Sequencing around 5-Hydroxyconiferyl Alcohol-Derived Units in Caffeic Acid *O*-Methyltransferase-Deficient Poplar Lignins[1][OA]

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Caffeic acid *O*-methyltransferase (COMT) is a bifunctional enzyme that methylates the 5- and 3-hydroxyl positions on the aromatic ring of monolignol precursors, with a preference for 5-hydroxyconiferaldehyde, on the way to producing sinapyl alcohol. Lignins in COMT-deficient plants contain benzodioxane substructures due to the incorporation of 5-hydroxyconiferyl alcohol (5-OH-CA), as a monomer, into the lignin polymer. The derivatization followed by reductive cleavage method can be used to detect and determine benzodioxane structures because of their total survival under this degradation method. Moreover, partial sequencing information for 5-OH-CA incorporation into lignin can be derived from detection or isolation and structural analysis of the resulting benzodioxane products. Results from a modified derivatization followed by reductive cleavage analysis of COMT-deficient lignins provide evidence that 5-OH-CA cross couples (at its β-position) with syringyl and guaiacyl units (at their *O*-4-positions) in the growing lignin polymer and then either coniferyl or sinapyl alcohol, or another 5-hydroxyconiferyl monomer, adds to the resulting 5-hydroxyguaiacyl terminus, producing the benzodioxane. This new terminus may also become etherified by coupling with further monolignols, incorporating the 5-OH-CA integrally into the lignin structure.

Lignins are polymeric aromatic constituents of plant cell walls, constituting about 15% to 35% of the dry mass (Freudenberg and Neish, 1968; Adler, 1977). Unlike other natural polymers such as cellulose or proteins, which have labile linkages (glycosides and peptides) between their building units, lignins' building units are combinatorially linked with strong ether and carbon-carbon bonds (Sarkanen and Ludwig, 1971; Harkin, 1973). It is difficult to completely degrade lignins. Lignins are traditionally considered to be dehydrogenative polymers derived from three monolignols, *p*-coumaryl alcohol **1H** (which is typically minor), coniferyl alcohol **1G**, and sinapyl alcohol **1S** (Fig. 1; Sarkanen, 1971). They can vary greatly in their composition in terms of their plant and tissue origins (Campbell and Sederoff, 1996). This variability is probably determined and regulated by different activities and substrate specificities of the monolignol biosynthetic enzymes from different sources, and by the carefully controlled supply of monomers to the lignifying zone (Sederoff and Chang, 1991).

Recently there has been considerable interest in genetic modification of lignins with the goal of improving the utilization of lignocellulosics in various agricultural and industrial processes (Baucher et al., 2003; Boerjan et al., 2003a, 2003b). Studies on mutant and transgenic plants with altered monolignol biosynthesis have suggested that plants have a high level of metabolic plasticity in the formation of their lignins (Sederoff et al., 1999; Ralph et al., 2004). Lignins in angiosperm plants with depressed caffeic acid *O*-methyltransferase (COMT) were found to derive from significant amounts of 5-hydroxyconiferyl alcohol (5-OH-CA) monomers **1$_{5H}$** (Fig. 1) substituting for the traditional monomer, sinapyl alcohol **1S** (Marita et al., 2001; Ralph et al., 2001a, 2001b; Jouanin et al., 2004; Morreel et al., 2004b). NMR analysis of a ligqnin from COMT-deficient poplar (*Populus* spp.) has revealed that novel benzodioxane structures are formed through β-*O*-4 coupling of a monolignol with 5-hydroxyguaiacyl units (resulting from coupling of

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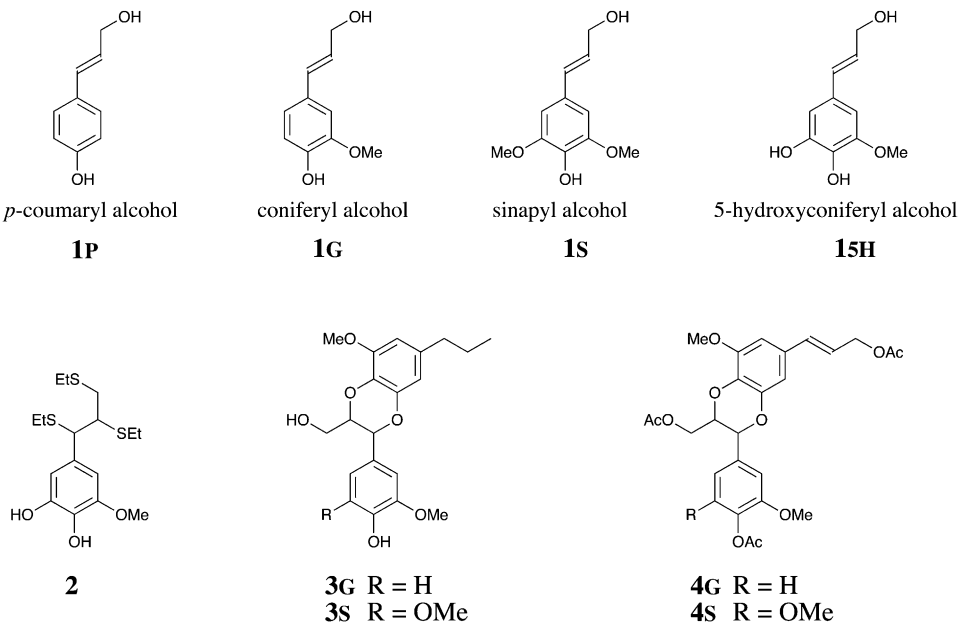

Figure 1. The monolignols **1**, and marker compounds **2** to **4** resulting from incorporation of novel monomer **1**$_{5H}$ into lignins: thioacidolysis monomeric marker **2**, dimers **3**, and DFRC dimeric markers **4**.

5-OH-CA), followed by internal trapping of the resultant quinone methide by the phenolic 5-hydroxyl (Ralph et al., 2001a). When the lignin was subjected to thioacidolysis, a novel 5-hydroxyguaiacyl monomer **2** (Fig. 1) was found in addition to the normal guaiacyl and syringyl thioacidolysis monomers (Jouanin et al., 2000). Also, a new compound **3**$_G$ (Fig. 1) was found in the dimeric products from thioacidolysis followed by Raney nickel desulfurization (Lapierre et al., 2001; Goujon et al., 2003).

Further study with the lignin using the derivatization followed by reductive cleavage (DFRC) method also confirmed the existence of benzodioxane structures, with compounds **4** (Fig. 1) being identified following synthesis of the authentic parent compounds **9** (Fig. 2). However, no 5-hydroxyguaiacyl monomer could be detected in the DFRC products. These facts imply that the DFRC method leaves the benzodioxane structures fully intact, suggesting that the method might therefore be useful as an analytical tool for determining benzodioxane structures that are linked by *β-O*-4 ethers. Using a modified DFRC procedure, we report here on results that provide further evidence for the existence of benzodioxane structures in lignins from COMT-deficient plants, that 5-OH-CA is behaving as a rather ideal monolignol that can be integrated into plant lignins, and demonstrate the usefulness of the DFRC method for determining these benzodioxane structures.

RESULTS AND DISCUSSION

Benzodioxane Products Released by DFRC

Our previous NMR and DFRC studies on COMT-deficient poplar lignins showed that the 5-OH-CA monomer cross couples with guaiacyl or syringyl lignin units into the growing lignin oligomer followed by further coupling with monolignols producing benzodioxane structures (Marita et al., 2001, 2003b; Ralph et al., 2001a, 2001b; Jouanin et al., 2004; Morreel et al., 2004a, 2004b; for review, see Boerjan et al., 2003b; Ralph et al., 2004). When COMT-deficient poplar lignins or whole plant cell wall fractions were submitted to DFRC degradation, a dimeric product containing the benzodioxane structure was released and identified to be compound **4**$_G$ (Fig. 1; Lapierre et al., 2001). Thioacidolysis also produced the corresponding benzodioxane products **3**$_{G/S}$; substantial amounts of monomer **2**, which may be the cleavage product of benzodioxanes, were also detected. The fact that 5-hydroxyconiferyl acetate has not been found in DFRC degradation products of COMT-deficient poplar lignin implies that all of the 5-OH-CA monomer is incorporated into lignin as benzodioxane structures and these structures totally survive DFRC conditions (without cleavage to monomeric products). To test this absolute survivability, lignin dimeric models **13** (Fig. 3) were synthesized and subjected to DFRC degradation.

The results (Fig. 3) showed that DFRC of benzodioxane compound **13**$_G$*f* (with a free phenol) gave benzodioxane compound **14**$_G$*f* with the hydroxyls on the A unit acetylated and the hydroxyl on the B unit reduced to methyl; a tiny amount of compound **15**$_G$*f* with all hydroxyls acetylated was also produced, but no monomeric units. With compound **13**$_G$*e* that models the internal (etherified) benzodioxane structures in lignin, similar results were obtained, i.e. the major product was compound **14**$_G$*e* and minor amounts of compound **15**$_G$*e* were also detected. The behaviors of compounds **13**$_G$*f* and **13**$_G$*e* during each step of DFRC treatment were monitored by NMR.

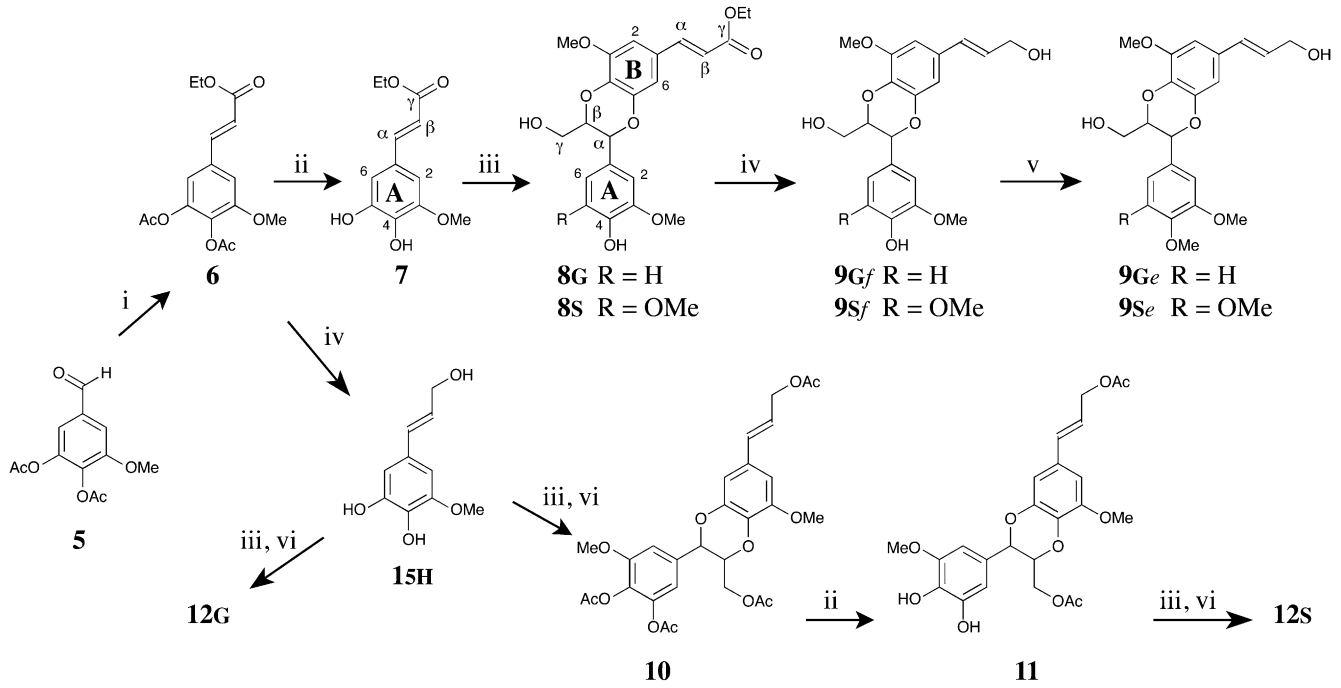

Figure 2. Synthesis of benzodioxane DFRC products **12** (see later in Fig. 6 for their structures). i, NaH, THF. ii, Pyrrolidine. iii, **1G** or **1S**, benzene/acetone (4/1, v/v). iv, DIBAL-H, toluene. v, Iodomethane-K_2CO_3, acetone. vi, Ac_2O pyridine.

Acetyl bromide in acetic acid cleanly acetylated γ-hydroxyls and free phenols, and brominated the benzylic hydroxyl groups. Zinc reduced the benzylic bromide on the B ring to methyl. The minor benzyl acetate remaining on the B unit was the result of hydrolysis of the benzylic bromide during the zinc reduction step, followed by acetylation. In general, the results shown in Figure 3 confirmed that benzodioxane structures totally survived DFRC conditions, suggesting that DFRC can be used to detect and determine benzodioxane structures in lignins of COMT-deficient plants, and also from F5H up-regulated plants such as Arabidopsis (*Arabidopsis thaliana*) that have been shown to contain lignins with benzodioxane structures (Ralph et al., 2001b).

Partial Sequencing around Benzodioxanes in COMT-Deficient Poplar Lignins

When a COMT-deficient poplar lignin was degraded by the standard DFRC procedure, the benzodioxane marker compound **4G** (Fig. 1) was detected by gas chromatography-mass spectrometry (GC-MS). The double bond present signifies that a β-*O*-4 bond was cleaved; the phenolic end may also result from cleaving a 4-*O*-β ether, or it could have already been free phenolic. But the absence of the corresponding analog **4S** raised a question as to whether the syringyl benzodioxane was not in this lignin, or if the marker compound **4S** simply could not get through the GC. With synthetic compound **4S**, we found that **4S** could not survive the GC conditions because of its thermoliability, but the more thermally stable trimethylsilyl (TMS)-derivatized **9S***f* and **9S***e* (Fig. 2), analogs of **4S**, proved to be amenable to GC quantitative analysis. So a modified DFRC procedure was developed (Fig. 4) to determine the benzodioxane structures to get more detailed structural picture of such structures in lignin polymers. First of all, the normal DFRC method results in cleavage of β-ethers but leaves the benzodioxanes intact. After zinc reduction, the phenols released due to β-ether cleavage are methylated, and the phenolic acetates derived from lignins' originally free phenols remain unaffected. So the benzodioxanes with free phenols on their G/S units in lignins will be acetylated by acetyl bromide and remain acetylated, whereas the benzodioxanes with etherified phenols on their G/S units become methylated. A solid-state extraction step, although not absolutely necessary, is effective in cleaning up the sample and enriching the dimeric products, allowing for more accurate GC analysis. The final step in this modified DFRC procedure, base hydrolysis to remove acetates, converts all benzodioxane dimeric products into compounds **9**. Their TMS derivatives were then analyzed by GC.

The partial GC-flame ionization detector profiles of the degradation products of four samples by the modified DFRC procedure are shown in Figure 5 (along with the reference model spectrum in Fig. 5A). The target compounds **9** were identified, as their TMS derivatives, by their mass spectra and GC retention time comparison with synthetic and authentic

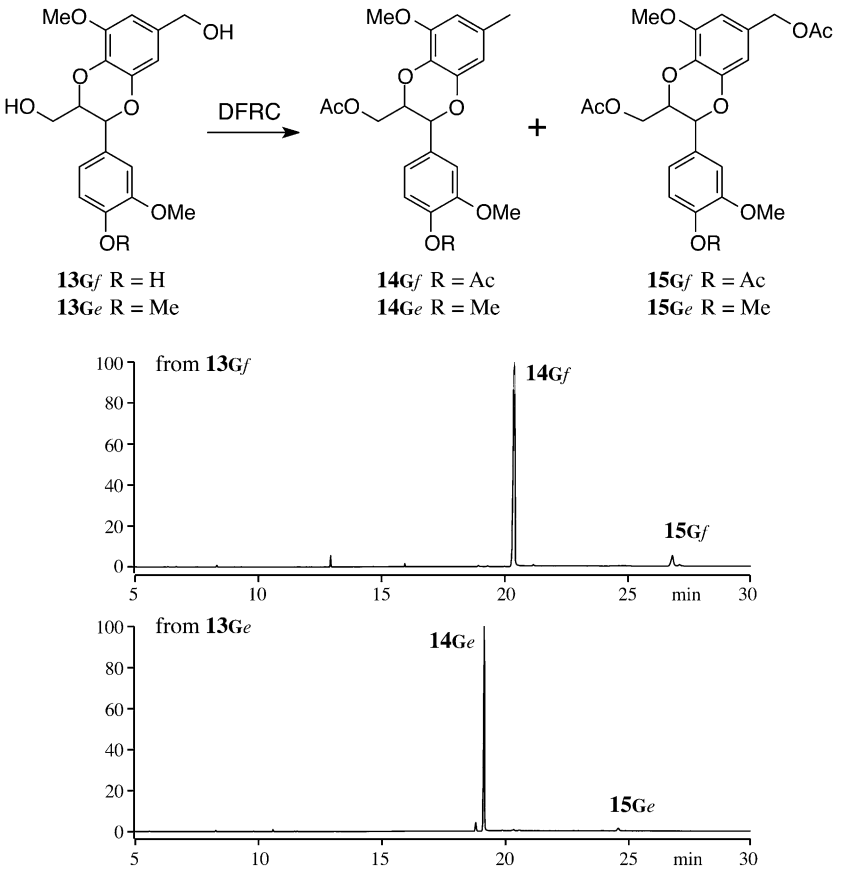

Figure 3. DFRC reactions of benzodioxane lignin model compounds **13**, and GC chromatograms of the DFRC products **14** and **15**.

model compounds in separate GC-MS runs. Theoretically, all of the compounds **9** can have four isomers, but only one or two isomers for each were significant enough to be detected. Based on results from NMR and model studies on free radical coupling of 5-OH-CA or 5-hydroxyferulate with hydroxycinnamyl alcohols, the benzodioxane ring having its two oxygen substituents in the trans-configuration (i.e. the *RR*/*SS* pair of enantiomers) would be major. The trans-double bond on the 5H unit is expected to be major according to the mechanism involved in DFRC reactions (Lu and Ralph, 1997b). Therefore the dominant benzodioxane products identified by GC profiles are the ones with a trans-ring and a trans-double bond, although small amounts of isomers having a cis-ring and a trans-double bond were also found by comparing

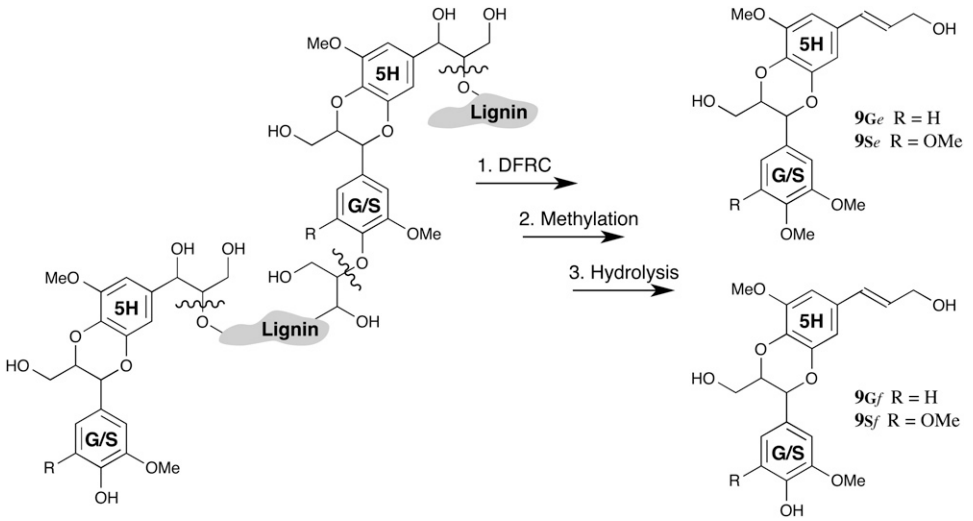

Figure 4. Benzodioxane compounds **9** released from DFRC degradation (using the modified post-treatment methods described here) of a hypothetical lignin-containing free-phenolic and etherified benzodioxane units.

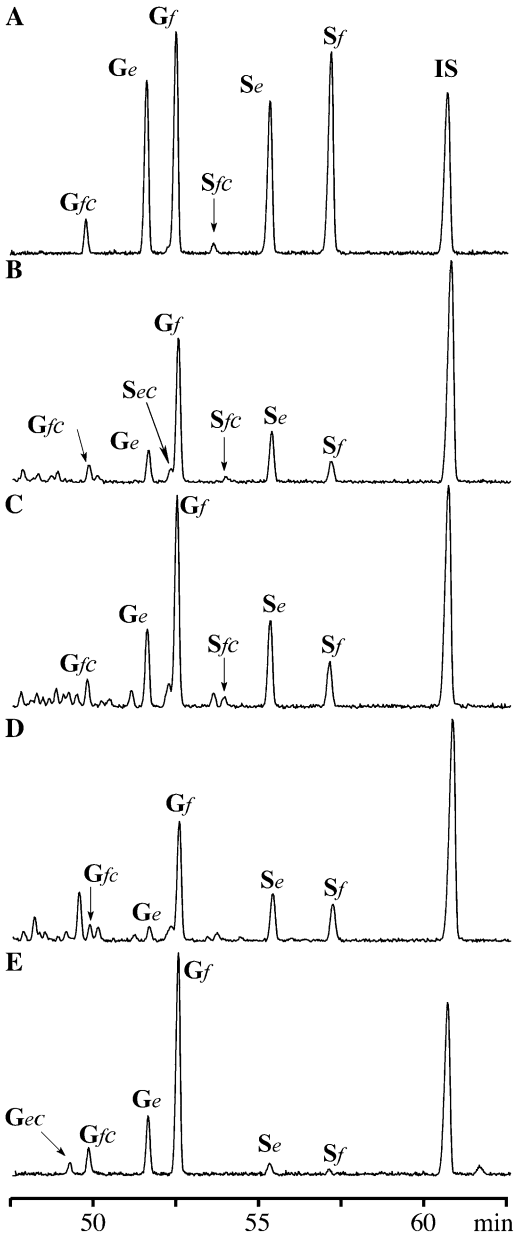

Figure 5. Partial GC-flame ionization detectors showing (TMS-derivatized) benzodioxane products **9** from degradation of COMT-deficient poplar lignins or whole cell wall fractions by the modified DFRC procedure. A, Synthesized models. B, Antisense COMT-deficient poplar lignin. C, The residue remaining after dioxane-water extraction of the lignin in B. D, Antisense COMT-deficient poplar cell walls. E, Gene-silenced COMT-deficient poplar lignin.

their retention times and mass spectra with those of authentic models. An internal standard, mono-4-*O*-methylated 5-5-diferulic acid, which has been used previously (Bunzel et al., 2003) but was synthesized here in pure form by an improved method (F. Lu, unpublished data), was added to samples right before TMS derivatization and GC analysis. At this moment, the GC results reflect the benzodioxanes actually recovered by this procedure after DFRC degradation, uncorrected for any losses. From the similarities among compounds **9**, it is expected that the relative ratios among them should not change through the procedure, i.e. they should not be subjected to any differential partitioning.

From the results summarized in the Table I, it can be seen that guaiacyl benzodioxanes released by DFRC were mostly (70%–86%) from the ends of lignin molecules and the released syringyl benzodioxanes were mostly (67%–77%) from internal units. In other words, the released guaiacyl benzodioxanes came from phenolic units whereas the released syringyl benzodioxanes came from those etherified through 4-*O*-β-linkages. This is fairly typical of observations from analysis of thioacidolysis products from methylated cell walls. For example, in the wild-type Arabidopsis samples in a recent report (Ralph et al., 2008), and in earlier pine (*Pinus* spp.) and poplar studies (Lapierre and Rolando, 1988), released *S*-monomers were more highly etherified than their guaiacyl counterparts. These results may be explained, in part, by the lignification mechanism. Guaiacyl units with an additional active site at their 5-positions could, during lignification, form 5-β-, 5-*O*-4-, and 5-5-linkages that are not cleavable by DFRC (or thioacidolysis). Therefore, there is less chance for guaiacyl units than syringyl ones to be etherified through 4-*O*-β-linkages that are cleaved by DFRC. Moreover, it has been shown that cross coupling between a guaiacyl unit and sinapyl alcohol is an unfavorable reaction (Landucci and Ralph, 2001), and that sinapyl alcohol dominates the monolignol supply at the later stages of lignification (Terashima et al., 1993). Therefore, if the supply of 5-OH-CA is similar to that of the sinapyl alcohol it replaces, most of the guaiacyl benzodioxanes that are released here are formed presumably at late stages. However, guaiacyl benzodioxanes formed earlier have more chance to be condensed at their 5-positions and so contribute less to the amount of released benzodioxanes. On the other hand, more internal syringyl benzodioxanes were released by DFRC because syringyl units are able to couple with sinapyl alcohol or cross couple with coniferyl alcohol only through 4-*O*-β-linkages that are cleavable by DFRC.

By comparing the results from dioxane-water-extracted lignins (so-called milled wood lignins [MWLs]) and the residual lignins remaining after the dioxane-water extraction, it is evident that some partitioning of benzodioxane structures between lignin fractions has occurred during the preparation of the MWL fraction, and that this partitioning is more significant for guaiacyl benzodioxanes than for syringyl ones.

Although guaiacyl benzodioxanes have more chance to have condensed linkages at their 5-positions, more guaiacyl benzodioxanes were released than syringyl ones. The reason for this may be that 5-hydroxy units react more favorably with coniferyl alcohol than with sinapyl alcohol. For example, the yield for compound **8**G from coupling of 5-hydroxyferulate with coniferyl

Table I. *Yields* and relative ratios of benzodioxanes released by modified DFRC*

*, The measured values listed here are means of replica experimental results and relative errors were within 10%; **, the amount was too low to be determined accurately.

COMT-Deficient Poplar Samples	Relative Ratios		Yields (% Lignin Sample)		G/S
	GOMe/GOH	SOMe/SOH	G	S	
	9G*e*/**9G***f*	**9S***e*/**9S***f*	(**9G***e*+**9G***f*)	(**9S***e*+**9S***f*)	
MWL-1 (antisense)	20.0/80.0	72.0/28.0	0.39	0.19	2.05
Residual lignin (antisense)	30.0/70.0	71.5/28.5	–	–	1.33
Cell wall (antisense)	13.5/86.5	66.7/33.3	–	–	0.93
MWL-2 (sense)	26.5/73.5	77.3/22.7	0.43	0.02**	21.5

alcohol (Fig. 2) is higher than that for compound **8S** from coupling of 5-hydroxyferulate with sinapyl alcohol. Research of this kind desperately needs a method to determine relative cross-coupling propensities between all combinations of natural and novel (from transgenics) units. Also of course, sinapyl alcohol and syringyl units are depleted by COMT deficiency.

One important aspect to note (that also applies to a lesser degree to the thioacidolysis products) is that the overall yields for benzodioxanes released by DFRC are relatively low compared to the high levels found via NMR analysis of the lignins. Since 5-hydroxy units could also couple with further 5-OH-CA monomers producing benzodioxane chains that are not cleavable by DFRC, trimers, tetramers, and higher oligomers of benzodioxane chains may exist in DFRC products and cannot be measured by the current GC method. In fact, evidence for such benzodioxane chains has already been demonstrated in a COMT-deficient alfalfa (*Medicago sativa*) transgenic (Marita et al., 2003a) and such oligomers have been found in the methanol-soluble phenolics fraction of xylem tissue from COMT-deficient poplar plants (Morreel et al., 2004b). As we discuss next, the isolation of those DFRC trimers or tetramers will give further evidence that 5-OH-CA is truly acting as a monolignol that is well integrated into lignins in COMT-deficient plants.

DFRC Trimers with Benzodioxane Structures from COMT-Deficient Poplar Lignins

From our work on synthesis of benzodioxane lignin model compounds by radical coupling reactions of coniferyl alcohol (**1G**) and 5-OH-CA (**15H**), it was evident that the **15H** radical generated from one-electron oxidation by silver (I) oxide, or by peroxidase-hydrogen peroxide, has similar chemical reaction propensities to **1G** or **1S** radicals. Therefore it is reasonable to expect that lignins from COMT-deficient plants in which significant amounts of **15H** are produced have such benzodioxane structures. The **15H** radical or radicals from 5-hydroxyguaiacyl units of oligomers could couple with **15H** radical producing 5H-5H benzodioxanes having 5-hydroxyguaiacyl end units, and these 5H-5H benzodioxanes could undergo further radical coupling reactions with **1G** or **1S** radicals forming G/S-5H-5H benzodioxane structures as we demonstrated in the synthesis of **12** (Figs. 2 and 6). DFRC reactions cleave normal *β-O*-4-ether linkages between two lignin (guaiacyl and/or syringyl) units but not the benzodioxane linkages. So monomers (acetylated **1H**, **1G**, and **1S**) are produced from DFRC degradation of lignins but not acetylated **15H**. Instead, benzodioxane (G/S-5H) dimers linked through *β-O*-4 ethers resulted from DFRC degradation of COMT-deficient poplar lignins. We surmised that benzodioxane (G/S-5H-5H) trimers linked with *β-O*-4 ethers would also be produced from DFRC degradation of COMT-deficient poplar lignins. Such acetylated trimers, if produced, will be not detected by GC-MS due to their low volatility and their likely thermoliability. However they could be isolated through a series of liquid chromatography and thin-layer chromatography (TLC) steps (Peng et al., 1998, 1999).

Large-scale DFRC experiments were performed with COMT-deficient poplar wood to isolate the expected trimeric products having benzodioxane structures. First of all, the final degradation products recovered by extractions were applied to normal-phase silica-gel flash chromatographic separation to produce fractions having monomers, dimers, trimers, and oligomers. After checking by TLC, fractions having similar compositions were combined. Using synthesized benzodioxane trimers to reveal TLC mobilities, fractions containing the expected trimers were pooled out, deacetylated by treatment with pyrrolidine in ethanol, followed by ethyl acetate extraction to remove the contaminating degraded carbohydrates. Second, the ethyl acetate-extracted trimers were acetylated (in acetic anhydride and pyridine) and subjected to further TLC separation. Two fractions (fraction A and fraction B) potentially having the benzodioxane trimers were recognized by comparing with synthesized models.

Two-dimensional (2D) NMR experiments were performed on such isolated samples. 2D COSY, HSQC, and HMBC NMR spectra were used to identify the expected benzodioxane trimers (Fig. 6). From COSY spectra of fraction A and fraction B (not shown here), two correlation signals, at 4.45/5.08 ppm and 4.39/4.95 ppm, were found. These are diagnostic COSY signals for benzodioxane trimeric compounds **12** (Fig. 6). Meanwhile, proton signals at 4.39 and 4.45 ppm

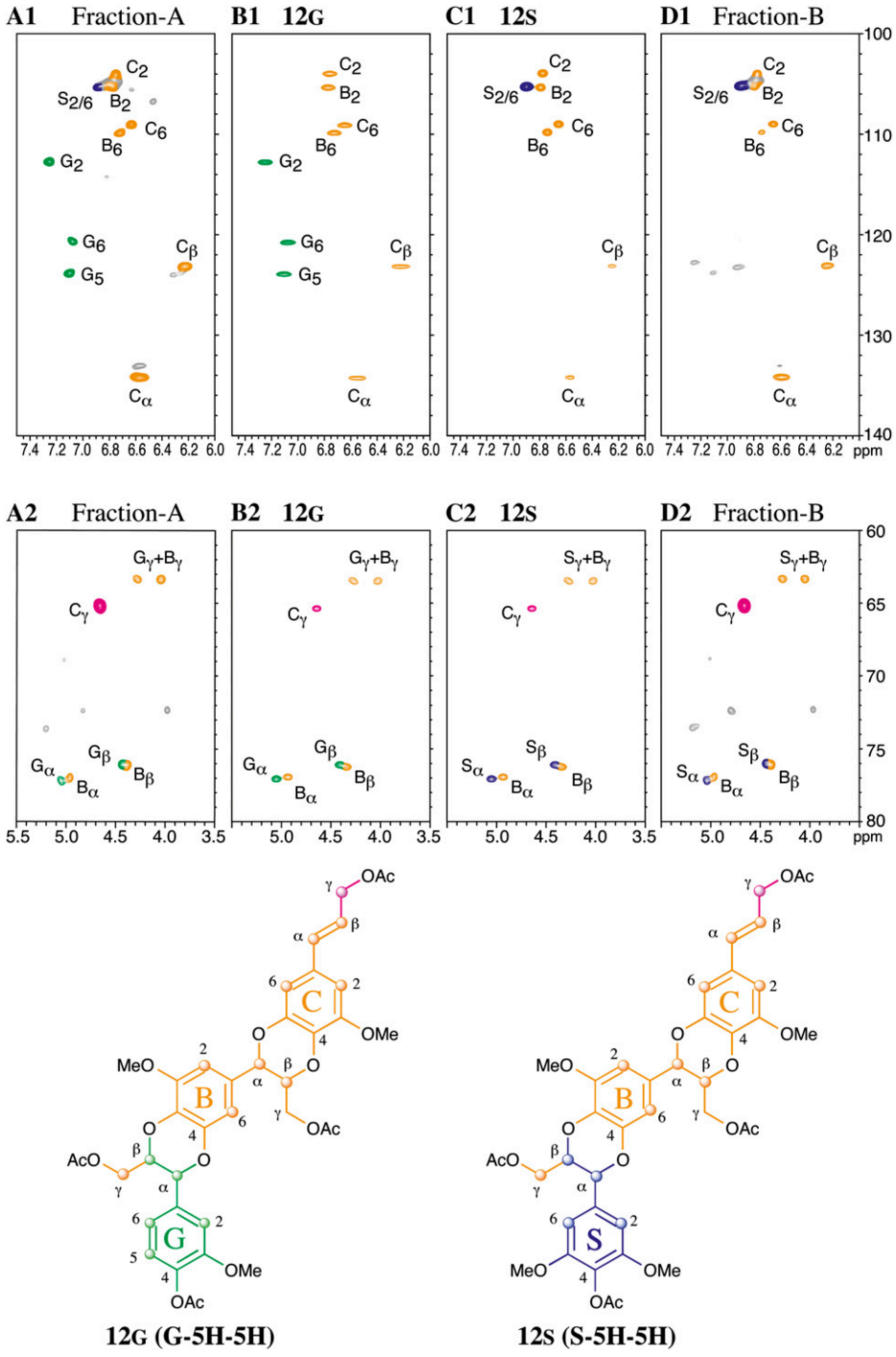

Figure 6. Chemical structures of **12** identified from DFRC degradation products of COMT-deficient poplar lignins, and HSQC NMR spectra showing the aromatic C-H correlations (top rows) and aliphatic C-H correlations (bottom rows) of synthesized models **12** (spectra B and C), and isolated DFRC degradation fractions A and B (spectra A and D).

also see signals at around 4.05 and 4.30 ppm (not shown here), which are consistent with model compounds **12**. As shown in HSQC NMR spectra (Fig. 6, A2 and D2), C-H correlations at around 77.0 to 77.5 (δ_C)/4.95 to 5.05 (δ_H) ppm and around 76.0 to 77.0 (δ_C)/4.36 to 4.50 (δ_H) ppm, are also characteristic of benzodioxane structures according to NMR data from model compounds **12** (Fig. 6, B2 and C2). Moreover, C-H correlations at around 77.0 to 77.5 (δ_C)/4.95 to 5.05 (δ_H) ppm were clearly separated into two distinct networks, suggesting that two kinds of benzodioxane structures exist in these compounds. Comparison of the HSQC NMR spectrum of fraction A (Fig. 6, A2) with that of synthesized benzodioxane trimers **12** (Fig. 6, B2 and C2) suggests that these two distinguishable correlations at around 77.0 to 77.5 (δ_C)/4.95 to 5.05 (δ_H) ppm belong to the Cα-Hα correlation of benzodioxanes constructed by a G/S unit and a 5H unit (B ring;

Table II. *NMR data for synthesized benzodioxane trimer acetates **12***

*, The chemical shifts for B5 and C5 are close to each other and may be interchangeable.

	G-5H-5H, **12G**			S-5H-5H, **12S**	
	^{1}H	^{13}C		^{1}H	^{13}C
Gα	5.06, 1H, d, J = 7.7 Hz	77.00, 77.03	Sα	5.05, 1H, d, J = 7.8 Hz	77.38, 77.44
Gβ	4.42, 1H, m	76.03, 76.06	Sβ	4.46, 1H, m	76.06, 76.12
Gγ	4.03, 1H, m, 4.30, 1H, m	63.31, 63.31	Sγ	4.06, 1H, m, 4.30, 1H, m	63.37, 63.37
G1	–	135.83, 135.83	S1	–	135.37, 135.37
G2	7.25, 1H, d, J = 1.7 Hz	112.72, 112.78	S2	6.90, 2H, s	105.30, 105.34
G3	–	152.53, 152.55	S3	–	153.50, 153.50
G4	–	141.43, 141.44	S4	–	130.12, 130.12
G5	7.12, 1H, d, J = 8.2 Hz	123.89, 123.91	S5	–	153.50, 153.50
G6	7.70, 1H, dd, J = 8.2, 1.7 Hz	120.72, 120.75	S6	6.90, 2H, s	105.30, 105.34
Bα	4.94, d, J = 7.6 Hz	76.83, 76.93	Bα	4.96, d, J = 7.6 Hz	76.87, 76.96
Bβ	4.35, 1H, m	76.20, 76.22	Bβ	4.39, 1H, m	76.23, 76.23
Bγ	4.03, 1H, m, 4.30, 1H, m	63.54, 63.54	Bγ	4.06, 1H, m, 4.30, 1H, m	63.59, 63.59
B1	–	129.56, 129.59	B1	–	129.64, 129.67
B2	6.77, 1H, d, J = 1.80 Hz	105.24, 105.39	B2	6.79, 1H, brd	105.30, 105.50
B3	–	150.19, 150.23	B3	–	150.24, 150.30
B4	–	134.32, 134.33	B4	–	134.41, 134.41
B5	–	145.14, 145.19	B5*	–	145.20, 145.26
B6	6.73, 1H, d, J = 1.8 Hz	109.78, 109.89	B6	6.74, 1H, d, J = 2.0 Hz	109.33, 109.36
Cα	6.56, 1H, brd, J = 15.9 Hz	134.23, 134.23	Cα	6.57, 1H, brd, J = 15.9 Hz	134.27, 134.27
Cβ	6.23, 1H, dt, J = 15.9, 6.3 Hz	123.12, 123.12	Cβ	6.24, 1H, dt, J = 15.9, 6.3 Hz	123.17, 123.17
Cγ	4.65, 2H, d, J = 6.3 Hz	65.32, 65.32	Cγ	4.66, 2H, d, J = 6.3 Hz	65.33, 65.33
C1	–	130.05, 130.06	C1	–	130.13, 130.20
C2	6.76, 1H, d, J = 1.8 Hz	103.94, 103.94	C2	6.77, 1H, d, J = 1.9 Hz	104.00, 104.00
C3	–	150.12, 150.12	C3	–	150.20, 150.20
C4	–	133.94, 133.96	C4	–	134.00, 134.03
C5	–	145.13, 145.13	C5*	–	145.17, 145.18
C6	6.65, 1H, d, J = 1.8 Hz	109.06, 109.06	C6	6.66, 1H, d, J = 1.9 Hz	109.10, 109.10

Fig. 6), and the Cα-Hα correlation of a benzodioxane constructed by two 5H units (B ring and C ring; Fig. 6), respectively. Further evidence for such benzodioxane structures can be found in the aromatic regions of HSQC spectra when we compare A1 (fraction A) and D1 (fraction B) with B1 (**12G**) and C1 (**12S**). Thus C-H correlations from the 2- and 6-positions of 5H units (B and C rings; Fig. 6) were readily identified. Also found from these spectra were C-H correlations from the 2-, 5-, and 6-positions of G units (**12G**; Fig. 6) and C-H correlations from 2/6-positions of S units (**12S**; Fig. 6), and even those from the α- and β-positions of cinnamyl acetate end groups (C ring; Fig. 6). All those C-H correlations found in these HSQC spectra of fraction A and fraction B are fully consistent with those from synthesized benzodioxane trimers **12**. However, integration of contour volumes from the well-separated aromatic correlations (at the 6-positions) in the HSQC spectra (Fig. 6, A1 and D1) suggests that fraction A contains almost only benzodioxane trimer **12G** whereas trimer **12S** was dominant in fraction B.

CONCLUSION

In summary, a combination of prior NMR and thioacidolysis data and this DFRC data demonstrates compellingly that 5-OH-CA is incorporating integrally into lignins like the traditional monolignols. 5-OH-CA is cross coupling (at its β-position) with the phenolic end of growing lignin oligomers, and new monolignols (**1G**, **1S**, and **15H**) can all cross couple with the newly formed 5-hydroxyguaiacyl end unit, extending the chain in a typical endwise fashion, and forming benzodioxane structures. Further monolignols will cross couple with the new end unit from this latest addition (G, S, or 5H unit), such that it too becomes further etherified forming new 4-*O*-β-bonds (for G and S end units) or another benzodioxane (for 5H end units). The isolated trimeric G/S-5H-5H benzodioxane products from DFRC degradation provide further evidence for such lignification mechanisms in COMT-deficient plants.

MATERIALS AND METHODS

General

All chemicals and reagents were from Aldrich and used as supplied. Solvents (analytical reagent grades) were from Mallinckrodt. Evaporations were conducted under reduced pressure at temperatures <40°C. Further removal of organic solvents, as well as drying of residues, was accomplished under higher vacuum (100–200 mTorr) at room temperature. Flash chromatography was performed using FLASH 40-M cartridges on a Biotage Isolera One flash chromatography system (Biotage, Dyax Corp.) equipped with a UA-6 UV-vis detector (ISCO). Preparative TLC plates (1 or 2 mm thickness, normal phase) were from Alltech.

^{1}H, ^{13}C, and 2D NMR (gradient COSY, HSQC, 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 0.5 to 60 mg of material in 0.5 mL of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). Carbon designations are based on conventional lignin numbering (Ralph et al., 1999). DFRC products from model compounds **13**, lignins, and poplar (*Populus* spp.) wood were analyzed by GC (Hewlett-Packard 5980) with a 0.20 mm × 25 m DB-1 (J&W Scientific) column, and electron impact-MS data were collected on a Hewlett-Packard 5970 mass-selective detector. GC conditions (He as carrier gas, 10 mL min^{-1}): initial column temperature 150°C, held for 1 min, ramped at 20°C min^{-1} to 280°C, then ramped at 2°C min^{-1} to 310°C, held for 15 min; injector 220°C, detector 300°C.

Two independent sets of COMT-deficient poplar lignin samples were used in this study. They have been described in previous publications. One is from antisense down-regulation (Van Doorsselaere et al., 1995; Lapierre et al., 1999); the other, with even lower COMT activity, is from a sense-suppression (gene-silencing) line (Jouanin et al., 2000). The COMT-deficient poplar (antisense line ASB10B) cell wall (ground to pass through a 0.85-mm screen) was used in large-scale DFRC experiments to isolate benzodioxane trimers.

Synthesis of Model Compounds

Compound **5** (Fig. 2), 5-hydroxyvanillin diacetate, was made quantitatively from 5-hydroxyvanillin by acetylation with acetic anhydride pyridine.

Compound **6**, ethyl 5-hydroxyferulate diacetate. Sodium hydride (750 mg, 31.25 mmol) was suspended in 250 mL dry triethyl phosphonoacetate (THF) in a 500 mL round-bottom flask, to which THF (7.01 g, 31.27 mmol) was added slowly (dropwise). A clear solution was formed when hydrogen gas evolution had ceased. Compound **5** (6.0 g, 23.80 mmol) was added into the solution while well stirred. Stirring was continued for another hour before adding 50 mL 3% HCl solution. THF solvent in the resultant mixture was removed by evaporation at 40°C under reduced pressure during which a white solid product precipitated. The crude product was extracted with ethyl acetate (2 × 200 mL). After being dried over anhydrous $MgSO_4$ and filtered, the ethyl acetate solution was evaporated to produce solid product (7.20 g, 94.0% yield). NMR analysis indicated that the crude product was pure enough for the next reaction. Compound **6**, NMR, δ_H: 1.27 (3H, t, J = 7.15 Hz, Me), 2.25 (3H, s, OAc), 2.26 (3H, s, OAc), 3.91 (3H, s, A-OMe), 4.20 (2H, q, J = 7.15 Hz, CH_2), 6.57 (1H, d, J = 15.97 Hz, Aβ), 7.15 (1H, d, J = 1.84, A6), 7.37 (1H, d, J = 1.84 Hz, A2), 7.61 (1H, d, J = 15.97 Hz, Aα); δ_C: 14.53 (Me), 20.07 (OAc), 20.39 (OAc), 56.79 (A-OMe), 60.84 (CH_2), 109.92 (A2), 116.19 (A6), 120.19 (Aβ), 133.66 (A1), 134.57 (A4), 143.81 (Aα), 144.86 (A5), 153.78 (A3), 166.76 (Aγ), 167.84 (OAc), 168.58 (OAc).

Compound **7** was made from 6 by deacetylation with neat pyrrolidine (Lu and Ralph, 1998). NMR, δ_H: 1.25 (3H, t, J = 7.14 Hz, Me), 3.86 (3H, s, A-OMe), 4.17 (2H, q, J = 7.14 Hz, CH_2), 6.34 (1H, d, J = 15.90 Hz, Aβ), 6.85 (1H, brs, A6), 6.88 (1H, brs, A2), 7.53 (1H, d, J = 15.90 Hz, Aα); δ_C: 14.56 (Me), 56.41 (A-OMe), 60.50 (CH_2), 104.22 (A2), 110.30 (A6), 115.95 (Aβ), 126.37 (A1), 137.31 (A4), 145.83 (Aα), 146.21 (A5), 149.01 (A3), 167.43 (Aγ).

Compound **8G**: Compounds **7** (870 mg, 3.65 mmol) and **1G** (788 mg, 4.38 mmol) were dissolved in benzene:acetone (45 mL, 2:1, v/v) in 100 mL round bottom flask and silver carbonate (Ag_2CO_3; 2.52 g, 9.13 mmol) was added. The resulting mixture was stirred overnight by which time no starting materials remained, as shown by TLC (cyclohexane:ethyl acetate, 3:1, v/v). After filtering off the solids, the organic solvents were evaporated at 40°C under reduced pressure. The residual products were redissolved in 5 mL dichloromethane and applied to a flash chromatography with cyclohexane:ethyl acetate (3:1, v/v) as eluting solvent. The major product, compound **8G** (white solid, 800 mg), was obtained in 53% yield. NMR, δ_H: 3.50 (1H, m, Aγ1), 3.80 (1H, m, Aγ2), 3.85 (3H, s, A-OMe), 3.89 (3H, s, B-OMe), 4.09 (1H, m, Aβ), 4.98 (1H, d, J = 8.0 Hz, Aα), 6.41 (1H, d, J = 15.92 Hz, Bβ), 6.85 (1H, d, J = 1.76 Hz, B6), 6.88 (1H, d, J = 8.0 Hz, A5), 6.95 (1H, d, J = 1.76 Hz, B2), 6.96 (1H, dd, J = 8.0, 1.73 Hz, A6), 7.10 (1H, d, J = 1.73 Hz, A2); δ_C: 56.19 (A-OMe), 56.29 (B-OMe), 61.55 (Aγ), 76.85 (Aα), 79.52 (Aβ), 104.70 (B2), 111.12 (B6), 111.73 (A2), 115.68 (A5), 116.98 (Bβ), 121.43 (A6), 127.37 (B1), 128.80 (A1), 136.44 (B5), 145.22 (Bα), 145.32 (B4), 147.83 (A4), 148.30 (A3), 150.10 (B3), and 167.22 (Bγ).

Compound **8S** was made in 45% yield from **1S** and **7**, in the analogous way as described above for compound **8G**. NMR, δ_H: 3.53 (1H, m, Aγ1), 3.81 (1H, m, Aγ2), 3.84 (6H, s, A/B-OMe), 3.91(3H, s, B-OMe), 4.10 (1H, m, Aβ), 4.98 (1H, d, J = 7.98 Hz, Aα), 6.41 (1H, d, J = 15.95 Hz, Bβ), 6.82 (2H, s, A2/6), 6.87 (1H, brd, B6), 6.98 (1H, brd, B2), 7.55 (1H, d, J = 15.98 Hz, Bα); δ_C: 56.40 (B-OMe), 56.66 (B-OMe), 61.62 (Aγ), 77.22 (Aα), 79.61 (Aβ), 104.98 (B2), 106.15 (A2/6), 111.17 (B6), 117.16 (Bβ), 127.51 (B1), 127.85 (A1), 136.64 (B5), 145.21 (Bα), 145.45 (B4), 148.73 (A3/5), 150.29 (B3), and 167.17 (Bγ).

Compound **9Gf**: Reduction of **8G** with DIBAL-H in toluene gave benzodioxane lignin models **9Gf** in 95% yield. NMR, δ_H: 3.49 (1H, m, Aγ1), 3.76 (1H, m, Aγ2), 3.84 (6H, s, A/B-OMe), 4.04 (1H, m, Aβ), 4.21 (2H, dd, J = 5.30, 1.34 Hz, Bγ), 4.95 (1H, d, J = 8.02 Hz, Aα), 6.26 (1H, dt, J = 15.87, 5.40 Hz, Bβ), 6.47 (1 h, dt, J = 15.87, 1.34 Hz, Bα), 6.59 (1H, d, J = 1.84 Hz, B6), 6.69 (1H, d, J = 1.84 Hz, B2), 6.87 (1H, d, J = 8.17 Hz, A5), 6.94 (1H, dd, J = 8.17, 1.80 Hz, A6), 7.08 (1H, d, J = 1.80 Hz, A2); δ_C: 56.17 (A-OMe), 56.17 (B-OMe), 61.64 (Aγ), 63.19 (Bγ), 76.88 (Aα), 79.29 (Aβ), 103.22 (B2), 108.51 (B6), 111.69 (A2), 115.68 (A5), 121.37 (A6), 129.01 (Bβ), 129.06 (A1), 130.08 (Bα), 130.34 (B1), 133.74 (B4), 145.22 (B5), 147.75 (A4), 148.30 (A3), and 149.78 (B3).

Compound **9Sf** was synthesized from 8S, in the analogous way as for **9Gf** from 8G, in 92% yield. NMR, δ_H: 3.50 (1H, m, Aγ1), 3.77 (1H, m, Aγ2), 3.84 (6H, s, A3/5-OMe), 3.85 (3H, s, B3-OMe), 4.04 (1H, m, Aβ), 4.20 (2H, dd, J = 5.40, 1.48 Hz, Aγ), 4.95 (1H, d, J = 7.92 Hz, Aα), 6.26 (1H, dt, J = 15.87, 5.40 Hz, Bβ), 6.47 (1H, dt, J = 15.87, 1.48 Hz, Bα), 6.59 (1H, d, J = 1.9 Hz, B6), 6.69 (1H, d, J = 1.90 Hz, B2), 6.80 (2H, s, A2/6); δ_C: 56.27(B-OMe); 56.71(A-OMe), 61.60 (Aγ), 63.08 (Bγ), 77.23 (Aα), 79.42 (Aβ), 103.52 (B2), 106.22 (A2/6), 108.54 (B6), 128.16 (A1), 129.54 (Bβ), 129.84 (Bα), 130.50 (B1), 134.03 (A4), 137.37 (A4), 145.37 (B5), 148.84 (A3/5), 150.02 (B3).

Compounds **9Ge** and **9Se** were made by methylation of **9Gf** and **9Sf** with iodomethane-potassium carbonate in acetone, respectively. Compound **9Ge**: NMR, δ_H: 3.49 (1H, m, Aγ1), 3.78 (1H, m, Aγ2), 3.821 (3H, s, A4-OMe), 3.824 (3H, s, A3-OMe), 3.85 (3H, s, B-OMe), 4.05 (1H, m, Aβ), 4.19 (2H, dt, J= 5.4, 1.42 Hz, Bγ), 5.00 (1H, d, J = 7.86 Hz, Aα), 6.26 (1H, dt, 15.84, 5.4 Hz, Bβ), 6.47 (1H, dt, J = 15.84, 1.45 Hz), 6.59 (1H, d, J= 1.92 Hz, B6), 6.69 (1H, d, J =1.92 Hz, B2), 6.98 (1H, d, J = 8.25 Hz, A5), 7.03 (1H, dd, J = 8.25, 1.92 Hz), 7.10 (1H, d, J = 1.92 Hz, A2); δ_C: 56.10 (A4-OMe), 56.16 (A-OMe), 60.31 (B3-OMe), 61.75 (Aγ), 63.25 (Bγ), 76.88 (Aα), 79.36 (Aβ), 103.60 (B2), 108.60 (B6), 112.22 (A2), 112.53 (A5), 121.09 (A6), 129.37 (Bβ), 130.03 (Bα), 130.45 (A1), 130.58 (B1), 134.03 (B4), 145.36 (B5), 150.06(B3), 150.43 (A4), 150.74 (A3). Compound **9Se**: NMR, δ_H: 3.52 (1H, m, Aγ1), 3.74 (3H, s, A4-OMe), 3.79 (1H, m, Aγ2), 3.84 (3H, s, B-OMe), 3.85 (6H, s, A3/5-OMe), 4.05 (1H, m, Aβ), 4.20 (2H, dt, J= 5.5, 1.50 Hz, Bγ), 4.99 (1H, d, J = 7.85 Hz, Aα), 6.26 (1H, dt, 15.87, 5.5 Hz, Bβ), 6.48 (1H, dt, J = 15.87, 1.50 Hz), 6.60 (1H, d, J= 1.84 Hz, B6), 6.70 (1H, d, J =1.84 Hz, B2), 6.84 (2H, s, A2/6); δ_C: 56.33 (B-OMe), 56.50 (A3/5-OMe), 60.53 (A4-OMe), 61.72 (Aγ), 63.26 (Bγ), 77.13 (Aα), 79.26 (Aβ), 103.66 (B2), 106.01 (A2/6), 108.58 (B6), 129.41 (Bβ), 130.01 (Bα), 130.61 (B1), 133.42 (A1), 134.01 (B4), 139.49 (A4), 145.23 (B5), 150.07(B3), 154.47 (A3/5).

Compound **12G** (Figs. 2 and 6) was synthesized from radical coupling reactions of **1G** and **1SH** via Ag_2CO_3 oxidation in toluene:acetone (4:1, v/v): **1SH** (1.5 g, 7.6 mmol) was dissolved in the solvents and Ag_2CO_3 (2.1g, 7.6 mmol) was added. This mixture was stirred for 1 h before **1G** (1.0 g, 5.5 mmol) and more Ag_2CO_3 (1.0 g, 3.64 mmol) were added in. Stirring the resultant mixture was continued for another 1 h. After filtering off the solid oxidant, the solvents were removed by evaporation and the resultant products were acetylated with Ac_2O:pyridine (20 mL, 1:1, v/v) at room temperature overnight. The excess reagents were removed by evaporation following addition of ethanol (20 mL), repeated four times. The resultant acetylated products were purified by normal-phase silica-gel flash chromatography using cyclohexane:ethyl acetate (3:1–2:1, v/v) as eluting solvents. Each fraction was checked by TLC and fractions with similar compositions were combined. The dimeric 5H-5H-benzodioxane acetate, **10** (200 mg) was obtained, and the desired pure product **12G** (G-5H-5H; 30 mg) was also isolated from the product mixture.

Compound **12S**: Compound **10**, isolated from the above reaction, was selectively deacetylated in neat pyrrolidine to produce benzodioxane catechol **11**. Radical coupling of **1S** and **11** via Ag_2CO_3 oxidation in benzene/acetone produced, after acetylation, trimeric S-5H-5H-benzodioxane **12S**. Thus compound **10** (200 mg) was dissolved in 1 mL pyrrolidine for 1 min then transferred with ethyl acetate into a separatory funnel containing 20 mL ethyl acetate and 10 mL 1 M aqueous HCl solution. After shaking the funnel and allowing to partition, the products in ethyl acetate were washed with saturated NH_4Cl and dried over $MgSO_4$. The ethyl acetate was removed by evaporation. The residues (150 mg) were dissolved in acetone and coupled with **1S** (100 mg, 0.48 mmol) via Ag_2O oxidation for 1 h. After filtering off the solid oxidant the products were acetylated with Ac_2O pyridine overnight. Evaporating off all reagents gave acetylated products. TLC separation of these products produced the desired trimeric S-5H-5H-benzodioxane acetate **12S** (10 mg) as pale oil.

The synthesized trimers **12** were each mixtures of two diastereomers (from the remote centers in each of the benzodioxane rings, i.e. RR/SS-RR/SS versus RR/SS-SS/RR isomers) with essentially a 1:1 ratio. They were characterized

by NMR and the data are listed in Table II (which shows the two peaks for each assigned carbon).

Synthesis of compound **13**G*f* (Fig. 3) has been reported previously (Ralph et al., 2001a). Compound **13**G*e* was made from **13**G*f* by methylation with iodomethane and K_2CO_3 in acetone. NMR, δ_H: 3.50 (1H, m, Aγ1), 3.80 (1H, m, Aγ2), 3.80 (9H, br-s, A/B-OMe), 4.02 (1H, m, Aβ), 4.51 (2H, s, Bα), 4.97 (1H, d, J = 7.85 Hz), 6.55 (1H, br-d, B6), 6.60 (1H, br-d, B2), 6.95 (1H, d, J = 8.30 Hz, A5), 7.01 (1H, dd, J = 8.30, 1.60 Hz, A6), 7.08 (1H, d, J = 1.60 Hz, A2); δ_C: 61.78 (Aγ), 64.62 (Bα), 76.81 (Aβ), 79.20 (Aα), 104.09 (B2), 108.56 (B6), 119.78 (A2), 112.51 (A5), 121.03 (A6), 130.53 (A1), 133.17 (B4), 1135.39 (B1), 145.09 (B5), 149.78 (B3), 150.18 (A4), 150.39 (A3).

Compounds **14** were obtained from DFRC treatment of compounds **13**. Since these were almost quantitative conversions, compounds **14** were characterized directly after DFRC reactions, without any purification. Compound **14**G*f*: NMR, δ_H: 2.22 (3H, s, Bα), 3.79 (3H, s, B-OMe), 3.84 (3H, s, A-OMe), 4.01 (1H, m, Aγ1), 4.26 (1H, m, Aγ2), 4.33 (1H, m, Aβ), 5.01 (1H, d, J = 7.65 Hz, Aα), 6.38 (1H, d, J =1.80 Hz, B6), 6.44 (1H, d, J = 1.80 Hz, B2), 7.06 (1H, dd, J = 8.04, 1.80 Hz, A6), 7.11 (1H, d, J = 8.04 Hz, A5), 7.24 (1H, d, J = 1.80 Hz, A2); δ_C: 21.18 (Bα), 56.29, 56.34, 63.48 (Aγ), 75.85 (Aβ), 77.01 (Aα), 110.18 (B2), 110.60 (B6), 112.74 (A2), 120.68 (A6), 123.84 (A5), 130.87 (B1), 131.65 (B4), 136.27 (A1), 141.37 (A4), 144.81 (B5), 149.78 (B3), 152.54 (A3). Compound **14**G*e*: NMR, δ_H: 2.22 (3H, s, Bα), 3.79 (3H, s, B-OMe), 3.84 (6H, s, A3/4-OMe), 3.96 (1H, m, Aγ1), 4.21 (1H, m, Aγ2), 4.29 (1H, m, Aβ), 5.01 (1H, d, J = 7.84 Hz, Aα), 6.36 (1H, br-d, B6), 6.43 (1H, br-d, B2), 6.99 (2H, br-s, A5+A6), 7.07 (1H, br-s, A2); δ_C: 21.20 (Bα), 56.07(A3-OMe), 56.23 (A4-OMe), 56.27 (B-OMe), 63.71 (Aγ), 76.09 (Aβ), 77.04 (Aα), 106.77 (B2), 110.62 (B6), 112.05 (A2), 112.58 (A5), 121.06 (A6), 129.82 (A1), 130.73 (B1), 131.75 (B4), 145.06 (B5), 149.74 (B3), 150.55 (A4), 150.94 (A3).

Compounds **15** (Fig. 3) were synthesized by acetylation of the corresponding compounds **13** with acetic anhydride and pyridine. Characterization of **15**G*f* and its NMR data have been reported (Ralph et al., 2001a). **15**G*e*: NMR, δ_H: 3.81 (3H, s, A3-OMe), 3.82 (3H, s, A4-OMe), 3.83 (3H, s, B-OMe), 3.95 (1H, m, Aγ1), 4.23 (1H, m, Aγ2), 4.34 (1H, m, Aβ), 4.95 (1H, d, J = 7.94 Hz), 4.96 (2H, s, Bα), 6.60 (1H, d, J = 1.50 Hz, B6), 6.65 (1H, d, J = 1.50 Hz), 6.97 (1H, d, J = 8.14 Hz, A5), 7.00 (1H, dd, J = 8.14, 1.74 Hz, A6), 7.08 (1H, d, J = 1.74 Hz, A2); δ_C: 55.95 (A3-OMe), 56.02 (A4-OMe), 56.27 (B-OMe), 63.55 (Ag), 66.36 (Bα), 76.12 (Aβ), 76.94 (Aα), 105.75 (B2), 110.39 (B6), 111.77 (A2), 112.33 (A5), 121.01 (A6), 129.35 (A1), 129.66 (B1), 133.59 (B4), 145.03 (B5), 149.82 (B3), 150.41 (A3), 150.84 (A4).

Modified DFRC Procedure for COMT-Deficient Lignins and Cell Wall Isolates

DFRC

Lignin (8–10 mg) or 40 mg extracted wood cell wall was used. The acetyl bromide treatment and zinc reduction steps were performed as per the standard DFRC procedure (Lu and Ralph, 1997a). After the zinc reduction step the degraded products were methylated as follows, instead of using the acetylation step from the original procedure.

Methylation

The above residue was methylated using iodomethane (50 μL) and cesium carbonate (100 mg) in 3 mL acetonitrile for 30 min. The excess reagents were quenched by addition of 1 mL acetic acid. The organic solvents were evaporated under reduced pressure before the methylated products were transferred into a separatory funnel containing 6 mL saturated sodium chloride aqueous solution with 15 mL dichloromethane. After shaking the funnel, the dichloromethane phase was collected and the water phase was extracted with further dichloromethane (2 $\times$ 6 mL). The combined dichloromethane solution was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure.

Solid Phase Extraction

The above residue was transferred with 2 mL dichloromethane into a 10 mL pear-shaped flask, concentrated to less than 50 μL, and loaded, with further dichloromethane (the total volume should not be larger than 150 μL), to a 3 mL preconditioned (cyclohexane:ethyl acetate, 5:1, v/v) normal-phase solid phase extraction column (LC-Si, Supelco). The monomers were eluted with 9 mL cyclohexane:ethyl acetate (5:1, v/v). Then the dimeric products were eluted with 9 mL cyclohexane:ethyl acetate (1:1.5, v/v). This dimeric fraction was evaporated under reduced pressure.

Hydrolysis

The above dimeric products were dissolved in 3 mL methanol in a 25 mL flask to which 3 mL 1 M aqueous potassium carbonate was added. This mixture was stirred for 1 h. The solution was concentrated to about 3 mL and acidified with 4 mL 1 M aqueous HCl solution and saturated with sodium chloride followed by extraction with dichloromethane (3 $\times$ 10 mL). The combined dichloromethane solution was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure.

TMS Derivatization and GC Analysis

The above residue was transferred with 2 mL dichloromethane into a 10 mL pear-shaped flask and internal standard (mono-4-*O*-methylated 5-5-diferulic acid; Bunzel et al., 2003), and 10 to 15 μL pyridine was added in. Finally, the sample was concentrated and transferred into a vial with 50 μL pyridine, and derivatized with 50 μL *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide for 30 min at 50°C before 5 μL was injected onto the GC.

Large-Scale DFRC on COMT-Deficient Poplar Cell Walls

To cryogenically (liquid N_2) milled-wood sawdust (extractive free, 8.5 g) were added 150 mL 20% (v/v) acetyl bromide/acetic acid solution in a 250 mL round-bottom flask. This mixture was gently stirred at 50°C for 3.5 h. After removal of all solvent and reagents by rotary evaporation at below 40°C, the residues were dissolved in 150 mL dioxane:AcOH:water (5:4:1, v/v/v) solution. Zinc dust, 8.0 g, was added in two parts while this solution was well stirred. Stirring was continued for 30 min, and then the mixture let stand for 30 min before the liquid contents were decanted with the help of some added acetone. The resulting liquid fraction was concentrated to about 100 mL and diluted with 200 mL water, the DFRC degradation products were extracted with dichloromethane (200 $\times$ 2 mL). After evaporation of the dichloromethane solution, the residues were acetylated with 20 mL acetic anhydride:pyridine (1:1, v/v) for 16 h.

Silica-Gel Flash Chromatographic Fractionation

The resulting products were purified by flash chromatography by applying to a normal-phase silica gel column (FLASH 40 M cartridge), and eluting with cyclohexane:ethyl acetate (3:1, 1 L; 1:1, 1 L; 1:2, 0.75 L; and 0:1, 0.5 L) successively. A total of 12 fractions was collected.

TLC Separation

TLC comparison of the isolated fractions with synthesized benzodioxane trimers **12** indicated that fractions 9 to 10 potentially contained the expected benzodioxane products. NMR examination of these fractions showed that they were heavily contaminated with degraded carbohydrates and further purification was needed. Thus fractions 9 and 10 were combined and deacetylated with 2 mL pyrrolidine in 10 mL ethanol overnight. The lignin degradation products were recovered by ethyl acetate extraction. After being dried over $MgSO_4$ and filtered off, the ethyl acetate solvent was removed on a rotary evaporator at 40°C under reduced pressure. The residues were acetylated in Ac_2O pyridine producing about 80 mg products, after removing the acetylating reagents by evaporation. These acetylated products were applied to a TLC plate (silica gel, 1 mm) using cyclohexane:ethyl acetate (1.5:1, v/v) as eluant. Two fractions (A, 2.5 mg and B, 2.0 mg) with the same R*f* values as trimers **12** were collected for NMR analysis.

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