INCORPORATION OF HYDROXY-CINNAMALDEHYDES INTO LIGNINS

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ABSTRACT

Peroxidase/H_2O_2-mediated radical coupling of hydroxycinnamaldehydes produced 8–O–4-, 8-5-, 8–8-, and 5-5dimers as had been documented earlier (although we found that the 8–5-dimer is produced in its cyclic phenylcoumaran form at neutral pH). Spectral data from dimers and oligomers has allowed a more substantive assignment of aldehyde components in lignins isolated from a CAD-deficient pine mutant and an antisense-CAD-downregulated transgenic tobacco. The CAD-deficient pine lignin shows the typical benzaldehyde and cinnamaldehyde peaks at levels significantly enhanced over the corresponding lignin from normal pine, along with evidence for two types of 8–O–4-coupled coniferaldehyde units. The CAD-downregulated tobacco has even higher levels of hydroxycinnamaldehyde (mainly sinapaldehyde) incorporation producing significant levels of the analogous two types of 8–O–4-coupled products. 8–8-Coupled units are also clearly evident. The isolated lignins do not appear to be artifacts: they are high molecular weight (~17 kDa), and there is clear evidence for coupling of hydroxycinnamaldehydes with each other and then incorporating into the lignin, as well as for the incorporation of coniferaldehyde monomers onto pre-formed lignin oligomers. The implication is that coniferaldehyde and sinapaldehyde (as well as vanillin and syringaldehyde) co-polymerize with the traditional monolignols, the hydroxycinnamyl alcohols (sinapyl, coniferyl, and 4-hydroxycinnamyl alcohol), into lignins and do so at markedly enhanced levels when the normal monolignol production is downregulated by CAD-deficiency.

INTRODUCTION

Aldehydes are well-known components in lignins[1-9] and are responsible for the characteristic phloroglucinol staining of lignified tissues.[10] Whether they are true components of lignin from co-polymerization (radical cross-coupling) with monolignols/oligolignols has recently become important to elucidate as the lignins from various CAD-deficient plants are examined.

Cinnamyl alcohol dehydrogenase (CAD) catalyses the final biosynthetic step from coniferaldehyde to coniferyl alcohol, the predominant monolignol from which softwood lignins are derived, as well as from sinapaldehyde to sinapyl alcohol. Although it has been suggested (from flux studies using suspension-cultured *Pinus taeda*) that CAD should not be rate limiting,[11] downregulation of CAD in a variety of mutants and transgenics clearly leads to an accumulation of hydroxycinnamaldehydes (at the apparent expense of the monolignols) and an apparent buildup of their content in resultant lignins,[1-5,12-17] (although confirming the association of these aldehydes with the polymeric "lignin" component has sometimes been difficult;[15] much of the aldehyde component remains as low molecular mass extractable compounds whose location is uncertain).

It has long been recognized[5,18] that coniferaldehyde is a viable substrate for free-radical coupling reactions analogous to those that occur with the standard monolignols. It is possible to make coniferaldehyde synthetic lignins (DHPs) for example,[5] as well as a range of dimers.[18] Here we examine dimerization and oligomerization of coniferaldehyde, and copolymerization of coniferaldehyde with coniferyl alcohol to produce synthetic lignins. Spectral data from these model reactions are used to provide a more substantive elucidation of the structures of aldehyde moieties in lignins, particularly those from mutant and transgenic plants that are CAD-deficient.

EXPERIMENTAL

Dimers, mixed oligomers, and synthetic lignins were made using peroxidase and H_2O_2 by rather standard methods that will not be detailed here. The mutant pine and transgenic tobacco lignins were isolated (by dioxane-water extraction of ball-milled cell wall material) as has been described.[12,13] More details on these lignins and a discussion of their nature and implications are found in other abstracts from this meeting.[19-21]

RESULTS AND DISCUSSION

Model Reactions. Radical coupling of coniferaldehyde using peroxidase/H_2O_2 produced mixtures from which the parent dimers of structures 1-4, Fig. 1, could be isolated and characterized by NMR, Fig. 2. Most of the products have been described previously, but without sufficient NMR data for the current study. Useful insight into the relative amounts and the nature of the dimers was gained from small-scale (5-10 mg) experiments using [9–^{13}C]coniferaldehyde. Assignments could be readily made from 1D ^{13}C-NMR of the mixture which, because of the [9–^{13}C]-labeling, could be acquired in minutes, and from 2D HSQC-TOCSY and HMBC experiments where the aldehyde carbon region could be selectively and quickly acquired. Analysis of the crude dimer/oligomer mixtures revealed an unexpected product.

When coniferaldehyde dimerizes with one of the radicals coupling at its 8-position, the resulting intermediate product is a quinone methide; this is analogous to the dimerization of coniferyl alcohol with one of the radicals coupling at its β-position. In the case of the aldehydes, however, the quinone methide has new options. The resultant 8-proton is particularly acidic because of the aldehyde group; elimination of the 8-proton allows re-aromatization. Thus, the 8–O–4-dimer produced is **1,** Fig. 1 – the addition of water to the quinone methide cannot compete with the faster 8-proton elimination. An 8–O–4/8–O–4 trimer was also isolated. The 8–8-dimer 3 similarly regains 7,8-unsaturation by 8-proton elimination from the di-quinone methide coupling intermediate. The products are both analogous to those produced by ferulate where the intermediate quinone methides also have acidic (but less so) 8-protons.[22] The surprise was that the 8–5-product was the cyclic phenylcoumaran structure **2c.** Although this product is analogous to that formed from coniferyl alcohol or ferulate, it had been thought that I-proton elimination was faster than intermolecular trapping of the 8–5-quinone methide intermediate. Indeed the previously reported product was the opened structure **2o.**[18] Attempted isolation of **2c** from the mixture produced only the ring-opened elimination product, dimer **2o.** Acetylation of the mixture also converts the phenylcoumaran **2c** to the (acetate of) acyclic dimer **2o,** as shown in Fig. 2, by the assumed mechanism in Fig. 3. Proof of the structural assignment for the phenylcoumaran 8–5-product **2c** is shown in Fig. 4, and HMBC experiment which also validates the other assignments (see caption).

Fig. 1. Aldehyde structures. *Structures of various units derived from radical coupling of hydroxycinnamaldehydes. Structures **1-4** result from homo-coupling of hydroxycinnamaldehydes. Units **1'** are from cross-coupling of a hydroxycinnamaldehyde radical with a radical from a preformed lignin oligomer (B-moiety). Hydroxycinnamaldehyde endgroups **V** can arise from incorporation of a hydroxycinnamaldehyde monomer into lignin or remain (as the B-moieties) following homo-coupling of hydroxycinnamaldehydes (in structures **1, 2,** and **4,** for example). Hydroxybenzaldehyde units (producing benzaldehyde endgroups U) result from hydroxycinnamaldehydes. It is not known whether they are result from direct incorporation of hydroxy-benzaldehyde monomers (vanillin and syringaldehyde) into lignin, or are produced post-lignification from hydroxycinnamaldehyde **V** units in lignin; the former is strongly implicated in the current work, but is not necessarily exclusive. It is not yet known whether units 4 exist as such in lignins – the dimer can clearly form, but radical coupling is still possible at the 8- and 4–O-positions; various types of 5–5-coupled products (including dibenzodioxocins) are possible. Finally, note that cross-coupling products other than 1' are possible and are presumably responsible for the broadening/ multiplicity seen in some of the ^{13}C-NMR peaks in the lignin spectra of Figure 2.*

Fig. 2. NMR spectra of aldehyde components.
***Left:** aldehyde sub-regions of ^{13}C-NMR spectra of: various coniferaldehyde dimers (relating to units 1-4); crude mixtures of dimers resulting from low-extent oligomerization of [P-^{13}C]-coniferaldehyde with horseradish peroxidase and H_2O_2 (in both acetone-d_6 and acetone-d_6/H_2O,6:1); a synthetic lignin (DHP) prepared from [9–^{13}C]-coniferaldehyde (10%) and coniferyl alcohol (90%); an isolated lignin from a CAD-deficient pine mutant: and an isolated lignin from uniformly ^{13}C-enriched (~13%) antisense-CAD-downregulated tobacco. Isolated dimers 1-4 and the first dimers/ oligomers mixture were run in acetone-d_6 The dimer/ oligomer sample run in 6:1 acetone-d_6/D_2O was from a different reaction than the one above it run in acetone-d_6. The DHP was in 9:1 acetone-d_6/D_2O. Note that radical coupling of coniferaldehyde via peroxidase/H_2O_2 clearly produces the cyclic (phenylcoumaran) 8–5-dimer corresponding to structure **2c,** as proven in Fig. 4; we have not yet been able to purify dimer **2c** – it ring-opens and eliminates (see Fig. 3) to the acyclic (open) form **2o** (for which we do have the dimeric model). We do not currently have. a model compound for the 8–**b**-cross-product **3'** seen in the copolymer DHP, so this peak remains unauthenticated. If it is the 8–b'-cross-product, it appears to be in the open form as shown in Fig. 1, in which the intermediate quinone methide on the aldehyde moiety is not internally trapped by the γ–OH (as occurs in **b**-**b** coupling of coniferyl alcohol to pinoresinol, for example). Substantial solvent and matrix-dependent shifts are noted for the unacetylated dimers, oligomers, and the polymers (which were run in ~6:1 acetone-d_6/ D_2O). The assignment lines linking the various peaks in the two lignin spectra have been anthenticated by further correlation experiments (not shown) on the lignin samples. The CAD-deficient pine lignin shows the typical benzaldehyde U and cinnamaldehyde V peaks (although significantly enhanced over the corresponding lignin from normal pine), along with evidence for 8–O–4-coupled coniferaldehyde units **1** and **1'.** The CAD-downregulated tobacco has significant levels of hydroxycinnamaldehyde (mainly sinapaldehyde) incorporation producing high levels of 8–O–4-coupled products **1** and **1'**– the peak at ~195 ppm, coincident with hydroxycinnamaldehyde endgroup units from a variety of structures, must derive almost entirely from the B-moieties of 8–O–4-homocoupled products **1** since the area of this peak matches that at ~189 ppm (belonging to the A-moiety); the 8–8-coupled product **3** is also clearly evident.*
***Right:** Corresponding spectra of acetylated oligomeric products and lignin isolates. Solvent shifts are not a problem in this case since all samples dissolved in acetone-d_6. Thus the assignments of 8–O–4- and 8–8-coupled products an verified (and further authenticated by diagnostic correlations in various **2D** NMR experiments). Note that the cyclic phenylcoumaran form of the 8–5-dimer **2c** converts to the opened product **2o** upon acetylation (see Fig. 3). The assignment of peaks at ~186.5 ppm (and 187 ppm in the unacetylated lignins) as cross-coupling products **1'** is from other NMR data (not shown) – we hope to have model data for this before the 1999 ISWPC symposium*

Fig. 1. Aldehyde structures. →

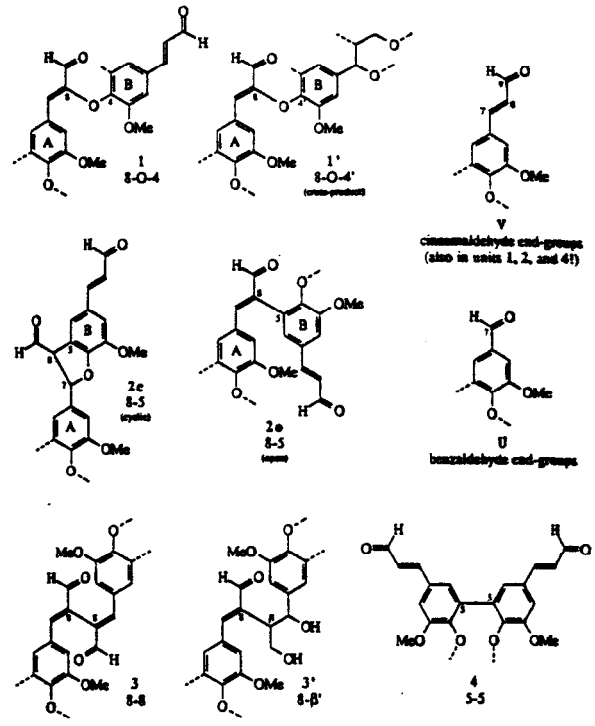


Fig. 2. NMR spectra of aldehyde components. ↓

In synthetic cross-coupling reactions, as seen in the DHP example, Fig. 2, that copolymerizes [9–^{13}C]-coniferaldehyde (10%) with coniferyl alcohol (90%), one new product arises. The peak labeled **3'** remains unauthenticated at present but is thought to be the 8–β'-cross-product **3'.** If so, its minor change in chemical shift indicates that, like the 8–8-coupled dimer **3,** the aldehyde moiety remains unsaturated. This means that the intermediate quinone methide produced following radical coupling was not efficiently trapped internally by the γ'–OH. Thus, unlike the 8–5-coupling product, 8-proton elimination is faster here than internal trapping. The copolymer synthetic lignin also contains 8–O–4-coupled aldehyde dimeric units **1** as well as the 8–O–4-cross-product **1'** that was (obviously) not seen in the coniferaldehyde-only reactions. One other feature of the synthetic lignification is notable: most of the coniferaldehyde appears not to have coupled at its 8-position. The dominant **V** peak indicates that any cross-coupling entered into by coniferaldehyde was predominantly at the ring 5- or 4–O-positions. As will be discussed below, this is not reflected in the isolated lignins. The reason likely results from the considerably more "bulk" nature of synthetic lignification. With excess coniferyl alcohol in the system, the likely coupling reaction is between coniferaldehyde and coniferyl alcohol monomer radicals. As has been seen countless time, coniferyl alcohol overwhelming couples at its β-position in cross-coupling reactions. Although the aldehyde may also couple at its 8-position, as seen from the (unauthenticated) **3'** peak it more commonly couples at the 4–O- or 5-position, as evidenced by the large **V** peak.

Due to severe matrix and solvent-dependent shifts, the model data (in acetone-d_6) and the lignin spectra do not appear to coincide – see caption to Fig. 2. The direction of the shifts upon adding water (necessary for the solution of unacetylated lignins) is illustrated with the crude dimer products and the copolymer DEE Dotted assignment lines on Fig. 2 have been authenticated by

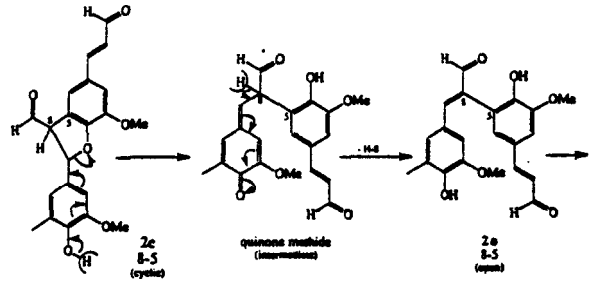
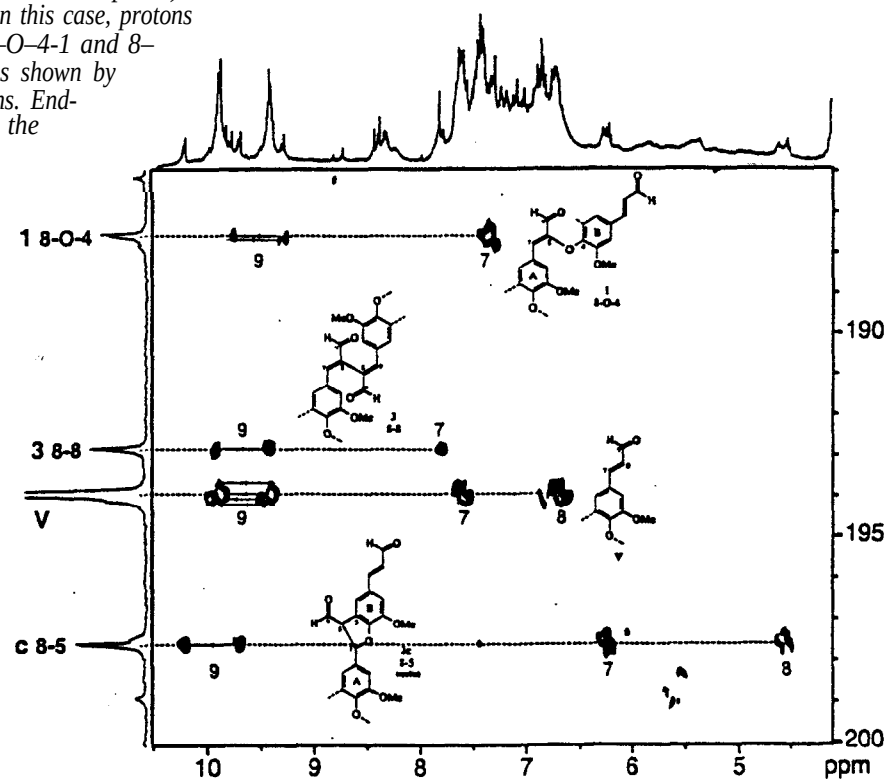


Fig. 3. Mechansim for opening 8–5-dimer. *The cyclic phenylcoumaran 8–5-dimer* ***2c,*** *produced at neutral pHs from coniferaldehyde via peroxidase/H_2O_2, undergoes ring-opening and 8-proton elimination under basic acetylation conditions (pyridine + acetic anhydride), presumably via the quinone methide as shown here, to produce the acyclic 8-5-dimer* ***2o*** *(which was isolated, Fig. 2). Analogous ring-opening likely occurs under acidic conditions. There is weak indication from the spectra in Fig. 2 that the cyclic form exists in isolated (and presumably in situ) lignins.*

Fig. 4. NMR of mixed dimers/oligomers. *NMR evidence for the dimer/oligomer assignments of aldehyde ^{13}C-NMR peaks to structures 1-4 and V, and proof that the 8–5-dimer is in the cyclic phenylcoumaran form following peroxidase-mediated radical coupling at around neutral pH. The The ^{13}C–^{1}H long-range correlation experiment (gradient-edited HMBC, solvent: acetone-d_6) correlates each aldehyde carbonyl carbon with its directly attached aldehyde proton (split by the 1-bond ^{13}C–^{1}H coupling constant – the proton chemical shift is halfway between the pair of correlation peaks) and other protons 2- or 3-bonds away (in this case, protons 7 and 8 on the sidechain). The 8–O–4-1 and 8–8-units 3 are clearly unsaturated as shown by their correlations to single 7-protons. End-units* ***V*** *(the peak also comprises the B-moieties of units* ***1, 2,*** *and* ***4)*** *show correlations to both unsaturated sidechain protons* ***(7*** *and* ***8).*** *The interesting 8–5-unit* ***2c*** *clearly shows its phenylcoumaran nature – the ring-opened elimination product* ***2o*** *has only one proton correlation (its aldehyde carbon and correlated 7-proton coinciding with those of units* ***V.*** *Clearly, therefore, internal trapping of the intermediate quinone methide product of radical coupling is faster than H–8 proton elimination (analogous to that seen following 8–O–4- and 8–8-coupling). The 8–5-coupling Product has always been reported as its opened form* ***2o;*** *then is weak evidence (Fig. 2) that (unacetylated) lignins contain some of the cyclic unit* ***2c.***



further **2D** correlation experiments (not shown). Once the models and the lignins are acetylated, data coincide much more closely (right-hand plots in Fig. 2).

Lignin Spectra. The aldehyde-carbon region of spectra from lignin isolates are also shown in Fig. 2, where the caption describes details of the assignments and findings. In the CAD-deficient pine mutant, the typical cinnamaldehyde **V** and benzaldehyde **U** peaks are present at enhanced levels, consistent with the build-up of coniferaldehyde in a plant deficient in CAD. More important are the peaks from 186-189 ppm which are due to 8–O–4-coupling products **1** of coniferaldehyde and 8-O-4'-cross-coupling products **1'** between coniferaldehyde and lignin oligomers. {These new aldehyde components were misidentified originally,[12] but corrected in subsequent publications.[13,23] (A book chapter on NMR of Lignins[23] even details how the incorrect assignment was made).} An accompanying abstract on mutants and transgenics also indicates that these aldehydes cross-couple nicely with the dihydroconiferyl alcohol components in the mutant pine, as shown by analysis of DFRC-dimers.[19]

In the CAD-downregulated tobacco transgenic, the isolated lignin contains striking aldehyde components. 8–O–4-Products **1** and **1'** abound and other NMR experiments determine that they are largely sinapaldehyde-derived in this case (tobacco produces syringyl-guaiacyl lignins).[13] Most of the peak at -195 ppm (194 ppm for the acetylated sample), usually ascribed generically to cinnamaldehyde end-units **V,** results from the 8–O–4-homocoupled product **1** (which also has a cinnamaldehyde endgroup) – the area of the A-moiety peak at 189 ppm (188 ppm for the acetylated sample) closely matches that of its B-moiety partner at 195 ppm. Presumably due to the large aldehyde concentrations, 8–8-coupled products **3** are also seen. Sinapaldehyde, like sinapyl alcohol, favors 8–8-coupling, in part because them are fewer options than for coniferaldehyde (which has a 5-position available for radical coupling).

Implications. The isolated phenolic polymers from the mutant pine and the transgenic tobacco contain significant aldehyde components, copolymerized in by radical coupling reactions that typify lignification. The polymers have significant molecular weight (~17 kDa for the pine isolate)[21] and contain cross-coupling products of coniferaldehyde/sinapaldehyde with lignin oligomers (e.g. 8–O–4'-structures **1'**) as well as homo-coupling products such as **1** and **3.** They therefore appear to be true components that are polymerized by radical coupling mechanisms into a phenylpropanoid polymer that may function as lignin. This is not the same polymer that would be produced (and can be isolated) when the plant does not have a CAD-deficiency. It contains significantly more aldehydes (as well as another major component, dihydroconiferyl alcohol units, in the pine mutant).[12,21] It appears, as originally proposed,[12] that the plants are utilizing these hydroxycinnamaldehyde precursors of the normal monolignols when the plant is unable to provide sufficient quantities of the monolignols for normal lignification. Hydroxycinnamaldehydes are logically anticipated to build up if the flux through the final reduction step, catalyzed by CAD, is reduced. The total levels of the phenylpropanoid polymers are close to those in the normal plants.[12-14,24] However, it appears from the build-up of extractable hydroxycinnam-aldehyde and benzaldehyde monomers in the plant stems that producing the polymer from these components is not straightforward. In synthetic systems too, the aldehydes don't appear to be as readily incorporated as the alcohols. Nevertheless, the hydroxycinnam-aldehydes, and their derived hydroxybenzaldehydes, become a significant part of the polymer fraction.

There has been considerable debate about whether these phenylpropanoid polymers are in fact lignin, and whether they are functioning as lignin in the plant. A great deal remains to be resolved, but some of the issues are raised in accompanying abstracts at this Symposium.[19,25] Aldehydes have always been considered to be associated with lignins.[26] Apart from their obvious presence ascertained from phloroglucinol staining, we see them in isolated lignins by NMR and other spectroscopies, and they are clearly present in intact cell walls of plants as seen by the release of characteristic products from the many degradative methods. Could they be artifacts? Indeed, but they are certainly in the polymers we think of as lignins in normal plants. Finding higher levels in CAD-deficient mutants and transgenics seems logical.

The important question to be answered from the observations reported here, however, is: are aldehydes incorporated, as monomers, into lignins or are they simply post-lignification artifacts of oxidation? Peroxidase/H_2O_2 is capable of producing aldehyde monomers from monolignols, so they may be expected in the lignifying zone even in the absence of CAD-deficiencies. The high levels on aldehydes in lignins from CAD-deficient mutants and transgenics and their incorporation of monomers by homo- and hetero-coupling reactions into polymeric fractions makes it clear that they can indeed enter into the phenylpropanoid polymer fractions by the mechanisms characteristic of lignification. For most researchers, there is little surprise in this. If phenols are present in the cell wall during lignification, and if lignification is not carefully enzymatically controlled (see the accompanying Abstract on "Optical Activity of Lignins" for a discussion on this aspect)[25], it is logical that they will incorporate into the polymer, depending only on their abilities to form radicals and their cross-coupling propensities under the conditions of lignification. Many other non-monolignols (e.g. ferulates, acylated monolignols) have been shown to be components of lignins, so there is ample precedence for the incorporation of non-monolignols into lignins. Further studies are required to determine if the aldehyde-rich

polymers function as lignins. However, it should be noted that, if these phenolic polymers (containing significant levels of aldehydes that have clearly incorporated hydroxycinnamaldehyde monomers by radical coupling reactions) are not to be classified as lignins, then these CAD-deficient mutant and transgenic plants are surviving nicely with very little lignin!!

CONCLUSIONS

Hydroxycinnamaldehyde monomers appear to incorporate well into synthetic and natural lignins as anticipated from the currently accepted lignification mechanism. The radicals couple in a variety of anticipated ways. Lignins isolated from plants with deficiencies in the enzyme CAD have elevated levels of aldehydes with bonding patterns discernable from NMR that provide evidence suggesting the incorporation of monomeric aldehydes (8–O–4-cross-coupled structures). Work is still required to determine whether the plant is truly producing a modified lignin by incorporating the aldehyde monolignol precursors to ensure the viability of the plant, or produces this polymer as some kind of a wound response. Either way, if such transgenic plants are to be utilized as forages for ruminants, or for chemical pulping, these polymers contribute to the non-cellulosic portion and it is logical to classify them broadly as lignins. Processing problems or advantages of such modified plants will be interesting to follow in the near future.

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DISCLAIMER

Responsibility for the opinions and commentary in this abstract lies with John Ralph and others at the US Dairy Forage Research Center, opinions are not necessarily be shared by the co-authors (who were involved in the scientific studies but not in the writing of this abstract).

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