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Structural characterization of feruloyl oligosaccharides from spinach-leaf cell walls †

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ABSTRACT

Hydrolysis of spinach-leaf cell walls with Driselase (a fungal enzyme preparation) released two arabino-oligosaccharides and one galactobiose, each carrying a ferulic acid moiety. The oligosaccharides were characterized by NMR spectroscopy, methylation analysis, and FABMS. They were *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1 → 5)-L-arabinofuranose, *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1 → 4)-D-galactopyranose, and *O*-α-L-arabinofuranosyl-(1 → 3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1 → 5)-L-arabinofuranose.

INTRODUCTION

The polysaccharides of plant cell walls contain a small proportion of phenolic material, which is present as ester linked side chains. Biochemical interest in these side chains arises from the fact that they apparently undergo in vivo oxidative coupling to yield cross-linked polysaccharides; such coupling may contribute not only to the control of cell wall extensibility and cell growth[1–9], but also to decreased digestibility by ruminants[10–12]. A more specific biochemical role is foreshadowed by the finding that a feruloyl arabinose-xylose trisaccharide, derived from feruloyl arabinoxylan, inhibited auxin-stimulated cell elongation in rice lamina joints[13].

The linkage between cinnamic acid derivatives and cell-wall polysaccharides has been well characterized in preparations from monocots. Feruloyl and *p*-coumaroyl arabinose-xylose oligosaccharides[14–21] and a feruloyl xylose-glucose disaccharide[20], derived from hemicellulosic arabinoxylan and xyloglucan, respectively, were isolated from the tissues of species of *Gramineae*.

† Dedicated to the late Mrs. Teruko Ishii, my mother.
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Fry[22] isolated a feruloyl arabinobiose and a feruloyl galactobiose from walls of suspension-cultured spinach (dicot) cells, and suggested that these disaccharides were probably derived from pectin. But the structural characterization of the compounds was incomplete. We have now isolated three feruloyl oligosaccharides from spinach-leaf cell walls and herein report their complete structural analysis.

EXPERIMENTAL

Plant material.—Fresh spinach (*Spinacia oleracea* L.) was purchased from a local grocery store in Ibaraki prefecture. To prepare cell walls from the leaf, the sample was homogenized in 50 mM Tris · HCl buffer (pH 7.6) at 4°C with a food mixer, and the homogenate was filtered. The residue was washed successively with the Tris · HCl buffer and water, extracted with MeOH for 16 h under reflux, and finally washed with acetone, then air dried. The resulting preparation contained 0.7 mg of phenolic acids per g (dry weight). The molar ratio of *p*-coumaric, ferulic, and diferulic acids, determined by HPLC, was 1 : 14 : 1.2.

Isolation of compounds **1**, **2** *and* **3**.—The cell walls (20 g) were suspended in 1.0 L of distilled water, the pH of the suspension was adjusted to 5.0 with AcOH, and it was incubated for 16 h at 30°C after the addition of 3 mL of a 60 mg mL^{-1} solution of Driselase (purchased from Kyowa Hakko, Tokyo, and purified as described[22]). The suspension was then heated for 15 min in a boiling-water bath to stop the reaction, and centrifuged. The supernatant solution was chromatographed on a column (2.5 × 40 cm) of Cosmosil 140 C_{18}-OPN (Nacalai Tesque, Tokyo) and eluted successively with water, aq 5% (v/v) MeOH, aq 50% MeOH, and MeOH. The fraction eluted with 50% MeOH contained feruloyl oligosaccharides. These were separated on a column (4.4 × 85 cm) of Sephadex LH-20 and eluted with water; portions of 15 mL were collected and assayed for total carbohydrate and phenolic acid. Fractions having K_{av} values of 2.1, 2.6, and 3.8 contained predominantly feruloyl oligosaccharides. Further purification was accomplished by analytical normal-phase HPLC using a 0.6 i.d × 15 cm column (Shim-Pack CLC-SIL from Shimadzu) at 40°C eluted with 150 : 150 : 1 (2-propanol–$CHCl_3$–AcOH at 0.5 mL min^{-1}. The effluent was monitored at 320 nm, and peaks of UV-positive material eluting at 7.6, 7.9, and 9.1 min were collected, and lyophilized to give **1**, **3**, and **2** respectively. Under the same conditions a feruloyl arabinobiose isolated from the walls of suspension-cultured spinach cells furnished by Dr. S.C. Fry eluted at 7.6 min.

General methods.—Evaporations were conducted under diminished pressure at < 40°C. Total carbohydrate was determined by the phenol–H_2SO_4 method[23]. Alditol acetates were prepared[24] and analyzed[25] as described, except that hydrolysis in 2 M CF_3CO_2H was done for 20 min; GLC was performed on a Shimadzu GC 14A instrument equipped with a 30 m × 0.25 mm SP-2330 fused-silica column (Supelco) and operating isothermally at 230°C. Absolute configurations were determined as described by Gerwig et al.[26]. Per-*O*-methylation was performed by a

modification[27] of the method of Hakomori[28], and per-*O*-methylated oligosaccharides and glycosyl alditols were purified as described by Waeghe et al.[29]. An authentic sample of *O*-α-L-arabinofuranosyl-(1 → 5)-L-arabinose was obtained from Megazyme Pty. Ltd., Sydney, Australia.

Alkaline hydrolysis and reduction of compounds **1**, **2**, *and* **3**.—Compounds **1**, **2**, and **3** (~ 300 μg of each) were separately dissolved in 250 μL of a 0.25 M solution of $NaBD_4$ in 1.5 M NH_4OH. After 3 h under N_2 in the dark at room temperature the solutions were acidified to pH 4.0 with Dowex 50W-X8 (H^+) and filtered, and the filtrates were extracted with ether. The ether phases were washed with water and evaporated. The glycosyl alditols **4**, **5**, and **6** in the aqueous phases were per-*O*-methylated and analyzed as described[19]. The ether extracts were fractionated as previously described[20].

Mass spectrometry.—GLC–MS was performed with a Shimadzu GC-MS QP2000A mass spectrometer. Per-*O*-methylated alditol acetates and per-*O*-methylated glycosyl alditols were separated as previously described[20].

FABMS was performed in the positive-ion and negative-ion modes on a Jeol JMS-DX 303HF mass spectrometer operating at an accelerating voltage of 3 kV. Samples were dissolved in water and loaded on a stainless-steel target with thioglycerol as matrix. Argon was used as the bombarding gas. Spectra were obtained by scanning the mass range from 100 to 1200 Da in 15 s and processing with the Jeol JMA-DA 5000 data system.

NMR spectroscopy.—NMR spectra were recorded at 27°C with a Bruker AM-500 spectrometer. Samples were dissolved in D_2O (99.996 atom%), and ^1H and ^{13}C chemical shifts were measured relative to internal acetone (δ 2.234) and internal CD_3OD (δ 49.30), respectively. One- and two-dimensional (2D) correlated spectroscopy (COSY), 2D *J*-resolved, 2D homonuclear Hartmann–Hahn (HOHAHA), and 2D nuclear Overhauser enhancement and exchange spectroscopy (NOESY), 2D heteronuclear correlated spectroscopy, and heteronuclear multiple bond correlation (HMBC) were performed with a Bruker AM-500 spectrometer using standard Bruker software. The normal mode probe was used for HMBC.

Paper chromatography.—Paper chromatography was done on Toyo filter paper No. 51 by the descending method with 12:3:5 butanol–AcOH–water as the irrigant. A radioactive feruloyl arabinobiose provided by Dr. Fry was detected by liquid scintillation counting and the nonradioactive feruloyl compound was visualized with alkaline silver nitrate[30].

RESULTS AND DISCUSSION

Characterization of compound **1**.—Glycosyl compositional analysis by GLC of the derivatized sugar showed that **1** consisted only of arabinose in the L absolute configuration. Ferulic acid was shown to be the only phenolic acid from **1** by alkali treatment and subsequent HPLC analysis. The FAB mass spectrum of **1** showed an intense peak at *m/z* 481 corresponding to the $(M + Na)^+$ quasimolecular ion.

TABLE I

Methylation analysis of compounds **1**, **2**, and **3** and their derived glycosyl alditols **4**, **5**, and **6**

Glycosyl residue	Methylated derivative	Linkage indicated	Mole percentages					
			1	2	3	4	5	6
Arabinofuranosyl	2, 3, 5	T-Ara	87		38	60		42
Arabinofuranosyl	2, 5	3-Ara	[a]		43			41
Arabinosyl	2, 3	5-Ara	13 [b]		19 [b]	[a]		[a]
Arabinitol	1, 2, 3, 4	5-Ara	[a]		[a]	40 [c]		17 [c]
Galactopyranosyl	2, 3, 4, 6	T-Gal		70			53	
Galactosyl	2, 3, 6	4-Gal		30			[a]	
Galactitol	1, 2, 3, 6	4-Gal		[a]			47	

[a] Not detected. [b] The reducing glycosyl residue was partly degraded during methylation. [c] The highly volatile derivative was probably lost.

Weak lines at m/z 459 and 497 corresponding respectively to the $(M + H)^+$ and $(M + K)^+$ quasimolecular ions were also observed. The negative-ion spectrum of **1** showed an intense peak at m/z 457 $[(M - H)^-]$, indicating that the molecular weight of **1** is 458. During positive-ion FABMS, fragment ions at m/z 309 and 177, generated by the loss of up to two pentose residues, were observed. These results suggest that **1** contains one ferulic acid group and two pentose residues. Methylation analysis (Table I) of **1** gave 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylarabinitol and 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol (derived from the nonreducing terminal arabinofuranosyl residue). Methylation analysis (Table I) of the glycosyl alditol **4** gave 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol and 5-*O*-acetyl-1,2,3,4-tetra-*O*-methylarabinitol, indicating that 5-linked arabinose was present at the reducing terminus of **1**. By comparison of the retention time and mass spectrum of the per-*O*-methylated glycosyl alditol **4** with those of the authentic compound, glycosyl alditol **4** was determined to be *O*-α-L-arabinofuranosyl-(1 → 5)-L-arabinitol.

Compound **1** was analyzed by ^{1}H NMR techniques to elucidate its primary structure (Fig. 1). Table II gives the complete assignments of the signals, which were made by COSY, 2D *J*-resolved spectroscopy, and HOHAHA spectroscopy. All chemical shifts and connectivities were clearly observable. A $J_{7,8}$ value of 16 Hz indicated that the ferulic acid occurs as the *trans* isomer. A comparison of the anomeric shifts and coupling constants for the arabinofuranosyl residue in **1** with those of methyl α- and β-L-arabinofuranoside[31] indicated that the nonreducing arabinofuranose is α-linked to the reducing arabinofuranose. Integration of the signals for anomeric protons for α-H-1, α-H-1, and β-H-1 gave the ratio 1 : 0.65 : 0.35. The nonreducing arabinofuranose was esterified at O-2, as indicated by the resonance of H-2 at 5.09 ppm, at lower field than that (4.04 ppm) for H-2 in methyl α-L-arabinofuranoside[31]. The reducing arabinofuranosyl residue was substituted at O-5; an H-5 of the α anomer was deshielded relative to the corresponding proton in free methyl arabinofuranoside[31] (0.11 ppm with respect to H-5α *pro-R*; 0.09 ppm with respect to H-5α *pro-S*). The NOESY spectrum of **1** showed an

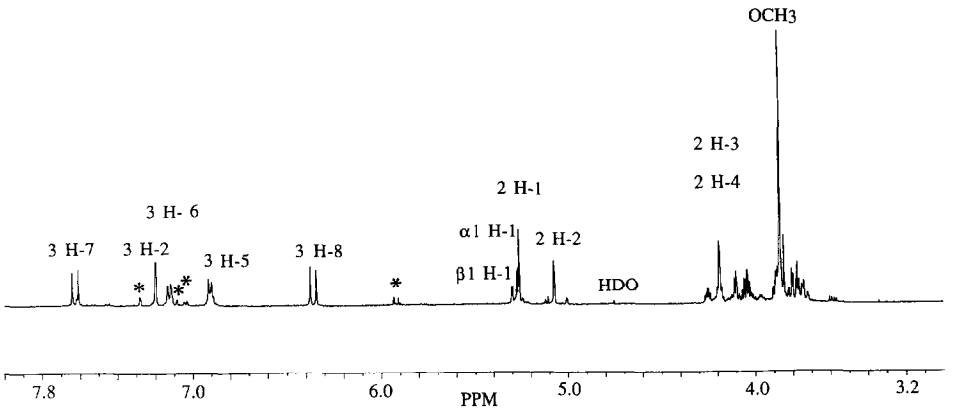


Fig. 1. 500-MHz ^{1}H NMR spectrum of **1**.

NOE between H-1 of nonreducing terminal arabinofuranose and H-5 of reducing α-L-arabinofuranose, confirming the sequence of **1**.

^{13}C NMR spectra of **1** corroborated the foregoing assignments (Table III). In the nonreducing arabinofuranosyl residue, C-2 (84.77 ppm) was deshielded by 2.62 ppm with respect to C-2 of α-L-arabinofuranose[20], indicating the linkage of ferulic acid at that position. The range of the chemical shift increments due to an ester linkage at CH_2OH and CHOH groups is −1.0 to +3.4 ppm. The resonance of the arabinofuranosyl C-1 at 106.79 ppm indicated that the nonreducing arabinofuranosyl residue is α-linked to the reducing arabinofuranose. For the α and β anomers of this residue, the signals for C-5 were shifted downfield by 5.5 and 5.0 ppm as compared with the corresponding signals from methyl α- and β-L-arabinofuranosides[32]. This indicated that the reducing arabinofuranose is 5-substituted. The location of the feruloyl group was clearly assigned by HMBC analysis. The feruloyl carbonyl–carbon signal at δ 169.49 was correlated with the proton signal at the site of esterification, H-2 (δ 5.09).

From these results, compound **1** has the structure *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1 → 5)-L-arabinofuranose, as shown in Fig. 2.

Comparison of **1** *with a previously isolated feruloyl arabinobiose.*—The feruloyl arabinobiose isolated by Fry[22] from walls of suspension-cultured spinach cells was characterized as *O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-(1 → 3)-L-arabinose. Compound **1** in this paper had the same R_f value on paper chromatography and the same retention time on HPLC as the previously isolated feruloyl arabinobiose. However, only a very limited amount of the feruloyl arabinobiose from suspension-cultured cells was available, so further comparison could not be performed.

Characterization of compound **2**.—Analysis by GLC of the derivatized sugar showed only galactose with the D absolute configuration. Ferulic acid was proved to be the only phenolic acid from **2** by alkali treatment and subsequent HPLC

TABLE II

^{1}H NMR data for compounds **1**, **2** and **3**

Compound and residue	Chemical shifts [a] (coupling constants [b])											
	H-1	H-2	H-3	H-4	H-5a	H-5b	H-5	H-6a	H-6b	H-6	H-7	H-8
1												
Reducing α-L-Ara*f*	5.28 (1.2)	4.05	4.05	4.26	3.79 (6.0)	3.89 (6.0–10.0)						
Reducing β-L-Ara*f*	5.31 (2.7)	4.11	4.11	3.98	3.81 (6.5)	3.88 (5.8–11.2)						
Nonreducing α-L-Ara*f*	5.27 (1.0)	5.09 (3.0)	4.20	4.20	3.76 (5.7)	3.87 (5.2–12.0)						
Ferulic acid		7.22					6.91 (7.8)			7.15 (7.8)	7.64 (16.0)	6.36 (16.0)
2												
Reducing α-D-Gal*p*	5.23 (3.1)	3.82 (10.4)	3.87 (3.5)	4.06 (1.0)			4.02	3.67 (4.5)	3.71 (7.2–11.9)			
Reducing β-D-Gal*p*	4.54 (7.7)	3.51 (10.0)	3.66 (3.4)	3.99 (1.1)			3.60	3.67 (4.4)	3.72 (6.7–10.3)			
Nonreducing β-D-Gal*p*	4.48 (7.5)	3.57 (9.6)	3.62 (3.5)	3.91 (1.0)			3.80	4.26 (3.9)	4.35 (8.2–11.4)			
Ferulic acid		7.12					6.66 (7.8)			7.06 (7.8)	7.53 (16.0)	6.28 (16.0)
3												
Reducing α-L-Ara*f*	5.24 (3.9)	4.00	4.05	4.22	3.75	3.85						
Reducing β-L-Ara*f*	5.27 (4.5)	4.06	4.10	3.90	3.78	3.84						
Internal α-L-Ara*f*	5.30 (1.3)	5.17 (2.0)	4.22 (5.5)	4.23	3.75 (2.4)	3.85 (5.6–11.1)						
Nonreducing α-L-Ara*f*	5.23 (2.2)	4.14 (2.5)	3.91 (6.0)	4.00	3.66 (2.4)	3.77 (5.9–12.4)						
Ferulic acid		7.22					6.80 (7.8)			7.13 (7.6)	7.64 (16.0)	6.37 (16.0)

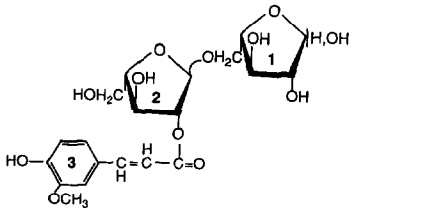
[a] In D_2O at 27°C. [b] Observed first-order splittings in Hz.

TABLE III

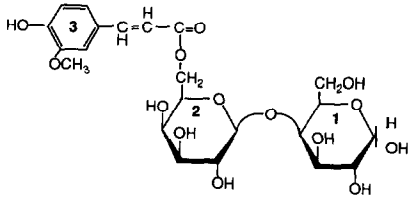
Assignments of signals in the ^{13}C NMR spectra of compounds **1**, **2**, and **3** [a]

Compound and residue	Chemical shifts (δ)									
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	OCH_3
1										
Reducing α-L-Ara*f*	102.41	82.61	77.13	82.61	67.91					
Reducing β-L-Ara*f*	96.58	77.29	75.71	80.64	69.29					
Nonreducing α-L-Ara*f*	106.79	84.77	76.36	85.67	62.18					
Furulic acid	127.64	112.88	149.00	149.60	116.94	124.78	148.16	114.58	169.49	57.18
2										
Reducing α-D-Gal*p*	94.49	71.10	71.61	81.36	72.00	63.39				
Reducing β-D-Gal*p*	95.58	74.50	75.39	80.29	76.54	63.22				
Nonreducing β-D-Gal*p*	106.74	73.46	74.89	70.78	74.85	65.83				
Ferulic acid	128.79	113.37	149.88	150.36	117.78	125.66	148.62	116.03	171.09	58.10
3										
Reducing α-L-Ara*f*	103.32	83.66 [b]	77.91	83.58 [b]	66.36					
Reducing β-L-Ara*f*	97.53	78.26	76.57	81.56	69.94					
Internal α-L-Ara*f*	107.63	83.58 [b]	82.27	85.95	62.82					
Nonreducing α-L-Ara*f*	109.19	83.42 [b]	78.66	86.15	63.35					
Ferulic acid	128.94	113.68	149.91	150.32	117.34	125.74	149.22	11592.	170.29	58.14

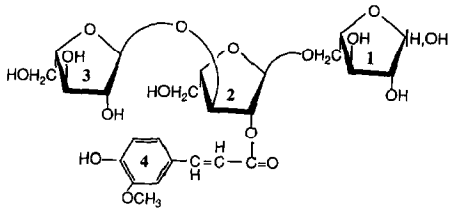
[a] In D_2O at 27°C. [b] These assignments may be interchanged.



1



2



3

Fig. 2. Structures compounds **1**, **2**, and **3**.

analysis. The positive-ion FABMS of **2** showed an intense peak at m/z 541 corresponding to the $(M + Na)^+$ quasimolecular ion of **2**. Weak ions at m/z 519 and 557 corresponding to $(M + H)^+$ and $(M + K)^+$, respectively, were also observed. The negative-ion spectrum of **2** showed an intense peak at m/z 517 $[(M - H)^+]$, indicating that the molecular weight of **2** is 518. During positive-ion FABMS, fragment ions at m/z 339 and 177, generated by the loss of up to two hexose residues, were observed. These results suggest that **2** contains one ferulic acid and two hexose residues. Methylation analysis (Table I) gave two derivatives, namely, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (derived from nonreducing terminal galactopyranose) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (derived from 4-linked galactopyranose). Methylation analysis of the reduced compound **5** (Table I) revealed terminal galactopyranosyl and 4-linked galactitol

residues, confirming that 4-linked galactose was present at the reducing terminus of **5**. By comparison of the retention time and mass spectrum of the per-*O*-methylated glycosyl alditol **5** with those of an authentic sample, glycosyl alditol **5** was identified as *O*-β-D-galactopyranosyl-(1 → 4)-D-galactitol.

^{1}H NMR and ^{13}C NMR spectroscopy were used to elucidate the primary structure of **2** (Tables II and III). Assignments were made as for the spectra of **1**. Examination of the signals of the ferulic acid protons in **2** again revealed a $J_{7,8}$ value of 16 Hz, diagnostic for the *trans* isomer. A comparison of the anomeric shifts and coupling constants for the nonreducing galactose residue with those of methyl α- and β-D-galactopyranoside[32], and methyl 6-*O*-feruloyl-β-D-galactopyranoside[33], indicated that this galactose is β-linked to the reducing galactose. The nonreducing galactose was esterified at O-6, as indicated by the downfield shift of the resonances of the H-6′s (4.26 and 4.35 ppm vs. ~ 3.6–3.7 ppm for galactose). The reducing galactose residue was substituted at O-4, as H-4 of the β anomer was deshielded relative to the corresponding proton in free galactose ($\Delta\delta$ 0.27 ppm). NOESY analysis confirmed the linkage position by showing an NOE between H-1 of the nonreducing galactose and H-4 of the β anomer of the reducing galactose.

In the ^{13}C NMR spectrum of **2**, C-6 of the nonreducing galactosyl residue was deshielded by 3.6 ppm, indicating that the linkage of ferulic acid is to O-6. HMBC analysis confirmed the linkage; the feruloyl carbonyl-carbon signal at δ 171.09 gave an HMBC peak correlated with the signals of the protons at the site of esterification, H-6a,b (δ 4.26 and 4.35). In the reducing galactose residue, the signals for C-4 (α and β anomers) were shifted 10 ppm downfield, as compared with the corresponding signals for galactose, indicating that the reducing galactose is 4-linked.

From these results, the structure of **2** is *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1 → 4)-D-galactopyranose (Fig. 2). The compound was earlier isolated by Fry[22] from walls of suspension-cultured spinach cells.

Characterization of compound **3**.—GLC analysis of alditol acetates from **3** gave only L-arabinose. Ferulic acid was proved to be the only phenolic acid from **3** by alkali treatment and subsequent HPLC analysis. The positive-ion FABMS of **3** showed an intense peak at *m/z* 613 corresponding to the $(M + Na)^+$ quasimolecular ion, and weak ions at *m/z* 591 $(M + H)^+$ and 629 $(M + K)^+$. The negative-ion spectrum of **3** showed an intense peak at *m/z* 589 $[(M - H)^-]$. During positive-ion FABMS, fragment ions at *m/z* 441 and 309, generated by the loss of up to two pentose residues, were observed. These results indicate that the molecular weight of **3** is 590, which is consistent with a molecule containing one ferulic acid and three pentose residues. Methylation analysis (Table I) of **3** gave [1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylarabinitol, (derived from 5-linked arabinofuranose), 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylarabinitol (derived from 3-linked arabinofuranose), and 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol (derived from terminal arabinofuranose). Methylation analysis (Table I) of diglycosyl alditol **6** gave 5-*O*-acetyl-1,2,3,4-tetra-

O-methylarabinitol, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylarabinitol, and 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, confirming the presence of 5-linked arabinose at the reducing terminus. EIMS of the reduced compound **6** gave ions at *m/z* 143 (aA_2, 100%), 175 (aA_1, 49), 192 ($aldJ_2$, 4.7), 238 ($aldJ_0$, 4.7), 303 (abA_2, 2.0, and 335 (abA_1, 2.0). The presence in the EI mass spectrum of an $aldJ_0$ fragment ion, and absence of an $aldJ_1$ fragment ion at *m/z* 252, indicated that the interior glycosyl residue was 3-linked[34].

Tables II and III show the complete assignments of the chemical shifts of **3**. The prior complete assignment of NMR spectra of **1** was useful in making these assignments. The ferulic acid occurs as the *trans* isomer. A comparison of the anomeric shifts and coupling constants with those of methyl α- and β-L-arbinofuranoside[31] indicated that the nonreducing terminal and internal arabinofuranoses were α-linked to adjacent arabinose residues. Integration of the signals for the anomeric protons of nonreducing terminal α-L-arabinofuranose, internal α-L-arabinofuranose, and reducing α-L- and β-L-arabinofuranose gave the ratio 1:1:0.65:0.35. The internal arabinofuranose residue was esterified at O-2, as indicated by the position of the H-2 signal at 5.17 ppm, well downfield of the H-2 signal for methyl α-L-arabinofuranoside (4.04 ppm)[31]. The internal arabinofuranosyl residue was 3-linked, as suggested by the presence of the H-3 signal at 4.22 ppm, rather than at 3.93 ppm as in α-L-arabinofuranose[31]. The NOESY spectrum showed NOEs between H-1 of the interior arabinofuranose and H-5 of the reducing α-L-arabinofuranose, and between H-1 of the nonreducing terminal arabinofuranose and H-3 of the interior arabinofuranose, confirming this sequence. In the ^{13}C NMR spectrum, C-3 of the interior arabinofuranosyl residue was deshielded by 4.42 ppm by comparison with the value for α-L-arabinofuranose (77.85 ppm)[20], showing that this residue is 3-linked. For the α and β anomers of the reducing residue, the signals for C-5 were shifted by 3.2 and 5.7 ppm downfield, respectively, as compared with the corresponding signals in methyl α- and β-arabinofuranoside[32], indicating linkage at O-5 for this residue. The presence of the nonreducing terminal and interior C-1 signals at 107.63 and 109.19 ppm, respectively, confirmed the α linkage of these residues to the adjacent residues. The location of the feruloyl group was confirmed by the HMBC analysis, where the feruloyl carbonyl-carbon signal at δ 170.29 correlated with the proton signal (5.17 ppm) at the site of esterification, H-2 of the interior residue.

These results characterize compound **3** as *O*-α-L-arabinofuranosyl-(1 → 3)-*O*-(2-*O*-*trans* feruloyl-α-L-arabinofuranosyl)-(1 → 5)-L-arabinofuranose (Fig. 2).

Contaminant in compounds **1**, **2**, *and* **3**.—Compounds **1**, **2**, and **3** were found to be accompanied by small amounts of isomers (Fig. 1). Analysis by GLC–MS of the ferulic acid liberated from **1**, **2**, and **3** by alkaki treatment showed *trans* and *cis* isomers in molar ratios of ~4:1, ~3:1, and ~3.5:1, respectively. The *cis* isomer might be an artifact formed from the *trans* isomer during isolation[20].

Approximately 4 mg of **1**, 1.5 mg of **2**, and 0.3 mg of **3** were purified from the enzymatic digest of 100 g of spinach cell walls. The quantities of **1**, **2**, and **3**

generated from cell walls of spinach leaves with Driselase accounted for 9.5% of the ferulic acid released by NaOH treatment.

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