JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Model Studies of Ferulate−Coniferyl Alcohol Cross-Product Formation in Primary Maize Walls: Implications for Lignification in Grasses

JOHN H. GRABBER,* JOHN RALPH, AND RONALD D. HATFIELD

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706

Ferulate and diferulates mediate cell wall cross-linking in grasses, but little is known about their cross-coupling reactions with monolignols and their role in lignin formation in primary cell walls. Feruloylated primary walls of maize were artificially lignified and then saponified to release ferulate and diferulates and their cross-products with coniferyl alcohol for analysis by GC-FID, GC−MS, and NMR spectroscopy. Ferulate and 5-5-coupled diferulate had a greater propensity than 8-coupled diferulates to copolymerize with coniferyl alcohol, forming mostly 4-*O*-β' and 8-β' and some 8-*O*-4′ and 8-5′ cross-coupled structures. Some 8-β' structures de-esterified from xylans, but these cross-links were subsequently replaced as 8-coupled diferulates formed stable cross-coupled structures with lignin. Based on the incorporation kinetics of ferulate and diferulates and the predicted growth of lignin, cross-products formed at the onset of lignification acted as nucleation sites for lignin polymerization.

KEYWORDS: ***Zea mays*; primary cell wall; ferulic acid; diferulic acids; coniferyl alcohol; cross-coupling; cross-product; cross-link; lignin; dehydrogenation polymer; nucleation site**

INTRODUCTION

Mild alkaline hydrolysis releases a variety of ferulate monomers and dimers from grass cell walls (**Figure 1**), which are esterified to the C5 hydroxyl of α-L-arabinosyl side chains on xylans. During cell-wall biosynthesis, feruloylated arabinoxylans are extensively cross-linked by the coupling of ferulate into diferulates and by the copolymerization of ferulate and diferulates with monolignols to form arabinoxylan−lignin complexes (*1*−*9*). It has been proposed that ferulates also act as initiation or nucleation sites for lignin formation in grasses—sites at which lignification begins in cell walls (*10*−*12*). The first structural evidence of this was obtained in HMBC NMR studies of lignin isolated from ^{13}C-labeled ryegrass where the initial coupling reactions of lignification involved ferulate coupled only to the β-position of monolignols (*7*). The lignins isolated from ryegrass were probably derived mainly from secondary cell walls (*13*), whereas lignification in grasses starts in the middle lamella and primary cell wall. As a result, we know little about the cross-coupling reactions of ferulate and its role as a nucleation site where lignification begins in cell walls.

As demonstrated in earlier studies (*5*, *9*), nonlignified primary walls isolated from maize cell suspensions are a valuable model system for studying the dimerization of ferulates and their subsequent incorporation into lignin. In the current study, this system was used to characterize the types of cross-products formed between ferulates and coniferyl alcohol and to further assess their role as nucleation sites for lignification. Coniferyl alcohol (**3**), with or without *p*-coumaryl and sinapyl alcohols, is the most abundant and consistently observed monolignol secreted into cell walls at the onset of lignification (*14*, *15*).

MATERIALS AND METHODS

Synthetic Lignification of Cell Walls. Cell suspensions (350 mL) of maize (*Zea mays* L. cv Black Mexican) were grown for 16 days with 33 mg of [1-^{13}C]-L-phenylalanine to produce [9-^{13}C]-labeled ferulate in cell walls (*5*). Cells collected at the early stationary growth phase were suspended in ice-cold Pipes buffer (pH 6.5; 10 mM) and ruptured by several explosive decompression cycles through a Parr nitrogen bomb pressurized at 1500 psi. Cell wall fragments were collected on a nylon mesh (20 μm) and washed with Pipes buffer followed by water to remove cytoplasmic debris. Cell walls were resuspended in four volumes of 200 mM $CaCl_2$ and stirred overnight at 2 °C. The following morning, cell walls were repeatedly pelleted by centrifugation (2000 × *g*) and resuspended in 200 mM $CaCl_2$ followed by water to remove loosely bound peroxidases. Cell walls (1.9 g dry weight, containing ca. 45 mg of ferulate) were then suspended in 120 mL of 25 mM Homopipes buffer (pH 5.25) with 400 units of glucose oxidase (Sigma). Glucose (150 mg) in 40 mL of Homopipes buffer was added to the stirred cell wall suspension during a 2-h period to generate (via glucose oxidase) ca. 3.7 equiv of H_2O_2 per mole of cell wall ferulate. Cell walls were pelleted and washed with Homopipes buffer several times by centrifugation. A subsample of cell walls (100 mg) was removed for analysis of ferulates and diferulates (*9*). Remaining cell walls (1.8 g) were resuspended in 120 mL of Homopipes buffer with glucose oxidase (400 units, Sigma G9010) and lignified by the addition of coniferyl alcohol (36 mg) and glucose (72 mg) in

* To whom correspondence should be addressed [telephone (608) 264 5820; fax (608) 264 5147; e-mail jgrabber@facstaff.wisc.edu.]

10.1021/jf0205312
Published on Web 09/14/2002

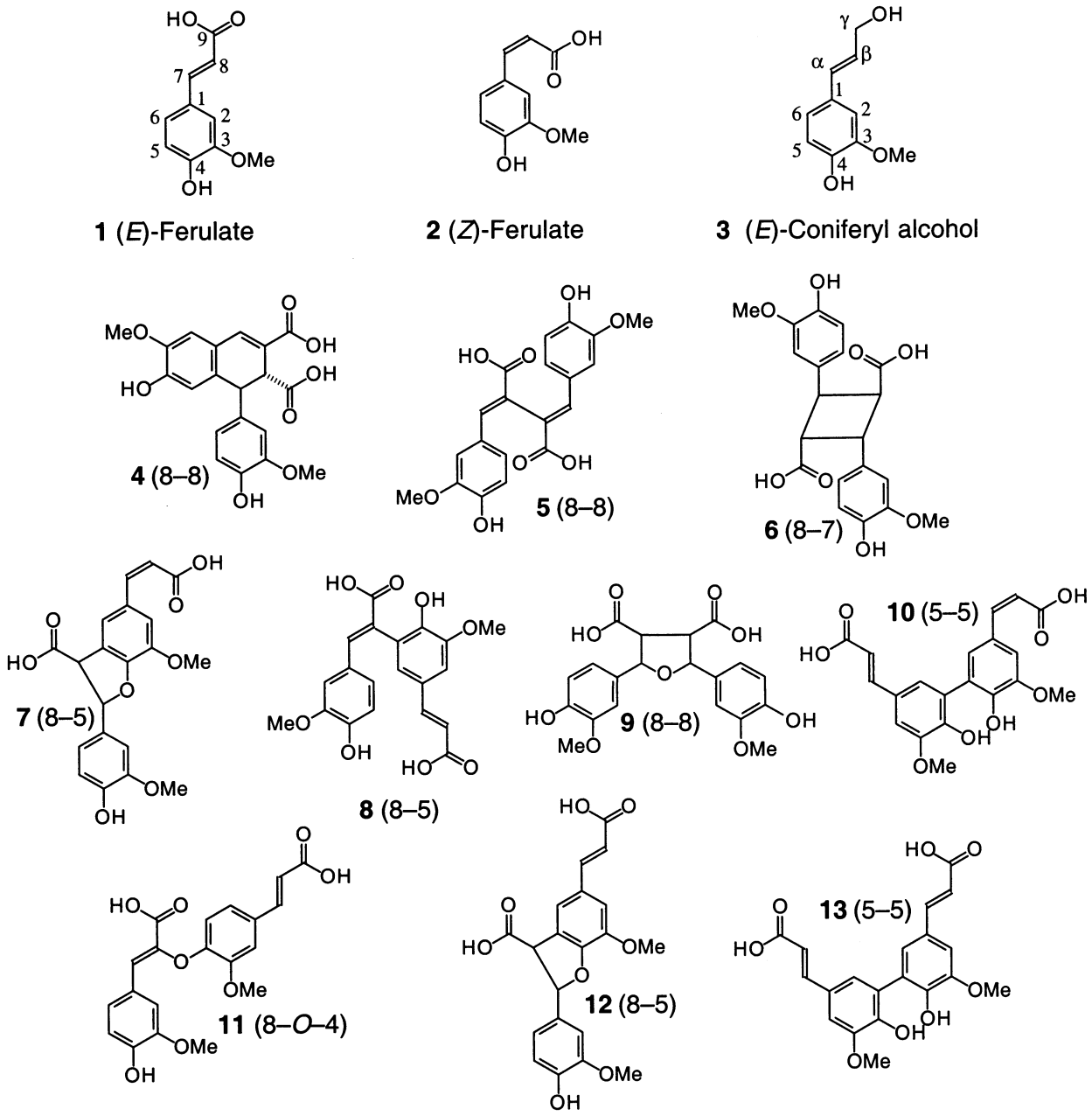

Figure 1. Ferulates (**1**, **2**) and diferulates (**4**–**13**) released from primary cell walls of maize by alkaline hydrolysis. During lignification, ferulate and diferulate xylan esters undergo oxidative coupling with coniferyl alcohol (**3**) and other monolignols.

40 mL of Homopipes buffer. Additions were made for a 1-h period, and then cell wall suspensions were stirred for an additional hour before being stored overnight at 2 °C with a few drops of mercaptoethanol to halt oxidative coupling.

To determine whether peroxidases leached out of cell walls during lignification, subsamples of cell wall suspensions were removed at the end of the coniferyl alcohol additions. After pelletization with a microcentrifuge (10 000 × *g*), the supernatant (400 μL) was mixed with Homopipes buffer (100 μL), ABTS (50 μL; 6 mM), and H_2O_2 (50 μL; 12 mM), and the reaction was monitored at 415 nm (*16*). Total peroxidase activity was determined by mixing 200-μL aliquots of the cell wall suspension with 19.8 mL of Homopipes buffer, 2.0 mL of ABTS, and 2.0 mL of H_2O_2. Periodically, subsamples of the reaction mixture were microcentrifuged and read at 415 nm.

The following morning, lignified cell walls were collected on glass-fiber filters (1.2 μm porosity), washed with water followed by acetone, and then air-dried. A subsample of the filtrate was evaporated in vacuo to remove acetone, acidified to pH 2.5 with HCl, and extracted with ethyl acetate to recover coniferyl alcohol and dehydrogenation products not bound to cell walls. Dried extracts were silylated and analyzed by GC-FID (*9*) and also dissolved in MeOH for scanning (250−400 nm) with a spectrophotometer. Similar methods were used to prepare 0.8 g of nonlignified cell walls with dimerized ferulates. The gradual lignification of cell walls over a wider range of coniferyl alcohol concentrations was described previously (*9*).

Isolation and Analysis of Cross-Products Formed within Cell Walls. Cell walls (1.6 g) lignified with coniferyl alcohol were hydrolyzed at room temperature for 16 h with 100 mL of aqueous 4 M NaOH to release ferulates, diferulates, and cross-products between these *p*-hydroxycinnamates and lignin. Alkaline-insoluble residues were collected by centrifugation (10 000 x *g* for 25 min) and then washed several times with 4 M NaOH followed by water. Supernatants were combined, acidified to pH 2 with HCl, saturated with NaCl, shaken with 1 volume of EtOAc, and centrifuged to aid in phase separation. Extractions were repeated a total of three times, and the combined EtOAc extracts were dehydrated with anhydrous $MgSO_4$ prior to being filtered and dried in vacuo (fraction 1). Similar methods were used to isolate ferulates and diferulates from nonlignified cell walls. Recovery of *p*-hydroxycinnamates and cross-products from cell walls lignified with coniferyl alcohol was lower than expected. Therefore, the $MgSO_4$

used to dry fraction 1 was dissolved in water, acidified, saturated with salt, and extracted with ethyl acetate to recover additional phenolics (fraction 2).

The 8-O-4′-ether model **26** was prepared from ethyl 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propionate by elimination of the 8-proton from the derived quinone methide followed by saponification (*17*). The 8-5′ models **27** and **28** were prepared from the ethyl ester of **12** (*18*) by hydrogenation (Pd−C, H_2, 1 atm, 95% EtOH) of the side chain followed by saponification. The 8-8-coupled diferulate **9** was identified here solely by its MS and NMR data. Diferulate **9** has now been independently synthesized, as will be reported elsewhere.

Diferulates **4**, **5**, **8**, **11**, **12**, and **13** and dimeric cross-products **16**, **18**, and **19** were prepared previously (*4*, *17*, *19*). A methanolic solution of **16** was exposed to UV light to produce the (*Z*)-isomer **14**. A new 8-*β*′ structure **15** was partially purified from fraction 1 by preparative TLC and characterized using the normal complement of 1D and 2D NMR experiments. Partial NMR data of **15** in acetone-d_6 (^{13}C chemical shift, ^{1}H chemical shift, assignment): 55.9, 2.75, F-8; 53.2, 3.05, G-*β*; 70.7, 3.94, G-*γ*1; 70.7, 4.25, G-*γ*2; 74.9, 4.63, G-α; 85.4, 4.82, F-7; 174.2, −, F-9.

Dried extracts from cell walls and dimeric cross-product models were silylated and analyzed by GC-FID and GC−MS. Cell walls (50 mg) were also hydrolyzed in aqueous 2 M NaOH and analyzed for ferulates, diferulates, and ferulate−lignin cross-products by GC-FID and GC−MS (*9*). Cell wall extracts (fractions 1 and 2) were surveyed by long-range correlative NMR spectroscopy (HMBC). Spectra were acquired using restricted acquisition in the ^{13}C dimension and a long-range coupling delay of 100 ms.

RESULTS AND DISCUSSION

Synthetic Lignification of Cell Walls. Nonlignified maize cell walls containing 22.8 mg/g of ferulate were pretreated with an excess of H_2O_2 to complete peroxidase-mediated dimerization of ferulates. These cell walls, containing 52 *μ*mol/g of ferulate monomers and 33 *μ*mol/g of diferulates, were then partially lignified with 110 *μ*mol/g of coniferyl alcohol for subsequent identification of ferulate− and diferulate−lignin cross-products by GC−MS and NMR spectroscopy. As with natural maize lignins, about one-half of the lignin in synthetically lignified maize walls is soluble in alkali (*20*). In the current study, cell walls were lignified only to a limited extent in the hope that most cross-products formed at the onset of lignification could be quantitatively released by alkali and recovered by extraction with ethyl acetate. To enhance NMR sensitivity for cross-products, 40% of cell wall ferulates were [9-^{13}C]-labeled by growing maize cell suspensions in the presence of [1-^{13}C]-labeled L-phenylalanine. Because peroxidases gradually leach out of cell walls during dehydrogenation polymer−cell wall (DHP−CW) complex formation (*21*), lignin precursors were added and mixed with cell walls for a 2-h period to help ensure that oxidative coupling occurred within the cell walls and not in the reaction medium. Assays of both the reaction medium and the cell walls indicated that >99% of the peroxidase activity remained within cell walls during the course of lignification. Thus, lignins were formed within the cell wall matrix and not in the reaction medium. Analysis of the filtrate collected after lignification indicated that about 95% of the coniferyl alcohol added to the reaction medium incorporated into cell walls. Even though very small amounts of dehydrogenation products were formed (∼2% of cell wall mass), they were not removed by washing with acetone, demonstrating that they were firmly bound to cell walls. Under these conditions, the only known means of attaching lignin to cell walls is via cross-coupling with ferulate and diferulate xylan esters. Coupling to other cell wall phenols, such as *p*-coumarate xylan esters or tyrosine in cell wall proteins, is inconsequential because their combined concentration in primary maize cell walls is less than 5% that of ferulate (*5*; J. H. Grabber, unpublished results). Other known mechanisms for binding lignin to cell walls, such as physical entrapment of cell wall polymers by lignin would reasonably occur only with substantially greater additions of monolignols. Another possible mechanism, the coupling of cell wall sugars or amino acids to lignin via quinone methide intermediates, requires hydrophobic conditions characteristic of only highly lignified cell walls (*22*−*25*).

Table 1. Concentration[a] and Incorporation[b] of Ferulate and Diferulates (DFA) into Lignin[c]

	nonlignified walls	lignified walls	incorporated into lignin		
	mg/g	mg/g	mg/g	%	*μ*mol/g
ferulate	10.15	3.54	6.61	65	34.1
8-8 DFA	2.22	1.56	0.65	30	1.7
8-5 DFA	6.44	4.66	1.78	28	4.6
8-*O*-4 DFA	1.68	0.87	0.80	48	2.1
5-5 DFA	2.29	0.36	1.93	84	5.0

[a] Acids released by room-temperature alkaline hydrolysis of walls. Walls were analyzed in duplicate with standard deviations averaging 5%. [b] Calculated by the difference in acids recovered from lignified and nonlignified walls. [c] Nonlignified primary walls of maize were treated with dilute H_2O_2 to predimerize the ferulates and then lignified by adding 20 mg/g of coniferyl alcohol.

Characterization of Cross-Products by GC-FID and GC−MS. Oxidative coupling of ferulate and diferulate esters with coniferyl alcohol prevents their release upon saponification of cell walls. Therefore, their incorporation into lignin can be estimated as the difference in alkali-labile acids recovered from nonlignified and lignified walls. About 50% of the total ferulate in cell walls (11.8 mg/g, 47 *μ*mol) copolymerized with coniferyl alcohol (**Table 1**), as revealed by alkaline hydrolysis and GC-FID analysis of cell walls collected before and after lignification. As noted previously (*9*), ferulate and 5-5-coupled diferulate had the greatest propensity to copolymerize with coniferyl alcohol, accounting for 82% of the cross-coupled structures formed (**Table 1**). Previous NMR studies have indicated that ferulate and 5-5-coupled diferulate form similar types of cross-coupled structures with coniferyl alcohol (*8*).

Alkaline hydrolysis and GC−MS analysis of saponification extracts and authentic compounds revealed that ferulate monomers were coupled to coniferyl alcohol by 8-*β*′, 5-*β*′, and 4-*O*-*β*′ linkages (**Figures 2** and **3**). Although 8-*β*′ couplings are prominent in HMBC NMR spectra of DHPs and ryegrass lignin (*3*, *7*), we did not expect to find 8-*β*′ structure **18** in alkaline hydrolysates. Rather, these structures should have become de-esterified from xylans during cyclization (lactonization) and rearomatization of the intermediate quinone methide and then released into the reaction medium. Indeed, EtOAc extraction of the reaction medium revealed that roughly 20% of 8-*β*′ cross-products formed were released as **18**. De-esterified 8-*β*′ dimers can become reincorporated into lignin via oxidative coupling at their 4-*O*- or 5-positions, but they would not then be released as dimers by saponification. The observed 8-*β*′ product **18** was probably present in the cell walls as **15** (see the following discussion), with the lactone in the pinoresinolide structure **18** being formed during saponification and acidification. The new 8-*β*′ dimer **15** was identified by its mass spectral fragmentation pattern (**Table 2**) and, as described later, by NMR analysis of the isolated compound. This 8-*β*′ product probably represents an intermediate structure in cell walls and was apparently released by saponification and (partially) recovered following

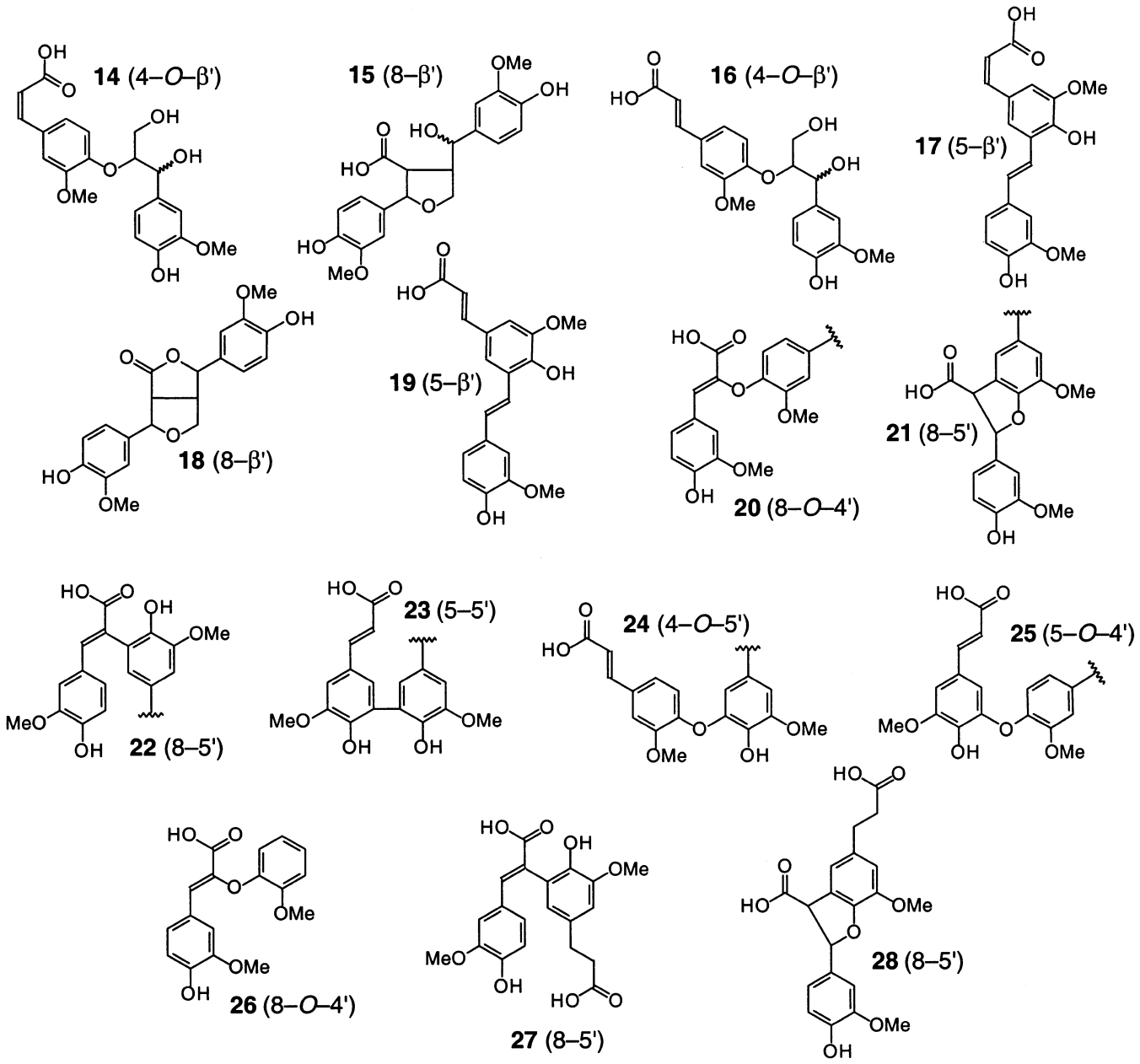

Figure 2. Potential cross-products between ferulate and coniferyl alcohol released by saponification of cell walls (**14**–**25**). Diferulates can form similar types of cross-products with coniferyl alcohol. Several cross-product models (**26**–**28**) were prepared for NMR studies.

acidification; again, it is assumed that the pinoresinolide **18** recovered from cell walls also derives from this structure.

Our data suggest that cross-products **15** and **18** were formed in roughly a 4:1 ratio in synthetically lignified maize cell walls. Recently, cross-product **15** also was detected during GC−MS analysis of alkaline hydrolysates from maize bran (unpublished results), confirming that it is a naturally occurring cross-product in grass cell walls. The 8-β' dimer **15** might have been previously detected in straw samples but incorrectly identified as a ferulate−coniferyl alcohol ether (*6*). In other studies with isolated ryegrass lignins and dehydrogenation polymers (*3*, *7*), 8-β' coupling of ferulate with coniferyl alcohol formed only cross-product **18**. Based on these observations, 8-β' coupling can, under some conditions, lead to the formation of stable cross-product **15** in addition to structure **18**, which undergoes de-esterification from arabinoxylans.

The 5-β' dimer **19** is frequently observed as a decarboxylation product of diferulate **8** (*4*). However, extensive decarboxylation

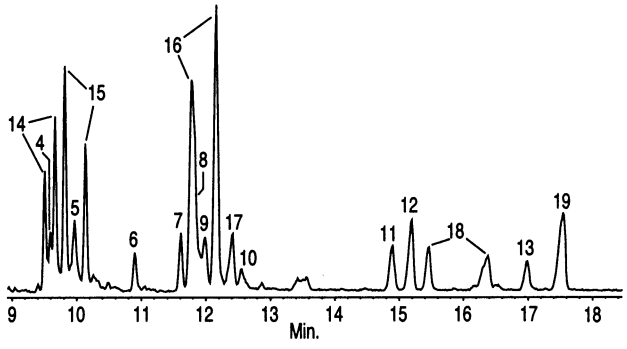

Figure 3. GC–MS total ion chromatogram of diferulates and ferulate–coniferyl alcohol dimers recovered following room-temperature alkaline hydrolysis of partially lignified primary cell walls from maize suspension cultures.

Table 2. Abbreviated Electron Ionization Mass Spectra of Silylated Cross-Products between Ferulate and Coniferyl Alcohol

structure	M^+ (*m*/*z* %)	other ions (*m*/*z* %)
14, **16** (4-*O*-*β*′)	678 (1)	663 (1), 485 (1), 412 (1), 351 (2), 338 (2), 323 (6), 297 (100), 265 (2), 235 (1), 223 (4), 209 (9), 179 (2), 147 (5), 103 (6), 73 (81)
15 (8-*β*′)	678 (1)	588 (1) 498 (3), 471 (3), 365 (3), 338 (4), 323 (2), 297 (66), 275 (4), 235 (15), 223 (21), 222 (16), 209 (10), 194 (6), 179 (4), 166 (3), 147 (5), 129 (5), 73 (100)
18 (8-*β*′)	516 (25)	501 (6), 403 (9), 263 (6), 243 (6), 235 (33), 223 (48), 209 (25), 194 (21), 193 (23), 179 (23), 131 (20), 73 (100)
17, **19** (5-*β*′)	558 (68)	543 (7), 528 (10), 484 (3), 380 (5), 306 (4), 256 (9), 223 (17), 193 (7), 179 (4), 147 (3), 89 (7), 73 (100)

of **8** usually occurs only under high-temperature (100−170 °C) alkaline hydrolysis (unpublished results). Although oxidative coupling of diferulates to lignin prevents their release as dimers during mild alkaline hydrolysis of cell walls, the quantity of **19** released tends to increase after the cell walls are synthetically lignified with coniferyl alcohol. Therefore, most **19** recovered following mild alkaline hydrolysis of lignified cell walls probably originates from 5-*β*′-coupled dimers of ferulate and coniferyl alcohol, which readily lose formaldehyde in a reverse aldol reaction. A (*Z*)-5-*β*′ dimer **17** was also tentatively identified by MS; it could be formed either by coupling of (*Z*)-ferulate to coniferyl alcohol or by photochemical isomerization from the (*E*)-isomer of **19**. The 4-*O*-*β*′ dimers **14** and **16** were previously observed in extracts from saponified wheat and oat straws (*6*). Mass spectra of 4-*O*-*β*′-, 8-*β*′-, and 5-*β*′-coupled cross-dimers are shown in **Table 2**. Mass spectra indicative of 8-*O*-4′-, 8-5′-, 5-5′-, 4-*O*-5′-, and 5-*O*-4′-crossed-dimers (**20**−**25**) were not observed in saponification extracts, providing evidence that dimeric cross-products were formed by the coupling of ferulate to only the *β*-position of coniferyl alcohol.

Based on relative GC−MS peak areas, 4-*O*-*β*′-, 8-*β*′-, and 5-*β*′-structures comprised an average of 52, 35, and 13%, respectively, of the dimeric cross-products recovered after alkaline hydrolysis. These proportions do not, however, include 8-*β*′ dimers released from cell walls into the reaction medium, in which case the abundance of 8-*β*′ dimers would rival that of 4-*O*-*β*′ dimers. If these proportions are representative of grass cell walls, then substantial amounts of ferulate-mediated cross-links between xylans and lignin could be lost via de-esterification if **18** were the major product of 8-*β*′ coupling. The 5-5-coupled diferulate can also become de-esterified by this mechanism, but complete loss of xylan−lignin cross-linking occurs only if both side chains form 8-*β*′ lactone structures (**18**) with monolignols. The role of 5-5-coupled diferulates as cell wall cross-linking agents is also diminished by the possibility that they are formed by intramolecular coupling within arabinoxylan chains (*26*). Because 8-8-, 8-5-, and 8-*O*-4-coupled diferulates do not undergo 8-*β*′-coupling and de-esterification, they assume a greater role in cell wall stiffening, forming stable cross-links between xylan chains and between xylans and lignin.

The preponderance of 4-*O*-*β*′- and 8-*β*′- over 5-*β*′-cross-coupled dimers in this study is in stark contrast to the predominance of 5-*β*′-coupled dimers observed from homocoupling of coniferyl alcohol or 5-8-coupled dimers observed from homocoupling of ferulate (*9*, *27*). These observations must be tempered by the fact that the recoverable dimers of ferulate and coniferyl alcohol represent only a portion of the cross-products formed in cell walls. Indeed, based on mass balance calculations, each molecule of ferulate or diferulate, on average, coupled with just over two molecules of coniferyl alcohol, yielding complexes with an average degree of polymerization (DP) of 3.2.

Characterization of Cross-Products by NMR Spectroscopy. Cell walls were hydrolyzed with aqueous NaOH and extracted with ethyl acetate to isolate cross-products for analysis by NMR spectroscopy. Based on the concentrations of ferulate and diferulates and the amount of coniferyl alcohol incorporated, we expected to recover a total of about 41 mg/g of ferulate, diferulates, and cross-products from cell walls, but only 21 mg/g were recovered by ethyl acetate extraction (fraction 1). An additional 12 mg/g (fraction 2) was recovered from the $MgSO_4$ used to dehydrate the EtOAc extract of fraction 1, increasing the recovery to about 80%. Acetyl bromide analysis of alkaline-insoluble residues indicated that phenolics were quantitatively released from lignified cell walls into the alkaline hydrolysate. Ultraviolet scans revealed that the balance of the phenolics remained in the aqueous phase in what appeared to be dehydrogenation products of coniferyl alcohol with little (if any) ferulate or diferulates.

GC and NMR spectroscopy indicated that fraction 1 contained ferulate monomers, most of the diferulates, and cross-products of low molecular weight. Fraction 2 contained some diferulates and higher-molecular-weight cross-products. HMBC NMR spectra of the fractions (**Figure 4**) contained correlations consistent with diferulates having 8-5- (**7**, **8**, **12**), 8-*O*-4- (**11**), and 8-8- (**5**, **9**) couplings but surprisingly correlations for the 8-8-coupled diferulate **4** were missing. Correlations for **4** were also missing from the alkaline hydrolysates isolated from nonlignified maize cell walls. In GC analyses, isomer **4** routinely accounts for about one-half of the 8-8-coupled diferulates recovered following alkaline hydrolysis from primary maize cell walls. Couplings involving the 4-*O*- and 5-positions of ferulate moieties (e.g., 5-5-coupled diferulate) could not be distinguished from ferulate monomers in NMR spectra. HMBC spectra contained peaks diagnostic of 8-*β*′-structure **18** (*3*, *8*) and correlations suggestive of the new 8-*β*′-product **15**. Further structural evidence for compound **15** was required to support the structure suggested by MS and NMR data. Preparative TLC of fraction 1 using EtOAc:$CHCl_3$:HCOOH 6:3:1 allowed the separation of fractions containing both 4-O-*β*′ isomers (**14** and **16**) and compound **15**. Further preparative TLC of this isolate in (6:5:1) eluant separated small amounts (<1 mg) of compound **15** from the 4-O-*β*′-isomers (~6 mg). Although not totally pure, the fraction containing compound **15** was characterized, and the side-chain assignments authenticated using the normal complement of 1D and 2D NMR experiments. Proof that the two side chains formed a contiguous proton-coupling network was most strikingly evident in the TOCSY experiment (100-ms spin-lock period) where, for example, proton G-*β* correlated with all of the ferulate [F-7 (weakly), F-8] and guaiacyl [G-α, G-*γ*'s] side-chain protons. Proof of the 8-*β*-linkage was readily evident from the long-range ^{13}C-^{1}H correlation experiment (HMBC), which correlates a carbon with protons 2−3 bonds away; the (enriched) F-9 carbonyl carbon correlated with proton F-7 and both protons F-8 and G-*β*. The stereochemistry could not be reliably assigned from these data. A trace of what was possibly another isomer was barely detectable in the ^{1}H NMR spectroscopy.

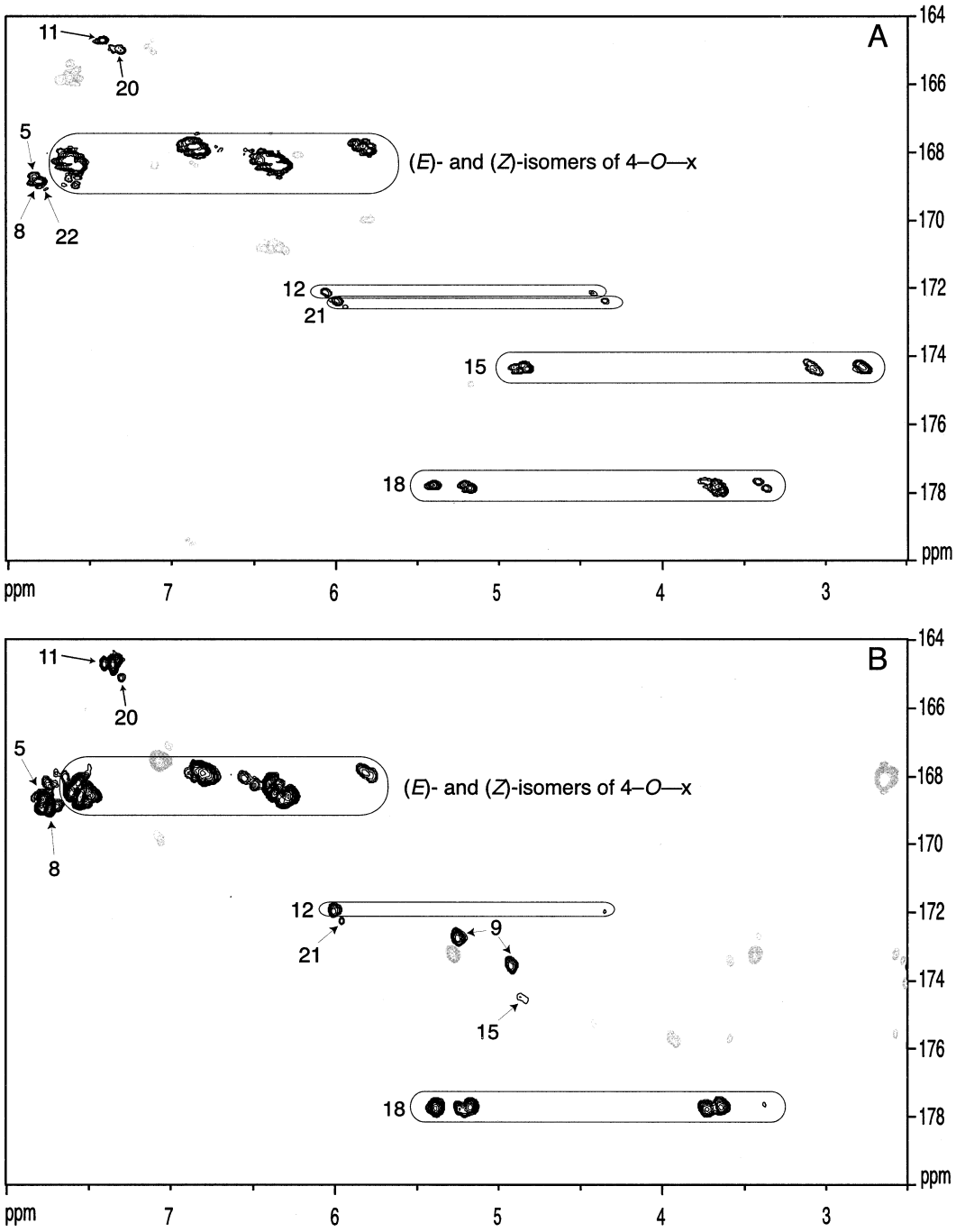

Figure 4. Partial long-range C–H correlation (HMBC) spectra of ferulate, diferulates, and their cross-products recovered following room-temperature alkaline hydrolysis of partially lignified primary cell walls from maize suspension cultures: (A) fraction 1 recovered by EtOAc extraction, (B) fraction 2 recovered from the $MgSO_4$ used to dehydrate the EtOAc extract.

Several minor peaks in the HMBC spectra (**Figure 4**) were assigned to 8-*O*-4′- (**20**) and 8-5′- (**21** and **22**) coupled cross-products due to their close proximity to analogous 8-*O*-4- (**11**) and 8-5′- (**8** and **12**) coupled diferulates and cross-product models (**26**, **27**, and **28**). The presence of 8-*O*-4′- and 8-5′-coupled cross-products indicates that a small proportion of ferulate or 5-5-coupled diferulate had coupled with adjacent complexes or possibly with dimers or oligomers of coniferyl alcohol formed in the apoplasic space. This is because coniferyl alcohol dimers or oligomers couple at their *O*-4′- and -5′-positions to form 8-*O*-4′- and 8-5′- (and probably 4-*O*-5′- and 5-5′-) coupled cross-products. In contrast, coniferyl alcohol monomers overwhelmingly favor coupling at its β-position. The presence of 4-*O*-5′- and 5-5′-coupled cross-products could not be confirmed by NMR spectroscopy because ^{13}C-labeling of the ferulate side chain does not provide adequate resolution of products involving coupling at the 4-*O*- and 5-positions.

Potential Role of Cross-Products in Lignin Formation. As noted above, only one-half of the total ferulate in cell walls copolymerized with one equivalent of coniferyl alcohol, yielding complexes with an average size of trimers. This nonuniform cross-coupling would suggest that coniferyl alcohol polymerization became localized in certain regions of the cell wall, with ferulate possibly acting as a nucleation site for lignin formation. To explore this possibility further, we reanalyzed data from a recent study examining the incorporation of various ferulate and diferulate isomers into lignin (*9*). For the current analysis, ferulate and diferulate isomers were combined into two groups,

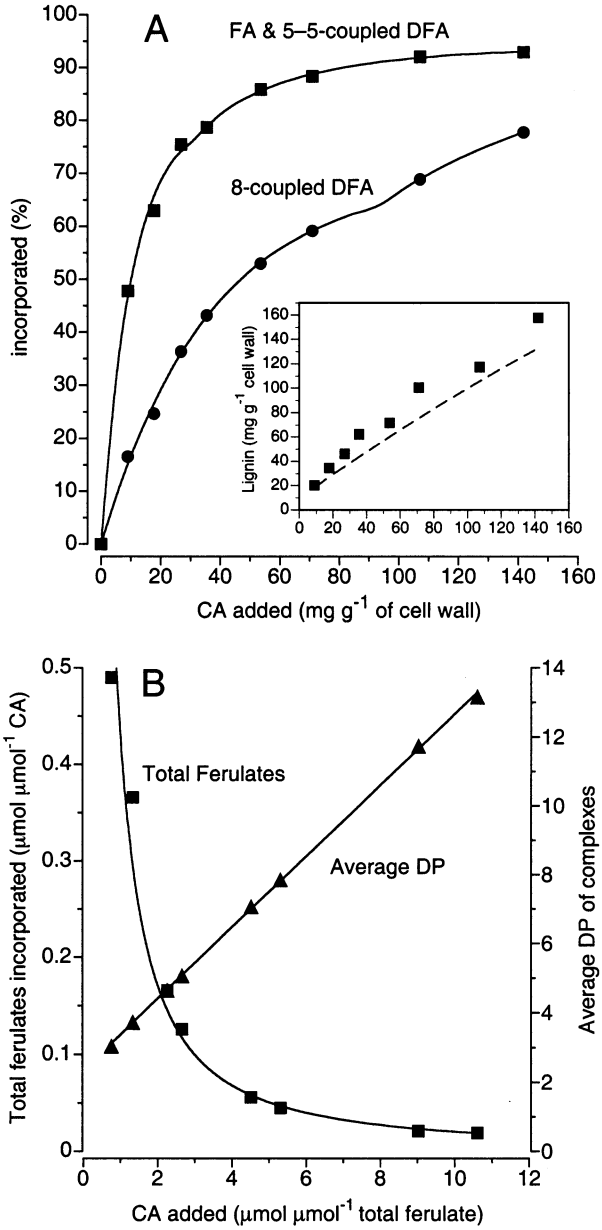

Figure 5. (A) Incorporation of ferulate (FA) and 5-5-coupled diferulate (DFA) and of 8-coupled DFA during lignification of primary cell walls of maize with coniferyl alcohol (CA). The inset shows the Klason lignin content of cell walls compared with the predicted lignin content of cell walls with complete incorporation of CA (dashed line). (B) Comparison of the incorporation of total ferulates (ferulate monomers plus dimers) with the apparent degree of polymerization (DP) of complexes formed between total ferulates and CA.

ferulate/5-5-coupled diferulate and 8-coupled diferulates, based on their kinetics of cross-product formation with coniferyl alcohol. Prior to lignification, cell walls contained 9.6 mg/g (44 mmol) of ferulate/5-5-coupled diferulate and 10.1 mg/g (26 mmol) of 8-coupled diferulates. During lignification, coniferyl alcohol was efficiently polymerized into cell walls, ultimately forming cross-coupled structures with, on average, 95% of the ferulates and diferulates in cell walls (**Figure 5A**; **Table 3**). As noted earlier (*9*), ferulate and diferulates incorporated as at least two pools, with the large pool (A1) incorporating earlier and more rapidly than the small pool (A2). Incorporation of the A1 pool for ferulate/5-5-coupled diferulate was 3.5-fold faster than that for 8-coupled diferulates. This confirms our earlier observation that cross-coupling reactions at the earliest stages of

Table 3. Kinetic Parameters Describing the Relationship between the Incorporation of Ferulate (FA) and Diferulates (DFA) into Lignin and the Quantity of Coniferyl Alcohol Polymerized into Maize Cell Walls[a]

constituent	L1	*k*1	A1	L2	*k*2	A2	fit
FA and 5-5-coupled DFA	0.0	0.097	80.1	31.7	0.026	13.8	0.998
8-coupled DFA	0.0	0.027	69.1	92.8	0.010	26.0	0.999

[a] Data were fitted to the dual-pool exponential model $y = A1[1 - e^{-k1(CA - L1 - |L1 - CA|)}] + A2[1 - e^{-k2(CA - L2 - |L2 - CA|)}]$, where CA = coniferyl alcohol added to cell walls (mg g^{-1}), A1 = rapidly incorporated pool (%), *k*1 = rate of A1 incorporation, L1 = CA added prior to A1 incorporation, A2 = slowly incorporated pool (%), *k*2 = rate of A2 incorporation, and L2 = CA added prior to A2 incorporation. Fit was calculated as 1 – (residual sum of squares/total degree-of-freedom-corrected sum of squares).

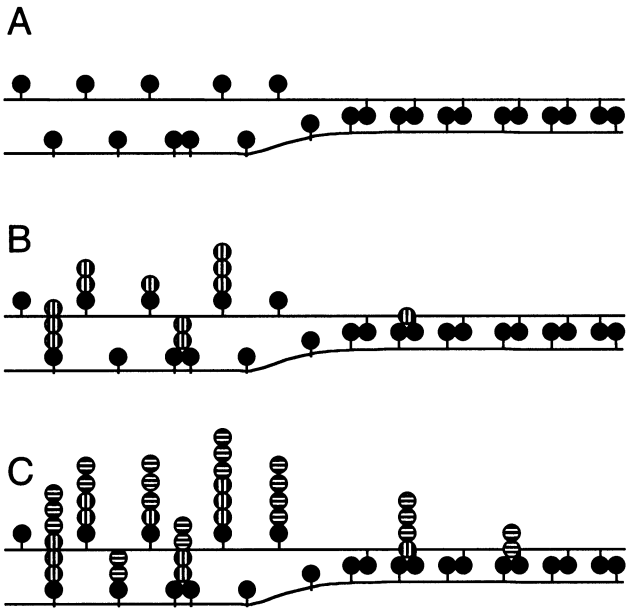

Figure 6. Schematic drawing illustrating the role of cross-products between ferulate/5-5-coupled diferulate and coniferyl alcohol as nucleation sites for continued lignin formation. (A) Nonlignified cell walls with xylans substituted by ferulate and cross-linked with diferulates (solid circles). (B) At the onset of lignification, coniferyl alcohol (circles with vertical stripes) couples mainly with ferulate monomers, 5-5-coupled diferulate, and their cross-products. (C) As lignification continues, additional coniferyl alcohol (circles with horizontal stripes) preferentially couples with existing cross-products, even though unincorporated ferulate monomers, 5-5-coupled diferulate, and particularly 8-coupled diferulates predominate as potential coupling sites.

lignification overwhelmingly involve ferulate and 5-5-coupled diferulate. During lignification, the average DP of complexes increased linearly even as the incorporation of total ferulates per unit of coniferyl alcohol declined dramatically (**Figure 5B**). As illustrated in **Figure 6**, these data suggest that the initial cross-products formed between coniferyl alcohol and ferulate/5-5-coupled diferulate act as preferred sites for continued lignin growth. Preferential growth of lignin occurred at these sites even though more numerous nonincorporated ferulates and diferulates were available for cross-coupling with coniferyl alcohol. Therefore, cross-products formed at the onset of lignification appear to act as nucleation sites for further lignin formation. This nucleation behavior appears to be consistent with the observation, albeit in pine, of lignin growth from point sources in the cell wall (*28*). As lignification continues, a portion of the cross-links formed by ferulate and 5-5-coupled diferulate

are lost due to de-esterification of 8-β-coupled cross-products. These cross-links, however, are replaced by stable cross-links formed by the subsequent incorporation of 8-coupled diferulates into the growing lignin polymer.

In plants, the types of cross-products formed between ferulates and monolignols might depend on whether lignins are formed under "bulk" or "end-wise" polymerization conditions (*29*). Lignins formed by the rapid addition and bulk polymerization of monolignols would favor coupling of ferulates to dilignols (or lignin oligomers) by 8-5′, 8-*O*-4′, and 5-5′ linkages. Under these conditions, ferulates and diferulates can act more as sites where preformed lignins are anchored within cell walls than as sites where lignification is initiated. In contrast, lignins formed by gradual diffusion and end-wise polymerization of monolignols—as in our experiments—would favor coupling of ferulates to monolignols by 4-*O*-β′, 8-β′, and 5-β′ linkages**.** In this case, ferulates and diferulates (or their initial cross-products) would appear to act as nucleation sites for continued lignin formation. Unfortunately, it is not known whether lignification in grasses starts as a bulk or end-wise type polymerization of monolignols. Although chemical, structural, and temporal aspects of lignification in grasses have been studied, little is known about the rate of lignin formation at the cellular level, particularly at the primary cell wall/middle lamella where lignification is thought to be initiated.

Overall, the results of this and previous studies (*5*, *9*) indicate that ferulate and diferulates readily form cross-products with monolignols. Ferulate and 5-5-coupled diferulate (or their initial cross-products) act primarily as nucleation sites for lignification, while 8-coupled diferulates serve as the major cross-linking agents within lignified cell walls. Our previous HMBC NMR experiments with ^{13}C-labeled ryegrass lignin revealed that ferulates coupled exclusively to the β-position of monolignols (*7*), providing the first structural evidence that ferulates can act as initiation or nucleation sites for lignification. The lignin used in that study was a dioxane/water-soluble fraction isolated from ball-milled and cellulase-treated cell walls, and it represented only about 25% of the lignin in ryegrass. Lignins isolated in this manner are probably derived from secondary cell walls where end-wise polymerization can occur, favoring coupling of ferulate to the β-position of monolignols. Our group is conducting additional model studies with maize cell walls and isolating additional lignin from the ^{13}C -labeled ryegrass, to more definitively establish whether ferulates or their cross-products act as nucleation sites for lignification in grasses.

LITERATURE CITED

(1) Ishii, T. Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls. *Carbohydr. Res.* **1991**, *219*, 15−22.

(2) Hartley, R. D.; Morrison, W. H., III. Monomeric and dimeric phenolic acids released from cell walls of grasses by sequential treatment with sodium hydroxide. *J. Sci. Food Agric.* **1991**, *55*, 365−375.

(3) Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. Lignin−feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961−2969.

(4) Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485−3498.

(5) Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **1995**, *40*, 1077−1082.

(6) Jacquet, G.; Pollet, B.; Lapierre, C.; Mhamdi, F.; Rolando, C. New ether-linked ferulic acid−coniferyl alcohol dimers identified in grass straws. *J. Agric. Food Chem.* **1995**, *43*, 2746−2751.

(7) Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin−ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995**, *275*, 167−178.

(8) Quideau, S.; Ralph, J. Lignin−ferulate cross-links in grasses. Part 4. Incorporation of 5-5-coupled diferulate into lignin. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2351−2358.

(9) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. *J. Agric. Food Chem.* **2000**, *48*, 6106−6113.

(10) Fry, S. C. Primary cell wall metabolism. In *Oxford Surveys of Plant Molecular and Cell Biology*; Milflin, B. J., Ed.; Oxford University Press: New York, 1985; pp 1−42.

(11) Yamamoto, E.; Bokelman, G. H.; Lewis, N. G. Phenylpropanoid metabolism in cell walls. An overview. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation*; Lewis, N. G., Paice, M. G., Eds.; American Chemical Society: Washington, DC, 1989; pp 68−88.

(12) Ralph, J.; Helm, R. F. Lignin/hydroxycinnamic acid/polysaccharide complexes: Synthetic models for regiochemical characterization. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 201−246.

(13) Whiting, P.; Goring, D. A. I. The morphological origin of milled wood lignin. *Svensk Papperstidn.* **1981**, *84*, R120−R122.

(14) Terashima, N.; Fukushima, K.; He, L.-F.; Takabe, K. Comprehensive model of the lignified plant cell wall. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 247−270.

(15) Joseleau, J. P.; Ruel, K. Study of lignification by noninvasive techniques in growing maize internodes. *Plant Physiol.* **1997**, *114*, 1123−1133.

(16) Makinen, K.; Terovuo, J. Observations on the use of guaiacol and 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) as peroxidase substrates. *Anal. Biochem.* **1982**, *126*, 100−108.

(17) Ralph, J.; Helm, R. F.; Quideau, S. Lignin−feruloyl ester cross-links in grasses. Part 2. Model compound syntheses. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2971−2980.

(18) Ralph, J.; Garcia-Conesa, M. T.; Williamson, G. Simple preparation of 8-5-coupled diferulate. *J. Agric. Food Chem.* **1998**, *46*, 2531−2532.

(19) Helm, R. F.; Ralph, J. Lignin−hydroxycinnamyl model compounds related to forage cell wall structure. 1. Ether-linked structures. *J. Agric. Food Chem.* **1992**, *40*, 2167−2175.

(20) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Severe inhibition of maize wall degradation by synthetic lignins formed with coniferaldehyde. *J. Sci. Food Agric.* **1998**, *78*, 81−87.

(21) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. Dehydrogenation Polymer−Cell Wall Complexes as a Model for Lignified Grass Walls. *J. Agric. Food Chem.* **1996**, *44*, 1453−1459.

(22) Ralph, J.; Young, R. A. Stereochemical aspects of addition reactions involving lignin model quinone methides. *J. Wood Chem. Technol.* **1983**, *3*, 161−181.

(23) Sipila, J.; Brunow, G. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Pt. 4: The reactions of a β-O-4 type quinone methide with carboxylic acids in the presence of phenols. The formation and stability of benzyl esters between lignin and carbohydrates. *Holzforschung* **1991**, *45*, 8−14.

(24) Toikka, M.; Sipila, J.; Teleman, A.; Brunow, G. Lignin−carbohydrate model compounds. Formation of lignin−methyl arabinoside and lignin−methyl galactoside benzyl ethers via quinone methide intermediates. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3813−3818.

(25) Toikka, M.; Brunow, G. Lignin−carbohydrate model compounds. Reactivity of methyl 3-O-(α-L-arabinofuranosyl)-*β*-D-xylopyranoside and methyl *β*-D-xylopyranoside towards a *β*-O-4-quinone methide. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1877−1883.

(26) Hatfield, R. D.; Ralph, J. Modeling the feasibility of intramolecular dehydrodiferulate formation in grass walls. *J. Sci. Food Agric.* **1999**, *79*, 425−427.

(27) Syrjanen, K.; Brunow, G. Regioselectivity in lignin biosynthesis. The influence of dimerization and cross-coupling. *J. Chem. Soc., Perkin Trans. 1* **2000**, 183−187.

(28) Donaldson, L. A. Mechanical constraints on lignin deposition during lignification. *Wood Sci. Technol.* **1994**, *28*, 111−118.

(29) Quideau, S.; Ralph, J. A biomimetic route to lignin model compounds *via* silver (I) oxide oxidation. 1. Synthesis of dilignols and non-cyclic benzyl aryl ethers. *Holzforschung* **1994**, *48*, 12−22.

Received for review May 7, 2002. Revised manuscript received July 29, 2002. Accepted July 30, 2002. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that might also be suitable.

JF0205312