5-HYDROXYCONIFERYL ALCOHOL AS A MONOLIGNOL IN COMT-DEFICIENT ANGIOSPERMS

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ABSTRACT

COMT is the enzyme responsible for methylating 5-hydroxyconiferyl aldehyde, on the way to producing syringyl units. It is deficient in recently examined transgenic poplars downregulated by two different methods, in F5H-upregulated transgenic arabidopsis, and in a brown-midrib maize mutant (bm3). In all cases, 5-hydroxyconiferyl alcohol incorporated intimately into the lignin. Benzodioxane (4-O-b/5-O-a) structures are produced and can be characterized by their beautiful NMR correlations, by their survival through DFRC-degradation, and their partial survival through thioacidolysis. The level of detail revealed by these methods provides evidence that the novel 5-hydroxyconiferyl alcohol monomer cross-couples with syringyl and guaiacyl units into the growing lignin oligomer, and that normal monolignols then add to the new 5-hydroxyguaiacyl terminus producing benzo-dioxanes. Demonstrated endwise polymerization into lignins suggests that 5-hydroxyconiferyl alcohol should be recognized as an authentic lignin monomer in these angiosperms.

INTRODUCTION

Recent advances in genetic engineering have, allowed researchers to perturb the monolignol biosynthetic pathway producing often significantly altered lignins. This approach provides valuable insights into the control of lignification and into the apparent bio-chemical flexibility of the lignification system.

Here we present NMR and DFRC data from recent studies on mutants and transgenics deficient in COMT (caffeic acid 0-methyl transferase, the favored substrate for which now appears to be 5-hydroxyconiferyl aldehyde (1). Downregulating these enzymes dramatically affects the composition of lignins and the structures contained in those lignins.

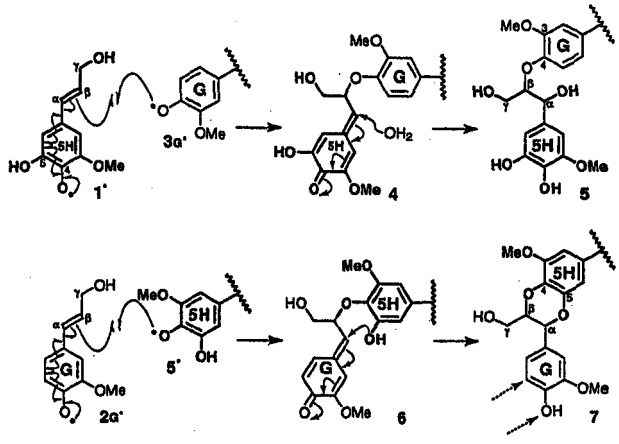

Fig. 1. Production of benzodioxanes 7 in lignins via incorporation of 5-hydroxyconiferyl alcohol 1 into a guaiacyl lignin. Only the pathways producing b-ether units are shown. Cross-coupling with syringyl units is less pronounced in lignins which have a low syringyl content due to COMT downregulation. Cross-coupling of 5-hydroxyconiferyl alcohol 1, via its radical 1; with a guaiacyl lignin unit 3G, via its radical 3G·, produces a quinone methide intermediate 4 which re-aromatizes by water addition to give the b-ether structure 5 (possessing a 5-hydroxyguaiacyl end-unit). This unit is capable of further incorporation into the lignin polymer via radical coupling reactions of radical 5·. Reaction with the monolignol coniferyl alcohol 2G, via its radical 2G·, produces a quinone methide intermediate 6. This time, however, quinone methide 6 can be internally trapped by the 5-OH phenol, forming a new 5–04-bond, and creating the benzodioxane ring system in 7. The presence of structures 7 in COMT-deficient transgenic plants is diagnostically revealed by NMR, units H in Fig. 2.

COMT-Deficient Poplar: Benzodioxanes from Incorporation of 5-Hydroxyconiferyl Alcohol

COMT is one of two enzymes required to 5-methoxylate guaiacyl monomeric units to produce sinapyl alcohol and eventually produce syringyl units in angiosperm lignins. If COMT is downregulated, 5-hydroxyconiferyl aldehyde might be expected to be reduced to 5-hydroxyconiferyl alcohol if the next enzyme, CAD, is sufficiently non-specific. In fact, it appears that 5-hydroxycohiferyl alcohol is indeed formed, shipped out to the wall, and incorporated into lignin analogously to other lignin monomers (although it produces some novel structures in the final lignin), Fig. 1. The incorporation of 5-hydroxyconiferyl alcohol into lignins has been documented several times (2-5). Benzodioxanes have been proposed to be in lignins previously (2, 4, 6), but until now the occurrence

of benzodioxanes in lignins has not been observed directly.

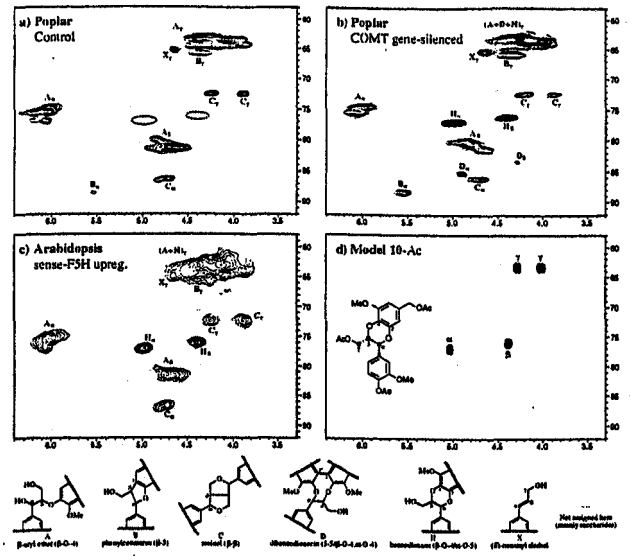

Fig. 2. Partial spectra from gradient HMQC NMR experiments highlighting new peaks for benzodioxane units H. Lignins were from a) a control poplar, b) a COMT-downregulated transgenic, c) an F5H-upregulated Arabidopsis, and d) similar correlations from a benzodioxane model. The major unit coding is the same as that used in (7) and other references.

NMR provides beautiful evidence that benzodioxane structures are produced in lignins which incorporate 5-hydroxyconiferyl alcohol. Figure 2b shows the side-chain region of an HMQC spectrum from an (acetylated) isolated lignin from a COMT-deficient poplar (*Populus tremula* x *Populus alba*) described recently (6). The transgenic was actually the result of an attempt to up-regulate COMT by sense-methods that 'instead resulted in gene silencing. The degree of COMT suppression was higher than in other trials using anti-sense suppression (8, 9). The benzodioxanes **H** are readily apparent in short-range $^{13}C–^{1}H$ correlation spectra (HMQC or HSQC) of acetylated isolated lignins. Well separated contours at d_C/d_H of 76.8/4.98 (a), and 75.9/4.39 (b) are diagnostic for the benzodioxanes **H;** the g-correlations overlap with those in other lignin units, but are elegantly revealed in 3D TOCSY-HSQC or 2D-HMQC-TOCSY spectra (10). The sidechain correlations are consistent with those in a model compound for the *trans*-benzodioxane, synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit (11).

A reasonable quantification of this unit can be done by measuring volume integrals in the 2D spectra (12), particularly if the similar Ca–Ha correlations are used. The ratios in the transgenic poplar (and in the wild-type control are given in Table 1. The 5-hydroxyconiferyl alcohol-derived benzodioxane H units are the second most abundant (–18%) interunit type in the COMT-gene-silenced sample. Since the lignins analyzed by NMR represent 65% of the total lignin in this transgenic, it is logical that the benzodioxane structures would remain a significant component even if the lignins were drastically partitioned by the isolation process. The total b-ether frequency (normal b-ether **A** plus dibenzodioxocins **D** plus benzodioxanes **H**) in the transgenic is around 78%, lower than in the control because of the higher guaiacyl content. However, there are a few units not covered by these percentages since they have no resonances in the aliphatic sidechain region of the NMR spectra (cinnamaldehyde endgroups, b–1-structures). Lignins from COMT antisense poplars also contain benzodioxane units (11) at a lower, level. Although the data are limited at present, it appears that 5-hydroxyconiferyl alcohol may quantitatively make up for the sinapyl alcohol deficiency.

Table 1. Subunit ratios derived from volume integrals of contours in the sidechain region of $^{13}C–^{1}H$ correlation spectra of acetylated isolated lignins.

Lignin	A	B	C	D	H	X	ß-O-4
Poplar							
WT	88	3	7	0	0	2	88
COMT-silenced	53	13	5	6	18	5	78
COMT-anti	65	12	5	3	10	4	78
Maize							
WT	86	7	0	0	0	6	86
bm3-mutant	60	7	0	0	25	8	85
Arabidopsis							
WT	67	13	6	6	0	7	73
F5H-sense	81	4	6	0	10	4	91

A = b-ether (b-O-4). **B** = phenylcoumaran (b-5), **C** = resinol (b-b). **D** = dibenzodioxocin (b-O-4/a-O-4), **H** = benzodioxane (b-O-4/a-O-5); the last column is the sum of all b-O-4 components **(A+D+H).**

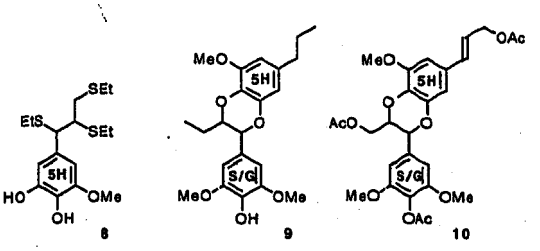

Fig. 3. Marker compounds for 5-hydroxyconiferyl alcohol incorporation into lignins (and COMT deficiency); thioacidolysis monomeric marker **8,** dimer **9** (following Raney-Ni desulfurization), and DFRC marker **10.**

Evidence for 5-hydroxyconiferyl alcohol incorporation and of benzodioxane units in the lignins also comes from degradative studies. A monomeric compound **8** is released following thioacidolysis products from COMT-deficient poplar transgenics (6), Fig. 3. A dimeric benzodioxane compound **9** was also observed following Raney-Ni desulfurization. The DFRC degradative method also produced benzodioxane dimers **10** in striking yields, but no monomeric 5-hydroxyguaiacyl products. Model studies showed that the DFRC method leaves benzodioxanes completely intact, whereas thioacidolysis partially cleaves them

(into monomeric products). The absence of monomeric 5-hydroxyguaiaicyl DFRC products strongly suggests that all such units in the lignins are present in benzodioxane structures. GC(-MS) data for the prominent DFRC dimeric products are shown in an accompanying abstract (13).

Benzodioxanes in F5H-Upregulated Arabidopsis

There is another interesting variant in the COMT-deficiency class. Arabidopsis transgenics with upregulated F5H have previously been shown to have only a minor guaiacyl component (14, 15). Contours previously unidentified in the 2D NMR spectra of their lignins now obviously result from benzodioxane structures, Fig. 2c (10). The observations here imply that, whereas syringyl production was enormously up-regulated in these transgenics, the methylation could apparently not keep pace with the accelerated production of 5-hydroxyunits (e.g. 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol). The result is a significant incorporation of 5-hydroxyconiferyl alcohol into the monolignol pool for the most heavily F5H-upregulated transgenics, ~10% as measured from contour volumes in the HMQC spectra – see Table 1.

Benzodioxanes in *bm3* Maize Mutants

Maize has a four of brown-midrib (*bm*) mutants, all of which have reddish-brown vascular tissue in the leaves and stems (16). Two are known to have mutations in the monolignol biosynthetic pathway; *bm1*, CAD; bm3, COMT (17).

Lignins in the bm3 mutant show the now characteristic signs of 5-hydroxyconiferyl alcohol incorporation. Very early on, thioacidolysis suggested that 5-hydroxyconiferyl alcohol was a constitutive unit of those lignins (3). The dimeric benzodioxane products from thioacidolysis and DFRC are also readily identified in the maize mutant (13). Preliminary NMR of isolated lignins shows the substantial presence of these new **H** units in the mutant (Table 1) where it accounts for some 25% of the interunit linkages characterized (although we have not yet examined how this structure is partitioned between the soluble fraction used for NMR and the residues).

Benzodioxane Dimers Released by DFRC: Mechanistic Implications

The release of compounds **1OG** and **10S** following DFRC degradation are more diagnostic than might at first be assumed. The following observations are relevant.

1. The double bond implies that the 5-hydroxyconiferyl alcohol unit had coupled b-O-4 to a syringyl or guaiacyl unit in lignin. It could not have been another 5-hydroxyguaiacyl unit or dimer **10** would not be released. In fact, it also is not likely to have been incorporated by reaction with another monolignol, since cross-coupling studies are showing that it is coniferyl or sinapyl alcohol that couples at the b-position and the 5-hydroxyconiferyl alcohol couples at the 4-O-position. This is borne out in the literature (18, 19) by the isolation of several lignans similar to 10 but none have been reported with reverse coupling modes (i.e. coupling at the b-position on the 5-hydroxyconiferyl alcohol unit).
2. Since we can readily identify both compounds **10S** and **10G,** we obviously know that monolignols, sinapyl or coniferyl alcohol, then react at their b-positions with the newly formed hydroxyguaiacyl unit (at its 4-O-position) on the growing polymer.
3. The NMR data already indicates that the phenol on the guaiacyl unit attached to the benzodioxane in structures **7** (or **10**) in the lignin is etherified. Since DFRC releases units 10, there must also be further etherification presumably by another monolignol (at its b-position) – again, it could not be another 5-hydroxyconiferyl alcohol or it would not release dimer 10. Further DFRC experiments will be able to establish what proportion of these released benzodioxanes were originally etherified.

What this data shows therefore, is that 5-hydroxyconiferyl alcohol is behaving like a normal monolignol does in lignification. It reacts at its p-position with the phenol at the end of the growing lignin polymer, and new monolignols then react with the resulting new 5-hydroxyguaiacyl phenolic end. This endwise polymerization is characteristic of the major lignification pathway. It also indicates that 5-hydroxyconiferyl alcohol appears to be incorporating intimately into the polymer in the same way that the traditional monolignols do. We see no reason therefore why 5-hydroxconiferyl alcohol should not be considered an authentic monolignol in these systems.

Implications for Pulping

How is offsetting syringyl units in lignins with 5-hydroxyguaiacyl units likely to affect pulping performance? The new benzodioxane units in the lignin are still ether structures (a,b-diethers), but will they cleave under pulping conditions? In preliminary studies with etherified benzodioxane model compounds, very little ether cleavage occurs under soda pulping conditions - the models are recovered intact in high yields. Kraft pulping conditions are unlikely to affect the outcome. With less cleavable b-ethers in the lignin, pulping efficiency would therefore likely be reduced. Recent pulping trials with COMT-deficient poplars confirm a lower pulping efficiency (6). In part this may also be attributed to the slightly higher guaiacyl content in the transgenic lignins compared with the wild-type control, but it is suspected that much of the effect can be attributed to the alkaline stability of the benzodioxanes.

Implications for Ruminant Digestibility

No clear correlations between lignin structure and cell wall digestibility in ruminants is evident. However,

various mutants and transgenics are of interest because of their potential to improve digestibility. At present, such studies remain empirical. No data have yet appeared in the literature but Dixon's group at the Noble Foundation are examining digestibility implications of various alfalfa transgenics.

CONCLUSIONS

As further evidence accumulates from degradative and NMR methods that 5-hydroxyconiferyl alcohol monomers integrate into the polymerization process, it becomes evident that 5-hydroxyconiferyl alcohol can be used as a lignin monomer by plants, in part offsetting the deficiency in sinapyl alcohol monomers. The recent statement that "There is, however, no known precedent for the free interchange of monomeric units in any biopolymer assembly, then or now, ..." (20) would appear to have been proven incorrect.

A salient observation is that the process of lignification appears to be flexible enough to incorporate phenolic phenylpropanoids other than the traditional monolignols. The incorporation of 5-hydroxyconiferyl alcohol as well as hydroxycinnamyl aldehyde monomers also implies that the plant is sending these products of incomplete monolignol biosynthesis out to the cell wall for incorporation. The resultant modified lignins apparently have properties sufficient to accommodate the water transport and mechanical strengthening roles of lignin and to allow the plant to be viable. 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, the plants' approach toward lignification, i.e. polymerizing monolignol precursors and derivatives along with the traditional monolignols, is a testament to a flexible survival strategy; in a single generation, these plants have circumvented genetic obstacles to remain viable. The recognition that monolignol intermediates and other novel units can incorporate into lignin provides expanded opportunities for engineering the composition and consequent properties of lignin for improved utilization of valuable plant resources.

ACKNOWLEDGEMENTS

Partial funding for the Dairy Forage Research Center authors was from USDA-NRI (Plant Growth and Development #96-35304-3864; Improved Utilization of Wood and Wood Fiber #97-02208 and #99-02351) and the Division of Energy Biosciences, U.S. Dept. of Energy (#DE-AI02-00ER15067; for French and Belgian collaborators, from the European Commission FAIR (OPLIGE, AGRE-0021-C), and "Tree improvement based on lignin engineering" (TIMBER-CT95-0424) projects; for the Purdue lab from the Division of Energy Biosciences, U.S. Dept. of Energy (#DE-FG02-94ER20138).

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