Elucidation of new pathways in normal and perturbed lignification

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ABSTRACT
It seems incomprehensible that new structures in lignins and new pathways in lignification remain to be discovered after all this time. With new technologies and the improved understanding of the process that comes from new ways to perturb lignification, such discoveries continue to be made, however. Some may be of rather academic interest only, while others underlie important new approaches to improved utilization of cell walls.

An assault on the existing theory of the process of lignification has lead not to its demise but its strengthening as it continues to account for old and new observations. That said, aspects of lignin structure still hold many biosynthetic mysteries. Although it is time to summarily discard the proposed new hypothesis/theory for the absolute control of lignin primary structure, lignification is complex and an all-encompassing theory remains elusive. Nevertheless, lignins and lignification are becoming increasingly well understood.

New details on the coupling processes are being revealed. The β–1-pathway produces novel spirodienone structures that are, now that we know how to look for them, surprisingly easy to detect in lignins. What used to be the well-accepted β–β-coupling pathway to produce resinols is now being shown to proceed by alternate pathways that give other structures in lignins. β–β-Coupling when the γ-alcohol is acylated in natural lignins is revealing a range of fascinating structures and, more importantly, that acylated lignins derive from acylated monolignols, which therefore must be recognized as authentic lignin monomers. To date, the benefit of such acylation to the plant is unknown, and the components and pathways remain unexploited.

A small range of monomers are now known to substitute for the conventional monolignols in various natural and transgenic plants. Substitution appears to be most successful when the replacement monomer behaves, in its chemical radical coupling and cross-coupling reactions, like a normal monolignol. The post-coupling reactions that may be altered by the different functionality on the monomer seem to have less impact. Thus massive changes in the lignin structure occur in COMT-deficient plants when 5-hydroxyconiferyl alcohol substitutes for sinapyl alcohol, for example — the coupling reactions are analogous, but post-coupling steps produce novel benzodioxane structures that drastically change the lignin structure. Such biosynthetic malleability functions well for the plant, but also provides significant opportunities for engineering the polymer. Already it has been demonstrated that natural and industrial processes ranging from ruminant digestibility to chemical pulping can be both positively and negatively impacted by alterations to lignin, and we are beginning to get a mechanistic handle to explain these effects. Future work should reveal opportunities beyond the interesting deviations achieved by up- and down-regulating genes on the monolignol pathway to date.

INTRODUCTION
In addition to the numerous reviews on lignins and lignification, we have recently published two reviews, one dealing more with the biosynthetic pathways and transgenics,[1] and one concentrating more on aspects of structure.[2] Consequently, this abstract will NOT be extensively referenced — references can be found in those two reviews, which are available electronically (including from the Dairy Forage Research Center's web site at http://www/dfrc.ars.usda.gov). The aim here is to provide a commentary on some of the recent revelations regarding lignin structure and the process of lignification, and to note some of the implications for fiber utilization.

THE MONOLIGNOL BIOSYNTHETIC PATHWAY
Various aspects of the pathway by which the monolignols (the primary lignin monomers, *p*-coumaryl, coniferyl, and sinapyl alcohol) are biosynthesized have been clarified. Briefly, the pathway through the previously entertained metabolic grid has been somewhat streamlined, particularly with outstanding work from several groups. In particular, 5-hydroxylation and methylation of the 5-OH (to ultimately produce sinapyl alcohol in angiosperms) has now been shown to occur principally at the aldehyde level, Fig. 1. Taken together with discoveries regarding 3-hydroxylation (see below), these data argue that none of the C3 and C5 substitutions of the aromatic ring take place at the cinnamic acid level in monolignol biosynthesis. Evidence suggests that wall ferulate esters may also be produced via the aldehydes rather than directly from their corresponding acids.[3]

For a synthetic organic chemist, one of the most pleasing recent discoveries concerns the 3-hydroxylation on the pathway from *p*-coumaric to caffeic acid. The pathway was always considered to proceed from *p*-coumaric acid via the CoA-thioester to caffeoyl-CoA (Fig. 1). But every organic chemist knows that you must be foolhardy to go and activate an acid, say as its acid chloride (c.f. the biological analog, the thioester), and then not directly utilize that group but go off and do some other modification elsewhere on the molecule, expecting your activated acid to remain intact. Of course plants are smarter than organic chemists, and perhaps the enzymes for hydroxylation require a rather common SCoA binding site. But it was always agonizing to have to teach this step in the pathway. It has been a delight to recently find, in arabidopsis but likely in all plants producing syringyl/guaiacyl lignins, that the SCoA ester is merely an intermediate and that the substrate for the hydroxylation is in fact a shikimic or quinic acid ester — see the expansion in Fig. 1. An HCT enzyme takes *p*-coumaroyl-CoA and makes the

shikimate ester which then becomes hydroxylated (via the C3H enzyme); the resultant caffeoyl shikimate is then activated back to the SCoA ester ready for the next transformations — methylation and reduction to the aldehyde. Although this process involves more steps, it is very much analogous to the use of protecting groups by the synthetic chemist. Nature makes sense again! Rather intriguingly, shikimate is a crucial intermediate in the pathway from carbohydrates to the aromatic pools (including the amino acid phenylalanine, and the monolignols) in the plant. It is interesting that shikimate is used again in this monolignol pathway, essentially as a temporary protecting group.

A practical benefit of the discovery of the new enzyme required on the pathway from *p*-coumarate to coniferyl and sinapyl alcohols is that there is now an additional target that can be used to down-regulate the biosynthesis

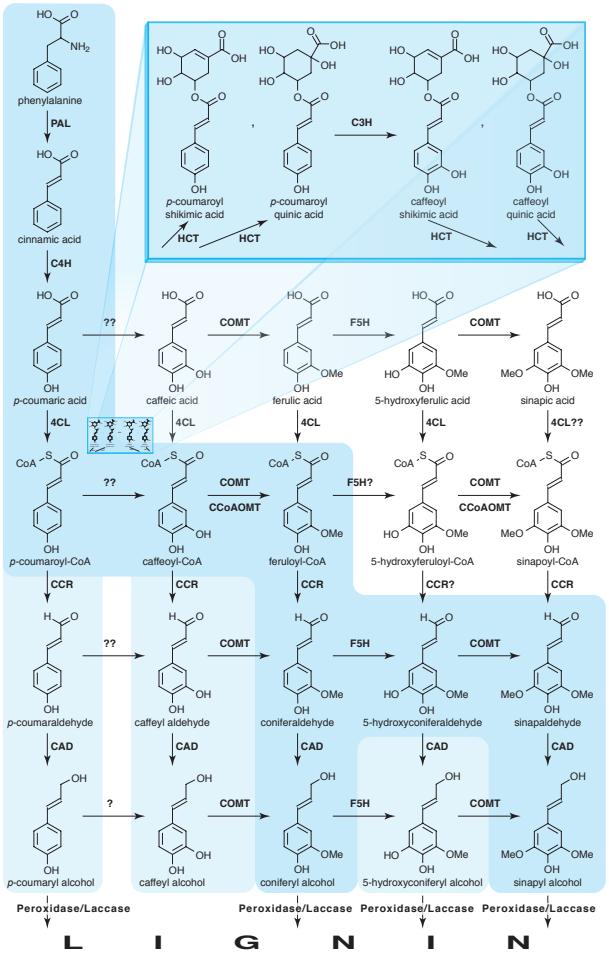


Fig. 1. Simplified monolignol biosynthetic pathway.[1] Note that, at least in Arabidopsis, the *p*-coumaric acid to caffeic acid conversion involves more steps than previously thought, as shown in the expansion. Traditional names for the enzymes involved are: 4CL = 4-coumarate:CoA ligase; C3H = *p*-coumarate 3-hydroxylase; HCT = *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; CCoAOMT = caffeoyl-CoA *O*-methyltransferase; CCR = cinnamoyl-CoA reductase; F5H = ferulate 5-hydroxylase; CAld5H = coniferaldehyde 5-hydroxylase; COMT = caffeic acid *O*-methyltransferase; AldOMT = 5-hydroxyconiferaldehyde *O*-methyltransferase; CAD = cinnamyl alcohol dehydrogenase. Many of these enzymes are no longer thought to function on the substrates for which they were originally named.

of these two primary monolignols. Thus downregulating either C3H or HCT can both produce similar effects. To the extent that inducing plants to produce a greater percentage of their lignins from *p*-coumaryl alcohol (rather than coniferyl and sinapyl alcohols) may have some advantages, there are now two distinct target genes for achieving this.

LIGNINS ARE RACEMIC POLYMERS

Although there is nothing new in the above heading, this concept bears bringing up once more as it continues to be overlooked and misunderstood. And yet this facet is crucial for understanding the biosynthesis of the polymer and its consequent chemical and physical properties.

In 1991 I tried to make the point that a regular but racemic β-ether polymer (not lignin!) with 110 units had about the same number of isomers as there were atoms in the Galaxy.[4,5] The molecular size was artificial and the statement somewhat silly, and in fact was a couple of orders of magnitude out. It has been repeatedly ridiculed by one scientist. Unfortunately, it remains true!! As any first year organic chemist knows, when you have a molecule with n optical centers you have 2^n possible optical isomers, and can have half that number of physically distinct isomers. Thus, with a β-ether 110-mer, there are actually only 218 optical center (not the 220 I originally assumed), and therefore 2^{117} physically distinct isomers. This is about $4x10^{65}$ — an astronomically large number in line with the original claim.

Since the racemic nature of the polymer continues to be underappreciated, we recently tried to make the point again using models for lignins from normal poplar and a COMT-deficient transgenic.[2] These are only models, but represent the compositional data available in a structure of 20 units in a manner similar to the famous models by Adler and the more recent advancement by Brunow. The two models are shown in Fig. 2; these and the molecular models themselves can be found on our website (http://www.dfrc.ars.usda.gov/LigninModels.html). For the single wild-type lignin structure drawn, without considering isomers based on altering the linkage sequence but merely the stereochemistry of each unit, and taking into account the isomer restriction in units such as the phenylcoumaran and dibenzodioxocin units (where the number of isomers is reduced by a factor of two from those theoretically possible), this simple little model 20-mer still has an astounding 17 billion isomers. The COMT-deficient lignin model has fewer isomers, a mere 134 million since it has more of the restricted phenylcoumaran and dibenzodioxocin structures (see Fig. 2 caption).

Any flaw in the above will hopefully be addressed by those choosing to ridicule these notions further! The major point of course is that lignins are produced by a process which creates two new optical centers with each coupling reaction when the coupling is at the favored β-position, and there really appears to be no stereo-control over their creation. Thus, unlike in the case reported for

the lignan pinoresinol,[6] and presumably other optically active lignans, dirigent proteins that control the coupling reaction do not appear to have any role in the formation of lignin units. Certainly, it has now been rather well established that a variety of units, including those β-β-units that could very well be formed in a lignan-like manner, have no optical activity, i.e. they are racemic. If the units are all racemic, the polymer of course is too and it is simply time to recognize that a great deal of lignins' complexity (including their broad NMR spectra) is due to the fact that each unit finds itself in a structurally and stereochemically distinct extended environment. In fact there may be no two molecules of lignin (over a certain, regrettably unspecified, size) in any plant or any isolate that are identical. While this seems to scare some, it is part of lignins' uniqueness and intrigue, and allows considerable scope in modifying the polymer.

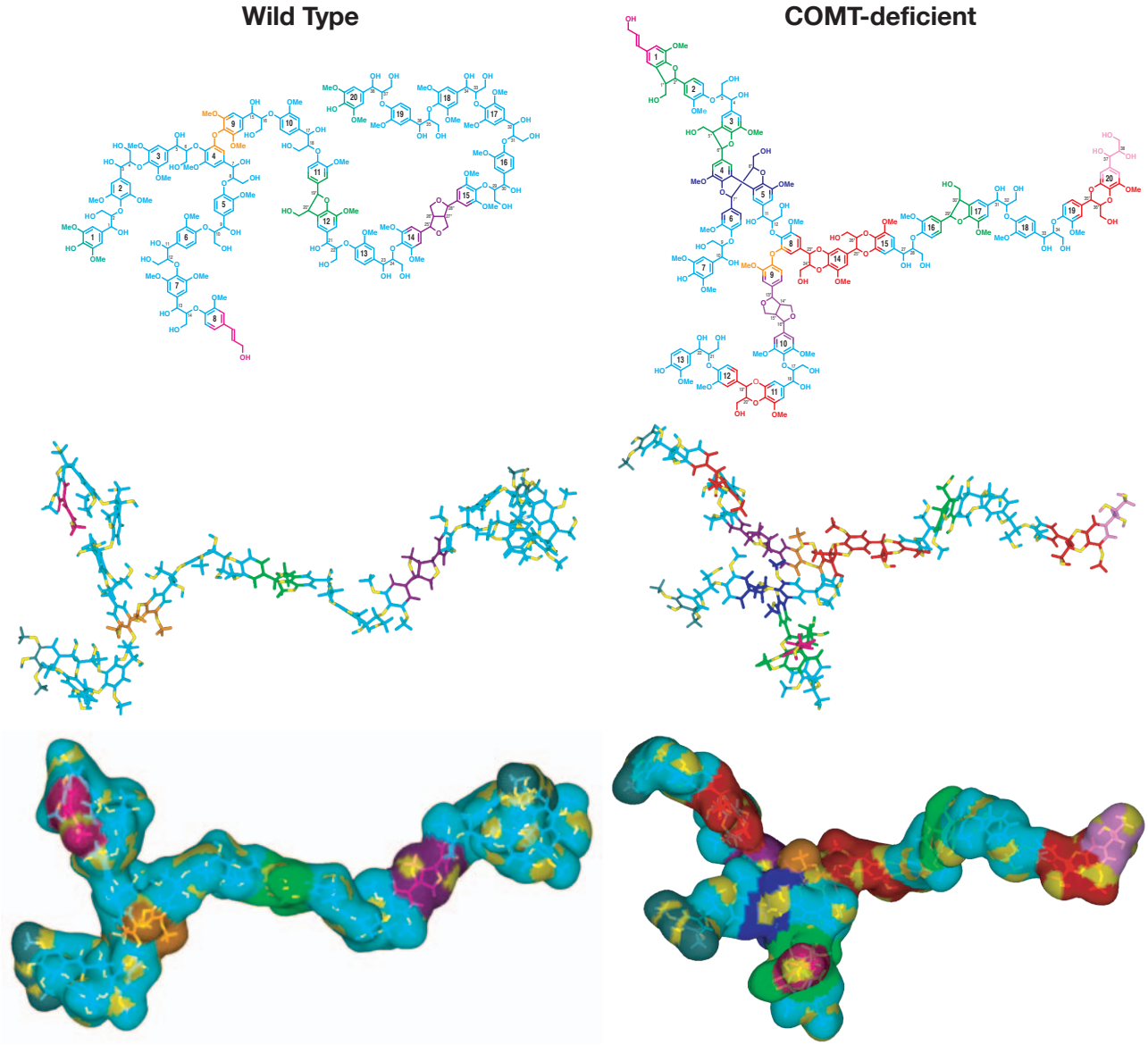


Fig. 2. Poplar lignin models.[2] These models, derived from 20 monomeric units. Color coding across all model structures are similar. This way it is readily seen, for example, that β-ether units (cyan) are prevalent in the wild-type lignins, but less prevalent in the COMT-deficient lignin. Since it is only these units that efficiently cleave by thioacidolysis, DFRC, or in pulping reactions, it is clear why the COMT-deficient lignin is less easily degraded. The new benzodioxane units in the COMT-deficient lignin are colored red. The branch points (4–O–5-units, orange; dibenzodioxocin units, dark blue) are also easily identified in the models. Each structure represents only one of many millions of isomers. For the model for milled wood lignin from the wild type poplar, containing 20 phenylpropanoid units, there are 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 3 pairs of centers are fixed (asterisked centers; e.g. *trans*-ring junctions in phenylcoumarans). We therefore insinuate that there are 2^{35} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{34} (17,179,869,184) real viable isomers for the structure shown. For the model for milled wood lignin from the COMT-deficient poplar, also containing 20 phenylpropanoid units, there are again 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 10 pairs of centers are fixed (asterisked centers; e.g. *trans*-ring junctions in phenylcoumarans, *trans*-ring junctures in the benzodioxanes) in this case; the significant incorporation of 5-hydroxyconiferyl alcohol leads to a high proportion of benzodioxane structures which are mostly in the *trans*-ring form. We therefore calculate that there are 2^{28} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{27} (134,217,728) real viable isomers for the structure shown. Clearly, their racemic nature alone confers incredible complexity and variability on polymeric lignins. The structures have been minimized in place, but it must be emphasized that each unit is probably just a local minimum – full optimization over all conformational space in a molecule of this size is impractical. They are ONLY MODELS! The pictures and the molecular models themselves may be downloaded from our website (http://www.dfrc.ars.usda.gov/LigninModels.html).

Refining the Theory of Lignification?

Here is an opportune place to reinforce statements we made in a recent review regarding the challenge to the existing theory of lignification.[2] As has been established, lignin formation occurs after the polysaccharides are laid down, providing structural integrity to those lignified cell walls, facilitating water transport, and providing defensive functions. But what makes the lignins enigmatic is their mode of formation. The existing theory has been that, unlike the polysaccharides and proteins, no exact chemical "sequence" of units is dictated by the cell. Although there is considerable control by the cell over aspects of the structure by the supply of the various lignin monomers to the wall and the supply and control of oxidants (in the peroxidase-H_2O_2 system), the assembly of the polymer is a combinatorial process, under the auspices of simple chemical control, i.e. governed by normal chemical concerns such as the concentrations of reactants, their natural coupling and cross-coupling propensities, the matrix, and the physical conditions during the polymerization.

That long-standing theory has been recently challenged,[7] and even a text book has pronounced that lignification is a process under absolute structural control.[8] Apparently the proposers still seem to believe in their revision, as evidenced by a recent abstract;[9] "This is further evidence for full biochemical control of lignin assembly, i.e. via monomer transport, oxidation and directed polymerization." However, this challenge is wholly without merit and should now be dismissed.[2] Arguments raised in an attempt to indicate that the old theory could not explain known facts were flawed. A prime example is the heavily quoted "anomaly" of high β-ether frequency. But as pointed out in our recent reviews and papers cited therein, the anomaly is a myth — once it is appreciated that lignification is predominantly a process of endwise coupling under limited monomer (and radical) supply, the high β-ether frequency is not only trivially explained but a readily demonstrable chemical event. In fact current observations are explained by the existing theory, whereas a great many either don't fit with the new hypothesis of controlled coupling or require convoluted anti-Occam arguments to attempt to explain what is readily explained by the existing theory. Attempts to unilaterally discard the existing theory appear to have therefore been somewhat overzealous.

On the positive side, there has been considerable value in the discussion the controversy has prompted. This is valuable in the design of new experiments and the interpretation of emerging data in the light of possible lignification theories. No simple unifying theory currently explains all of the features seen in lignin structural analyses, some of which will be noted below, so researchers should still feel free to advance new concepts and hypotheses. However, as with all scientific hypotheses and theories, they must be allowed to be tested before declaring victory over a functional existing theory.

LIGNIFICATION IN PERTURBED SYSTEMS

Over the past decade, researchers have become increasingly adept at applying genetic engineering methods to up- and down-regulate known lignin genes and to identify mutants in natural lines such as maize and in the model plant arabidopsis. This has allowed the lignification system, particularly the pathway for monolignol production, to be perturbed in an unprecedented and defined manner.

Most of the revelations from structural elucidations on lignins from plants in which monolignol pathway genes have been altered have already been reviewed. One significant aspect remains. That is the two possibilities alluded to above for thwarting the biosynthesis of the two primary monolignols, coniferyl and sinapyl alcohol. A C3H-deficient mutant has been identified in arabidopsis where, in line with its crucial role on the pathway, the resulting lignins have no discernible guaiacyl or syringyl components; only *p*-hydroxyphenyl units have been identified.[10] However, the plants are extremely stunted and have essentially no woody stems. There has therefore not been enough material available for more detailed structural analysis by NMR, for example. With the C3H and HCT genes (Fig. 1) now available, researchers are exploring the effects of down-regulation, even in commercially important crops such as alfalfa. These materials have only just become available to us so, while findings can't be reported in this abstract, it is hoped to be able to do so by the time of the ISWPC meeting. Note that the levels of *p*-coumaryl units in these plant tissues are significantly higher than in pine compression wood lignins, the perturbed lignins identified previously as being derived from higher *p*-coumaryl alcohol levels.

Along with the important structural findings from studies of other monolignol pathway mutants and transgenics has emerged the notion that lignification may have the malleability to incorporate non-traditional precursors, as monomers, into the lignin polymers, particularly when the production of the conventional monolignols is curtailed. This concept was introduced in a deliberately provocative manner (but in a carefully delineated section in the Abstract) at an ISWPC in Montreal in 1997.[11] It came from incipient observations on one CAD-deficient pine mutant and some early tobacco transgenics that seemed to indicate that the plants might be augmenting their under-supply of lignin monomers by utilizing other available phenolics to make their "lignin" polymers. The exact statement, which has often been misquoted or the source carefully hidden in following attacks should leave no doubt as to its tongue-in-cheek nature: "I suggest the blasphemous idea that the plant simply needs a polymer with required properties and that lignin's composition really is not particularly significant!!" [The double exclamations are in the original quote]. However, the reaction from one sector was quite extraordinary. Suffice it to say, the notion has been rather more prophetic than is usually given credit. Indeed data continues to emerge that plants attempt monolignol substitution with a small range of phenolics (some of which are shown in Fig. 3), and some are particularly successful.

Hydroxycinnamaldehydes in CAD-deficient plants

A pertinent example of apparently attempted monomer substitution is in a viable but unhealthy CAD-deficient mutant pine. CAD is the last enzyme on the monolignol biosynthetic pathway, reducing coniferaldehyde to the monolignol coniferyl alcohol in softwoods. Coniferaldehyde might be anticipated to build up in this plant, and evidence is that it does. On paper, coniferaldehyde appears to be a good candidate for a lignin monomer, Fig. 3c. It doesn't have the alcohol group, but it has the requisite conjugated double bond of the cinnamyl unit, and it was early on shown that it undergoes the same range of monomer-monomer coupling reactions as coniferyl alcohol, forming its own versions of β–O–4-, β–5-, and β–β-dehydrodimers. It could even make a dehydrogenation polymer. The drawback, as was not revealed until later studies on angiosperms, is that coniferaldehyde simply will not chemically β–O–4-cross-couple with phenolic guaiacyl units in the polymer. It is purely a chemistry problem; it will undergo such coupling with suitably unsaturated units, but with a normal guaiacyl unit, the reactants are not sufficiently chemically compatible to react either *in vitro* or, apparently, *in vivo*. Since the CAD-deficient pine still makes some coniferyl alcohol, the polymer needs to incorporate monomers compatible with a normal guaiacyl lignin. Coniferaldehyde simply does not comply. It therefore becomes relegated to homo-coupling reactions and cross-coupling reactions solely with the monolignol coniferyl alcohol, and is therefore left adorning only the periphery of the lignin molecule (as end-groups) and not significantly incorporating into the polymer chain.

Interestingly, this pine also produces, for unknown reasons, high levels of dihydroconiferyl alcohol, DHCA. DHCA also finds itself in the lignin. Obviously DHCA cannot couple at its β-position — there is no way to get the required single electron density to the β-position without the presence of the double bond. It therefore is also limited to a set of reactions that do not incorporate it into the lignin chain, again relegating it to the periphery of the structure. Nevertheless, it is found in the lignin at striking levels due to its ability to still 5- and 4–O-couple with guaiacyl units, as well as with the monolignol, coniferyl alcohol. This pine then appears to have attempted to augment its polymer by substituting available coniferaldehyde and DHCA monomers for some of the deficient coniferyl alcohol. It remains viable, but not vigorous.

The above example would be unfulfilling if it were not for the fact that coniferaldehyde is a well-behaved monomer in angiosperms. How can this be? Again, it is simple chemistry. Angiosperms have guaiacyl/syringyl lignins (deriving from both coniferyl and sinapyl alcohols). Coniferaldehyde, which does not β–O–4-cross-couple with guaiacyl units, readily undergoes β–O–4-cross-coupling reactions with syringyl phenolic end-groups. As it turns out, sinapaldehyde readily undergoes cross-coupling with either guaiacyl or syringyl end-groups. As a result, both coniferaldehyde and sinapaldehyde couple in a similar way as the monolignols do, and can therefore incorporate integrally into the body of the polymer in CAD-deficient angiosperms. These details are revealed by NMR studies on the lignins, and by the release of thioacidolysis marker compounds specifically from these hydroxycinnamaldehyde-β–O–4-linked units (shown in Fig. 3c).[12] The plants appear to grow essentially normally, but the hydroxycinnamaldehydes may not be quite perfect monomers. It appears that coupling to the new type of conjugated β–O–4-phenolic end-units that these create, the next step in the polymerization (from A–B to A–B–C), becomes difficult. The result is that many of the incorporated coniferaldehyde/sinapaldehyde units remain as free-phenolic end-groups (Lapierre, unpublished), limiting the degree of polymerization of the lignin. In addition to the property changes caused by the structural changes, these lower molecular weight lignins are presumably less ideal for the plant. An interesting side-benefit however is that the lignins are much more easily broken down and removed in chemical pulping,[13] so plants with limited CAD-deficiency are being pursued for their enhanced pulping potential.

5-Hydroxyconiferyl alcohol in COMT-deficient plants

Beyond the (partial) substitution of the hydroxycinnamaldehydes for their hydroxycinnamyl alcohol monolignol analogs in CAD-deficient angiosperms is a particularly successful substitution in the case of COMT-deficiency.[14] COMT (Fig. 1) is a methyl transferase enzyme necessary for the biosynthesis of sinapyl alcohol and ultimately syringyl groups in lignins. Knock-out mutants are essentially or totally devoid of syringyl components, and COMT down-regulation will reduce the syringyl content. As CAD-deficient angiosperms incorporated the immediate CAD precursors (the hydroxycinnamaldehydes), COMT-deficient plants must deal with the un-methylated 5-hydroxyconiferaldehyde precursor. Apparently CAD is able to reduce this aldehyde as it is 5-hydroxyconiferyl alcohol, not the aldehyde, that is exported to the wall and incorporated into lignins. 5-Hydroxyconiferyl alcohol has all the makings of an ideal monolignol substitute (Fig. 3d). It beautifully β–O–4-couples with guaiacyl, syringyl, or new 5-hydroxy-guaiacyl phenolic endgroups, integrating into the polymer as would a primary monolignol. The lignin structure becomes strikingly different however. The presence of the extra phenolic-OH, the 5-OH, drastically affects the post-coupling reactions. Novel benzodioxane units are formed in the polymer as a result of incorporating 5-hydroxy-coniferyl alcohol, at striking levels (essentially replacing the syringyl units in the control plants). The plant does not seem to mind; COMT-deficient plants appear to grow normally. In this case, the severe structural changes are a serious detriment to chemical pulping. Despite still being β-ethers, benzodioxanes will not efficiently cleave under pulping conditions, as do the syringyl β-ethers which they displace. However, COMT-deficient plants appear to be more digestible.[15] A possible reason is that, by providing a rapid alternative internal pathway for rearomatizing the quinone methide intermediate, these units cannot cross-link with polysaccharides in the wall (via addition to

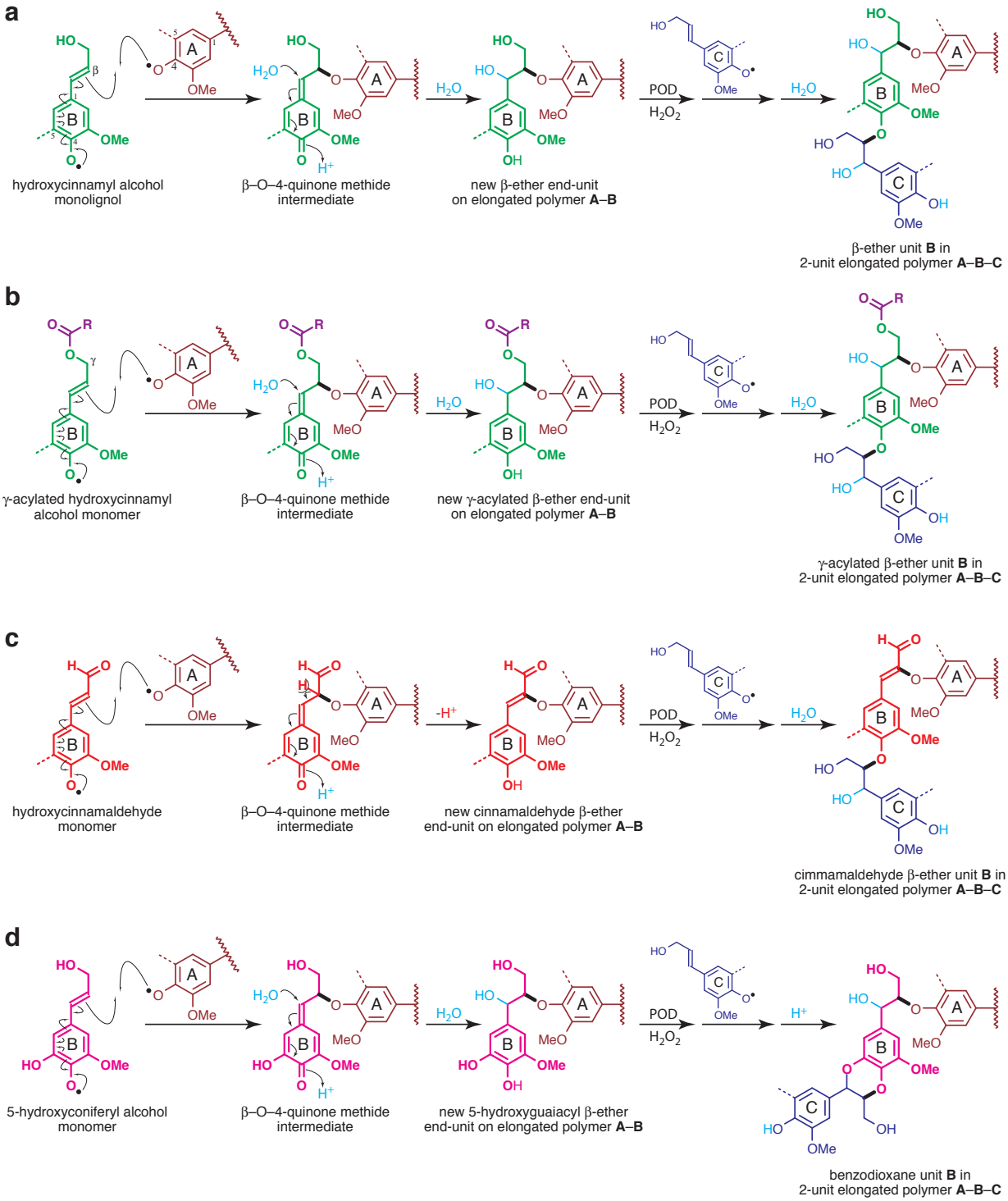


Fig. 3. Differences in cross-coupling and post-coupling reactions for various well-suited "monomers" incorporated into lignification. Illustration is for the major β–O–4-coupling only. a) Normal hydroxycinnamyl alcohol radicals **B** cross-couple with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide which rearomatizes by nucleophilic water addition to produce the elongated lignin chain **A**–**B**. The subsequent chain elongation via a further monolignol radical **C** etherifies the unit created by the prior monomer **B** addition, producing the 2-unit-elongated polymer unit **A**–**B**–**C**. b) Various γ-acylated monolignols (*p*-coumarate, *p*-hydroxybenzoate, and acetate) cross-couple equally well producing analogous products but with the β-ether unit **B** γ-acylated in the lignin polymer unit **A**–**B**–**C**. c) Hydroxycinnamaldehydes **B** may also cross-couple with the phenolic end of the growing polymer **A**, again mainly by β–O–4-coupling, to produce an intermediate quinone methide again, but one which rearomatizes by loss of the acidic β-proton, producing an unsaturated cinnamaldehyde-β–O–4-linked **B** end-unit. Incorporation further into the polymer by etherification is analogous to a). The unsaturated aldehyde units **B** give rise to unique thioacidolysis markers. d) 5-Hydroxyconiferyl alcohol monomer **A** also cross-couples with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide as usual which rearomatizes normally by nucleophilic water addition to produce the elongated lignin chain **A**–**B** bearing a novel 5-hydroxyguaiacyl phenolic end-unit. The subsequent chain elongation via a further monolignol radical **C** coupling β–O–4 to the new phenolic end of **A**–**B**, but this time the rearomatization of the quinone methide (not shown) is via internal attack of the 5-OH producing novel benzodioxane units **B**–**C** in the 2-unit-elongated polymer unit **A**–**B**–**C**. 5-Hydroxyconiferyl alcohol incorporation produces a lignin with a structure that deviates significantly from the "normal" lignin. The bolded bonds are the ones formed in the radical coupling steps.

quinone methides). Lignin-polysaccharide cross-linking has been shown to have a detrimental effect on cell wall digestibility.

So, what makes a good monolignol substitute?
A small range of monomers are now known to substitute for the conventional monolignols in various natural and transgenic plants. Monolignol substitution appears to be most successful when the novel monomer behaves, in its chemical radical coupling and cross-coupling reactions, like a normal monolignol. Most important is the β–O–4-coupling reaction with the phenolic end of the growing polymer to extend the polymer chain, as shown in Fig. 3. The post-coupling reactions that may be altered by the different functionality on the monomer seem to have less effect. Thus massive changes in the lignin structure occur when 5-hydroxyconiferyl alcohol substitutes for sinapyl alcohol, for example — the coupling reactions are analogous, but post-coupling steps produce novel benzodioxane structures that drastically change the lignin. And although hydroxycinnamaldehyde incorporation resulting from CAD-deficiency is not well tolerated in gymnosperms, the incorporation is well tolerated in angiosperms.

Observations that plants with monolignol substitution and profoundly altered lignin structure can fare well supports the heretical notion that the *exact* structure of lignins is not that important to the functioning of the plant.[11] The plant requires certain properties and functionality of its lignins, but does not expend resources dictating those properties by exactly stipulating lignin primary structure. Such biosynthetic malleability functions well for the plant, and also provides significant opportunities for engineering the polymer. Already it has been demonstrated that natural and industrial processes ranging from ruminant digestibility to chemical pulping can be both positively and negatively impacted by alterations to lignin composition and structure. It is also apparent that phenolic components from beyond the monolignol pathway itself (such as acylated monolignols) may be incorporated into lignins if they have compatible reaction chemistry and are transportable to the wall. Future work should reveal opportunities beyond the interesting deviations achieved by up- and down-regulating genes on the monolignol pathway to date.

OTHER "NEW" FEATURES OF LIGNIN STRUCTURE

One doesn't have to look to perturbed lignins in genetically altered plants to find new, sometimes unexpected, and occasionally difficult to explain features of lignin structure. Indeed "normal" plants are replete with many remaining mysteries.

Spirodienones (β-1-units)
Although β–1-coupling products are readily observed in dimer fractions from acidolysis, thioacidolysis, or DFRC, the "conventional" units have been difficult to observe in isolated lignins by NMR. In part this has more recently been assumed to be because the lignification produces dienone or spirodienone precursors to the conventionally-assumed product. Spirodienone units are now trivial to find in NMR spectra thanks to the beautiful NMR investigations by Zhang, which have documented the complete assignments.[16,17] High-syringyl lignins such as Kenaf bast fiber lignins contain remarkable amounts of the syringyl spirodienones Fig. 4.[17]

What is pleasing again about these findings is that the structures could have been chemically predicted. We all know that quinone methide intermediates forego reaction with external water nucleophiles when they can be internally trapped by hydroxyls situated so as to produce 5-membered (phenylcoumarans, resinols), 6-membered (benzodioxanes, from 5-hydroxyguaiacyl units) and even, in a special case, 8-membered (dibenzodioxocin) ring products. Yet the logical (in hindsight!) pathway to the spirodienone was apparently not considered until Brunow's proposal, supported by observation of a sinapate analog.[18] This is a rare case in that good model data are not available and the synthesis continues to be elusive. Although some of the peaks could have been detected in NMR spectra previously, it was not until the sensitive inverse-detected gradient-edited experiments became available, aided in part by the added sensitivity from cryogenically-cooled probes, that allowed the full assignments to be tracked down. With the data now available, such products are easy to detect in essentially any lignin isolate.

Naturally acylated lignins
A class of successful lignin precursors are the variously acylated monolignols implicated in an assortment of plants, Fig. 3b. Sinapyl acetate has been demonstrated to be a monomer in kenaf bast fiber and palm lignification, and at low levels in other hardwoods. Sinapyl *p*-hydroxybenzoate is similarly a monomer in palm, aspen, poplar and willow lignification. And sinapyl *p*-coumarate (as well as lower levels of coniferyl *p*-coumarate)

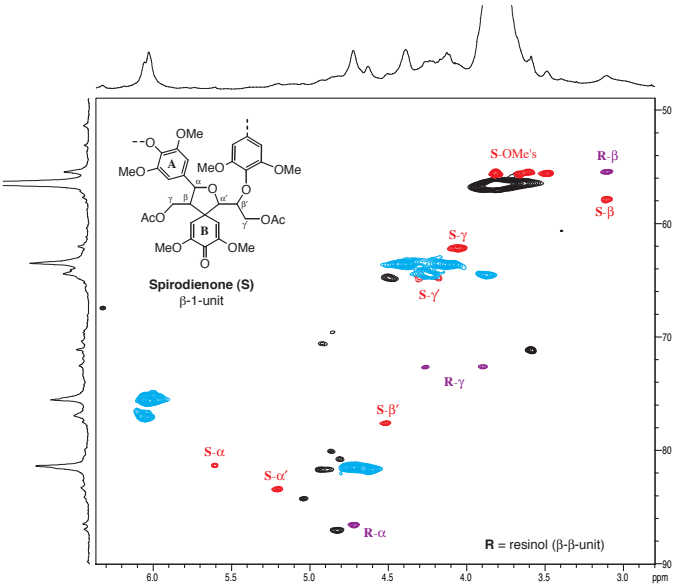


Fig. 4. Partial aliphatic (side-chain) region of 2D HSQC NMR spectrum of acetylated kenaf lignin. Signals from the spirodienone (**S**, red) along with, for reference, the signals from the resinol units (**R**, magenta), are marked and identified.

analogously contribute to lignification in grasses. What benefit the plant receives from lignin acylation is little understood. *p*-Hydroxybenzoate and *p*-coumarate are excellent substrates for many of the peroxidases that only slowly oxidize sinapyl alcohol. And, since they form less stable radicals, they readily undergo radical transfer with sinapyl alcohol, generating the sinapyl alcohol radical required for its incorporation into the polymer.[19] Their roles as radical transfer agents make them a sort of catalyst for lignification, especially with respect to sinapyl alcohol monomers. These phenolic esters then may have a role in facilitating the oxidation of sinapyl alcohol and oligomer substrates. Despite being phenolic, they themselves do not undergo coupling reactions and are found adorning lignins as pendant free-phenolic entities. In model reactions, it is not until other phenolics in the system are depleted that these components couple. Their presence in free-phenolic form in lignins provides evidence that the cell limits radical concentrations. The role of the over 50% levels of acetylated sinapyl alcohol in kenaf is even less understood. Presumably the resultant polymer is more hydrophobic than normal lignins, so the acetylation may be associated with drought tolerance.

Whatever the reasons for these acylated components, the three types of acylated monolignols (acetate, *p*-hydroxybenzoate, and *p*-coumarate) are becoming well implicated as authentic lignin precursors in their respective plants. *In vitro* studies indicated that the acylation does not significantly affect the course of the coupling reactions (Fig. 3b). That means that the acylated monolignols also behave nicely as lignin monomers, most importantly undergoing the β–O–4-coupling reactions that incorporate them into the chain of the polymer. Acylated monolignols alter the structure of the lignins by more than just adorning them with pendant groups, however. This is because the γ-OH group on a monolignol, Fig. 3a, functions in some post-coupling reactions, internally trapping the quinone methide following β–β-coupling, for example (Fig. 5). With the γ-OH group acylated, such internal reactions are no longer possible and the quinone methide must be rearomatized by trapping an external nucleophile, usually water, and forming quite different products **4**-**7** in the lignin as a result.[20] Additionally, the

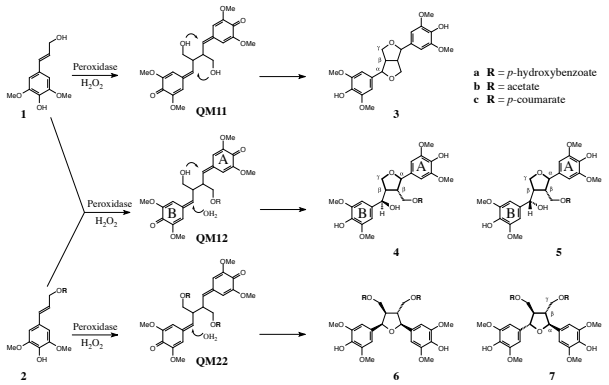


Fig. 5. Radical coupling reactions of sinapyl alcohol **1** and γ-acylated sinapyl alcohols **2** produce β–β-coupled products **3**-**7**. Such structures are found in lignins incorporating γ-acylated monolignols. Compound **5a** has been found in actively lignifying xylem tissue in poplar.[30]

stereochemistry of water attack on the quinone methide intermediate following β–O–4-coupling is altered. The lignins therefore differ from "normal" lignins in both substantial and subtle ways.

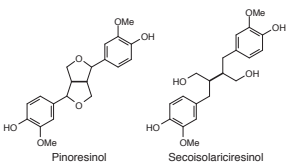
β–β-*Tetrahydrofurans (THFs)*

For some time, our group has been interested in determining whether acylated lignins derive from acylated monolignols or from post-lignification acylation reactions. It is now fairly certain that monolignol γ-acetates, γ-*p*-hydroxybenzoates, and γ-*p*-coumarates are all indeed lignin monomers in a variety of plants. Part of the evidence comes from the post-coupling reactions alluded to above, particularly following β–β-coupling (Fig. 5). Clearly in when the γ-OH is acylated, forming the usual resinol structure will no longer be possible. As a poster abstract for this meeting[21] describes, we can now detect a range of β–β-tetrahydrofuran products resulting from lignification utilizing the variously acylated monolignols, either *in vitro* (semi-synthetically) or *in vivo*.

What a shock, however, to find that Zhang is describing, with substantial NMR proof, the same kinds of tetrahydrofurans (c.f. the guaiacyl analogs of **6** and **7**, R = H, Fig. 5) in lignins that are not naturally acylated.[22] β–β-Structures other than the resinols **C** were not expected monolignol coupling products since it has always been assumed that internal trapping of the quinone methide by the γ-OH would be rapid and complete. It suggests that lignification may be consistent with low pH conditions where the protonation-dependent addition of water is more rapid; the paucity of acyclic α-aryl ethers in lignins has been noted to be consistent with low-pH conditions.[23] If these products are actually in the native lignin, must we conclude that internal trapping of the quinone methide intermediate by the γ-OH does not always occur faster than (acid-catalyzed) water addition? The THFs are at only a fraction of the level of the pinoresinol units in softwoods, but are nevertheless readily detected. Does this mean that lignification at the time of their creation is under more acidic conditions than we have envisioned? Incidentally, we have not been successful in seeing β–β-coupling products other than pinoresinol with *in vitro* coupling reactions at various pHs.

Secoisolariciresinol units in lignins

Another dilemma to be touched on here is the rather well authenticated appearance of secoisolariciresinol units in softwood lignins. Subsequent to β–β-coupling in lignans, the reductive transformations to (optically active) secoisolariciresinol are well known. So are these lignans somehow incorporated into lignins? Despite admonitions about such possibilities in the past, it has been difficult to find evidence that true lignans contaminate lignin preparations. Indeed, evidence suggests that the lignan and lignin pathways, although they share common monolignols, are kept quite distinct (see below).



The appearance of secoisolariciresinol structures in lignins is problematic. How do they get there? They must arise from post-β–β-coupling reactions, possibly directly from reductive trapping of the quinone methide intermediates,[24] or via reductase transformations on pinoresinol as have been well demonstrated in lignans.[25] But why are they in the lignin polymer? Were they exported to the wall, for lignification, as preformed dimers? The evidence that secoisolariciresinol units are in lignins comes from their release by acidolytic methods (acidolysis, thioacidolysis, DFRC) and now their detection by NMR. NMR investigation showed that secoisolariciresinol units are present in isolated spruce lignins at about half the level of pinoresinol units.[24] The level of the secoisolariciresinol product releasable by DFRC is about 4 times the level of releasable pinoresinol units. [Incidentally, the product originally attributed to β–β-dimers from pinoresinol in early DFRC work[26] in fact originated from reduced units in the lignins, i.e. secoisolariciresinol units that must have been ether linked].

The released products are not optically active, whereas the lignan products from softwoods are, so they appear not to be lignan derived. To date, their appearance in lignins has therefore been well authenticated, but the method by which secoisolariciresinol find themselves in lignins is unknown.

Lignan vs Lignin Pathways

It has long been a contention that lignans, dimeric products of *p*-hydroxycinnamyl alcohols, and lignins (polymers of the same *p*-hydroxycinnamyl alcohols, the monolignols) are products of independent biochemical processes. However, the situation has become blurred with recent allegations that so-called poly-lignans are being confused with lignins. The charge is that it is difficult to remove the lignan extractives components from the lignin. And yet this problem appears to be far less prevalent than espoused. After all, the cell walls are extensively solvent-extracted prior to lignin isolation, and lignins cannot be removed in any significant quantity without depolymerization by ball-milling. And in numerous labs attempts to find optical activity in isolated lignins or in low molecular mass products released from them (under conditions which have been proven to retain any optical activity), have never been successful, even for the most obvious units likely to arise from lignans, the resinol units.

It has been contended that the best proof that lignins and lignans are differentially compartmentalized, or separated in time and space, are the softwoods that produce syringyl lignans but no syringyl lignins (Prof. Hou-min Chang has pointed this out at several ISWPC meetings). [Normal softwoods have lignins derived predominantly from coniferyl alcohol, with small amounts of *p*-coumaryl alcohol, but no sinapyl alcohol; their lignins therefore contain predominantly guaiacyl units, and no syringyl units]. However, the syringyl compounds reported, particularly in the cedars, have ambiguous derivation and it has been difficult to find compelling supporting data in the literature. Kawai et al.[27] concluded, although they couldn't find evidence for the required intermediates, that the syringyl lignans (–)-4-*O*-demethylyatein **2**, (–)-thujaplicatin methyl ether **3** and 8-hydroxythujaplicatin methyl ether **4** (Fig. 6a) probably all derived from coniferyl alcohol coupling reactions followed by various ring-opening, reduction, hydroxylation and methylation reactions, rather than directly from sinapyl alcohol.

We are not aware of any reports of a direct sinapyl alcohol coupling product itself being found in a gymnosperm, i.e. a product which has not undergone extensive post-coupling modifications. Such a finding, while still falling short of proving that the lignan is produced from the monolignol sinapyl alcohol (rather than from coniferyl alcohol followed by subsequent hydroxylations and methylations), is evidence required to support the hypothesis that lignans are not involved in lignin biosynthesis.

A softwood with a primary syringyl lignan

As discovered by Joe Karchesy, Oregon State U., methanol extracts from Alaskan Cedar shavings can be readily shown to contain the lignan syringaresinol **1S**, the β–β-dehydrodimerization product of sinapyl alcohol (Fig. 6a). The product is readily isolated by column or thin-layer chromatography, or HPLC. The proton NMR spectrum (not shown) is identical to authentic synthetic syringaresinol. The GC retention time of the trimethylsilylated product is the same (Fig. 6b), as shown by co-injection with the standard, and its mass spectrum is identical. It is nevertheless a lignan, since the syringaresinol from the Alaskan Cedar, unlike the synthetic sample, is optically active. No such syringaresinol could be detected in similar extracts from Port Orford Cedar (which serves here as a control). More extensive studies will be required to determine whether the syringaresinol **1S** (Fig. 6a) is a primary product, formed directly from sinapyl alcohol, or whether it derives via pinoresinol **1G** following a series of hydroxylation and methylation reactions.

Since the Alaskan Cedar may ostensibly produce sinapyl alcohol, it is opportune to ask whether sinapyl alcohol is used as a monolignol for its lignification. The most sensitive methods to reveal the composition are degradative. Thioacidolysis and the DFRC method are more relevant since the products retain the propanoid sidechain and derive only from cleavage of β-ether units, units that are diagnostic for polymeric lignins. DFRC of either pre-solvent-extracted Alaskan or Port Orford Cedar wood cleanly produced the guaiacyl product, coniferyl acetate, and traces of *p*-coumaryl acetate, Fig. 6c, but no detectable sinapyl acetate, even via selected-ion GC-MS spectra. The simple conclusion therefore is that the Alaskan Cedar has a normal softwood-type lignin, devoid of syringyl units.

To address the question of whether lignans can become incorporated into lignins, we next sought evidence for the incorporation of syringaresinol **1S** (or any syringyl

product) into the lignin. This is readily achieved, without the need for lignin isolation, in 2D NMR spectra of the aromatic region of solubilized whole cell walls.[28] Syringyl units, with two electron-releasing methoxyl groups, have their identical S2/6 $^{13}C–^{1}H$ correlations at ~104/6.55 ppm, in a clean region of the spectrum well separated from the unsymmetrical G2 and G6 correlations at ~111/6.75 and 119/6.9. Thus softwood lignins typically have no resonances in the 104/6.55 region. As seen in Fig. 6dii, no syringyl units can be detected (even at contour levels below those plotted, down to the noise level) in the Alaskan Cedar (nor in the Port Orford Cedar, not shown). The conclusion therefore is that syringaresinol is not utilized in lignin polymerization, nor are other syringyl components. The important implication is that these prevalent syringyl lignans find themselves as neither contaminating artifacts nor integral components of the polymeric lignins.

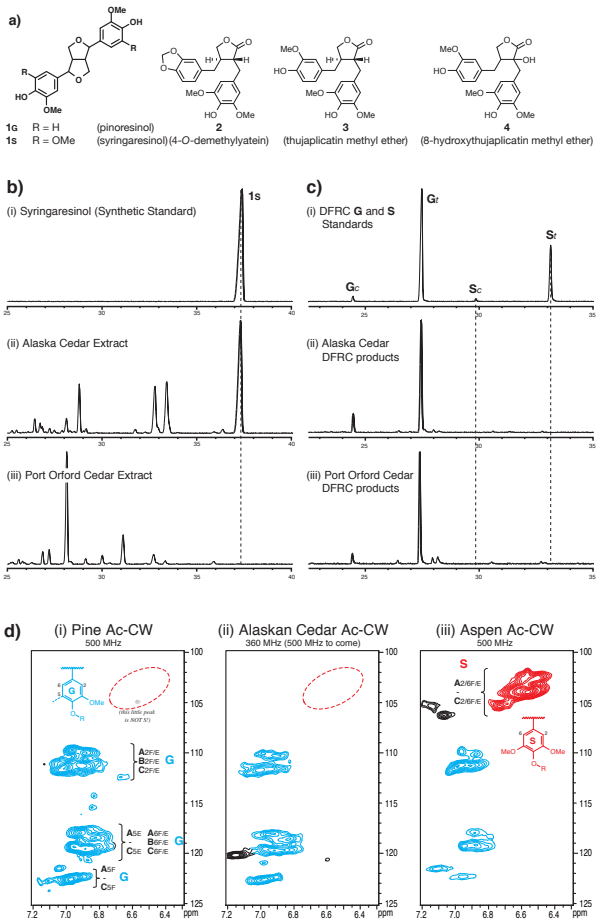


Fig. 6. a) Structures of the resinols, syringaresinol **1S** (as found here in Alaskan Cedar) and pinoresinol **1G**, and other lignans **2-4** containing syringyl moieties previously found in cedars. b) GC traces showing (i) standard syringaresinol and its presence or absence in methanol extracts of (ii) Alaskan Cedar, and (iii) Port Orford Cedar. c) DFRC products showing (i) the guaiacyl **G** and syringyl **S** standards coniferyl acetate and sinapyl acetate (subscript *t* = *trans*, *c* = *cis*); and the crude DFRC products from (ii) Alaskan Cedar and (iii) Port Orford Cedar, showing only guaiacyl and no syringyl DFRC monomers. d) HSQC NMR sections showing the aromatic $^{13}C–^{1}H$ correlations from samples of pre-extracted, ball-milled, and solubilized whole cell walls from: (i) loblolly pine, a reference softwood (showing correlations from solely guaiacyl units), (ii) the Alaskan Cedar (also showing only guaiacyl correlations; no syringyl correlations are seen even at base-plane level) and (iii) Aspen (a reference hardwood, showing clear syringyl correlations). Figure d(i) is annotated with crude assignments from the 3 major unit types, **A** = β-ether, **B** = phenylcoumaran, **C** = resinol (subscripts F = free-phenolic, E = etherified units).

Alaskan cedar therefore produces at least one syringyl lignan, syringaresinol as a substantial component, but absolutely no detectable syringyl lignin. These long-sought findings support the contention that lignan and lignin biosyntheses are independent pathways, despite the commonality of their monomers.

Where are the 5–O–4-units in softwood lignin NMR spectra?
Softwood lignins are supposed to have 1-7% 5–O–4-biphenyl ether units resulting from oligomer-oligomer coupling. The final conundrum to be addressed here is one raised by Sally Ralph, more fully described by a Poster Abstract at this meeting,[29] comes from looking again at the aromatic region of a softwood HSQC spectrum, e.g that in Fig. 6di. The 5–O–4-units are electron-rich, somewhat like syringyl units, and should have their $^{13}C–^{1}H$ correlations in the nice clear region of the spectrum at around 105/6.5-7.0 ppm. Even down at the base-plane level, there are no significant correlations in this region from whole-cell-wall or isolated lignin samples. Diagnostic HSQC correlations from anticipated 5–O–4-units in lignins should be readily detected if such structures were present in the lignins. Better model compound data are still needed, but the complete lack of such correlations remains a conundrum for discussion and research!

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REFERENCES

1. Boerjan, W.; Ralph, J.; Baucher, M., Lignin Biosynthesis. *Ann. Rev. Plant Biol.* **2003,** 54, 519-549.
2. Ralph, J.; Lundquist, K.; Brunow, G.; Lu, F.; Kim, H.; Schatz, P. F.; Marita, J. M.; Hatfield, R. D.; Ralph, S. A.; Christensen, J. H.; Boerjan, W., Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem. Reviews* **2004,** 3, (1), 29-60.
3. Nair, R. B.; Bastress, K. L.; Ruegger, M. O.; Denault, J. W.; Chapple, C., The Arabidopsis thaliana REDUCED EPIDERMAL FLUORESCENCE1 gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis. *Plant Cell* **2004,** 16, (2), 544-554.
4. Ralph, J.; Rodger, C. NMR of Lignin Model Trimers, or why you will never find crystalline regions in lignin. Sixth ISWPC, Melbourne, Australia, 1991; pp 59-64.
5. Ralph, J., ^{1}H NMR of acetylated β-ether/β-ether lignin model trimers. *Magn. Reson. Chem.* **1993,** 31, (4), 357-363.

6. Davin, L. B.; Wang, H.-B.; Crowell, A. L.; Bedgar, D. L.; Martin, D. M.; Sarkanen, S.; Lewis, N. G., Stereoselective biomolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* **1997,** 275, 362-366.
7. Gang, D. R.; Costa, M. A.; Fujita, M.; Dinkova-Kostova, A. T.; Wang, H. B.; Burlat, V.; Martin, W.; Sarkanen, S.; Davin, L. B.; Lewis, N. G., Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. *Chem. Biol.* **1999,** 6, (3), 143-151.
8. Croteau, R.; Kutchan, T. M.; Lewis, N. G., Chapter 24. Natural Products (Secondary Metabolites). In *Biochemistry and Molecular Biology of Plants*, ed.; Buchanan, B.; Gruissem, W.; Jones, R., Am. Soc. Plant Physiologists: Rockville, MD, 2000; pp 1250-1318, specifically pp 1294-1299.
9. Patten, A. M.; Anterola, A. M.; Chung, B.-Y.; Davin, L. B.; Lewis, N. G. Confocal microscopy and laser excision microdissection/chemical analyses of Arabidopsis lignifying xylem and interfascicular fibers genetically altered in lignin monomer composition: evidence for differential control of monolignol polymerization. 14th International Conference on Arabidopsis Res., Madison, WI, USA, June 20-24, 2003, Poster No. 68.
10. Franke, R.; Hemm, M. R.; Denault, J. W.; Ruegger, M. O.; Humphreys, J. M.; Chapple, C., Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of Arabidopsis. *Plant J.* **2002,** 30, (1), 47-59.
11. Ralph, J. Recent advances in characterizing 'non-traditional' lignins and lignin-polysaccharide cross-linking. 9th ISWPC, Montreal, Quebec, June 9-12, 1997, pp 1-7, paper PL2.
12. Kim, H.; Ralph, J.; Lu, F.; Pilate, G.; Leplé, J. C.; Pollet, B.; Lapierre, C., Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. *J. Biol. Chem.* **2002,** 277, (49), 47412-47419.
13. Pilate, G.; Guiney, E.; Holt, K.; Petit-Conil, M.; Lapierre, C.; Leple, J. C.; Pollet, B.; Mila, I.; Webster, E. A.; Marstorp, H. G.; Hopkins, D. W.; Jouanin, L.; Boerjan, W.; Schuch, W.; Cornu, D.; Halpin, C., Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnol.* **2002,** 20, (6), 607-612.
14. Ralph, J.; Lapierre, C.; Marita, J.; Kim, H.; Lu, F.; Hatfield, R. D.; Ralph, S. A.; Chapple, C.; Franke, R.; Hemm, M. R.; Van Doorsselaere, J.; Sederoff, R. R.; O'Malley, D. M.; Scott, J. T.; MacKay, J. J.; Yahiaoui, N.; Boudet, A.-M.; Pean, M.; Pilate, G.; Jouanin, L.; Boerjan, W., Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochem.* **2001,** 57, (6), 993-1003.
15. Guo, D. G.; Chen, F.; Wheeler, J.; Winder, J.; Selman, S.; Peterson, M.; Dixon, R. A., Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin *O*-methyltransferases. *Transgenic Res.* **2001,** 10, (5), 457-464.
16. Zhang, L.; Gellerstedt, G., NMR observation of a new lignin structure, a spiro-dienone. *Chem. Commun.* **2001**, (24), 2744-2745.
17. Zhang, L.; Gellerstedt, G.; Lu, F.; Ralph, J., NMR studies on the occurrence of spiro-dienone structures in lignins. *J. Wood Chem. Technol.* **2004**, in press.
18. Setälä, H.; Pajunen, A.; Rummakko, P.; Sipilä, J.; Brunow, G., A novel type of spiro compound formed by oxidative cross-coupling of methyl sinapate with a syringyl lignin model compound. A model system for the β-1 pathway in lignin biosynthesis. *J. Chem. Soc., Perkin Trans. 1* **1999**, (4), 461-464.
19. Takahama, U.; Oniki, T.; Shimokawa, H., A Possible Mechanism for the Oxidation of Sinapyl Alcohol by Peroxidase-Dependent Reactions in the Apoplast: Enhancement of the Oxidation by Hydroxycinnamic Acids and Components of the Apoplast. *Plant Cell Physiol.* **1996,** 37, (4), 499-504.
20. Lu, F.; Ralph, J., Preliminary evidence for sinapyl acetate as a lignin monomer in kenaf. *J. Chem. Soc., Chem. Commun.* **2002**, (1), 90-91.
21. Lu, F.; Ralph, J. Novel β–β-structures in natural lignins incorporating acylated monolignols. Thirteenth ISWPC, Auckland, New Zealand, 2005; these proceedings.
22. Zhang, L.; Gellerstedt, G. Observation of a novel β–β-structure in native lignin by high resolution 2D NMR techniques. Eighth European Workshop on Lignocellulosics and Pulp, Riga, Latvia, Aug. 22-25, 2004.
23. Brunow, G.; Sipilä, J.; Mäkelä, T., On the mechanism of formation of non-cyclic benzyl ethers during lignin biosynthesis. Part 1: The reactivity of β-0-4 quinone methides with phenols and alcohols. *Holzforschung* **1989,** 43, (1), 55-59.
24. Zhang, L.; Henriksson, G.; Gellerstedt, G., The formation of β-β structures in lignin biosynthesis - are there two different pathways? *Org. Biomol. Chem.* **2003,** 1, (20), 3621-3624.
25. Katayama, T.; Davin, L. B.; Chu, A.; Lewis, N. G., Novel benzylic ether reductions in lignan biogenesis in *Forsythia intermedia*. *Phytochem.* **1993,** 33, (3), 581-591.
26. Peng, J.; Lu, F.; Ralph, J., The DFRC method for lignin analysis. Part 4. Lignin dimers isolated from DFRC-degraded loblolly pine wood. *J. Agric. Food Chem.* **1998,** 46, (2), 553-560.
27. Kawai, S.; Sugishita, K.; Ohashi, H., Identification of Thuja occidentalis lignans and its biosynthetic relationship. *Phytochem.* **1999,** 51, (2), 243-247.
28. Lu, F.; Ralph, J., Non-degradative dissolution and acetylation of ball-milled plant cell walls; high-resolution solution-state NMR. *Plant J.* **2003,** 35, (4), 535-544.
29. Ralph, S. A. Conundrums regarding 5–O–4-linkages in softwood lignins. Thirteenth ISWPC, Auckland, New Zealand, 2005; these proceedings.
30. Lu, F.; Ralph, J.; Morreel, K.; Messens, E.; Boerjan, W., Preparation and relevance of a cross-coupling product between sinapyl alcohol and sinapyl *p*-hydroxybenzoate. *Org. Biomol. Chem.* **2004,** 2, 2888-2890.