acid. A portion (10 mL) of the 50% methanol-soluble fraction (~80 mL) was applied to a column (2.3 × 67 cm) of Sephadex LH-20 pre-equilibrated with 50% methanol and eluted with 50% methanol. Tubes 21–35, 36–58, 59–68, 70–82, 91–109, 111–136, and 161–180 were separately pooled to give fractions I-2–I-8.

a maximum absorption at 345 nm in M NaOH and at 325 nm in water. Sugar analysis by g.l.c. of the alditol acetates obtained from the acid hydrolyzate of substance B showed that it consisted of arabinose and xylose in the molar ratio of 1:2.1. Alkali treatment of substance B yielded ferulic acid and a substance having R_F 0.49 (solvent *A*), in a weight ratio of 1:2.1. The latter was designated substance B′. Substance B was treated with carboxyl esterase from porcine liver and the product analyzed by paper chromatography (solvent *A*). Ferulic acid and substance B′ were detected on the chromatogram. These results indicate that ferulic acid is bonded to substance B′ by an ester linkage.

Substance B′ consisted of three glycosyl units, as determined by the ratio of reducing sugar/total sugar and from its elution position from a Bio-Gel P-2 column calibrated with xylo-oligosaccharides from a hydrolyzate of larch xylan. Partial acid-hydrolysis of substance B′ and successive analysis of the hydrolyzate by paper chromatography showed that it contained arabinose, xylose, and 4-*O*-β-D-xylopyranosyl-D-xylopyranose. G.l.c. of the alditol acetates obtained from a hydrolyzate of the methylated substance B′ showed that it consisted of one non-reducing terminal arabinofuranosyl residue, one 4-substituted xylopyranosyl residue, and one 3-substituted xylopyranosyl residue (Table II). Methylation analysis of the reduced substance B′ revealed one 4-substituted xylitol residue and the concomitant disappearance of one 4-substituted xylosyl residue (Table II), indicating that a 4-substituted xylosyl residue is present at the reducing terminal of substance B′. On

TABLE I

YIELDS AND SEVERAL PROPERTIES OF FRACTIONS OBTAINED FROM *Zea* SHOOT CELL-WALLS UPON TREATMENT WITH 30mM OXALIC ACID

Fraction	*Yield (dry weight, mg)*	*Total sugar content (mg)*[a]	*Phenolic compound content (mg)*[b]	*Sugar composition (mol%)*					R_F[c]			
				Ara	*Xyl*	*Man*	*Gal*	*Glc*				
I-1	[d]	777	15.2	3.8	58.5	[e]	12.5	25.2				
I-2	[d]	1497	31.1									
I-3	3500	795	3.4	85.4	8.0	[e]	4.0	2.6				
I-4	26.5	4.9	1.9	32.3	47.3	[e]	20.4	[e]				
I-5	14.1	3.1	1.7	35.7	50.7		13.6	[e]	*0.33*	0.37		
I-6	10.6	2.3	2.5	47.9	52.1		[e]	[e]	*0.50*	0.64		
I-7	35.7	6.3	17.8	98.9	1.1		[e]		0.64	*0.69*	0.78	0.82
I-8	4.7	0.3	2.4			[d]						

[a]Determined by the phenol–sulfuric acid method and expressed as xylose equivalents. [b]As ferulic acid. [c]Preparative paper chromatography of fractions I-5, I-6, and I-7 in Solvent *B* (see experimental section). [d]Not determined. [e]Trace.

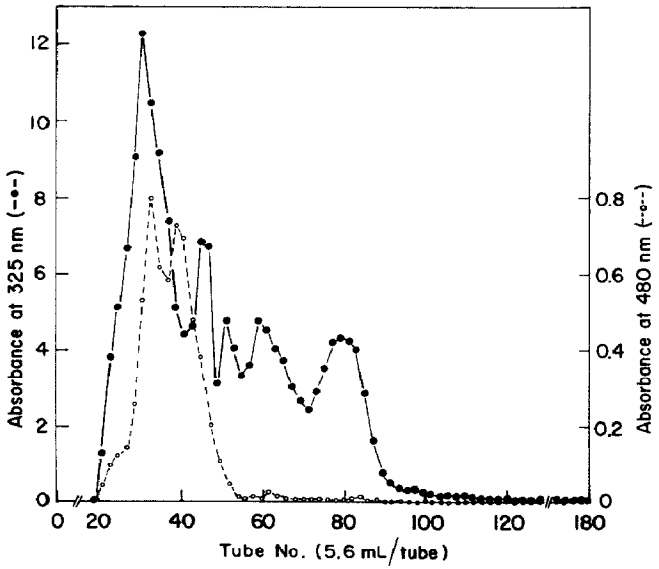

Fig. 3. Sephadex LH-20 chromatography of the enzymic hydrolyzate of combined I-1 and I-2. Tubes 75–85 were combined to give fraction I-12. Details are given in the text.

TABLE II

METHYLATION ANALYSIS OF SUBSTANCE B′ AND REDUCED SUBSTANCE B′

Alditols	*Relative retention times*[a]	*Peak area (%)*	
		B′	*Reduced B′*
1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol	0.71	26.9	32.5
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methylxylitol	0.90	40.8	51.0
1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methylxylitol	0.94	32.3	0
4-*O*-Acetyl-1,2,3,5-tetra-*O*-methylxylitol	0.47	0	16.6

[a]Relative to retention time of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

the basis of these results, substance B′ was assigned the structure α-L-arabinofuranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-D-xylopyranose (Fig. 4). Oligosaccharides similar to substance B′ have been isolated from other xylans[25–29].

To elucidate the position involved in the ester-bonding, substance B′ was analyzed by ^{13}C-n.m.r. spectroscopy. After the initial ^{13}C-n.m.r. spectrum of substance B had been recorded, the sample was recovered by column chromatography on Sephadex LH-20 and then treated with M NaOH. Substance B′ obtained from the alkali-hydrolyzate of substance B was then subjected to ^{13}C-n.m.r. analysis. Assignments of the signals in the 75.46-MHz ^{13}C-n.m.r. spectra in D_2O are given in Table III. The signals of carbon atoms of substance B′ were assigned by using the earlier data for arabinose, xylose, xylobiose, and so on[30–33]. The presence of a signal at 107.9 p.p.m. in the region for anomeric carbon atoms suggests that

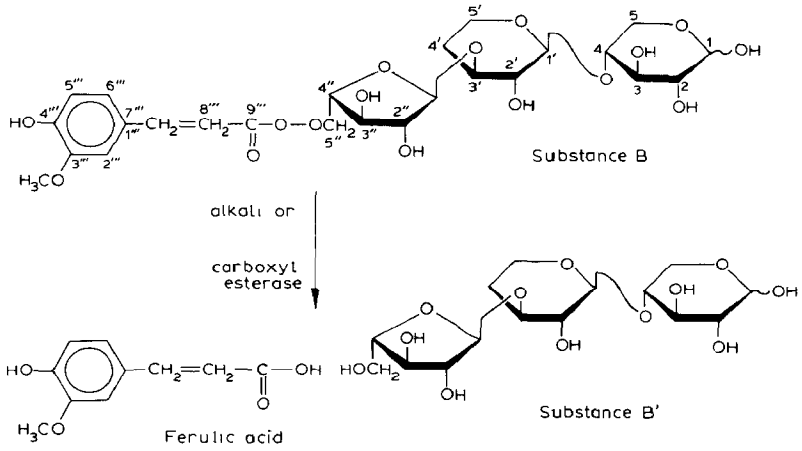

Fig. 4. Structures proposed for substances B and B'.

TABLE III

ASSIGNMENT OF SIGNALS IN ^{13}C-N M.R SPECTRA OF SUBSTANCE B AND SUBSTANCE B'

δ		*Assignment*[a]	δ		*Assignment*[a]
Substance B	*Substance B'*		*Substance B*	*Substance B'*	
125.73		C-1‴	101.52	101.54	C-1′
110.56		C-2‴	72.48	72.67	C-2′
147.29		C-3‴	81.14	81.05	C-3′
147.29		C-4‴	67.54	67.62	C-4′
113.06		C-5‴	64.79	64.84	C-5′
123.44		C-6‴			
146.39		C-7‴	96.28	96.32	C-1β
115.38		C-8‴	91.79	91.84	C-1α
168.76		C-9‴	73.71	73.74	C-2β
55.45		O-CH_3	70.73	70.77	C-2α
			73.76	73.82	C-3β
107.99	107.95	C-1″	71.75	71.19	C-3α
80.89	81.29	C-2″	76.46	76.31	C-4β
76.62	76.46	C-3″	76.28	76.31	C-4α
81.73	83.80	C-4″	62.78	62.84	C-5β
67.26	61.10	C-5″	58.59	58.69	C-5α

[a]For the source of carbon atoms, see Fig. 4.

arabinofuranose in substance B′ is α-linked. In the ^{13}C-n.m.r. spectrum of substance B, signals derived from the aromatic carbon atoms of ferulic acid were well separated from signals of carbon atoms of carbohydrate, and readily assigned by using published data[34,35]. A comparison of the spectrum of substance B with that of substance B′ revealed an appreciable shift (+6.16 p.p.m.) in the C-5 signal of the arabinofuranosyl residue. From this result, the proposed structure of substance B

is *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (Fig. 4).

We have isolated feruloylated arabinoglucuronoxylan from the *Zea* cell walls[23]. Therefore, isolation and identification of substance B from *Zea* shoot cell-walls in the present study shows that ferulic acids occur at 3-*O*-arabinosyl branch-points of linear backbones of (1→4)-linked β-D-xylopyranosyl residues in *Zea* shoot arabinoglucuronoxyulan.

EXPERIMENTAL

Plant material. — *Zea* shoot cell-walls were prepared and pre-treated with 3M LiCl to remove a fraction of wall-associated proteins as described previously[36,37].

Enzymes. — Driselase from *Basidiomycetes* containing various carbohydrate *exo*- and *endo*-hydrolases[19] including cellulase, pectinase, β-xylanase, and mannanase was purchased from Sigma: 400 mg of Driselase was dissolved in 4 mL of 50mM sodium acetate buffer (pH 5.0) and centrifuged. To the supernatant solution, 2.064 g of $(NH_4)_2SO_4$ was added with stirring at 4°. After 15 h, the precipitate was collected by centrifugation, redissolved in 4 mL of the same buffer, and centrifuged. The supernatant was dialyzed against the same buffer and the dialyzate was used in Driselase treatments. Carboxyl esterase from porcine liver (esterase type II) was also obtained from Sigma.

General methods. — Concentration of the carbohydrate solutions by rotary evaporation was performed under diminished pressure at 35–40°. Paper chromatography, both analytical and preparative, was performed on Whatman filter paper No. 1 by the ascending method with butanol–pyridine–water (6:4:3, v/v/v, solvent *A*) or with butanol–acetic acid–water (15:3:5, v/v/v, solvent *B*). Neutral sugars on the chromatograms were detected with alkaline silver nitrate[38], and phenolic compounds on the chromatograms were located with the aid of u.v. light. Total carbohydrate was determined by the phenol–sulfuric acid method[24] and reducing sugars measured by the Nelson–Somogyi method[39,40]. Ferulic acid content was determined by u.v.-absorption as sodium ferulate at λ_{max} 340 nm.

Hydrolysis of LiCl-pretreated Zea *shoot cell-walls with 30mM oxalic acid.* — LiCl-pretreated *Zea* shoot cell-walls (20 g, dry wt) was suspended in 1 L of 30mM oxalic acid and hydrolyzed under reflux in a boiling water-bath for 3 h. The resultant suspension was filtered through Miracloth, and the residue was washed twice with 100 mL of water. The filtrate and washings were combined and made neutral with 0.1M NaOH to give fraction I (1100 mL). Fraction I was concentrated to ~40 mL, and 40 mL of methanol was added to the concentrate with stirring. The mixture was centrifuged to separate insoluble (fraction I-1) and soluble fractions. The oxalic acid-insoluble material was dried to give fraction II.

Resolution of the soluble fraction by chromatography on Sephadex LH-20. — A portion (10 mL) of the soluble fraction (total of ~80 mL) was applied to a column (2.3 × 67 cm) of Sephadex LH-20 pre-equilibrated with 50% methanol and eluted

with 50% methanol; 5.6-mL fractions were collected and assayed for carbohydrate and phenolic compounds (Fig. 2). Tubes 21–35, 36–58, 59–68, 70–82, 91–109, 111–136, and 161–180 were separately combined and concentrated to dryness to give fractions I-2–I-8. This procedure was repeated eight times.

Enzymic hydrolysis of combined I-1 and I-2 and resolution of the hydrolyzate. — Fractions I-1 and I-2 were combined, dissolved in 450 mL of 10mM sodium acetate buffer (pH 5.0), and incubated with 1 mL of Driselase for 72 h at 32°. After incubation, the mixture was heated in a boiling-water bath for 20 min and then evaporated. The dried material was dissolved in 20 mL of 50% methanol and centrifuged. One-half of the supernatant solution was applied to a column (2.3 × 67 cm) of Sephadex LH-20 pre-equilibrated with 50% methanol and eluted with 50% methanol; 5.6-mL fractions were collected and assayed for carbohydrate and for phenolic compounds (Fig. 3). Tubes 75–85 were combined and evaporated to give fraction I-12. This procedure was performed twice.

Enzymic hydrolysis of fraction II and resolution of the hydrolyzate. — Fraction II was suspended in 800 mL of 50mM sodium acetate buffer (pH 5.0) and incubated with 1 mL of Driselase at 32°. After 24 h of incubation, an additional 1 mL of Driselase was added and the incubation was continued. After a total of 120 h of incubation, the reaction was stopped by heating in a boiling-water bath for 20 min. The resultant suspension was filtered with the aid of Miracloth. The filtrate was concentrated to dryness. The dried material was dissolved in 10 mL of 50% methanol and centrifuged. The supernatant solution was applied to a column (2.3 × 67 cm) of Sephadex LH-20 pre-equilibrated with 50% methanol and eluted with 50% methanol; 5.6-mL fractions were collected and assayed for carbohydrate and phenolic compounds. Tubes 20–29, 30–38, 39–48, 49–60, and 63–78 were separately combined and evaporated to give fractions II-1–II-5.

Isolation of substance A and substance B from fraction I-7 and fractions I-12 and II-15, respectively. — Fractions I-7, I-12, and II-5 were separately rechromatographed on the Sephadex LH-20 column in a manner identical with that already described. Fraction I-7, thus obtained, showed four spots (detected with u.v. light) at R_F 0.82 (faint), 0.78 (faint), 0.69, and 0.64 (faint) upon paper chromatography with solvent *B*. Fraction I-12 showed three spots at R_F 0.49 (faint), 0.44 (faint), and 0.38. Fraction II-5 showed three spots at the same R_F values of fraction I-12. Each component was separated by preparative paper-chromatography with solvent *B*. The zones having R_F values 0.69 (fraction I-7), 0.38 (fraction I-12), and 0.38 (fraction II-5) were excised, eluted with 50% methanol, and finally evaporated to give substances A, B, and C, respectively. Substance C was combined with substance B because its properties were similar to those of substance B.

Analysis of neutral sugars in fractions I-1–I-7 and substances A and B. — Fractions I-1–I-7 (50–70 μg as xylose equivalent) and substances A and B (20–30 μg as xylose equivalent) were separately hydrolyzed with 1 mL of M HCl for 3 h at 100°. The hydrolyzate was then evaporated with a stream of air. Sugars were converted into their corresponding alditol acetates and then analyzed[41] by g.l.c. on a

glass column (0.2 × 190 cm) packed with 3% SP-2340 (Supelco) at 210° at a helium-flow rate of 35 mL/min.

Alkaline hydrolysis of substances A and B. — Substance A (100 μg) and substance B (100 μg) were separately dissolved in 1 mL of M NaOH. The solutions were maintained for 24 h at room temperature and then acidified with HCl. The resultant solution was extracted with ether. The ether phase was washed with water and evaporated. The water phase was treated with Dowex 50W (H^+ form) resin and evaporated. Both were subjected to paper chromatography with solvent *A* or *B*. Identification was made by comparison with the following standards, which are given with their respective R_F values in solvents *A* and *B*: arabinose, 0.52 and 0.24; xylose, 0.58 and 0.27; β-D-xylopyranosyl-(1→4)-D-xylose, 0.40 and not determined; and ferulic acid, 0.79 and 0.83. Two spots (R_F 0.52 and 0.79 in solvent *A* and R_F 0.24 and 0.83 in solvent *B*) were detected in the hydrolyzate of substance A, whereas two spots (R_F 0.48 and 0.79 in solvent A) were detected in that of substance B.

Hydrolysis of substance B by carboxyl esterase. — Substance B (60 μg) was dissolved in 0.2 mL of sodium phosphate buffer (pH 7.7) and incubated with 5 μL of carboxyl esterase (Sigma) at 25°. After 12 h, the solution was concentrated and subjected to paper chromatography with solvent *A*. Two spots (R_F 0.49 and 0.79) were detected.

Methylation analysis of substance B′. — Substance B′ (~200 μg) in dimethyl sulfoxide (0.2 mL) was methylated with methylsulfinyl carbanion (0.1 mL) and methyl iodide (0.1 mL) by the method of Hakomori[42]. The methylated substance B′, extracted into chloroform followed by evaporation of the extract, was hydrolyzed with 90% HCO_2H for 1 h at 100°. The acid was removed with a stream of air, and the residue treated with 0.5M HCl for 4 h at 100°. The acid was removed with a stream of air. Substance B′ (~300 μg in 0.2 mL of 50mM NH_4OH) was also reduced with 1 mg of $NaBH_4$ at room temperature. After 3 h, the solution was acidified with CH_3CO_2H, evaporated with a stream of air, and methanol-distilled to remove borate. The reduced substance B′ was then methylated and hydrolyzed as just described. The methylated sugars were converted into their corresponding alditol acetates, followed by g.l.c. analysis[43] on a 30-m DB-1 fused-silica capillary column with a split ratio of 50:1. The sample in dichloromethane was injected at a column temperature of 150°, and after 4 min the temperature was raised at 4°/min to 230°. For g.l.c.–m.s., a combined gas chromatograph–mass spectrometer, Finnegan 4000 with INCOS data system, fitted with a DB-1 fused-silica capillary column (25 m) was used.

Partial acid-hydrolysis of substance B′. — Substance B′ (990 μg) was hydrolyzed with 0.5 mL of 0.1M HCl for 1 h at 100°. After hydrolysis, the acid was removed with a stream of air and the hydrolyzate subjected to paper chromatography with solvent *A*. Identification was made by comparison with the following standards; arabinose, xylose, and β-D-xylopyranosyl-(1→4)-D-xylose.

^{13}C-N.m.r. spectra. — The ^{13}C-n.m.r. spectra were recorded at room temper-

ature at 75.46 MHz with a Nicolet NT-300 spectrometer, in the deuterium-lock mode. Before each measurement, the sample was dissolved in deuterium oxide (D_2O) and air-dried, and this procedure was repeated three times. The material was finally dissolved in 0.5 mL of D_2O. Chemical shifts (δ) are referenced to an external standard of 1,4-dioxane (66.5 p.p.m.)

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