Detection and Determination of *p*-Coumaroylated Units in Lignins

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The **d**erivatization **f**ollowed by **r**eductive **c**leavage (DFRC) method cleaves α- and β-ethers in lignins but leaves lignin γ-esters intact. When applied to grasses, which contain *p*-coumarate esters on their lignins, esterified monolignol derivatives are released. Saturation of the *p*-coumarate double bond occurs during DFRC, so the released products are 4-acetoxycinnamyl 4-acetoxyphenylpropionates. Synthesis of the esters allowed determination of response factors for the released products. Maize and bamboo lignins released 221 and 38 μmol/g of *p*-coumarate-derived esters. The sinapyl ester was much more abundant than the coniferyl one. The bamboo and maize lignin S/G ratios in the conjugates were 12 and 38 times greater than those of the normal monomers released by DFRC, evidence of a strong selectivity for acylation of syringyl units. Of three possible biochemical mechanisms for incorporating *p*-coumarates into lignin, evidence is mounting that the process involves incorporation of preacylated monolignols into the normal lignification process.

Keywords: *Acetyl bromide; lignin; grass; Gramineae; maize; bamboo; bromegrass; p-coumarate; phenolic acid; β-aryl ether; cleavage; quantitative analysis; gas chromatography; reductive elimination; acetylation; bromination; DFRC*

INTRODUCTION

In grasses (Gramineae) hydroxycinnamic acids are highly involved in the lignification process during plant cell wall development. Ferulates in ferulate—polysaccharide esters are intimately incorporated into lignin via the free-radical coupling processes that typify lignification, producing strong lignin—polysaccharide cross-linking (Ralph et al., 1995, 1998). Lignins in grasses also contain *p*-coumaric acid connected to lignin residues by ester linkages (Smith, 1955; Nakamura and Higuchi, 1976, 1978a,b; Ralph and Helm, 1993). Elucidation of the sites of *p*-coumarate attachment to lignin is important to understand mechanisms by which *p*-coumaric acid is incorporated into lignins. Three pathways (Figure 1) could be responsible for acylation of lignins by *p*-coumaric acid resulting in two different regiochemistries: (a) free *p*-coumaric acid **10** reacts with quinone methide lignin intermediates **4**, forming lignin acylated by *p*-coumarate at the α-positions of the lignin side chains **9**; (b) preformed hydroxycinnamyl *p*-coumarates **2** are incorporated by free-radical coupling into lignins during lignification so that *p*-coumarates are exclusively on the γ-positions of lignin side chains, **7** and **8**; and (c) acylation by an activated *p*-coumarate **11** (e.g., *p*-coumaroyl-SCoA) occurs postlignification, resulting in esters at α- (**9**) and/or γ-positions (**7**, **8**), depending on the selectivity of the (presumably required) transferase.

NMR studies on isolated lignins from maize (Ralph et al., 1994), wheat (Crestini and Argyropoulos, 1997), and many other grasses including bamboo (Ralph, unpublished data) reveal that *p*-coumarates are exclusively at the γ-positions of lignin side chains, suggesting that pathway b (and/or perhaps c) is involved. Radiotracer/microscopy (Terashima et al., 1993) and solvolytic studies (Grabber et al., 1996) suggested that *p*-coumarates are attached dominantly to syringyl units in grass lignins. Thioacidolysis detected *p*-coumarates on a maize lignin containing ~18% *p*-coumarate (Grabber et al., 1996) but cleaved some esters, lowering its sensitivity to lignins with low contents of such esters.

DFRC (**d**erivatization **f**ollowed by **r**eductive **c**leavage) is a procedure that produces analyzable monomers and dimers by cleaving α- and β-ethers in lignins (Lu and Ralph, 1997a,b, 1998a,b, 1999; Peng et al., 1998, 1999; Ralph and Lu, 1998). One advantage of the method is that γ-ester groups on lignins remain largely intact (Lu and Ralph, 1998b). A modified DFRC protocol allowed successful proof of the occurrence of acetate groups on lignin side chains in some species (Ralph and Lu, 1998); kenaf bast fiber lignins, in particular, are extensively acetylated in nature (Ralph, 1996). Thus, the method should allow us to confirm that *p*-coumarate groups are at the γ-positions of grass lignins and determine their distribution on syringyl (S) and guaiacyl (G) β-ether units **8**. Three isolated (bamboo, bromegrass, and maize) lignins were subjected to DFRC degradation to provide information about sites of *p*-coumarate attachment and the likely pathway for its biosynthetic incorporation.

EXPERIMENTAL PROCEDURES

General. All reagents were from Aldrich (Milwaukee, WI) and used as supplied. Solvents (AR grades) were from Mallinckrodt (Paris, KY). ^{1}H, ^{13}C, and 2D NMR (gradient HMQC and HMBC) spectra were taken on a Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The conditions used for all samples were 2—60 mg of material in 0.4 mL of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). Carbon/proton designations are based on conventional lignin numbering (Figure 1).

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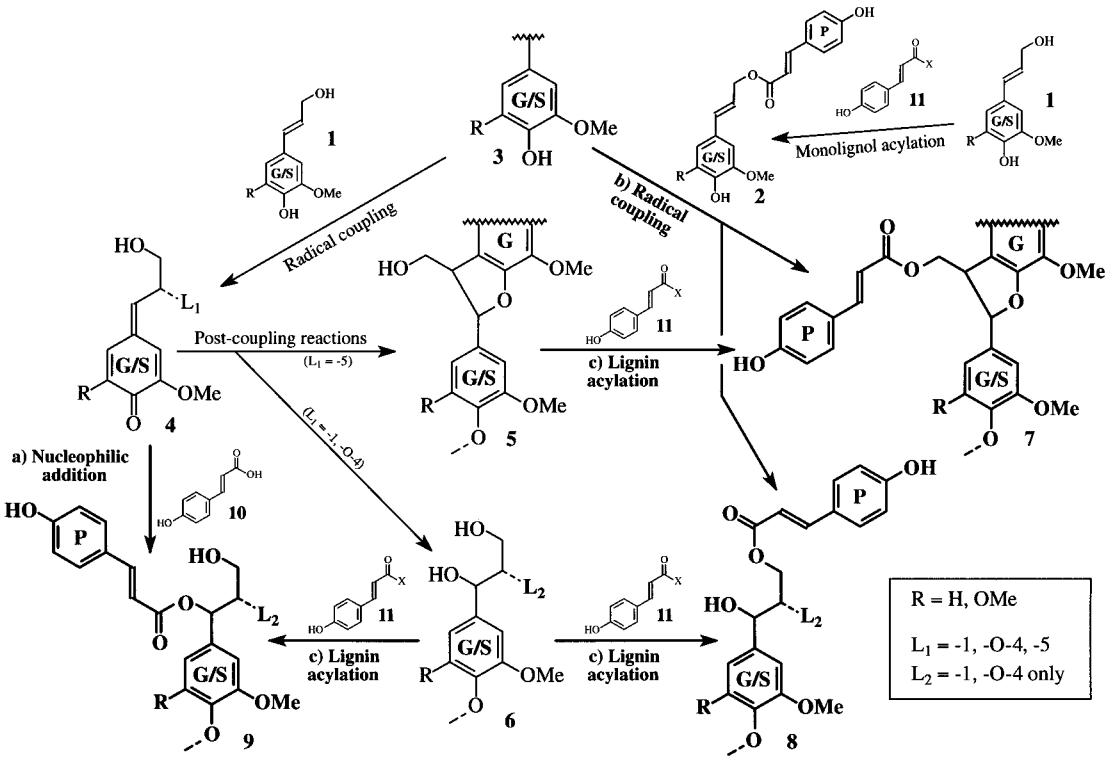

Figure 1. Three possible pathways for incorporation of *p*-coumarates into lignins. Because >95% of lignification arises from coupling of a monolignol **1** with a preformed oligolignol **3**, monolignol dimerization is neglected here. (a) Nucleophilic addition of free *p*-coumaric acid **10** to a quinone methide **4** (formed by coupling of a monolignol **1**, at β, with an oligolignol **3**, at −O-4, -1, or -5; weak external nucleophiles (such as *p*-coumaric acid **10**) cannot compete with internal ring closure (such as **4** → **5**), so only quinone methides **4** formed by β-1- or β-O-4- (and not β-5- or β-β-) coupling can give α-esters **9**. (b) Incorporation of preformed hydroxycinnamyl *p*-coumarates **2** into lignin via free-radical coupling giving acylated lignins **7** and **8**; any β-coupling product of **2** (β-O-4, β-5, or β-1) is possible for this pathway. (c) Postlignification acylation by an activated *p*-coumarate **11** (via a transferase); this pathway can acylate β-O-4-, β-1-, and β-5-linked units at γ (to give **7** and **8**), but only β-O-4- and β-1-linked units at α (to give **9**).

Isolated Lignins. Lignins were isolated from bamboo (*Bambusa*), bromegrass (*Bromus*), and maize (*Zea mays*) according to described methods (Björkman, 1954; Ralph and Hatfield, 1991; Ralph et al., 1994, 1995). Plant material was ground in a Wiley mill (1-mm screen), and soluble phenolics, carbohydrates, and other components were removed by successive extractions with water, methanol, acetone, and chloroform. The ground wood was then ball-milled, treated with crude cellulases to degrade most of the polysaccharides, and extracted with 96:4 dioxane/water. Saccharides and metal ions were removed from the lyophilized crude lignin using 5 mM (pH 8) EDTA (Ralph et al., 1994).

The DFRC Procedure. The DFRC method is described in part 1 of this series (Lu and Ralph, 1997b) and in the initial protocol (Lu and Ralph, 1997a), but 4,4′-ethylidenebisphenol (Aldrich Chemical Co.) was used as the internal standard. Response factors for monomers **P**, **G**, and **S** were 1.76, 1.37, and 1.46, respectively. [**P** is the DFRC monomeric product from *p*-coumaryl units, 4-acetoxycinnamyl acetate, not otherwise mentioned in this paper but included here for completeness.] Response factors for the acylated moieties, 4-acetoxy-3-methoxycinnamyl 4-acetoxyphenylpropionate **13a** and 4-acetoxy-3,5-dimethoxycinnamyl 4-acetoxyphenylpropionate **13b**, were 2.58 and 3.00, respectively. Molar yields were calculated on the basis of molecular weights of (underivatized) **P** =150, **G** =180, and **S** =210; acetates **13a** = 412 and **13b** = 442.

GC and GC/MS. The monomers from degraded lignins were determined by GLC (Hewlett-Packard 5980) using a 0.20-mm × 25-m DB-1 (J&W Scientific, Folsom, CA) column and a flame ionization detector with He as carrier gas (10 cm^3/min). GC conditions for monomers were as follows: initial column temperature, 60 °C, held for 1 min, ramped at 6 °C/min to 300 °C, held for 15 mim; injector temperature, 250 °C; FID detector temperature, 300 °C. For **13a** and **13b**, GC conditions were as follows: initial column temperature, 100 °C, held for 1 min, ramped at 15 °C/min to 300 °C, held for 25 min; injector temperature, 250 °C; FID detector temperature, 300 °C. EI-MS data were collected on a Hewlett-Packard 5970 mass-selective detector with the same type of column using the same temperature program.

Synthesis. Syntheses of 4-acetoxy-3-methoxycinnamyl 4-hydroxyphenylpropionate (**13a**) and 4-acetoxy-3,5-dimethoxycinnamyl 4-hydroxyphenylpropionate (**13b**) were accomplished according to published methods (Lu and Ralph, 1998c) using 4-hydroxyphenylpropionic acid, Figure 3. **13a** was a pale yellow oil: δ_H 2.22 (6H, s, OAc), 2.68 (2H, t, J = 7.5 Hz, P-8), 2.94 (2H, t, J = 7.5 Hz, P-7), 3.85 (3H, s, OMe), 4.70 (2H, dd, J = 6.4, 1.4 Hz, G-γ), 6.32 (1H, dt, J = 15.8, 6.4 Hz, G-β), 6.65 (1H, dt, J = 16.0, 1.4 Hz, G-α), 7.00 (2H, m, G-5/G-6), 7.01 (2H, m, P-3/5), 7.18 (1H, s, G-2), 7.27 (1H, s, P-2/6); δ_C 30.8 (P-8), 36.2 (P-7), 56.2 (OMe), 65.2 (G-γ), 111.2 (G-2), 110.0 (G-6), 122.4 (P-2/6), 123.7 (G-5), 124.8 (G-β), 130.0 (P-3/5), 133.7 (G-α), 136.20 (G-1), 139.0 (P-1), 140.8 (G-4), 150.3 (P-4), 152.4 (G-3), 169.0 (P-OAc), 169.7 (G-OAc), 172.7 (P-9). **13b** was a clear oil: δ_H 2.21(3H, s, S-OAc), 2.22 (3H, s, P-OAc), 2.68 (2H, t, J = 7.6 Hz, P-8), 2.94 (2H, t, J = 7.6 Hz, P-7), 3.82 (6H, s, OMe's), 4.70 (2H, dd, J = 6.4, 1.3 Hz, S-γ), 6.35 (1H, dt, J = 15.8, 6.4 Hz, S-β), 6.63 (1H, dt, J = 16.0, 1.2 Hz, S-α), 6.82 (2H, s, S-2/6), 7.0 (2H, m, P-3/5), 7.27 (2H, s, P-2/6); δ_C 30.9 (P-8), 36.2 (P-7), 56.5 (OMe's), 65.2 (S-γ), 104.2 (S-2/6), 122.4 (P-2/6), 124.9 (S-β), 129.1 (S-1), 130.1 (P-3/5), 134.2 (S-α), 139.1 (P-1), 135.7 (S-4), 150.3 (P-4), 153.4 (S-3/5), 168.5 (P-OAc), 169.7 (S-OAc), 172.7 (P-9).

β-Aryl ether models **17** were prepared according to the procedure of Helm and Ralph (1993). NMR data are deposited in the NMR Database of Lignin and Cell Wall Model Compounds (Ralph et al., 1999).

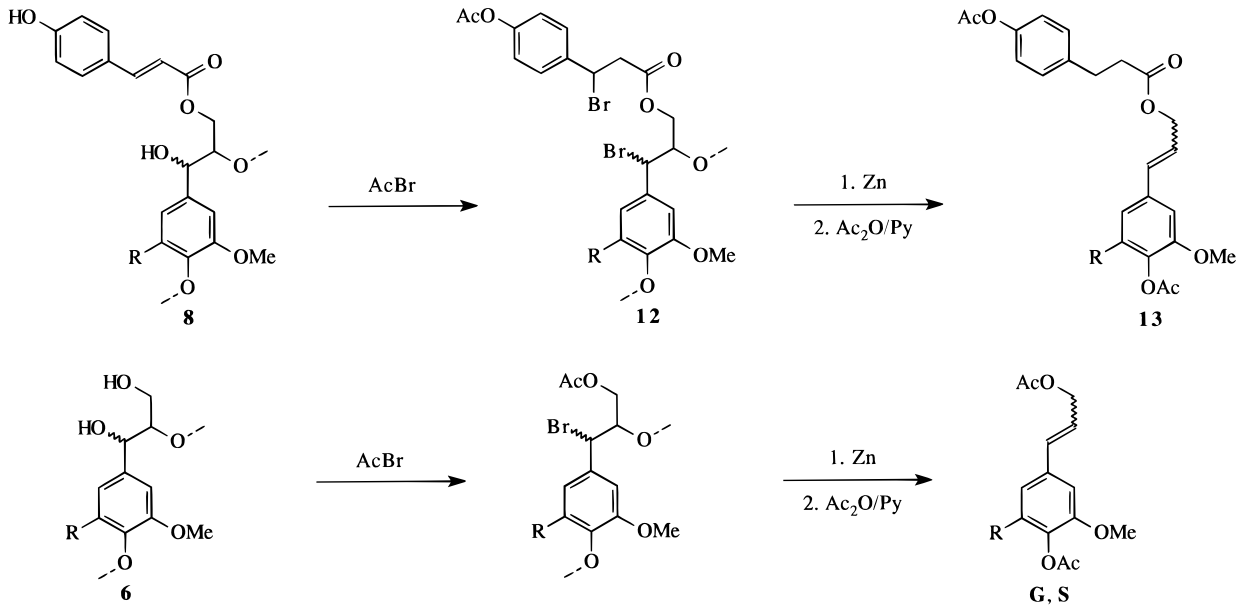

Figure 2. Pathway for formation of analyzable ester conjugates **13** from acylated β-aryl ether units **8** in lignins following DFRC.

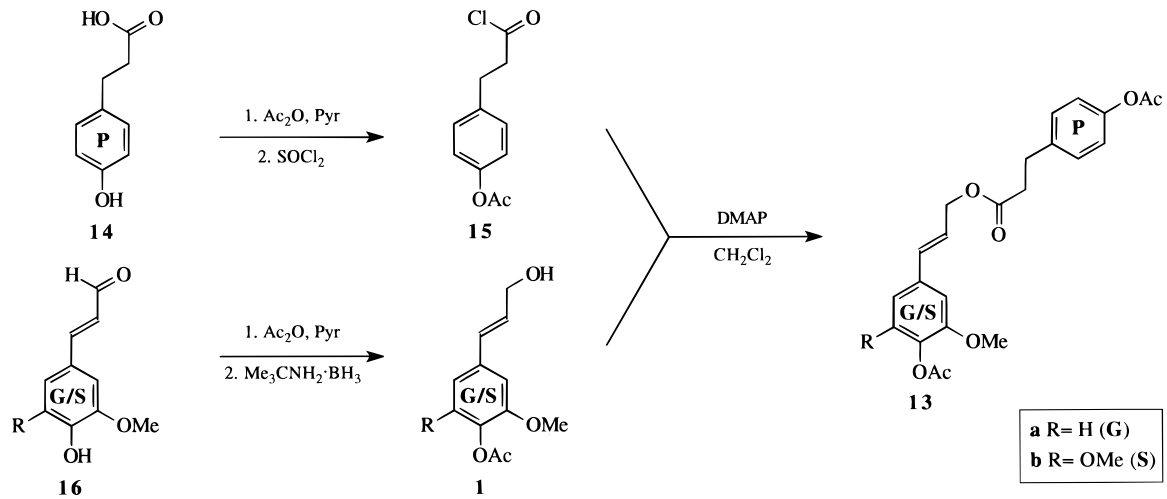

Figure 3. Synthesis of 4-acetoxycinnamyl 4-acetoxyphenylpropionates **13**.

RESULTS AND DISCUSSION

When DFRC was first developed, lignins representing softwoods, hardwoods, dicotyledons, and grasses were used for testing its capabilities. Chromatograms of DFRC products from maize and bamboo lignins showed peaks in the dimeric compound region that were not present in products from other lignins. From NMR studies, maize lignin contains large amounts of *p*-coumarate exclusively at the γ-positions of lignin side chains (Figure 1, lignin fragments **7** and **8**). The new dimeric compounds were therefore suspected to be dimeric degradation products containing *p*-coumarate (or a derived product). Some *p*-coumarate ester linkages to lignin survived thioacidolysis of the same material, although results were varied and poorly reproducible (Grabber et al., 1996). Because DFRC does not affect lignin γ-esters in models, it seemed reasonable that this method could detect and determine *p*-coumarates attached to the γ-positions of β-aryl ether units in lignin (Figure 2, lignin fragment **8**). It should be noted that the method explored here cannot be used to gain any information about α-esters because they would be cleaved in the AcBr step of DFRC. However, such cleavage could form the basis of a method to detect and quantify α-esters plus α-ethers of *p*-coumaric acid (Lu and Ralph, 1998b).

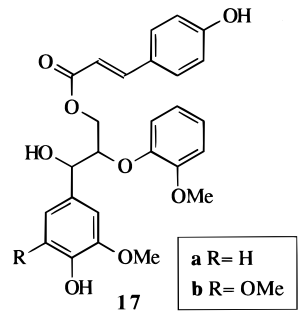

Figure 4. β-Aryl ether γ-*p*-coumarate ester models used for DFRC release studies.

Studies with β-aryl ether models **17** (Figure 4) tested the efficiency of DFRC for releasing hydroxycinnamyl *p*-coumarate derivatives and allowed identification of their degradation products. The β-aryl ether linkages in models **17** were cleaved in ~60−65% yield, lower than the 95% yields from normal unacylated β-aryl ether models (Lu and Ralph, 1997b). The major product from **17b** was sinapyl 4-acetoxyphenylpropionate **13b**; no de-

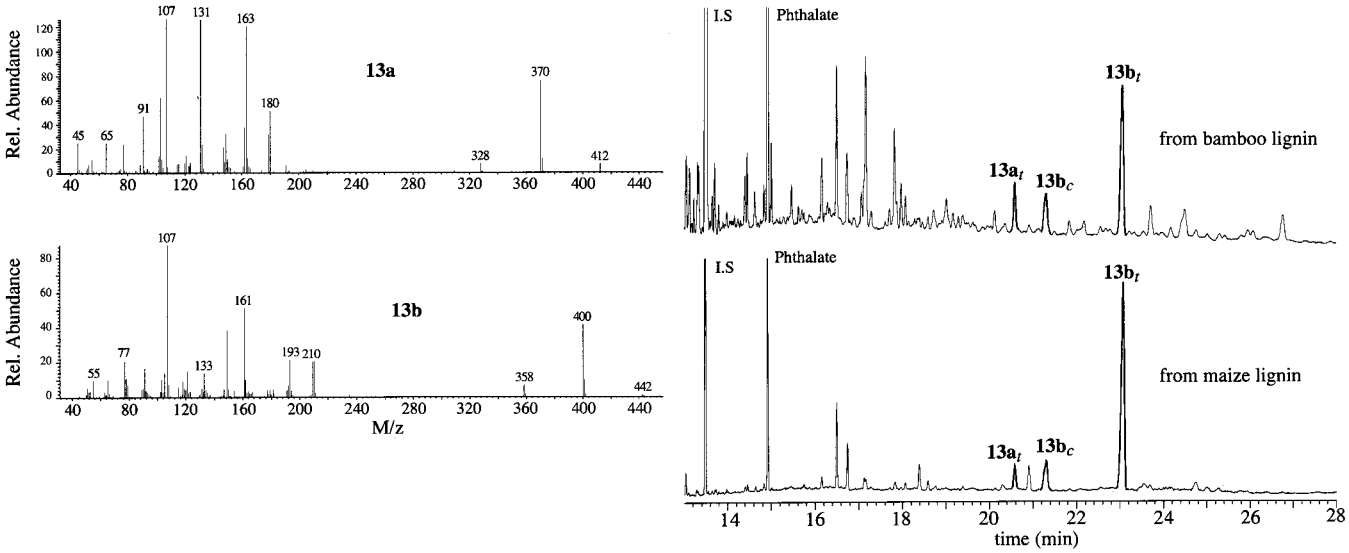

Figure 5. GC-FID chromatograms of DFRC dimers from bamboo and maize lignins. Products **13a** and **13b** from β-aryl ether γ-*p*-coumarate esters **8** are well resolved and easily detected. Their mass spectra are diagnostic. c = *cis*, t = *trans*.

Table 1. Yields of Monomers and Acylated Monomers from DFRC of Bamboo, Maize, and Bromegrass Lignins

	monomers (μm/g)			*p*-coumarate products (μm/g)		
sample	G	S	S/G	**13a**	**13b**	**13b/13a**
bamboo lignin	504	222	0.44	6	32	5.33
maize lignin	342	124	0.36	15	206	13.73
bromegrass lignin	454	327	0.72	nd[a]	d[b]	

[a] nd, not detected. [b] d, detected, by GC/MS.

esterified monomer was detected. Coniferyl 4-acetoxyphenylpropionate (**13a**) was obtained from model **17a**. Minor amounts of coniferyl/sinapyl *p*-coumarates found by GC/MS were not included in the yield. Compounds **13a** and **13b** were identified by GC/MS and comparison of their retention times with those of genuine compounds, which were synthesized (Figure 3) and authenticated by NMR. Compound **13b** was a major component from ether extracts of DFRC degradation products of maize lignin, as confirmed by 2D NMR experiments. Mass spectra of **13a** and **13b** were diagnostic (Figure 5). In each, the molecular ion was observed, and even-massed ion radicals from one phenolic acetate loss [(M $-$ 42)$^+$] were abundant. The most abundant ion fragment with *m*/*z* 107 is characteristic of the 4-hydroxybenzyl moiety from ring P (following loss of ketene from the acetate). Coniferyl and sinapyl alcohol fragment ions (*m*/*z* 180, 210) are prominent, and other fragment ions are logical.

Syntheses of **13a** and **13b** allowed measurement of response factors so that *p*-coumarates in lignins released by DFRC could be quantified by GC. Table 1 shows DFRC results from lignins isolated from bamboo, maize, and bromegrass. In bromegrass, the amounts of esters were too low, but selected-ion mass spectrometry identified the sinapyl *p*-coumarate product **13b**; **13a** could not be detected. Evidently syringyl units of bamboo and maize lignins are acylated by *p*-coumarate to a much greater degree than guaiacyl units. In bamboo and maize, the S/G ratios in the esters were 12 and 38 times greater than those of the normal monomers released by DFRC. More syringyl units than guaiacyl units are linked by labile β-aryl ethers, but this would explain only a $\sim$2-fold difference in S/G molar ratios of DFRC products from both lignins. Studies with model compounds showed that acylation did not differentially affect the release of syringyl or guaiacyl units from β-aryl ethers. Preferential acylation of syringyl units by *p*-coumarate before their incorporation into lignin is therefore likely. Whole-cell-wall samples were not used in this study but are expected to produce similar results, although some fractionation of the *p*-coumarate into the extractable lignins has been noted (Ralph et al., 1994). Whole-cell-wall studies will be aided by solid-phase extraction cleanup steps currently under development.

Considering the accessibility difference for acylation of an α-OH versus that of a γ-OH, selective enzymatic acylation of γ-OH's by *p*-coumarates postlignification would explain why no α-ester has been detected by NMR. However, this cannot explain why syringyl β-aryl ether units are far more esterified by *p*-coumarate than guaiacyl β-aryl ethers; any γ-acylation should be more sensitive to the S/G nature of the β-O-aryl unit than to the unit detected as bearing the *p*-coumarate. The observation (Ralph et al., 1994) that *p*-coumarates on grass lignins are on many types of units (both isomers of β-O-4 units, β-5 units, and even cinnamyl alcohol end groups) suggests that enzyme-assisted acylation of the lignin polymer (pathway c) is unlikely—the enzyme would have to be remarkably nonspecific. The selectivity required could easily be expected from a transferase acylating sinapyl versus coniferyl alcohol with an activated *p*-coumaric acid. Evidence is therefore mounting that the pathway leading to preferential acylation is b (Figure 1), in which sinapyl alcohol is preacylated by *p*-coumarate and laid down in the cell wall late in the lignification process when sinapyl alcohol appears most predominantly (Terashima et al., 1986a,b, 1993; Terashima and Fukushima, 1988; He and Terashima, 1990; Chabbert et al., 1994a,b). Pathway b was the pathway originally proposed by Nakamura and Higuchi (1978a).

CONCLUSIONS

Because DFRC leaves lignol γ-esters essentially intact, it is valuable for identifying esterified lignin components and for determining the sites of acylation. This study confirmed that grass lignins are acylated at the γ-position by *p*-coumarates; primarily syringyl units are acylated in maize, bamboo, and bromegrass. Of

three possible pathways, evidence is mounting that the biochemical process involved is the incorporation into normal lignification of preacylated monolignols.

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