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Cross-Coupling of Hydroxycinnamyl Aldehydes into Lignins

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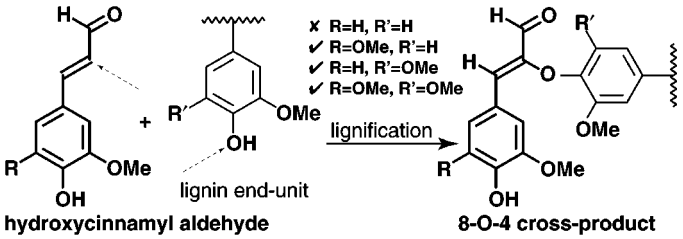
ABSTRACT



Pathways for hydroxycinnamyl aldehyde incorporation into lignins are revealed by examining transgenic plants deficient in cinnamyl alcohol dehydrogenase, the enzyme that converts hydroxycinnamyl aldehydes to the hydroxycinnamyl alcohol lignin monomers. In such plants the aldehydes incorporate into lignins via radical coupling reactions. As diagnostically revealed by long-range $^{13}C-^{1}H$ correlative NMR, sinapyl aldehyde (3,5-dimethoxy-4-hydroxy-cinnamaldehyde) 8-O-4-cross-couples with both guaiacyl (3-methoxy-4-hydroxyphenyl-propanoid) and syringyl (3,5-dimethoxy-4-hydroxyphenyl-propanoid) units, whereas coniferyl aldehyde cross-couples only with syringyl units.

Lignins are polymeric natural products present in large quantities in the cell walls of terrestrial plants. They arise principally from one or more of the three monolignols, *p*-coumaryl (4-hydroxycinnamyl), coniferyl (4-hydroxy-3-methoxy-cinnamyl), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohols, analogues varying in their degrees of methoxylation.[1] However, many other phenolic components also intimately incorporate into lignins;[2] acylated hydroxycinnamyl alcohols[3] and hydroxycinnamate esters[4] are notable examples. Various monolignol precursors including hydroxycinnamyl aldehydes[5] and 5-hydroxyconiferyl alcohol[6] are incorporated into lignins, particularly when the plant is deficient in certain enzymes on the monolignol biosynthetic

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pathway. Lignin-biosynthetic-pathway mutants and transgenics provide useful perturbations to "normal" lignification that allow normally minor components to be substantially enhanced and therefore structurally analyzed, to better understand the lignification process.

The lignin-biosynthetic-pathway enzyme CAD (cinnamyl alcohol dehydrogenase) is the last enzyme on the pathway to the monolignols coniferyl and sinapyl alcohols **2G** and **2S**, from which lignins are normally derived (Figure 1). When CAD is downregulated, the hydroxycinnamyl aldehyde precursors to the monolignols, coniferyl aldehyde **1G** and sinapyl aldehyde **1S**, build up and may be incorporated into the polymer by radical coupling.[5] Indeed, it was readily shown that coniferyl aldehyde would incorporate into synthetic lignins under biomimetic conditions.[5f] Hydroxycinnamyl aldehydes **1** and hydroxybenzyl aldehydes **5** derived from them are well-known components of lignins isolated from normal plants,[7] as well as mutant or transgenic plants.[5] In a CAD-downregulated tobacco transgenic, the aldehyde incorporation into the lignin was significant.[5b] Detailed proof that the aldehydes intimately incorporate into the polymer, cross-coupling with normal lignin oligomers, will be published elsewhere[8] (brief arguments are given below). In this paper, we report on the cross-coupling propensities of coniferyl and sinapyl aldehydes with guaiacyl and syringyl units in lignins. Data revealing such details of plant lignification are rarely obtained.

Radical cross-coupling of monolignols **2** with the growing lignin oligomer/polymer **3** is the major reaction occurring during lignification. Thus the hydroxycinnamyl alcohol **2** (primarily at its β-position) couples with phenolic units **3** (at the 4-O- or 5-position for guaiacyl units **3G**, and almost exclusively at the 4-O-position for syringyl units **3S**)[9] to form a chain-extended oligomer/polymer, also with the general formula **3**. Recent studies have begun to elucidate the propensity for the monolignols to cross-couple with (free-phenolic) guaiacyl and syringyl units in lignins.[10] No data on cross-coupling of the hydroxycinnamaldehydes into lignins are available.

With the availability of a ^{13}C-enriched lignin from a CAD-deficient tobacco transgenic,[5b] NMR methods can be used to ascertain hydroxycinnamyl aldehyde in vivo cross-coupling propensities. Figure 1 (right) shows a selected region of the HMBC spectrum from the CAD-deficient tobacco lignin,[11] with peaks in the ^{13}C-projection and the resultant contours in the HMBC spectrum colored to match structures on the left for easy identification. In fact, the aldehyde structures in the lignins likely have been further incorporated into the polymer by primarily 4-O-coupling with the next monolignol, so they are not strictly the phenolic compounds **4** indicated. Available coupling sites are indicated in Figure 1 by the dashed arrows. Correlation of the aldehyde carbonyl carbons in crossed dimers **4** to the H7-protons 3-bonds away identifies the type of lignin units involved. Protons H7 resonate at ~7.3 ppm for hydroxycinnamyl aldehydes (either **1G** or **1S**) coupled 8-O-4 to guaiacyl units **3G** (compounds **4GG** and **4SG**, $\delta_C = 188.1$ ppm), whereas they resonate considerably upfield at ~6.7 ppm when coupled 8-O-4 to syringyl units **3S** (compounds **4GS** and **4SS**, $\delta_C = 186.8$ ppm). Correlations from these H7 protons into the ring identify the hydroxycinnamyl aldehyde involved in the coupling; 3-bond correlations identify equivalent S2/6 carbons derived from sinapyl aldehyde **1S** units at higher field than the nonequivalent G2 and G6 carbons from units derived from coniferyl aldehyde **1G**. Therefore, for cross-coupled units **4** in lignins, the G/S nature of both the hydroxycinnamyl aldehyde component (coupled 8-) and the lignin unit (coupled 4-O-) are diagnostically revealed. Other aromatic protons in the complex lignin polymer resonate in the H7 regions, so correlations that are not of interest here will result; an absence of correlations is therefore more diagnostic, revealing the absence of a component.

Of the four possible aldehyde incorporation products **4**, only three can be detected in the lignin by NMR methods. Product **4GG** is conspicuously absent. The data imply that sinapyl aldehyde **1S** cross-couples with both guaiacyl and syringyl units (to form cross-coupled structures **4SG** and **4SS**) but that coniferyl aldehyde **1G** cross-couples only with syringyl units **3S** and not with guaiacyl units **3G**. Thus cross-coupling product **4GS** is readily detected, whereas **4GG** cannot be detected even when spectra are viewed at close to the baseplane noise level. Attempting to prepare compounds modeling **4GG** by biomimetically cross-coupling coniferyl aldehyde **1G** (at the 8-position) with coniferyl alcohol **2G** or with a simple guaiacyl model 1-(4-hydroxy-3-methoxy)-

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(9) Another type of coupling is possible from monolignols **2** (at the β-position) with guaiacyl or syringyl units **3** at the aromatic 1-position (β-1-coupling); see ref 1.

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(11) The acetylated CAD-deficient transgenic tobacco lignin was that described previously (ref 5b). Gradient-HMBC spectra of the lignin and model compounds were run on a Bruker DRX-360 using a 5 mm inverse {^{1}H-broadband} probe equipped with 3-axis gradients. The long-range coupling delay was 80 ms.

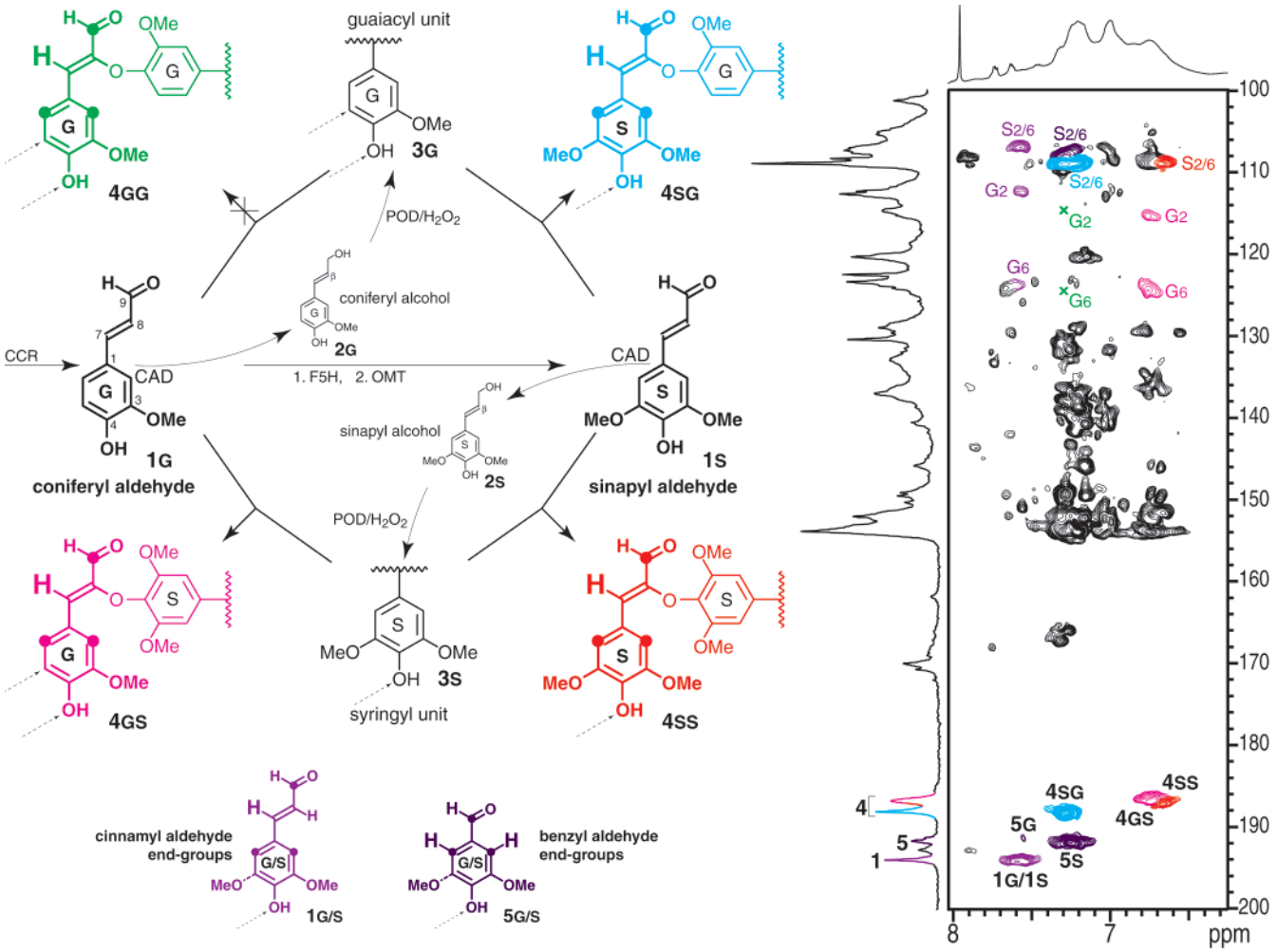

Figure 1. Cross-coupling of hydroxycinnamyl aldehydes **1G** and **1S** with guaiacyl and syringyl lignin units **3G** and **3S** can potentially produce four 8-O-4-cross-coupled structures **4**. Only three of the four cross-products (**4GS**, **4SG**, and **4SS** but not **4GG**) are significantly represented, as determined by analysis of the gradient-HMBC partial spectrum to the right. The HMBC section shows, in a lignin isolated from CAD-deficient tobacco stems, correlations from the aldehyde carbonyl carbons (185−195 ppm) to the H7 protons within 3-bonds in structures **1** and **4** that have been further incorporated into lignin at positions indicated by the dashed arrows. (For compounds **5**, it is H2 and H6, rather than H7, that are 3-bonds from the aldehyde carbonyl carbon). These H7 protons diagnostically correlate with the 2- and 6-carbons of the cinnamyl aldehyde ring; the G2 and G6 carbons for guaiacyl units, and the higher-field equivalent S2/6 carbons are identified and colored correspondingly. Colored dots on the structures indicate the carbons correlated (by $^{3}J_{C-H}$) to the H7-protons (which are shown in a larger font size) shown in the HMBC spectrum. The aldehydes at ∼188.1 ppm, with their corresponding H7s at ∼7.3 ppm result from hydroxycinnamyl aldehydes **1** 8-O-4-coupled to guaiacyl units **3G** in lignin; correlations from H7 indicate, however, that sinapyl aldehyde **1S**, but not coniferyl aldehyde **1G**, couples with guaiacyl units **3G**; the green X's show where model **4gg** correlations exist.[12] The aldehydes at ∼186.7 ppm, with their corresponding H7s at ∼6.7 ppm result from hydroxycinnamyl aldehydes **1** 8-O-4-coupled to syringyl units **3S** in lignin; correlations from H7 indicate that sinapyl aldehyde **1S** and coniferyl aldehyde **1G** each couple with syringyl units **3S**. Both hydroxycinnamaldehydes **1G** and **1S** couple at the 4-O-position with monolignols **2** at β to produce structures **1G** and **1S** in lignin, as seen by the aldehyde peaks at 193.9 ppm, their correlation with H7-protons at ∼7.6 ppm, and their correlations with both S2/6 and G2 and G6 carbons (colored). Benzyl aldehydes **5**, principally syringyl aldehyde **5S**, are also 4-O-coupled by monolignols at β (peaks labeled **5**). S = syringyl, G = guaiacyl, CCR = cinnamoyl-CoA reductase, CAD = cinnamyl alcohol dehydrogenase, F5H = ferulate 5-hydroxylase (which has recently been renamed CAld-5H = coniferyl aldehyde 5-hydroxylase, to reflect the preferred in vivo substrate[16]), OMT = *O*-methyl transferase, POD = peroxidase. Fully authenticated peak assignments will be reported elsewhere.[12] Convention for compound numbering: e.g., **4GS** = a guaiacyl aldehyde (coniferyl aldehyde **1G**) coupled 8-O-4 to a syringyl lignin unit (**3S**).

ethanol (at the 4-O-position), also failed to produce 8-O-4-coupled cross-products. Apparently the same factors that promote cross-coupling in vitro apply in vivo, providing new evidence that radical coupling reactions in lignification are likely to be under simple chemical control in the plant.[2b] In the crossed dimers, the chemical shifts assigned match with model compounds synthesized by traditional means.[12]

Examination of the spectra provides significant information regarding the incorporation of hydroxycinnamyl aldehydes into "bulk" vs "endwise" polymers.[13] Bulk lignins are characterized by frequent dimerization reactions, whereas endwise polymerization, the normal process occurring during lignification in the secondary cell wall, is from cross-coupling of monomers with the growing polymer. Dimerization of

coniferyl aldehyde produces the 8-O-4-coupled dimer (analogous to **4GG**, Figure 1), along with the other anticipated 8-8- and 8-5-coupled dimers.[14] The absence of signals for coniferaldehyde coupled with G-units (of which the coniferaldehyde dimer **4GG** is an example), is evidence that coniferaldehyde does not dimerize and therefore is not significantly involved in bulk lignification. Further evidence will be described elsewhere.[8]

Also available from the spectrum in Figure 1 is some information regarding the incorporation of the hydroxycinnamyl aldehydes **1** and their derived hydroxybenzyl aldehydes **5** at ring 4-O-positions. Syringyl aldehyde **5S**, as seen from the contours correlating the aldehyde carbon with the 2/6-protons (which are now the protons within 3-bonds of the aldehyde carbonyl carbon), appears to be well incorporated, but there is little evidence for incorporation of vanillin **5G**. Syringyl aldehyde **5S** can only couple at its 4-O-position with a monomer radical, not with the growing polymer. Vanillin **5G** can react either 4-O- with a monomer or 5- with either a monomer or an oligomer **3**. The hydroxycinnamyl aldehydes **1** can cross-couple to give products **4** as described above and can also couple at their 4-O-positions with monolignols (at β). Contours correlating the H7-proton in these units with both **1S**-2/6 carbons, as well as **1G**-2 and **1G**-6 carbons, indicate that both sinapyl aldehyde **1S** and coniferyl aldehyde **1G** will incorporate at their 4-O-positions (by radical coupling with monolignols at Cβ). NMR does not readily reveal information regarding the nature of the cross-coupling partner in this case.

In summary, in 8-O-4-coupling reactions, coniferyl aldehyde cross-couples only with syringyl units,[15] whereas sinapyl aldehyde cross-couples with both guaiacyl and syringyl units. Syringyl aldehyde incorporates efficiently into lignins, whereas vanillin units are either not produced extensively or are incorporated poorly into 4-O-structures. Sinapyl and coniferyl aldehydes **1** both appear to 4-O-incorporate by radical coupling with monolignols. These observations require no departure from the existing theory of radical coupling of phenols into polymeric lignins since they appear to reflect simple chemical coupling propensities.

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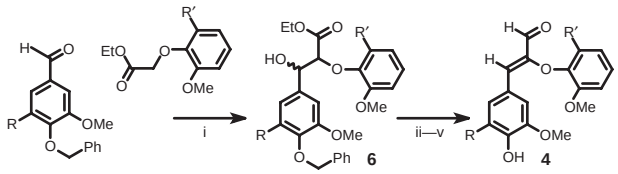
(12) Model compounds for the hydroxycinnamaldehyde-lignin cross-coupled products were synthesized following normal lignin β-O-4 model pathways (Nakatsubo, F.; Sato, K.; Higuchi, T. *Holzforschung* **1975**, *29*, 165−168). Elimination from the β-hydroxyester **6**, as previously published (Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485−3498) accompanied by debenzylation, DIBAL reduction of the ester to the alcohol (Quideau, S.; Ralph, J. *J. Agric. Food Chem.* **1992**, *40*, 1108−1110.), and final allylic oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (Becker, H. D.; Bjoerk, A.; Adler, E. *J. Org. Chem.* **1980**, *45*, 1596−600) produced the required aldehydes **4**.



4gg: R = R′ = H **4gs**: R = H, R′ = OMe **4sg**: R = OMe, R′ = H **4ss**: R = R′ = OMe

(i) LDA, THF, -70 °C; (ii) TMSBr, CH_2Cl_2, 12 h; (iii) DBU; CH_2Cl_2; (iv) DIBAL, $PhCH_3$; (v) DDQ, THF.

Relevant NMR data for samples in acetone-d_6 follow; solvent peaks used as internal reference (^{1}H 2.04, ^{13}C 29.8 ppm). The fully authenticated data for the four compounds and their acetates will be deposited as compound numbers 3034−3041 in the "NMR Database of lignin and cell wall model compounds" available via the Internet at *http://www.dfrc.ars.usda.gov/software.html*; Ralph, S. A.; Ralph, J.; Landucci, W. L.; Landucci, L. L. **2000**. **4gg** (acetate, model for **4GG** units): δ_C/δ_H (assignment) 188.2/9.55 (9), 149.9/- (8), 135.4/7.30 (7), 133.3/- (1), 114.7/7.68 (2) 124.5/7.45 (6). **4sg** (acetate, model for **4SG** units): δ_C/δ_H (assignment) 188.3/9.57(9), 149.8/- (8), 136.1/7.31(7), 131.4/- (1), 108.1/7.29 (2,6). **4gs** (acetate, model for **4GS** units): δ_C/δ_H (assignment) 187.0/9.32(9), 152.1/- (8), 126.7/6.71(7), 133.4/- (1), 115.2/7.68 (2), 124.0/7.54 (6). **4ss** (acetate, model for **4SS** units): δ_C/δ_H (assignment) 187.0/9.32(9), 152.1/- (8), 126.8/6.69 (7), 132.7/- (1), 108.1/7.34 (2,6).

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(14) Connors, W. J.; Chen, C.-L.; Pew, J. C. *J. Org. Chem.* **1970**, *35*, 1920−1924.

(15) There is some suggestion in spectra from guaiacyl-only lignins and synthetic lignins (not shown) that coniferyl aldehyde may cross-couple with other 5-substituted guaiacyl units.

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