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Arylpropane-1,3-diols in Lignins from Normal and CAD-Deficient Pines

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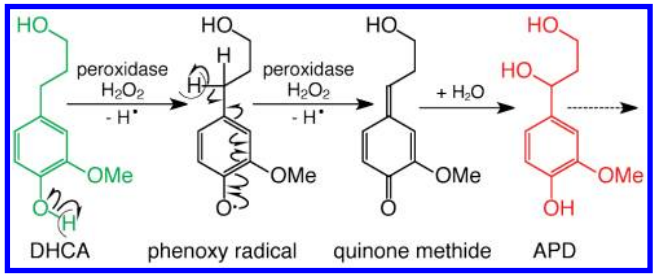
ABSTRACT



Significant quantities of arylpropane-1,3-diols have been identified in lignins isolated from a CAD-deficient pine mutant; smaller amounts are also present in lignins from normal pine. They arise from dihydroconiferyl alcohol via the action of peroxidases which are responsible for the radical generation steps of lignification. The structures in the complex lignin polymers are proven using 2D and 3D NMR of isolated lignin fractions.

The hydroxyphenylpropanoid polymeric component of plants, lignin, can be dramatically altered when lignin-biosynthetic-pathway enzymes are downregulated in mutant or transgenic plants.[1−3] We recently characterized a pine mutant deficient in cinnamyl alcohol dehydrogenase (CAD),[2,4] the enzyme catalyzing the conversion of 4-hydroxycinnamaldehdes to 4-hydroxycinnamyl alcohols, the monomers principally used in lignification. During lignification, these monomers are converted to radicals where they undergo radical coupling reactions with radicals from other monomers or, more commonly, from the growing lignin oligomer/polymer to build up a complex macromolecular structure.[5] Because of the various possible coupling sites on these radicals, the resulting polymer is complex and contains a variety of interunit linkages.

Several mutants and transgenics in which the CAD level is downregulated incorporate the precursor aldehydes of the normal monolignols into their lignins.[6] Thus in a CAD-deficient tobacco transgenic, sinapaldehyde becomes a major component of the lignin, radically coupling with other such aldehyde units and cross-coupling with traditional lignin components.[2] Similarly, in the CAD-deficient mutant pine, coniferaldehyde units become significant in the polymer.[7] But the more striking observation in the pine was the clear incorporation of significant quantities of an unexpected unit, dihydroconiferyl alcohol (DHCA), into the lignin.[8] DHCA is found at lower levels in normal plant lignins,[9,10] but its derivation is not completely clear. Despite claims that the

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(7) This has now been shown by various methods which act on the whole cell wall material,[6,9] not just the isolated lignin examined by NMR methods.[1]

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unit can only arise as a modified metabolic product *following* dimerization of traditional lignin monomers,[11] we have presented ample evidence that it is formed as a monomer and that the monomer is incorporated into the lignin via radical coupling processes.[1,6,9] Additionally, DHCA monomer is found in solvent extracts from these plants.[6] It is however still not known whether it derives from coniferyl alcohol or can come via other pathways from coniferaldehyde. Since all indications in the pine mutant are that coniferaldehyde builds up and is not efficiently reduced to the alcohol, the significant DHCA component was conjectured to arise from coniferaldehyde rather than coniferyl alcohol,[1] but this has not yet been elucidated.

Here we identify another significant component of the mutant pine's lignin, arylpropane-1,3-diols, whose source was originally puzzling. It is now clear that these units derive directly from DHCA by peroxidase-mediated reactions.

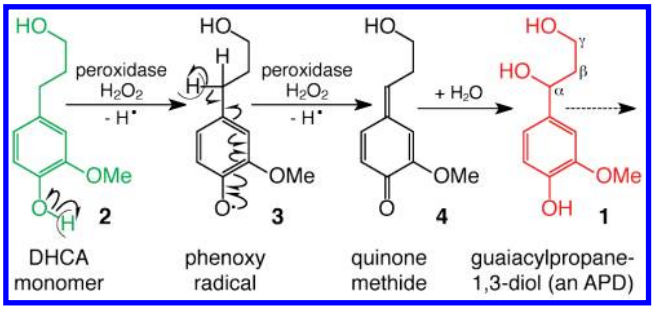
Figure 1a shows a subplot of the side chain region from a 2D HMQC-TOCSY NMR experiment on acetylated lignin isolated from the mutant pine,[12] highlighting the new arylpropane-1,3-diacetates (red) along with the previously identified DHCA units (green). Data from a model compound, 1,3-diacetoxy-1-(4-*O*-benzyl-3-methoxyphenyl)propane, **5**-Ac,[13] are at the center of the yellow circles and obviously match well.

Perhaps more diagnostic is the data from a 3D TOCSY-HSQC experiment.[14] 2D F_2-F_3 planes from this experiment show a complete HSQC spectrum of any units bearing protons resonating at the frequency of the taken slice.[9] In spectra as complex as those from lignins, finding unique resonances in the proton spectrum is often difficult. However, slices through each of the three side chain protons in APD units show rather clear HSQC spectra of that unit, along with correlations from other units with protons resonating in the same regions. Thus, resonances at 2.2 ppm arise only from the new unit (β-proton) and DHCA (β-proton) (as well as the strong acetone solvent signal). Figure 1e is therefore a composite HSQC of those units, namely, the acetone, as well as DHCA (green) and APD (red). The plane through H_α (5.8 ppm, Figure 1d) is quite clean but the TOCSY transfer between H_α and H_β is poor (under the chosen acquisition conditions), giving rise to only weak H_β/C_β and H_γ/C_γ cross-peaks. γ-Protons in lignin seriously overlap; consequently the plane through an H_γ (4.1 ppm, Figure 1f) shows the nice correlations for the APD unit, but also with DHCA (green), lignin's β-aryl ether units (blue), and the intense methoxyl. The observation of complete HSQC spectra for the ADP unit in all three planes corresponding to the APD side chain protons (Figures 1d−f), along with the evidence from the 2D-HMQC-TOCSY (Figure 1a), provides sufficiently compelling proof of the structure in the isolated lignin from the CAD-deficient pine mutant. A model compound for etherified APD structures, **5**,[13] provides data for comparison, Figure 1g; note however that there are a large number of different bonding environments in lignin so the peaks are broader and more disperse in lignin spectra.[9]

As with other novel units found in lignins from transgenic or mutant plants, traces of the same components can be found in lignins from control plants. Figure 1b shows the α-C/H region of HSQC spectra of the mutant's lignin, where the β-aryl ether units (blue) and the strong new ADP unit (red) appear most cleanly. Figure 1c shows the same region in lignin from a normal pine control. Although the peak is weak, it is diagnostic and, with the other correlations evident (not shown), well authenticated. The peak is also in lignins isolated from mature pine clear sapwood.

Where do APD units come from? They derive from DHCA **2** via the action of peroxidase and hydrogen peroxide, Scheme 1. The mechanism, via a vinylogous quinone methide **4**, involves two H-radical abstractions. Abstraction from a benzylic CH_2 to produce quinone methides from phenoxy radicals has been noted previously.[15] When DHCA is subjected to peroxidase−H_2O_2, monomeric APD **1** as well as the range of homo dimers and crossed dimers **7**−**11** (Scheme 2) involving DHCA and APD are found, as will be detailed elsewhere.

Scheme 1



(8) Lignin is the term used for the phenylpropanoid polymer in the cell walls of normal plants and will be used here although there remain issues regarding the biochemical and functional roles of "nontraditional" lignins in mutants and transgenics (see refs 6 and 11).

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(12) NMR 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 ~80 mg of acetylated isolated lignin in 0.4 mL of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). Experiments used were standard Bruker implementations of inverse (^{1}H-detected) 2D-gradient-HSQC, 2D-HMQC-TOCSY, and 3D-gradient-TOCSY-HSQC experiments. The TOCSY spin lock period was 100 ms. Carbon/proton designations are based on conventional lignin numbering. Lignin 2D and 3D spectra have been extensively detailed in a recent book chapter.[9] The CAD-deficient pine mutant and the isolation of lignin fractions have been described previously.[1] For this study, the lignins were acetylated, extracted into EtOAc, and washed with 6 mM EDTA to improve NMR relaxation properties (cf. ref 2).

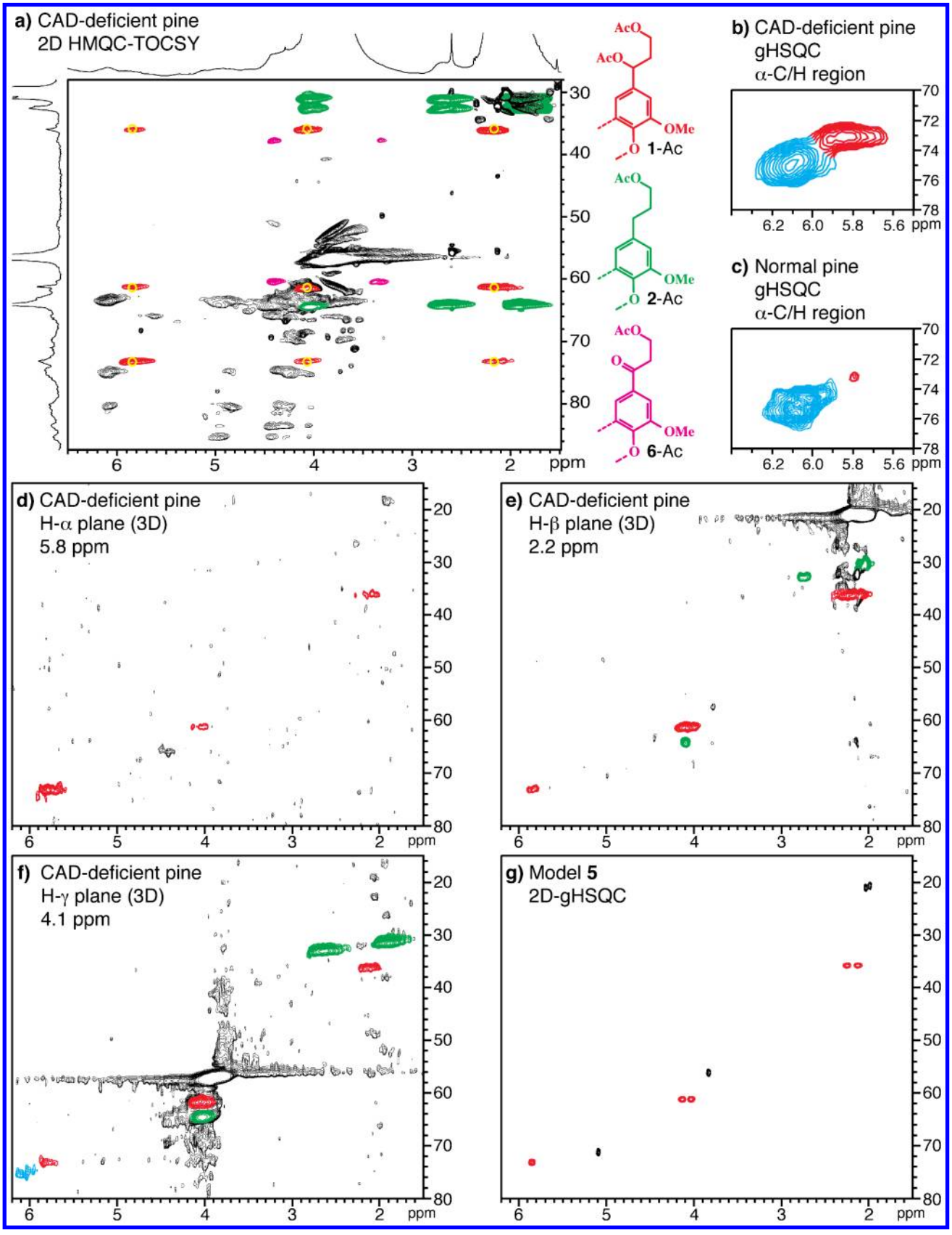
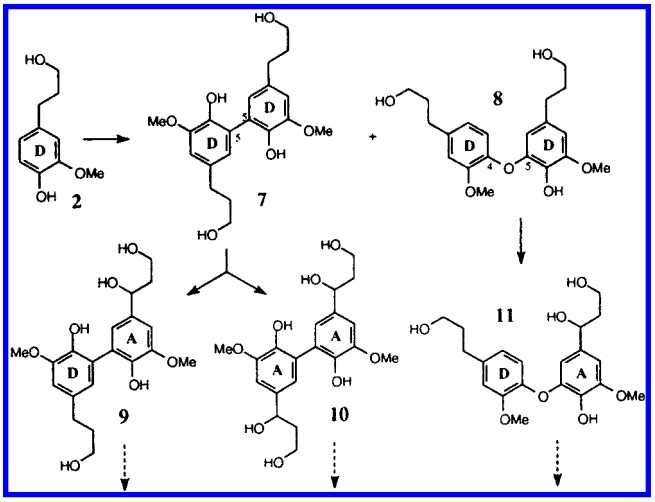


Figure 1. NMR spectra[12] showing new APD structures (red) and derived benzylic ketone analogues (magenta), along with DHCA units (green); some β-aryl ether units are also shown (blue); data from acetylated model **5** are in yellow: **a**−**c** and **g** are from 2D experiments; **d**−**f** are 2D planes from a gradient-selected 3D-TOCSY-HSQC experiment.

Scheme 2



Also evident in the HMQC-TOCSY spectrum, Figure 1a, are (acetylated) ketones **6**[16] (magenta contours). Products of benzylic alcohol oxidation are seen in various isolated lignins, notably from syringyl (3,5-dimethoxy-4-hydroxyphenyl) units;[9] they may arise during lignin isolation (particularly in the ball-milling step). Ketones **6** provide additional confirmatory evidence for the APD structures **1** described above.

The finding of significant quantities of APD units in the mutant's lignin is further evidence for the involvement of DHCA monomers in this plant's lignification. Nevertheless, some APD units likely arise *following* radical homo-coupling of DHCA **2** to give 5-5- or 4-O-5-coupled units **7** and **8**, Scheme 2; each of these units retains a free-phenolic DHCA moiety **D** which can be hydroxylated to give units **A**, structures **9**−**11**, by the same mechanism as shown in Scheme 1. As additional proof, degradation of the mutant's lignin by "derivatization followed by reductive cleavage" (the DFRC method)[17] produces the monomer APD **1** as well as numerous 5-5- and 4-O-5- products of homo- and cross-coupling (manuscript in preparation).

In conclusion, another previously unidentified unit present in small quantities in normal lignins is a major component of the hydroxyphenylpropanoid polymeric component of a pine mutant deficient in CAD. Those units, arylpropane-1,3-diols, arise from dihydroconiferyl alcohol monomers by radical reactions. Lignin-biosynthetic-pathway mutants and transgenics provide a rich source of insight into details of the chemistry and biochemistry of hydroxyphenylpropanoid polymer formation.

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Supporting Information Available: Full NMR data for diacetate of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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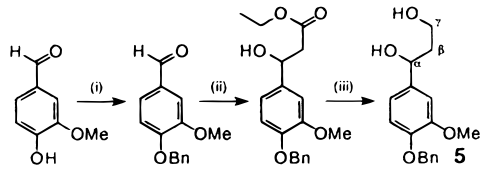
(13) **Preparation of APD model 1,3-dihydroxy-1-(4-*O*-benzyl-3-methoxyphenyl)propane 5**:



(i) BnCl, Me_2CO, K_2CO_3; (ii) $BrCH_2COOEt$, Zn, NH_4Cl, THF (Tanaka, K.; Kishigami, S.; Toda, F. *J. Org. chem.* **1991**, *56*, 4333−4334); (iii) DIBAL, toluene. Diacetate of compound **5**, δ_C/δ_H: 73.2/5.85 (α), 35.8/2.18 (*β*), 61.2/4.08 (*γ*). Unambiguously assigned full NMR data will be deposited in the Internet-accessible *NMR Database of Lignin and Cell Wall Model Compounds* (Ralph, S. A.; Ralph, J.; Landucci, W. L.; Landucci, L. L.; http://www.dfrc.ars.usda.gov/software.html **1999**, entry #3029) Various APD-containing products have also been isolated from radical coupling reactions of DHCA, as will be described elsewhere.

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(16) NMR data for units **6**-Ac, δ_H/δ_C (assignment): 3.31/37.5 (*β*), 4.41/60.4 (*γ*), 196.3 (α).

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