Lignin-Hydrolyzable Tannin Interactions in Wood

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A series of investigations were carried out to ascertain the relationship between lignin and hydrolyzable tannins in white oak (*Quercus alba*) and American chestnut (*Castanea dentata*) milled wood lignins as well as synthetic co-DHPs. Acidolysis and chromatographic analysis of chestnut and white oak milled wood lignins revealed the presence of ellagic acid in the chestnut sample but not in the white oak sample. Synthetic protocols were developed to prepare theorized lignin−gallate dimers, and these compounds served as a database for assessing American chestnut and white oak milled wood lignin by NMR spectroscopy. While dehydrogenative co-polymerization of ^{13}C-labeled pentagalloyl D-glucopyranoside (PGG) with coniferyl alcohol and sinapyl alcohol showed evidence for hydrolyzable tannin/lignin interactions, unambiguous identifications in native milled wood lignin could not be made. The observed results are discussed in relation to natural wood durability.

Keywords: *DHP; ellagic acid; ellagitannins; lignin; gallic acid; oak; chestnut; NMR*

INTRODUCTION

Heartwood formation in trees is the final biochemical event associated with woody plant growth and development. While the signaling and biochemical processes of heartwood formation are still poorly understood (Hillis, 1987), the net result of heartwood formation is the deposition of relatively large quantities of secondary metabolites, the types and amounts of which are species and site dependent. While previous theories suggested that this production of metabolites was due to the elimination of excess carbohydrate (Stewart, 1966), current thought is that these compounds provide a deterrent to pathogen attack in regions of the tree where a biochemical response is not possible (Zucker, 1983; Loehle, 1988).

Natural restriction to wood decay in many species is due to the secondary metabolites broadly classified as tannins (Scalbert, 1992). Durable hardwoods such as the oaks and chestnuts are thought to obtain much of their resistance through the deposition of ellagitannins, compounds in which gallic acid (3,4,5-trihydroxybenzoic acid) is attached via an ester bond to a D-glucose core and also biaryl-coupled to an adjacent galloyl group (Figure 1). Cleavage of biaryl-coupled ester bonds in molecules such as potentillin (**3**) would provide the dilactone ellagic acid. Resistance to fungal invasion is due to the ability of the tannins to precipitate protein (astringency; Luck *et al*., 1994) and/or removing metal cofactors through their strong affinity for metal ions (Mila *et al*., 1996).

The biosynthetic pathway for ellagitannin formation has not been determined, although it is generally assumed that all ellagitannins are derived from pentagalloyl β-D-glucopyranoside (**1**) by a stepwise, enzyme-mediated oxidative coupling pathway, followed by acyclic C-glucoside formation (Haslam and Cai, 1994) as shown in Figure 1. An alternative pathway to castalagin (**5**) and vescalagin (**6**) from **1** has recently been proposed (Vivas *et al*., 1995). As discussed by Hillis (1987), tannin accumulation is generally observed microscopically to occur in parenchyma cell vacuoles, and tannin production can be related to depletion of starch reserves. Formation of heartwood is under biological control, therefore it is not surprising that changes in ethylene levels have been associated with changing the hormonal balance of auxin and other phytohormones, leading to polyphenol biosynthesis. Once tannin accumulation has reached a certain level, the vacuole disrupts, mixing the tannins with the cytostolic constituents. Cell disintegration follows, allowing the tannins to migrate by diffusion processes, effectively dispersing ellagitannins throughout the woody tissue (Masson *et al*., 1994). These visual observations have led to the development of the concept of stereospecific and non-stereospecific tannin reactions. Enzyme-mediated transformations occur in the vacuole, and random enzymatic and chemical reactions can occur subsequent to vacuole/cell disruption (Klumpers *et al*., 1994; Viriot *et al*., 1994). Tree wounding can also induce heartwood formation, although it has been demonstrated that the extractive profile of this material is generally different from that of regular heartwood (Hillis, 1987).

Since ellagitannins are formed in a highly lignified region by an oxidative coupling pathway, incorporation into the lignin structure is a distinct possibility, either by enzyme-mediated processes or by random chemical oxidation. It has been established by several studies that concentration of soluble ellagitannins in oaks and chestnuts decreases from the sapwood−heartwood transition zone to the pith, with a concomitant increase in the concentration of insoluble ellagitannins (Peng *et al*., 1991; Masson *et al*., 1995). If an enzymatic process were operative, a logical pathway for incorporation would be via the free radical coupling/addition sequence shown in Figure 2. The net result of this process would be both the 8-*O*-3 lignin gallates (**IV**) as well as the benzodioxanes (**V**). Due to the expected stability of structures such as **V**, and the unique chemical shift difference of the α-proton and carbon before and after acetylation (the position cannot be acetylated), it may be possible to detect this structure in wood isolates.

In an effort to more fully understand the processes associated with ellagitannin chemistry, biochemistry and heartwood formation, we have embarked on a series of investigations in this general area. Our initial investigations concerned the nature of the association of ellagitannins with the polymeric constituents of the woody cell wall and, more specifically, the potential

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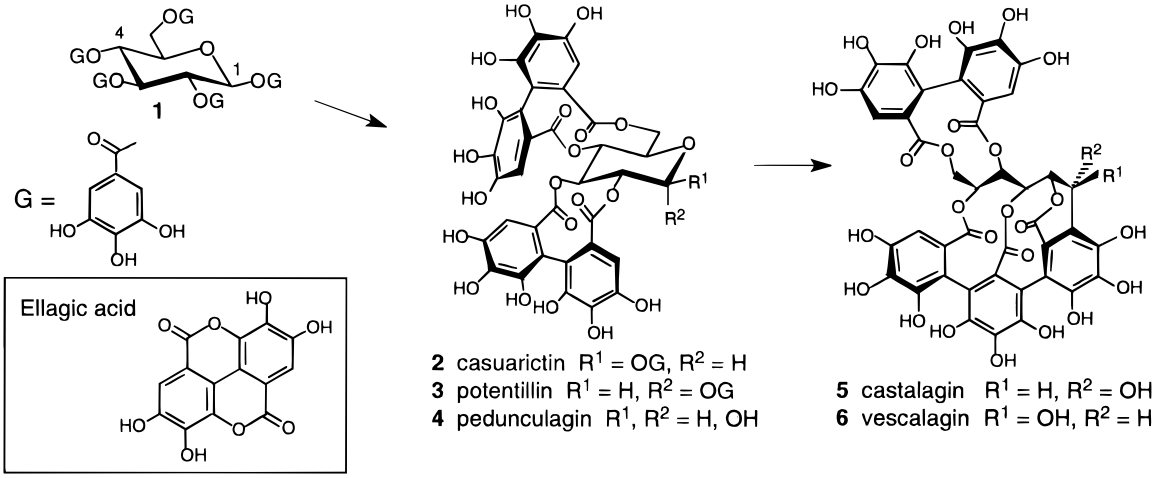


Figure 1. Simplified version of the proposed pathway for ellagitannin biosynthesis.

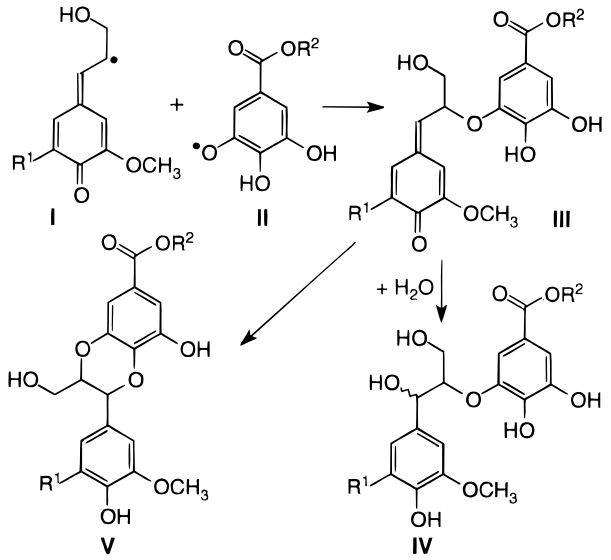


Figure 2. Hypothetical pathway for the enzymatic incorporation of a gallate moiety into a lignin macromolecule. Nucleophilic attack of the quinone methide can lead to 8-*O*-3′ (**IV**) or benzodioxane (**V**) structures. R^1 = H, OCH_3, or the lignin polymer; R^2 = remainder of the tannin molecule.

covalent interaction of ellagitannins and lignin. Organic synthesis provided several lignin−gallate model compounds for characterization by NMR spectroscopy. This database served as a starting point for the NMR analysis of chestnut milled wood lignin as well as several DHPs prepared with and without ^{13}C-labeled pentagalloyl β-D-glucopyranoside. Chemical methods in combination with HPLC also provided important evidence for the relationship between ellagitannins and lignin.

EXPERIMENTAL METHODS

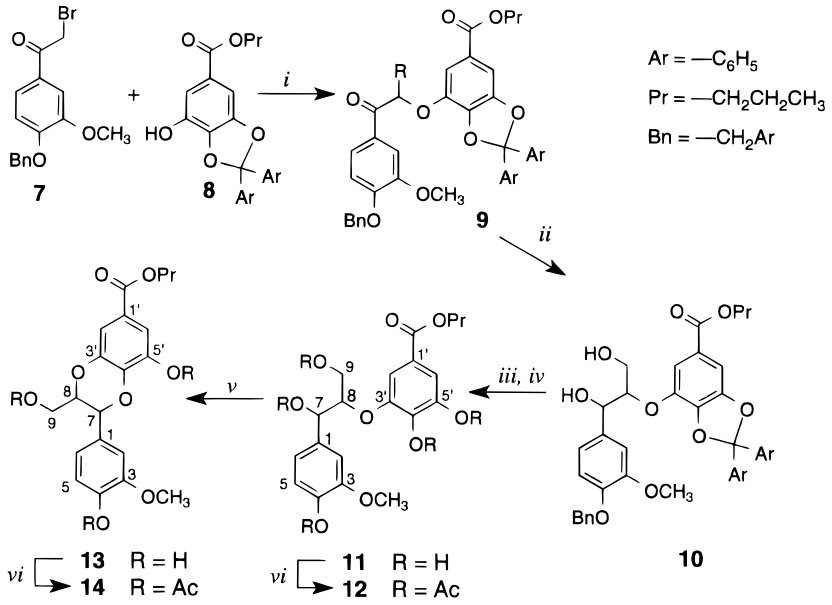
General. Evaporations were accomplished under reduced pressure at temperatures not exceeding 42 °C. NMR spectra were recorded with either a 400 or 360 MHz instrument at ambient temperatures, and chemical shifts (ppm) are relative to the central solvent peak of acetone-d_6 (^{13}C, 29.8 ppm; ^{1}H, 2.04 ppm). Column chromatography was done with Silica Gel 60 (230−400 mesh) using a standard flash chromatography apparatus. All synthetic transformations (Schemes 1 and 2) were performed several times and on different scales, and typical protocols are given for each transformation. Standard processing implies drying with either sodium or magnesium sulfate, filtration, and elimination of solvent (eventually under high vacuum) to provide a syrup, solid, or foam. Coniferyl and sinapyl alcohols (Quideau and Ralph, 1992) were prepared as described previously, as were **7** (Landucci *et al.*, 1981) and **8** (Jurd, 1959). Compound **16** (Scheme 2) was prepared according to Armitage *et al.* (1961) starting with propyl gallate. Small-scale acetylations were performed with acetic anhydride/4-(dimethylamino)pyridine (Ralph *et al.*, 1992b) in dichloromethane or THF, depending on the solubility of the starting material. DHPs were prepared with Type II horseradish peroxidase (Sigma).

Gallate Addition. Compounds **7** (2.03 g, 6.06 mmol), **8** (2.33 g, 6.19 mmol), and powdered K_2CO_3 (620 mg) were refluxed in acetone (60 mL) with stirring until TLC indicated complete disappearance of starting material (12 h). The solution was filtered, the filtrate was evaporated under diminished pressure, and **9** was subsequently crystallized from acetone (3.00 g, 78%). Selected NMR data (acetone-d_6): δ_H 0.95 (3H, t, J = 7.4, Pr), 1.70 (2H, dt, J = 7.2 and 14.0 Hz, Pr), 3.87 (3H, s, OMe), 4.17 (2H, t, J = 6.6 Hz, Pr), 5.25 (2H, s, $-CH_2$Ar), 5.70 (2H, s, H-8), 7.17−7.73 (Ar); δ_C 10.7 (Pr), 22.7 (Pr), 56.2 (Pr), 67.0 (Pr), 71.2 ($-C$H$_2$Ar), 72.4 (C-8), 165.8 (ester C=O), 192.8 (C-7). Recrystallization of the mother liquor can provide additional material (400 mg) of slightly less purity.

Hydroxymethylation. The reaction was performed using a previously described protocol (Helm and Ralph, 1992). Compound **9** (1.02 g, 1.62 mmol) was dissolved in dioxane (25 mL), and powdered K_2CO_3 (1.68 g) and aqueous formaldehyde (37% by wt, 230 μL, 1.9 mmol) were added. The reaction was stirred until complete disappearance of starting material. The reaction time for this step can vary between 4 and 10 h depending on the degree of stirring as well as the particle size of the potassium carbonate. The reaction should be monitored closely as prolonged reaction times can lead to dihydroxymethylated products. Processing of the mixture provided a crude syrup which was purified by silica gel chromatography (9:1, $CHCl_3$:EtOAc) to provide **10** which crystallized from CH_2Cl_2/hexane as small needles in 77% yield (mp 120−123 °C). Selected NMR data (acetone-d_6): δ_H 0.94 (3H, t, J = 7.4, Pr), 1.69 (2H, dt, J = 7.3 and 14.0 Hz, Pr), 3.82 (3H, s, OMe), 4.0−4.18 (2H, m, H-9 and H-9′), 4.16 (2H, t, J = 6.6 Hz, Pr), 4.48 (0.7H, t, J = 6.4 Hz, alkyl OH), 5.24 (2H, s, $-CH_2$Ar), 5.70 (2H, s, H-8), 6.06 (1H, dd, J = 3.4 and 6.0 Hz, H-8), 7.13−7.84 (Ar); δ_C 10.7 (Pr), 22.7 (Pr), 56.1 (Pr), 64.3 (C-9), 67.0 (Pr), 71.2 ($-C$H$_2$Ar), 84.2 (C-8), 165.7 (ester C=O), 192.8 (C-7).

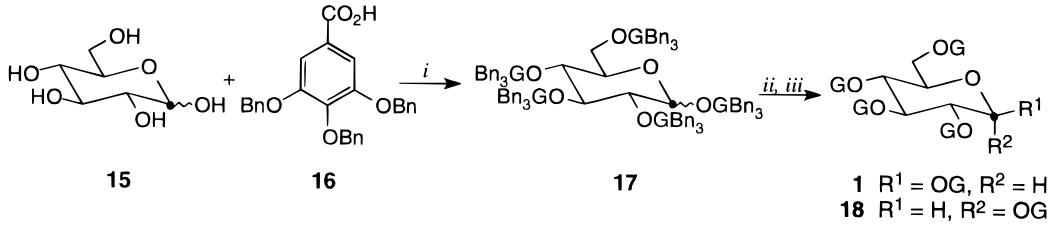
Reduction and Deprotection. Reduction of the α-ketone was accomplished with $Zn(BH_4)_2$ in EtOAc/Et_2O as reported previously (Helm and Ralph, 1993). Compound **10** (1.00 g, 1.52 mmol) was dissolved in a mixture of EtOAc (15 mL) and Et_2O (20 mL) and cooled to 0 °C. A solution of ethereal $Zn(BH_4)_2$ (0.15 M, 10 mL) was added, and the reaction was kept at 0 °C for an hour. The reaction was quenched with HOAc/H_2O (1:1) and processed by washing with $NaHCO_3$ followed by NH_4Cl. Standard processing gave an 80:20 *threo*:*erythro* mixture of the protected β-*O*-3 model. This material could be submitted

Scheme 1. Synthesis of the 8-*O*-3′ Gallates and the Benzodioxanes[a]



[a] Reactions: *i*, K_2CO_3/acetone/reflux; *ii*, H_2CO/K_2CO_3/dioxane; *iii*, $Zn(BH_4)_2$; *iv*, H_2-Pd/C; *v*, H_2SO_4/Ac2O; *vi*, Ac_2O/DMAP.

Scheme 2. Synthesis of the Pentagalloyl [1-^{13}C]-D-Glucopyranosides[a]



[a] Reactions: *i*, DCC/DMAP/DMAP-HCl; *ii*, silica gel chromatography; *iii*, H_2-Pd/C then HW40F.

directly to the deprotection step or small amounts could be purified into enriched *threo* and *erythro* fractions by silica gel chromatography ($CHCl_3$:EtOAc, 3.5:1). Selected NMR data (acetone-d_6): δ_C (*threo*) 10.7 (Pr), 22.7 (Pr), 56.0 (OMe), 62.1 (C-9), 66.9 (Pr), 71.4 (Bn), 73.4 (C-7), 86.2 (C-8), 165.9 (ester C═O); (*erythro*) 10.7 (Pr), 22.7 (Pr), 56.0 (OMe), 62.2 (C-9), 66.9 (Pr), 71.4 (−CH_2Ar), 73.7 (C-7), 85.6 (C-8), 165.9 (ester C═O).

The borohydride-reduced material (312 mg, 0.47 mmol) was dissolved in 95% EtOH (15 mL), and Pd/C (5%, 71 mg) was added. The flask was flushed with hydrogen and sealed with a balloon filled with hydrogen. TLC indicated the reaction was done in 2 h, at which time the mixture was filtered through a 0.2 μm filter, evaporated to a syrup, and purified by silica gel chromatography (EtOAc) to remove the diphenylmethane. Processing of the appropriate fractions gave **11** (173 mg, 90%) as a foam. The *threo* and *erythro* fractions could not be readily purified at this stage. NMR data (acetone-d_6): δ_H (**11**-*threo* 0.97 (3H, t, J = 7.5 Hz, Pr), 1.73 (2H, dt, J = 7.4 and 14.0 Hz, Pr), 3.52 (1 H, dd, J = 5.8 and 11.9 Hz, H-9a), 3.74 (1H, dd, J = 3.2 and 11.9 Hz, H-9b), 3.82 (3H, s, OMe), 4.07 (1H, ddd, J = 4.1, 5.5, and 7.5 Hz, H-8), 4.18 (2H, t, J = 6.6 Hz, Pr), 4.98 (1H, d, J = 7.5 Hz, H-7), 6.80 (1H, d, J = 7.9 Hz, H-5), 6.91 (1H, dd, J = 1.9 and 8.1 Hz, H-6), 7.08 (1H, d, J = 2.0 Hz, H-2), 7.27 (1H, d, J = 2.0 Hz, H-2′ or H-6′), 7.43 (1H, d, J = 2.0 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 22.8 (Pr), 56.2 (OMe), 61.9 (C-9), 66.5 (Pr), 74.4 (C-7), 89.3 (C-8), 111.1, 112.8, 114.9, 115.5, 120.6, 121.6, 133.4, 142.9, 146.6, 147.1, 147.8, and 148.2 (Ar), 166.6 (C═O); δ_H (**11**-*erythro*) 0.98 (3H, t, J = 7.5 Hz, Pr), 1.73 (2H, dt, J = 7.4 and 14.0 Hz, Pr), 3.79 (3H, s, OMe), 3.80 (1H, dd, J = 3.7 and 11.6 Hz, H-9a), 3.88 (1H, dd, J = 7.2 and 11.6 Hz, H-9b), 4.14−4.21 (3H, m, Pr and H-8), 4.99 (1H, d, J = 4.7 Hz, H-7), 6.78 (1H, d, J = 8.1 Hz, H-5), 6.90 (1H, dd, J = 2.0 and 8.1 Hz, H-6), 7.09 (1H, d, J = 2.0 Hz, H-2), 7.17 (1H, d, J = 2.0 Hz, H-2′ or H-6′), 7.23 (1H, d, J = 2.0 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 22.8 (Pr), 56.2 (OMe), 61.8 (C-9), 66.5 (Pr), 73.7 (C-7), 88.4 (C-8), 111.3, 112.7, 114.3, 115.3, 120.5, 121.7, 133.2, 142.7, 146.7, 146.9, 147.3, and 148.1 (Ar), 166.5 (C═O).

The peracetates of **11** were prepared by acetylation with an acetic anhydride/DMAP/THF system. Processing and purification by preparative TLC provided the acetylated derivatives in greater than 90% yield. NMR data (acetone-d_6): δ_H (**12**-*threo*) 1.00 (3H, t, J = 6.9 Hz, Pr), 1.77 (2H, dt, J = 7.4 and 14.1 Hz, Pr), 1.96, 2.01, 2.22, 2.29, and 2.30 (3H, s, Ac), 3.82 (3H, s, OMe), 4.11 (1H, dd, J = 6.1 and 12.2 Hz, H-9a), 4.19 (1H, dd, J = 3.5 and 12.2 Hz, H-9b), 4.26 (2H, t, J = 6.6 Hz, Pr), 5.10 (1H, m, H-8), 6.12 (1H, d, J = 7.3 Hz, H-7), 7.06 (1H, d, J = 8.1 Hz, H-5), 7.10 (1H, dd, J = 1.8 and 8.1 Hz, H-6), 7.26 (1H, d, J = 1.6 Hz, H-2), 7.50 (1H, d, J = 1.8 Hz, H-2′ or H-6′), 7.75 (1H, d, J = 1.8 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 20.1, 20.4, 20.4, 20.5, and 20.9 (Ac), 22.7 (Pr), 56.3 (OMe), 63.3 (C-9), 67.5 (Pr), 75.2 (C-7), 80.8 (C-8), 112.7, 114.8, 118.2, 120.6, 123.6, 123.8, 129.3, 136.0, 141.2, 144.8, 152.4, and 152.5 (Ar), 165.4 (C═O), 167.8, 168.7, 168.8, 170.1, and 170.6 (Ac); δ_H (**12**-*erythro*) 1.00 (3H, t, J = 7.5 Hz, Pr), 1.77 (2H, dt, J = 7.4 and 14.1 Hz, Pr), 1.92, 2.13, 2.21, 2.22, and 2.28 (3H, s, Ac), 3.82 (3H, s, OMe), 4.23 (1H, dd, J = 4.0 and 12.0 Hz, H-9a), 4.26 (2H, t, J = 6.6 Hz, Pr), 4.34 (1H, dd, J = 6.6 and 12.0 Hz, H-9b), 5.12 (1H, m, H-8), 6.07 (1H, d, J = 4.4 Hz, H-7), 7.05−7.07 (2H, m, H-5 and H-6), 7.20 (1H, d, J = 1.4 Hz, H-2), 7.49 (1H, d, J = 1.8 Hz, H-2′ or H-6′), 7.71 (1H, d, J = 1.8 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 20.1, 20.4, 20.5, and 20.9 (Ac), 22.7 (Pr), 56.3 (OMe), 62.9 (C-9), 67.5 (Pr), 74.3 (C-7), 80.1 (C-8), 112.6, 114.7, 118.4, 120.1, 123.6, 123.8, 129.2, 135.9, 140.9, 144.8, 151.9, and 152.2 (Ar), 165.4 (C═O), 167.7, 168.6, 168.8, 169.8, and 170.7 (Ac).

Benzodioxane Formation. Compound **11** (*t*/*e* mixture, 319.2 mg, 0.78 mmol) was dissolved in glacial HOAc (8 mL), and a solution of concentrated sulfuric acid in HOAc (75 μL, 60 mg/mL) was added (Goyal *et al.*, 1993). The mixture was stirred for 2.5 h and subsequently transferred to a separatory

funnel and diluted with EtOAc. The organic phase was washed with aqueous NH_4Cl (2×) followed by aqueous $NaHCO_3$ (2×). Standard processing provided crude **13** which was separated into *cis*- and *trans*-isomers by silica gel chromatography (2:1 EtOAc:hexane). Three fractions were taken: the *trans*-isomer (190.6 mg), a *cis*/*trans* mixture (43.4 mg), and a predominantly *cis* fraction (27.0 mg). Overall yield was 85.5%, with the major impurities being C-9 acetylated benzodioxanes. The *trans*-isomer crystallized from EtOAc/hexane was needles (mp 155 °C). NMR data: δ_H (**13**-*trans*) 1.01 (3H, t, J = 7.6 Hz, Pr), 1.75 (2H, dt, J = 7.3 and 14.0 Hz, Pr), 3.15 (1H, bddd, J = 4.2, 6.3 and 12.5 Hz, H-9a), 3.37 (1H, bddd, J = 2.3, 5.0 and 12.4 Hz, H-9b), 3.86 (3H, s, OMe), 4.11 (0.7H, bd, J = 6.0 Hz, 9-OH), 4.15 (1H, m, H-8), 4.19 (2H, t, J = 6.7 Hz, Pr), 5.01 (1H, d, J = 8.0, H-7), 6.88 (1H, d, J = 7.9 Hz, H-5), 6.96 (1H, d, J = 1.9 and 7.9 Hz, H-6), 7.11−7.12 (2H, m, H-2 and H-2′ or H-6′), 7.14 (1H, d, J = 1.9 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 22.8 (Pr), 56.3 (OMe), 61.7 (C-9), 66.7 (Pr), 77.7 (C-7), 79.4 (C-8), 110.1, 110.3, 112.0, 115.7, 121.8, 123.6, 128.5, 137.5, 144.8, 147.0, 148.1, and 148.5 (Ar), 166.4 (C═O); δ_H (**13**-*cis*) 1.00 (3H, t, J = 7.5 Hz, Pr), 1.75 (2H, dt, J = 7.4 and 14.0 Hz, Pr), 3.47 (1H, dd, J = 3.9 and 12.1 Hz, H-9a), 3.62 (1H, dd, J = 7.8 and 12.1 Hz, H-9b), 3.81 (3H, s, OMe), 4.20 (2H, t, J = 6.6 Hz, Pr), 4.53 (1H, m, H-8), 5.36 (1H, J = 2.7 Hz, H-7), 6.84 (1H, d, J = 8.1 Hz, H-5), 6.92 (1H, dd, J = 1.8 and 8.1 Hz, H-6), 7.08 (1H, d, J = 1.8 Hz, H-2), 7.13 (1H, d, J = 7.2 Hz, H-2′ or H-6′), 7.17 (1H, d, J = 1.8 Hz, H-2′ or H-6′); δ_C 10.8 (Pr), 22.8 (Pr), 56.3 (OMe), 59.5 (C-9), 66.7 (Pr), 77.1 (C-7), 78.6 (C-8), 110.5, 110.9, 111.2, 115.8, 120.3, 123.8, 137.1, 143.4, 146.9, 147.6, and 148.3 (Ar), 166.4 (C═O).

The peracetates of **13** were prepared by acetylation with an acetic anhydride/DMAP/CH_2Cl_2 system. Processing and purification by preparative TLC provided the acetylated derivatives in greater than 90% yield. NMR data (acetone-d_6): δ_H (**14**-*trans*) 1.00 (3H, t, J = 7.4 Hz, Pr), 1.76 (2H, dt, J = 6.6 and 14.1 Hz, Pr), 2.03, 2.21, and 2.25 (3H, s, Ac), 3.84 (3H, s, OMe), 4.05 (1H, dd, J = 4.6 and 12.5 Hz, H-9a), 4.23 (2H, t, J = 6.6 Hz, Pr), 4.34 (1H, dd, J = 3.2 and 12.5 Hz, H-9b), 4.64 (1H, m, H-8), 5.23 (1H, d, J = 7.7 Hz, H-7), 7.07 (1H, dd, J = 1.9 and 8.1 Hz, H-6), 7.13 (1H, d, J = 8.1 Hz, H-5), 7.24 (1H, d, J = 1.9 Hz, H-2), 7.39 (1H, d, J = 2.0 Hz, H-2′ or H-6′), 7.50 (1H, d, J = 2.0 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 20.4, 20.5, and 20.5 (Ac), 22.7 (Pr), 56.3 (OMe), 62.9 (C-9), 67.1 (Pr), 76.4 (C-7), 77.6 (C-8), 112.5, 116.3, 117.6, 120.5, 123.7, 124.0, 135.2, 140.2, 141.2, 141.5, and 152.6 (Ar), 165.6 (C═O), 168.7, 168.9, and 170.6 (Ac); δ_H (**14**-*cis*) 1.00 (3H, t, J = 7.5 Hz, Pr), 1.77 (2H, dt, J = 6.6 and 14.0 Hz, Pr), 1.89, 2.24, and 2.31 (3H, s, Ac), 3.82 (3H, s, OMe), 4.03 (1H, dd, J = 7.4 and 12.1 Hz, H-9a), 4.09 (1H, dd, J = 4.4 and 12.1 Hz, H-9b), 4.25 (2H, t, J = 6.6 Hz, Pr), 4.93 (1H, m, H-8), 5.57 (1H, d, J = 3.0 Hz, H-7), 7.07 (1H, dd, J = 1.9 and 8.1 Hz, H-6), 7.13 (1H, d, J = 8.1 Hz, H-5), 7.23 (1H, d, J = 1.9 Hz, H-2), 7.41 (1H, d, J = 2.1 Hz, H-2′ or H-6′), 7.51 (1H, d, J = 2.1 Hz, H-2′ or H-6′); δ_C 10.7 (Pr), 20.4, 20.4, and 20.4 (Ac), 22.7 (Pr), 56.2 (OMe), 60.7 (C-9), 67.2 (Pr), 74.8 (C-7), 76.4 (C-8), 111.1, 116.8, 117.5, 118.7, 124.0, 124.1, 134.9, 140.2, 140.7, 140.9, 143.3, and 152.5 (Ar), 165.6 (C═O), 168.8, 168.9, and 170.6 (Ac).

Pentagalloyl β-D-Glucopyranoside (PGG). [1-^{13}C]-D-Glucose (**15**, 110 mg, 0.58 mmol) was dissolved in DMF (4.5 mL) under nitrogen. Compound **16** (1290 mg, 2.93 mmol, 5 equiv) was added followed by methylene chloride (10 mL), DMAP (180 mg, 1.49 mmol, 2.5 equiv), and DMAP-HCl (230 mg, 1.48 mmol, 2.5 equiv; Boden and Keck, 1985). The reaction was initiated by the addition of a solution of dicyclohexylcarbodiimide (DCC, 620 mg, 3.02 mmol, 5 equiv) in methylene chloride (3 mL). A precipitate formed within 5 min, and the reaction flask was equipped with a reflux condensor and placed in an oil bath (40 °C). The reaction was allowed to proceed overnight with stirring and was subsequently filtered through a bed of Celite. The filtrate was washed with cold 3% HCl (1×) followed by aqueous NH_4Cl (2×), and the organic phase was dried with anhydrous sodium sulfate and evaporated to a syrupy solid. This crude product was taken up in methylene chloride (5 mL) and filtered through a glass wool plug to remove a portion of the DCC byproducts. The filtrate was evaporated to a syrup and purified by silica gel chromatography to separate α- and β-enriched fractions of **17** (83.6% yield). NMR data (acetone-d_6): δ_C 93.8 (C-1); for **17**α, δ_C 91.2 (C-1).

Compound **17** (α/β mixture, 370 mg, 0.16 mmol) was dissolved in THF (3 mL) with stirring. Ethanol (95%, 3 mL) was then added followed by Pd-C (5% Pd, 180 mg), and the flask was flushed with hydrogen gas and subsequently fitted with a balloon containing hydrogen gas. The reaction was monitored by silica TLC (3:3:1:toluene:acetone:formic acid), and upon completion the reaction mixture was filtered through a bed of Celite, evaporated to a syrup, and cleaned by passage through a column of Toyopearl HW40F (acetone:H_2O, 7:3). Evaporation of the acetone under reduced pressure and water removal by freeze-drying provided a mixture of **1** and **18** as a white solid (121.2 mg, 80%). Selected NMR data (acetone-d_6): for **1**, δ_H 6.33 (1H, dd, J = 8.4 and 168.4 Hz, H-1), 6.96, 7.00, 7.04, 7.10, and 7.17 (2H, s, gallate protons), δ_C 93.3 (C-1); for **18**, δ_H 6.72 (1H, dd, J = 4.0 and 170.2 Hz, H-1), 6.99, 7.00, 7.19, and 7.27 (2H, s, gallate protons), δ_C 90.3 (C-1).

Dehydrogenative Polymerization (DHPs). All DHPs were prepared according to Zutropfverfahren method in the dark with constant stirring (Ralph *et al.*, 1992a), using degassed 0.1 M phosphate buffer solutions (pH 6.5). Solution A: substrates and peroxidase in buffer. Solution B: hydrogen peroxide in buffer. These solutions were added simultaneously to a flask containing stirred buffer. The addition was done with the help of a two-channel variable speed peristaltic pump (model EP-1 Econo Pump, Bio-Rad) set at 0.13 mL/min. After the additions were complete, the reaction was allowed to proceed for another 70 h, at which time, the reaction mixture was filtered through a 0.2 μm Nylon filter. The solid was washed with water and subsequently freeze-dried to yield the desired DHP.

Coniferyl Alcohol DHP (pH 6.5). Solution A: coniferyl alcohol (100 mg, 0.56 mmol) and horseradish peroxidase (0.6 mg) in phosphate buffer (50 mL). Solution B: hydrogen peroxide (0.07 mL) diluted with phosphate buffer (55 mL). Solutions A and B were added to a flask containing phosphate buffer (25 mL). Solids recovery: 70−85 mg (three preparations).

CA-[1-^{13}C]PGG Co-DHP (pH 6.5). Solution A: coniferyl alcohol (66 mg, 0.37 mmol) and a 1:1 mixture of labeled **1** and **18** (22 mg, 0.02 mmol) dissolved in 40 mL of phosphate buffer containing horseradish peroxidase (0.6 mg). Solution B: phosphate buffer (45 mL) containing hydrogen peroxide (0.08 mL). Solutions A and B were added to a flask containing phosphate buffer (20 mL). Solids recovery: 57−73 mg (three preparations).

[1-^{13}C]PGG DHP (pH 6.5). Solution A: compound **1** (80 mg, 0.08 mmol) in phosphate buffer (40 mL) containing horseradish peroxidase (0.8 mg). Solution B: phosphate buffer (45 mL) containing hydrogen peroxide (0.16 mL). Solutions A and B were added to a flask containing phosphate buffer (20 mL). Solids recovery: 10−26 mg (three preparations).

Propyl Gallate DHP (pH 6.5). Solution A: propyl gallate (79.3 mg, 0.084 mmol) dissolved in phosphate buffer (40 mL) containing horseradish peroxidase (0.5 mg). Solution B: phosphate buffer (45 mL) containing hydrogen peroxide (0.16 mL). Solutions A and B were added to a flask containing phosphate buffer (20 mL). No solids were recovered.

Ellagic Acid Determinations. Milled wood lignins (MWLs) were prepared in a previous study (Glasser *et al.*, 1983) and kindly provided by Prof. Glasser (VA Tech). The procedures used for ellagic acid determinations are essentially those described by Peng *et al.* (1991) except that an external standard analysis was used. In a Teflon-lined screw-capped vial, MWL (40−60 mg) was dissolved in 5 mL of MeOH−aqueous 6 M HCl (9:1). The tubes were kept at 120 °C for 160 min with stirring. The samples were filtered, diluted, and subjected to gradient HPLC analyses using a Gilson HPLC system equipped with a C18 column (Whatman RAC-II, 5 mm particle size). The linear gradient used for the analysis utilizes methanol (solvent A) and 0.2% trifluoroacetic acid (TFA) in water (solvent B). An initial concentration of solvent B, 100%, was modified beginning at 1 min such that at 40 min the concentration of solvent B decreased to 5%. The system was

held for 5 min at this concentration, followed by a 10 min gradient to the initial solvent concentration. The flow rate was maintained at 0.75 mL/min throughout, and UV detection was carried out at 280 nm (retention time of ellagic acid was 26.7 min). A calibration curve was constructed using ellagic acid standards (60−160 μg/mL) prepared by dissolving ellagic acid in methanol. The amounts of ellagic acid in acid-degraded samples were estimated by linking to the calibration curve in the Gilson Unipoint software package.

RESULTS AND DISCUSSION

If ellagitannins are incorporated into the lignin polymer, both nonenzymic and enzymatic routes are possible. While a nonenzymic route would provide a vast array of coupling products, an enzymatic pathway would provide discrete products which could theoretically be detected by NMR spectroscopy. Peroxidases, polyphenol oxidases, and amylases have been detected in the heartwood of several species, including oak (Ebermann and Stich, 1982; Stich and Ebermann, 1984). Therefore transformations similar to lignification are possible, which would result in free radical coupling. One of the most logical modes of attachment would be between the 8-position (β) of a lignin moiety and one of the phenolic oxygens of a gallate ring (Figure 2). If such a reaction was to occur, an intermediate quinone methide structure would result (**III**) which could either react with water to form an 8-*O*-3′ structure (**IV**) or cyclize to form a benzodioxane (**V**). In order to detect these possible structures by NMR, it was necessary to prepare model compounds to develop a small database.

Procedures for the preparation of the lignin−gallate models were based on a standard lignin dimer synthetic scheme and are shown in Scheme 1. Acetovanillone is protected and brominated to provide **7**. This material serves as the acceptor of a nucleophilic substitution reaction by the protected gallate derivative **8** in refluxing acetone/powdered K_2CO_3. The resulting β-ether (**9**) was submitted to a formaldehyde addition and subsequently reduced with $Zn(BH_4)_2$ to provide the benzylic alcohol **10** as an 80:20 *threo*:*erythro* ratio. Assignment of the *threo* and *erythro* isomers was based on NMR chemical shifts and coupling constants (Ralph and Helm, 1991; Helm and Ralph, 1993). Deprotection provided the 8-*O*-3′ compounds **11**, and an acid-catalyzed cyclization reaction (Goyal *et al*., 1993) provided the benzodioxanes **13**, with the *trans*-isomer predominating. The *cis*/*trans*-isomers were assigned on the basis of the H-7 coupling constants (Antus *et al*., 1989), which were significantly smaller for the *cis*-isomer (3 Hz) than the *trans*-isomer (8 Hz).

Another essential model compound is **1**. While this material can be isolated from commercial tannic acid (Yoshizawa *et al*., 1992), not only would [1-^{13}C]-labeled PGG provide an easily identifiable signal in complicated ^{13}C NMR spectra, but the anomeric carbon signal is quite sensitive to the presence or absence of biaryl coupling (Okuda *et al*., 1989). The protocol for preparation of PGG is shown in Scheme 2, with the key step being the acylation reaction utilizing the modified Steglich procedure of Boden and Keck (1985). The coupling provides a mixture of the perbenzylated PGG in a 7:3 β:α ratio. The anomers can be separated by silica gel chromatography to generate enriched fractions of **17**β and **17**α or be submitted directly to the debenzylation reaction.

Analyses of Milled Wood Lignins. While the NMR approach can provide regiochemical information on the nature of the tannin−lignin interaction, simple acidolysis of the milled wood lignin can provide direct evidence for the presence or absence of tannin moieties. Peng *et al*. (1991) have developed a protocol for the determination of "insoluble" ellagitannins using acid hydrolysis followed by HPLC analysis. The average of four separate hydrolyses indicated that the chestnut MWL had 1.04% ± 0.04% ellagic acid, whereas the oak heartwood MWL had undetectable levels (Figure 3).

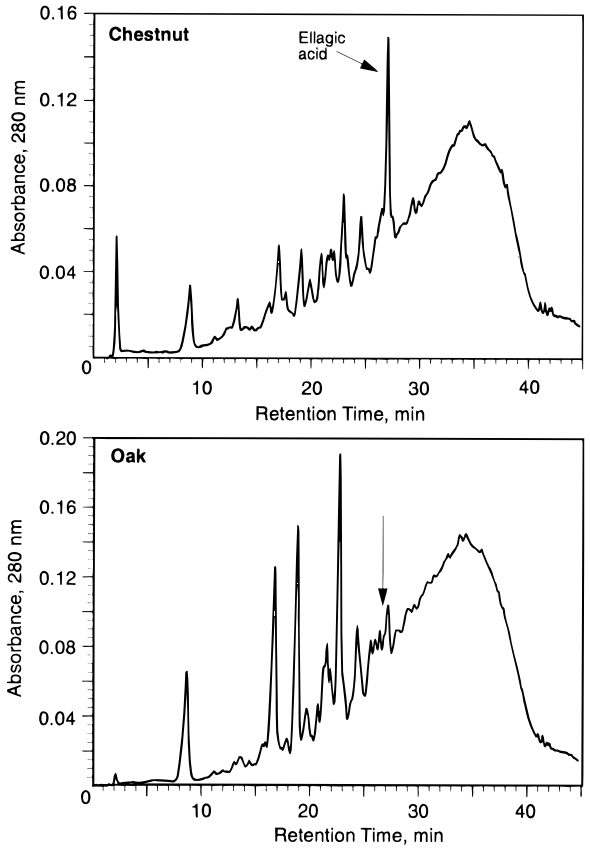


Figure 3. HPLC chromatograms of acid-treated milled wood lignins of American chestnut and white oak heartwood. The arrow for the oak chromatogram indicates the retention time of ellagic acid.

This is an interesting observation, especially when considered in relation to the known durabilities of the two woods. American chestnut is considered one of the most durable North American temperate zone hardwoods, and its durability is considered higher than that of white oak. It may be that the tannins in oak wood undergo chemical degradation upon ageing where the gallate moieties become oxidized and can no longer be detected as gallic or ellagic acid. Chestnut wood, on the other hand, may be more stable to wood ageing and resists this gallate/ellagate oxidation. The possibility also exists that lignin−tannin bonds are more prevalent in chestnut than oak, suggesting biochemical processes may be responsible. While none of the above hypotheses can be advocated at this time, acidolysis clearly shows the presence of ellagic acid in chestnut milled wood lignin−strong evidence for lignin-hydrolyzable tannin interactions.

Unfortunately, NMR analyses of both the free and acetylated chestnut milled wood lignins did not provide unambiguous proof of ellagate presence or of potential linkage sites. This could have been predicted by the low ellagate content (1%), but MWL fractionation via chromatography and re-analysis using standard 2D methods may provide unambiguous regiochemical information.

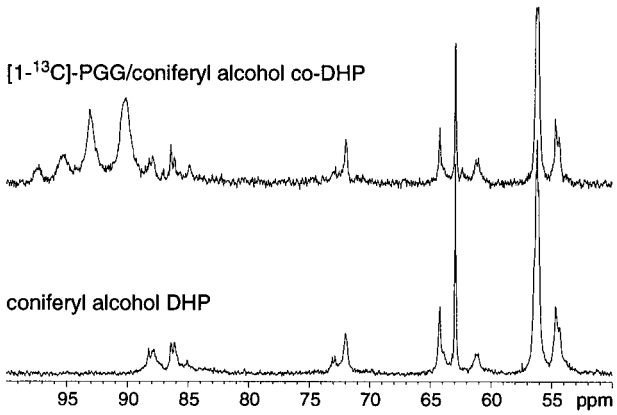


Figure 4. ^{13}C NMR spectra of coniferyl alcohol and [1-^{13}C]-PGG/coniferyl alcohol DHPs.

DHP Analyses. A series of DHPs were prepared using mixtures of monolignols and an anomeric mixture of pentagalloyl [1-^{13}C]-D-glucopyranoside (α:β, 2:3) and characterized by both NMR and wet chemical techniques. Typical NMR spectra for a coniferyl alcohol DHP and a α/β-PGG and coniferyl alcohol co-DHP are shown in Figure 4. The two larger peaks at 91 and 93 ppm are centered around the chemical shifts of the labeled C-1 carbon in α- and β-PGG, respectively. While the broadened signals above 90 ppm are indicative of labeled gallates in the water-insoluble DHP product, the data cannot be used to indicate whether or not the gallate moieties are oxidatively coupled either to a lignin moiety or another gallic acid group. Nonetheless, it is clear from the spectrum that ^{13}C-labeled material is present in the DHP.

Application of the acid hydrolysis procedure for the determination of ellagic acid was performed on a lignin−gallate DHP product; only a trace of gallic acid was found, and no ellagic acid could be detected. This can be explained by either degradation of the gallate moieties by "over-oxidation", or active incorporation of the gallate moieties by free radical processes, providing a material that is no longer recognizable as originating from gallic or ellagic acid.

Over-oxidation is a phenomenon that has been noted previously (Pospisil *et al*., 1983; Mayer *et al*., 1984). Indeed, when propyl gallate alone was submitted to a DHP preparation, the solution turned deep purple with no isolable solid produced. This purple color is indicative of purpogallin, an over-oxidation product of gallic acid. Furthermore, while a solid residue could be isolated from the [1-^{13}C]PGG DHP, ^{13}C NMR analysis indicated a very minor amount of label incorporation, and the major material in the solid product was unreacted PGG. Its presence in the solid form can potentially be explained by precipitation of a PGG/peroxidase complex, brought about by the affinity of hydrolyzable tannins for proteins.

Active incorporation appears at the onset to be a more plausible explanation. There was a distinct lack of a purple discoloration in any DHPs prepared besides those of using propyl gallate. Precedence for catechin incorporation into DHPs has been demonstrated previously (Kodera *et al*., 1979), although the redox potentials of catechin and PGG would be expected to be different. Nonetheless, nonselective ether and/or biaryl couplings between coniferyl alcohol moieties and gallate groups would provide linkages that may not be susceptible to acidolysis. Further work should help to clarify this issue.

CONCLUSIONS

Co-DHPs of lignin monomers and [1-^{13}C]PGG indicated that polymers containing both lignin and the labeled glucose could be obtained, although ellagic acid formation was not detected. American chestnut and white oak milled wood lignins were analyzed for their ellagic acid content. While the chestnut sample was found to contain 1% ellagic acid, the white oak sample had none. This suggests that the ellagic acid originally present in the oak heartwood is not bound to the lignin, that the ellagates have degraded over time, or that the ellagates are not present in MWL preparations. NMR spectroscopic analysis of the co-DHPs as well as chestnut milled wood lignin did not reveal signals indicative of a classical coupling product, suggesting that if tannin and lignin are covalently linked, the process is somewhat complicated. However, the possibility does exists that the high durability of American chestnut relative to white oak could be the result of a coupling of tannin and lignin providing a "fixed preservative" where the ellagate moiety is resistant to oxidative degradation. Further chemical and biochemical work underway in this laboratory is pursuing this possibility and may eventually lead to a better understanding of the resistance of ellagitannin-containing woods to biological decay.

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