NMR Evidence for Benzodioxane Structures Resulting from Incorporation of 5-Hydroxyconiferyl Alcohol into Lignins of *O*-Methyltransferase-Deficient Poplars

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Benzodioxane structures are produced in lignins of transgenic poplar plants deficient in COMT, an *O*-methyltransferase required to produce lignin syringyl units. They result from incorporation of 5-hydroxyconiferyl alcohol into the monomer supply and confirm that phenols other than the three traditional monolignols can be integrated into plant lignins.

Keywords: *Lignin; benzodioxane; 5-hydroxyconiferyl alcohol; lignan; O-methyltransferase; antisense; sense-upregulation; transgenic plants; NMR*

INTRODUCTION

Lignins are traditionally described as plant polymers resulting from dehydrogenative polymerization (via radical coupling reactions) of three primary phenylpropanoid monomers, *p*-coumaryl (4-hydroxycinnamyl), coniferyl (4-hydroxy-3-methoxycinnamyl), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohols, analogues varying in their degree of methoxylation (*1*). They represent a class of complex polymeric natural products present in large quantities in the cell walls of terrestrial plants.

Recent advances in genetic engineering have allowed researchers to perturb the monolignol biosynthetic pathway (*2*−*5*), allowing normally minor components to be substantially enhanced and therefore structurally analyzed (*6*−*10*). This approach provides valuable insights into the control of lignification and into the remarkable biochemical flexibility of the lignification system. For example, *Arabidopsis* plants completely devoid of syringyl units in their lignins were found in a chemical-mutagenesis-derived mutant (*11*, *12*) deficient in ferulate 5-hydroxylase, for which the preferred substrate is now recognized to be coniferyl aldehyde rather than ferulate (*13*, *14*). More strikingly, when the gene was reintroduced into the mutant background, with a suitable promoter, the guaiacyl level was <3%, far lower than in any plant reported to date (*15*). Similarly, when the final enzyme on the monolignol pathway [cinnamyl alcohol dehydrogenase (CAD)] was downregulated, incorporation of hydroxycinnamyl aldehydes into the lignins increased and new products were found (*10*).

Benzodioxane lignans resulting from cross-coupling of monolignols (coniferyl or sinapyl alcohol) with 5-hydroxyconiferyl alcohol have been reported. Compound **1**, in Figure 1a, the cross-product of sinapyl alcohol and 5-hydroxyconiferyl alcohol, was isolated from the wood of *Xanthoxylum nitidum* (*Fagara nitida*) (*16*), a plant used as a folk medicine in the tropics. NMR methods (selective INEPT) were used to identify the coupling regiochemistry (4-O-*β*/5-O-α versus 5-O-*β*/4-O-α). Glucosides of the cross-products of coniferyl alcohol and 5-hydroxyconiferyl alcohol, isomers **2a** and **2b**, were isolated from whole plants of *Pedicularis verticillata* (*17*), a Chinese medicinal plant.

The first suggestion that 5-hydroxyconiferyl alcohol can be incorporated into lignins was the observation of the benzodioxane **3** (Figure 1b) in hydrogenolysis products from *Fraxinus mandshurica* var. *japonica* (*18*). Later, analytical thioacidolysis produced 5-hydroxyguaiacyl monomers from a brown-midrib mutant of maize (*19*), specifically the *bmr3* mutant deficient in caffeic acid *O*-methyltransferase (COMT) (*20*), an *O*-methyltransferase operating late in the lignin biosynthetic pathway. The enzyme, which now appears to preferentially methylate 5-hydroxyconiferyl aldehyde (*21*) but will also methylate 5-hydroxyconiferyl alcohol (*22*), is one of two enzymes required to produce lignin syringyl (3,5-dimethoxy-4-hydroxyphenyl) units. Jacquet (*23*) postulated benzodioxane structures in lignins following extensive work demonstrating the role of 5-hydroxyconiferyl alcohol in the lignification of COMT-deficient plants. 5-Hydroxyguaiacyl compounds were also noted in pyrolysates of a wider variety of plants, mainly tropical grasses and tropical angiosperm woody plants (*24*). More recently, with one of the COMT-deficient

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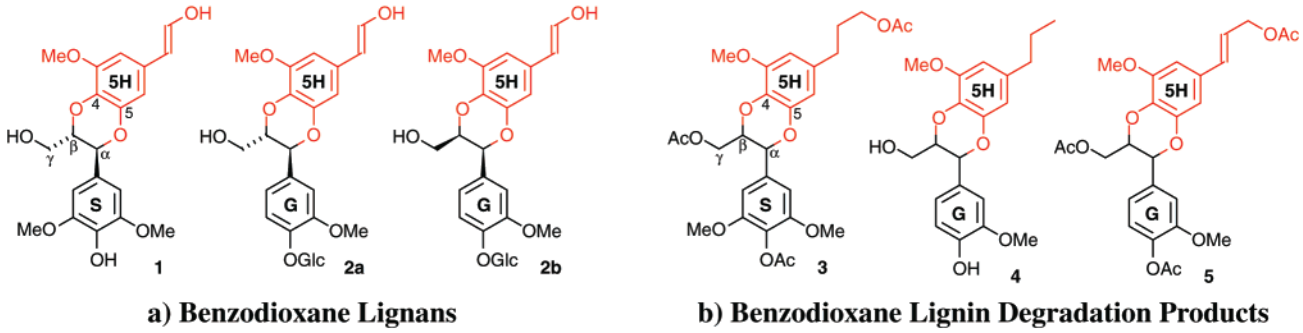

Figure 1. (a) Benzodioxane lignans **1** and **2** previously reported. (b) Benzodioxanes **3**−**5** observed in lignin degradation products.

poplar transgenics used in the study reported here, a new compound was found among the products of thioacidolysis followed by Raney nickel desulfurization (*9*). The mass spectrum suggested that it was compound **4** (Figure 1b) or an isomer. We also have preliminary evidence for the release of compound **5** by derivatization followed by reductive cleavage, the "DFRC" method (*25*, *26*). None of these methods, however, establishes the presence of benzodioxanes as such in the lignin polymer.

Here we provide NMR evidence for benzodioxane units in lignins isolated from poplar (*Populus tremula* × *Populus alba*) transgenics deficient in COMT. The presence of benzodioxane structures strongly suggests that 5-hydroxyconiferyl alcohol (a hydroxycinnamyl alcohol not normally associated with lignification) incorporates as a monomer into the lignin.

MATERIALS AND METHODS

General. Commercially available chemicals were from Aldrich, Milwaukee, WI, and used without further purification unless otherwise stated. Ion-trap mass spectra were run on a Thermoquest (Austin, TX) Trace 2000 GC (Austin, TX) coupled to a Thermoquest GCQ ion-trap MS.

Transgenic Poplars. The transgenic poplars examined were (a) an attempted sense-overexpressed transgenic line with a double 35S promoter in which the COMT activity was close to zero due to a gene silencing phenomenon, as described recently (*9*)—similar sense suppression was earlier observed in quaking aspen (*27*) and (b) an antisense COMT poplar also previously described (*28*, *29*).

Lignin Isolation. Three to four stems from each line of 7-month-old poplars (control and the two transgenics) were pooled in their respective groups. Before isolation of the poplar stem cell walls, the stems were cut into 1−2-cm pieces and ground to pass a 1.0-mm screen of a cyclone mill (Udy Corp., Fort Collins, CO). Lignin isolations were essentially as described previously (*6*, *30*). Soluble phenolics, carbohydrates, and other components were removed by successive extractions with water, methanol, acetone, and chloroform. Most of the colored material was removed by the water and methanol extraction cycles. The isolated cell wall material was ball-milled (three cycles of 0.5 h on, 0.5 h off; stainless steel ball bearings, stainless steel vessels, under ambient atmosphere), suspended in 50 mM acetate buffer (pH 5.0), and treated with 30 mg of cellulase (Cellulysin Calbiochem-Novabiochem) per 1 g of ball-milled material. Cell wall digestions ran for 8 days with fresh enzyme and buffer being added after 2.5 and 5 days of incubation at 30 °C. The resulting lignin polysaccharide complex was subjected to fractionation in 96:4 v/v dioxane/water, reflective of standard "milled wood lignin" conditions (*31*). The final yields of the dioxane soluble lignin fractions were 65% of the total (Klason) lignins in all three samples. Isolated lignins (200 mg of each) were acetylated overnight using acetic anhydride/pyridine, and the solvents were removed by coevaporation with 95% ethanol. Traces of ethanol were removed by coevaporation with acetone. The acetylated lignins were then extracted into $CHCl_3$ and washed with aqueous EDTA (6 mM, pH 8.0) to remove trace metal contaminants.

NMR Spectroscopy. NMR spectra were acquired 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). Acetylated lignins (EDTA-washed, 100 mg) were dissolved in 0.4 mL of acetone-d_6; unacetylated lignins (not EDTA-washed, 60 mg) were dissolved in acetone-d_6 (0.35 mL) and deuterium oxide (D_2O, 0.05 mL). The central acetone solvent peak was used as internal reference (δ_C 29.80, δ_H 2.04). We used the standard Bruker implementation of the gradient-selected inverse (^{1}H-detected) HMQC experiment (*32*, *33*) for the spectra in Figure 3.

Synthesis of Benzodioxane Model Compound 13 {3,5-Bis(hydroxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-methoxybenzo[1,4]dioxane}. Methyl 4,5-dihydroxy-3-methoxybenzoate (methyl 3-*O*-methylgallate) was prepared from methyl gallate by methylation of the borate complex (*34*). Coniferyl alcohol was prepared from coniferaldehyde by reduction using sodium triacetoxyborohydride in ethyl acetate (*35*). Radical coupling was accomplished using the method of She et al. (*36*). Ag_2CO_3 (2.53 g, 9.20 mmol) was added to a solution of methyl 3-*O*-methylgallate (900 mg, 4.55 mmol) and coniferyl alcohol (954 mg, 5.3 mmol) in acetone/benzene (1:2, 100 mL) and the mixture stirred for 18 h at room temperature. Following filtration through silica gel and solvent evaporation, the product was purified by flash chromatography using 2:1 petroleum ether (bp range 30−60 °C)/ethyl acetate to yield the *β*-*O*-4/α-*O*-5-coupled product {methyl 2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-5-methoxybenzo[1,4]dioxane-7-carboxylate} in 65% yield: δ_C/δ_H (assignment) 76.8/4.99 (α), 79.6/4.12 (*β*), 61.5/3.82,3.52 (*γ*). Reduction of the ester (150 mg) with DIBAL (8 equiv) (*37*) and collection of the white solid triturated from acetone produced the required compound **13** (115 mg, 85%). *trans*-**13**: δ_H 3.47 (1H, m, γ_1), 3.76 (1H, m, γ_2), 3.81 (3H, s, 5H-3-OMe), 3.85 (3H, s, G-3-OMe), 3.99 (1H, ddd, J = 7.9, 3.9, 2.5 Hz, *β*), 4.49 (2H, m, 5H-α), 4.95 (1H, d, J = 7.9 Hz, α), 6.53 (1H, ddd, J = 1.8, 0.75, 0.75 Hz, 5H-6), 6.60 (1H, d, J = 1.8 Hz, 5H-2), 6.86 (1H, d, J = 8.1 Hz, G-5), 6.94 (1H, ddd, J = 8.1, 1.9, 0.5 Hz, G-6), 7.09 (1H, d, J = 1.9 Hz, G-2), 7.80 (1H, s, G-4-OH); δ_C 56.26 (5H-3-OMe), 56.32 (G-3-OMe), 61.8 (*γ*), 64.6 (5H-α), 77.0 (α), 79.3 (*β*), 104.1 (5H-2), 108.6 (5H-6), 111.9 (G-2), 115.7 (G-5), 121.5 (G-6), 129.5 (G-1), 133.2 (5H-4), 135.5 (5H-1), 145.2 (5H-5), 147.9 (G-4), 148.4 (G-3), 149.8 (5H-3). Acetylation with 1:1 pyridine/acetic anhydride produced the triacetate **13**-Ac as a colorless oil: δ_H 1.99 (3H, s, 5H-α-OAc), 2.03 (3H, s, *γ*-OAc), 2.24 (3H, s, G-4-OAc), 3.83 (3H, s, 5H-3-OMe), 3.84 (3H, s, G-3-OMe), 4.02 (1H, dd, J = 12.4, 4.3 Hz, γ_1), 4.28 (1H, dd, J = 12.4, 3.4 Hz, γ_2), 4.40 (1H, ddd, J = 7.7, 4.3, 3.4 Hz, *β*), 4.98 (2H, s, 5H−α), 5.04 (1H, d, J = 7.7 Hz, α), 6.61 (1H, d, J = 1.9 Hz, 5H-6), 6.66 (1H, d, J = 1.9 Hz, 5H-2), 7.08 (1H, dd, J = 8.1, 1.7 Hz, G-6), 7.11 (1H, d, J = 8.1 Hz, G-5), 7.26 (1H, d, J = 1.7 Hz, G-2); δ_C 20.45 (G-4-OAc), 20.49 (*γ*-OAc), 20.8 (5H-α-OAc), 56.36 (G-3-OMe), 56.44 (5H-3-OMe), 63.4 (*γ*), 66.4 (5H-α), 76.0 (*β*), 77.0 (α), 106.1 (5H-2), 110.4 (5H-6), 112.8 (G-2), 120.8 (G-6), 123.9 (G-5), 129.9 (5H-1), 133.7 (5H-4), 136.0 (G-1), 141.5

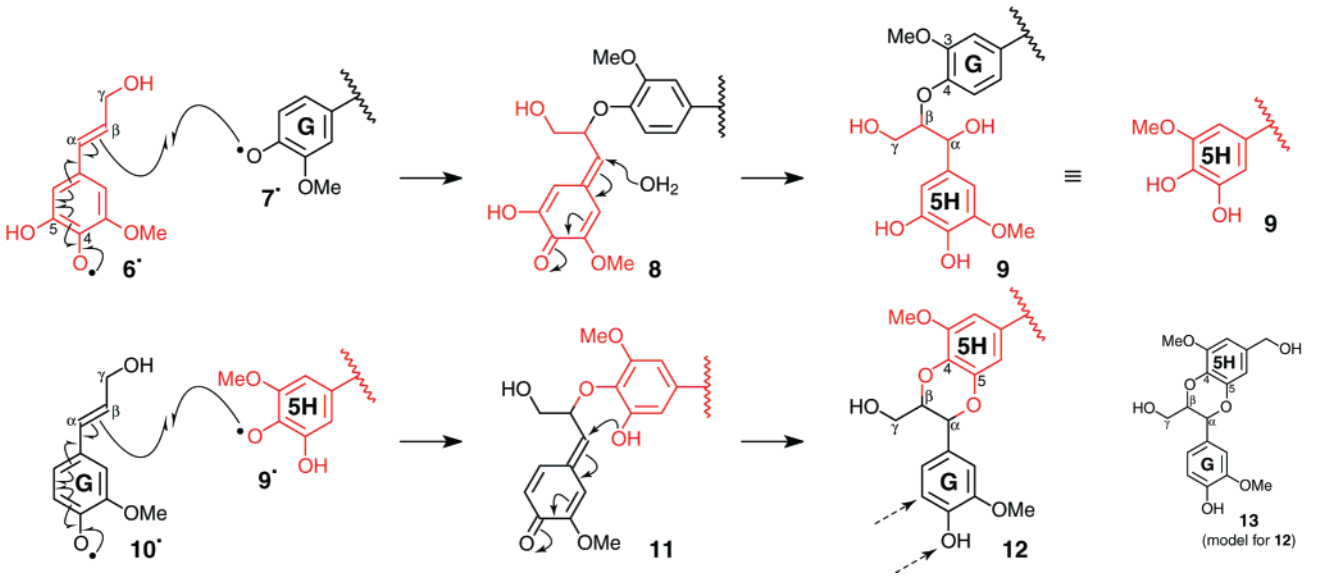

Figure 2. Mechanistic scheme for the production of benzodioxanes **12** in lignins via incorporation of 5-hydroxyconiferyl alcohol **6** into a guaiacyl lignin. Only the pathways producing β-ether units are shown, but β-5- (phenylcoumaran) units are also possible. Cross-coupling with syringyl units is also possible but must be less pronounced in lignins which have a low syringyl content due to strong COMT downregulation. G = guaiacyl unit; 5H = 5-hydroxyguaiacyl unit.

(G-4), 144.9 (5H-5), 145.0 (5H-3), 152.6 (G-3), 168.9 (G-4-OAc), 170.6 (γ-OAc), 170.8 (5H-α-OAc); ms, *m/z* (rel abundance) 474 (100, M^+), 432 (25), 415 (11), 372 (26), 313 (25), 271 (45), 222 (19), 179 (10). Authentication of the 4-O-β/5-O-α-regiochemistry was from long-range $^{13}C-^{1}H$ (HMBC) correlations between proton-α (4.98 ppm) and the 5H-5-carbon (144.9 ppm); the small ^3J $C_{5H-5}-H_\alpha$ coupling constant (apparently ~1 Hz) required an HMBC coupling delay of at least 200 ms to readily observe this correlation. This regiochemistry in the rapidly relaxing lignins could not be authenticated as the required long-range coupling delay was beyond their proton relaxation times. A *trans* configuration was assigned from coupling constants ($J_{\alpha\beta}$ = 7.7 Hz); traces of the presumed *cis* isomer (δ 5.38, $J_{\alpha\beta}$ = 2.7 Hz, α) were also found.

RESULTS AND DISCUSSION

Radical cross-coupling of monolignol radicals **10•** (Figure 2) with the growing lignin oligomer/polymer (radicals) **7•** is the major reaction occurring during lignification (*1*). Consequently, it is anticipated that if 5-hydroxyconiferyl alcohol **6** incorporates into lignins, it will likely do so via radical **6•** reacting at its favored β-position with lignin radicals **7•**, at their 4-*O*- or 5-positions for guaiacyl radicals (only the 4-*O*-coupling is shown in Figure 2); syringyl lignin units can react only at their 4-*O*-positions. Following trapping by water of the quinone methide intermediate **8** in the conventional way, a new 5-hydroxyguaiacyl unit **9** is produced. These bis(phenol)s can also generate radicals via oxidative processes, typically peroxidase and hydrogen peroxide, or laccase and oxygen in vivo. Radicals **9•** react readily at their 4-*O*- positions with monolignol radicals **10•** (at their favored β-positions) as seen in the synthesis of **13**. What follows is a logical trapping of the quinone methide internally by the phenolic 5-OH, effecting rearomatization and generating a benzodioxane structure **12**. These units may then incorporate more fully into the lignin by further radical coupling reactions with normal lignin monomers **10•** or radicals from the novel 5-hydroxyconiferyl alcohol monomer **6**.

NMR spectra (Figure 3) provide elegant proof that benzodioxanes are major structures in the two COMT-deficient poplar lignins. As with the recently identified dibenzodioxocins (*38*−*40*), the benzodioxanes are readily apparent in short-range $^{13}C-^{1}H$ correlation spectra (HMQC or HSQC) of acetylated isolated lignins. Well-resolved contours centered at δ_C/δ_H of 76.8/4.98 (α) and 75.9/4.39 (β) are diagnostic; the γ-correlations overlap with those in other lignin units. Anticipated shifts occur following acetylation; in the unacetylated lignins (not shown), the correlations are centered at δ_C/δ_H of 76.5/4.87 (α) and 78.9/4.06 (β). As seen in Figure 3d, the side-chain correlations agree well with those in a model compound for the acetylated *trans*-benzodioxane, **13**-Ac, synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit. The α-proton shift deviated the most, presumably because this is an acetylated (and therefore originally free-phenolic) model rather than a phenol-etherified structure, which would correspond to most of the units in the lignin polymer. The presumed *cis*-benzodioxane model *cis*-**13**-Ac, had its α-proton at 5.38 ppm, significantly downfield of that from the *trans* isomer (at 4.98 ppm); its location beyond the Cα/Hα contour in either of the HMQC spectra (Figure 3b,c) suggests that the *trans* isomer is the major benzodioxane isomer in the lignins as well. The 4-O-β/5-O-α- versus 5-O-β/4-O-α-regiochemistry of the benzodioxane model **13**-Ac was established from long-range $^{13}C-^{1}H$ (HMBC) correlations between proton-α (4.98 ppm) and the 5H-5-carbon (144.9 ppm). Strictly, this regiochemical assignment could not be authenticated in the lignins as the required long-range coupling delay of >200 ms was well beyond their proton relaxation times. The assignment here is therefore by analogy with the coupling reactions used to produce model **13** and lignan **1** (*16*) and those required to produce the structure in lignins. Independent authentication must be sought. The possibility that the correlations might be due to catechol structures (without the methoxylated aromatic) known to be in the extractives of poplars is unlikely to be due to the absence of such structures and the presence of the 5-hydroxyguaiacyl monomer and putative benzodioxane **4** in thioacidolysis products (*9*).

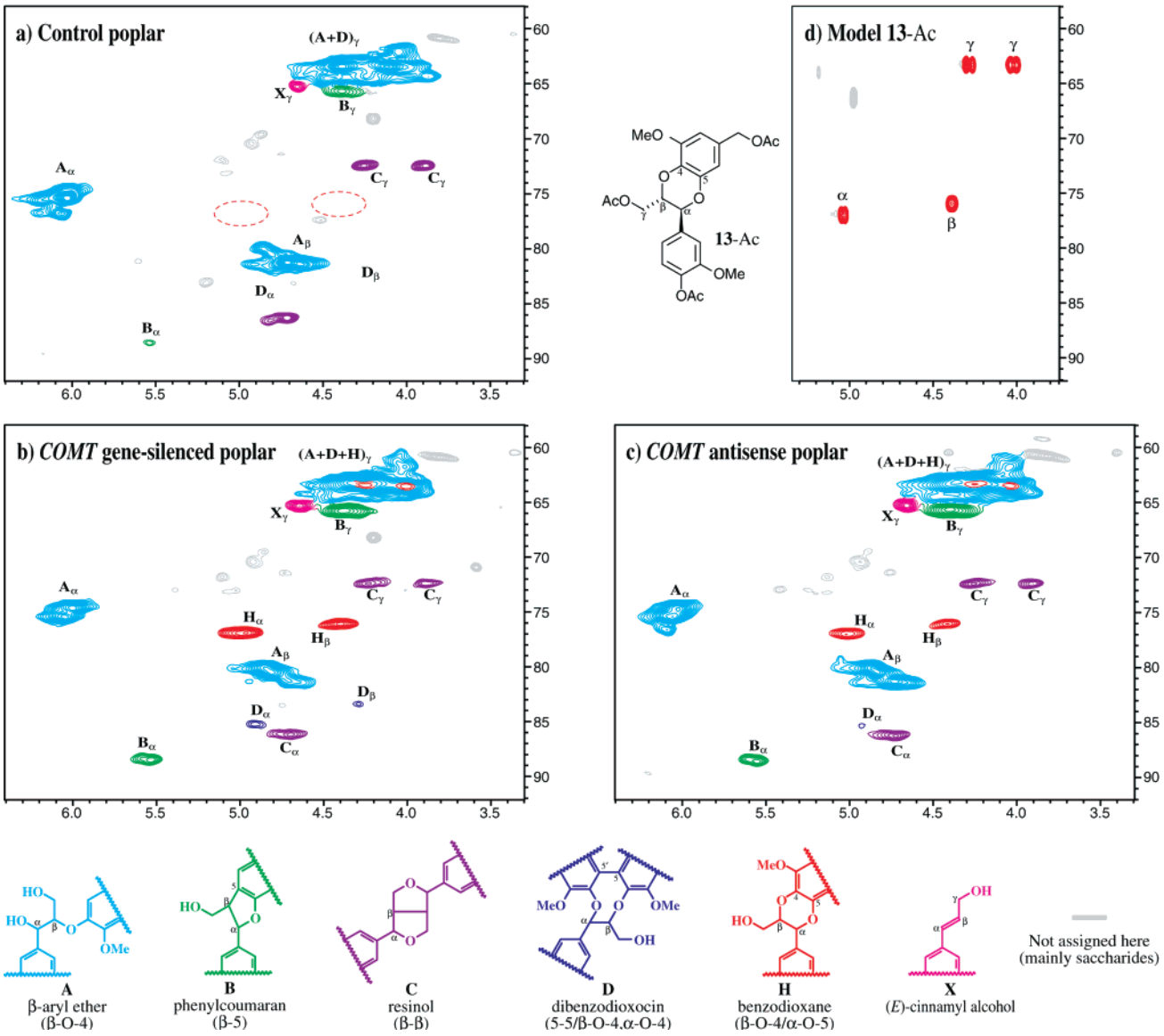

Figure 3. Presence of benzodioxane structures in COMT-deficient transgenic plants diagnostically revealed by NMR. Partial spectra from gradient HMQC NMR experiments highlight new peaks (not in the control) for benzodioxane units **H** in acetylated lignins from COMT-downregulated transgenic poplars and similar correlations from a benzodioxane model **13**-Ac. The major unit coding is the same as that used in a recent review on NMR of lignins (*40*). It is suggested that **H** be reserved for these 5-hydroxyguaiacyl benzodioxane units in lignin NMR spectra.

Recent studies have begun to elucidate the propensity for the monolignols to cross-couple with (free-phenolic) guaiacyl and syringyl units in lignins (*41*, *42*). Cross-coupling of hydroxycinnamaldehydes into lignins has been recently demonstrated (*10*). The cross-coupling of 5-hydroxyconiferyl alcohol into the lignins of the COMT-deficient poplar plants, by the logical mechanism in Figure 2, is further evidence for our contention that lignification is metabolically plastic (*43*). Degradation experiments are planned to complement these NMR findings and to establish how intimately 5-hydroxyconiferyl alcohol is incorporated into the lignin polymer.

COMT-deficient poplars incorporate 5-hydroxyconiferyl alcohol into the lignin polymer whereas the wild-type controls do not. The statement on lignification by Lewis (*44*) that "There is, however, no known precedent for the free interchange of monomeric units in any biopolymer assembly, then or now, and no biochemical evidence..." must therefore be revisited. When the flux to the final syringyl monolignol is restricted, a phenolic intermediate other than the traditional monolignols can be utilized to make modified lignin polymers with properties apparently sufficient to accommodate the water transport and mechanical strengthening roles of lignin and to allow the plant to be viable, as we have noted previously (*6*, *10*, *43*). Whether such plants will be able to confront the rigors of a natural environment replete with a variety of pathogens remains to be determined. However, having the metabolic flexibility to allow polymerization of products of incomplete monolignol synthesis along with the traditional monolignols is a significant advantage to plants; in a single generation these plants have circumvented genetic obstacles to remain viable.

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