PERGAMON

Phytochemistry 57 (2001) 993–1003

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR

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Received 7 July 2000; received in revised form 16 November 2000

Abstract

Studying lignin-biosynthetic-pathway mutants and transgenics provides insights into plant responses to perturbations of the lignification system, and enhances our understanding of normal lignification. When enzymes late in the pathway are downregulated, significant changes in the composition and structure of lignin may result. NMR spectroscopy provides powerful diagnostic tools for elucidating structures in the difficult lignin polymer, hinting at the chemical and biochemical changes that have occurred. COMT (caffeic acid *O*-methyl transferase) downregulation in poplar results in the incorporation of 5-hydroxyconiferyl alcohol into lignins via typical radical coupling reactions, but post-coupling quinone methide internal trapping reactions produce novel benzodioxane units in the lignin. CAD (cinnamyl alcohol dehydrogenase) downregulation results in the incorporation of the hydroxycinnamyl aldehyde monolignol precursors intimately into the polymer. Sinapyl aldehyde cross-couples 8–O–4 with both guaiacyl and syringyl units in the growing polymer, whereas coniferyl aldehyde cross-couples 8–O–4 only with syringyl units, reflecting simple chemical cross-coupling propensities. The incorporation of hydroxycinnamyl aldehyde and 5-hydroxyconiferyl alcohol monomers indicates that these monolignol intermediates are secreted to the cell wall for lignification. The recognition that novel units can incorporate into lignins portends significantly expanded opportunities for engineering the composition and consequent properties of lignin for improved utilization of valuable plant resources.

Keywords: NMR; Transgenic; Mutant; *O*-Methyltransferase; Monolignol; Coniferyl aldehyde; Sinapyl aldehyde; 5-Hydroxyconiferyl alcohol

1. Introduction

NMR continues to become increasingly valuable as a tool for lignin structural elucidation. A number of powerful experiments stand out as being exceptionally useful. An extensively illustrated overview of solution-state NMR of lignins was recently published (Ralph et al., 1999c), so experimental details will not be given here. This paper will instead present recent NMR applications elucidating the structures of novel units in lignins from plants deficient in two monolignol biosynthetic pathway genes/enzymes.

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0031-9422/01/$ - see front matter
PII: S0031-9422(01)00109-1

Lignins are traditionally described as plant polymers resulting from dehydrogenative polymerization (via radical coupling reactions) of three primary phenylpropanoid monomers, *p*-coumaryl (4-hydroxy-cinnamyl), coniferyl (4-hydroxy-3-methoxy-cinnamyl), and sinapyl (3,5-dimethoxy-4-hydroxy-cinnamyl) alcohols, analogs varying in their degrees of methoxylation (Harkin, 1967; Freudenberg and Neish, 1968). They represent a class of complex polymeric natural products present in large quantities in the cell walls of terrestrial plants. The strict definition has, however, been long recognized as too narrow (Sarkanen and Ludwig, 1971). Other phenolic components, such as acylated hydroxycinnamyl alcohols and hydroxycinnamate esters may also intimately incorporate into lignins (Boudet, 1998; Sederoff et al., 1999; Ralph et al., 1999c). There has been considerable debate over what constitutes a "real" lignin (Lewis, 1999; Sederoff et al., 1999). We do not wish to revisit those issues here. We shall simply make the following observation. If a phenolic phenylpropanoid that is likely derived from the basic monolignol biosynthetic pathway couples (via radicals), as a monomer, with a growing oligomer/polymer of phenylpropanoids, and is further integrated into the polymer by endwise coupling with a new monolignol (or monomer), then we shall call this product lignin here. Ideally, histochemical studies should also be done to demonstrate that the polymer incorporating these units is in the cell wall.

Recent advances in genetic engineering have allowed researchers to perturb the monolignol biosynthetic pathway (Dixon and Ni, 1996; Baucher et al., 1998; Boudet, 1998; Whetten et al., 1998), allowing the abundance of normally minor components to be substantially enhanced and therefore structurally analyzed (Van Doorsselaere et al., 1995; Ralph et al., 1997, 1998, 1999b; Lapierre et al., 1999; Kim et al., 2000; Jouanin et al., 2000). This approach provides valuable insights into the control of lignification and into the remarkable biochemical flexibility of the lignification system.

The monolignol biosynthetic pathway in angiosperms has undergone considerable reevaluation recently. Fig. 1 derives from recent clarifications of the primary pathways, as deduced by Chiang's and Chapple's groups (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000). Studies to date indicate that downregulating any of the enzymes of the major pathways can affect the course and/or extent of lignification. General down-regulation of lignification (producing plants with reduced lignin levels) is possible. For example, NMR and other methods showed that the lignins from plants deficient in either a CoA ligase, Pt4CL1 (Hu et al., 1999) or an *O*-methyl transferase, CCoAOMT (Meyermans et al., 2000), were essentially normal in their composition and structure, despite having significantly lower than normal lignin levels. In both cases however, the levels of *p*-hydroxybenzoates on the lignins increased suggesting that the pathways to the monolignol esters might not be fully coupled to general monolignol synthesis (Li et al., 2000).

The most striking example of compositional flexibility in the normal monolignols came from the F5H (ferulate 5-hydroxylase) Arabidopsis mutants and transgenics produced by Chapple's group; F5H is sometimes now referred to as CAld5H to implicate coniferaldehyde as the preferred substrate for that enzyme (Humphreys et al., 1999; Osakabe et al., 1999). As shown by NMR (Marita et al., 1999) and other methods (Meyer et al., 1998), a chemical mutagenesis mutant of Arabidopsis had lignins devoid of syringyl units. More strikingly, when a suitably promoted *F5H* gene was introduced into the F5H-deficient mutant, up-regulation of sinapyl alcohol production was at such a high level that the lignin became extremely syringyl-rich. With an approximately 3% guaiacyl component, these plants' lignins have a far lower guaiacyl content than any plant reported to date (Marita et al., 1999). The up-regulation of F5H is therefore strikingly effective at diverting the monolignol pool toward sinapyl alcohol. In these examples massive compositional shifts in the traditional lignin components are apparent. As will be seen, compositional shifts can range considerably wider.

Here we present NMR data from recent studies on mutants and transgenics deficient in two of the later pathway enzymes, CAD (cinnamyl alcohol dehydrogenase) and COMT (caffeic acid *O*-methyl transferase, the favored substrate for which now appears to be 5-hydroxyconiferyl aldehyde (Li et al., 2000) although 5-hydroxyconiferyl alcohol must also be a viable substrate (Matsui et al., 1994; Chen et al., 1999; Humphreys et al., 1999; Maury et al., 1999). Downregulating these enzymes dramatically affects the composition of lignins and the structures contained in those lignins. We also reexamine the F5H-upregulated Arabidopsis transgenics where the methylation apparently can't keep pace with the increased hydroxylation rate.

2. CAD-deficient tobacco: aldehyde incorporation

CAD is the last enzyme on the pathway to the monolignols coniferyl and sinapyl alcohols **2**G and **2**S, from which lignins are normally derived, Figs. 1 and 2. When CAD is downregulated, the hydroxycinnamyl aldehyde precursors to the monolignols, coniferyl aldehyde **1**G and sinapyl aldehyde **1**S, build up and may be incorporated into the polymer by radical coupling (Halpin et al., 1994, 1998; Higuchi et al., 1994; Provan et al., 1997; Ralph et al., 1997, 1998; Stewart et al., 1997; Yahiaoui et al., 1998; Vailhe et al., 1998). Indeed, coniferyl aldehyde readily incorporated into synthetic lignins under biomimetic conditions (Higuchi et al., 1994). Recent studies have begun to elucidate the propensity for the mono-

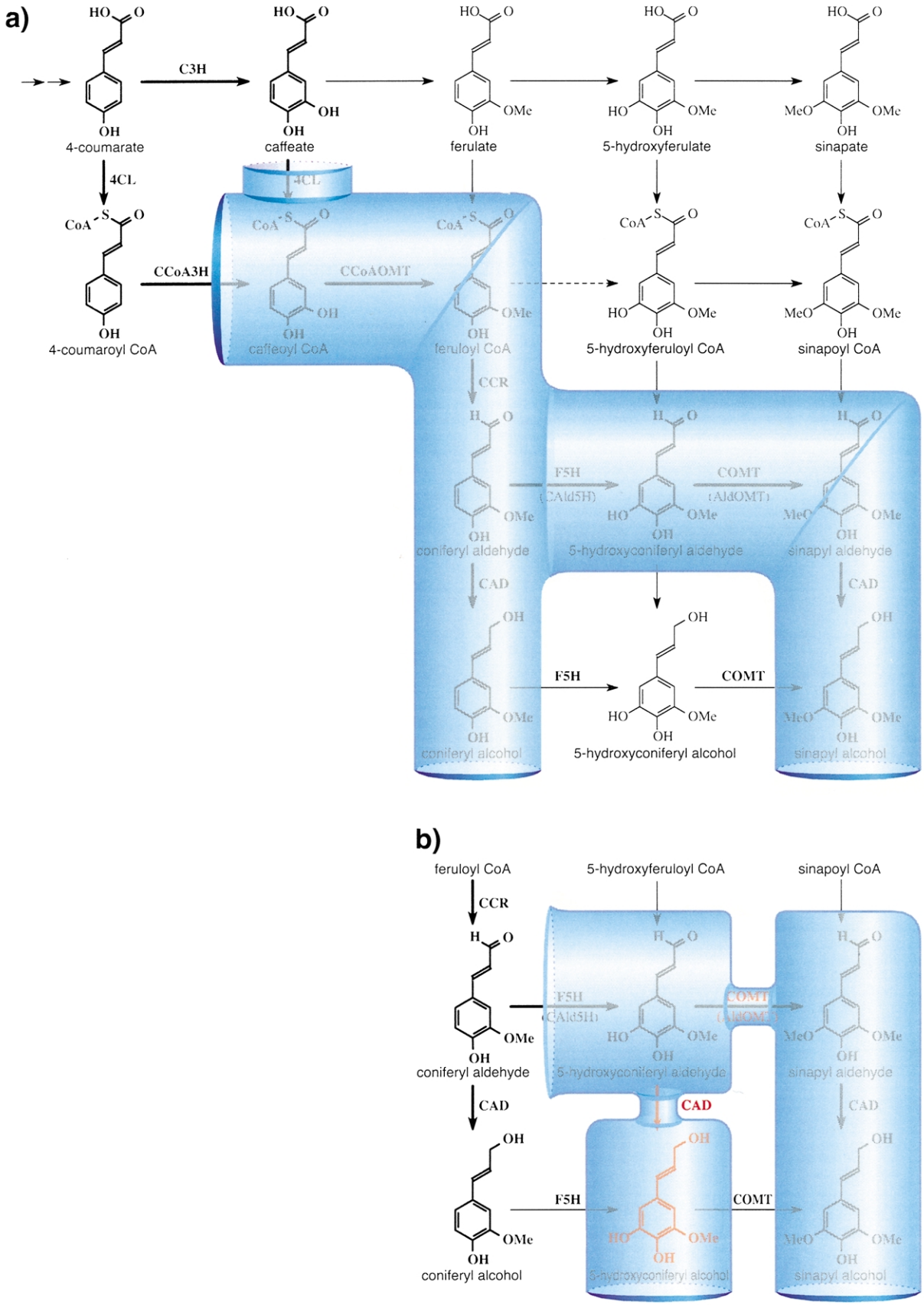

Fig. 1. (a) The monolignol biosynthetic pathway in angiosperms. Bolder structures and arrows (with large-diameter pipes) represent preferred pathways as recently clarified (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000). (b) When COMT activity is deficient, sinapyl alcohol production backs up the 5-hydroxyconiferyl aldehyde intermediate which can apparently be reduced by CAD; 5-hydroxyconiferyl alcohol then becomes a significant monomer in lignin biosynthesis.

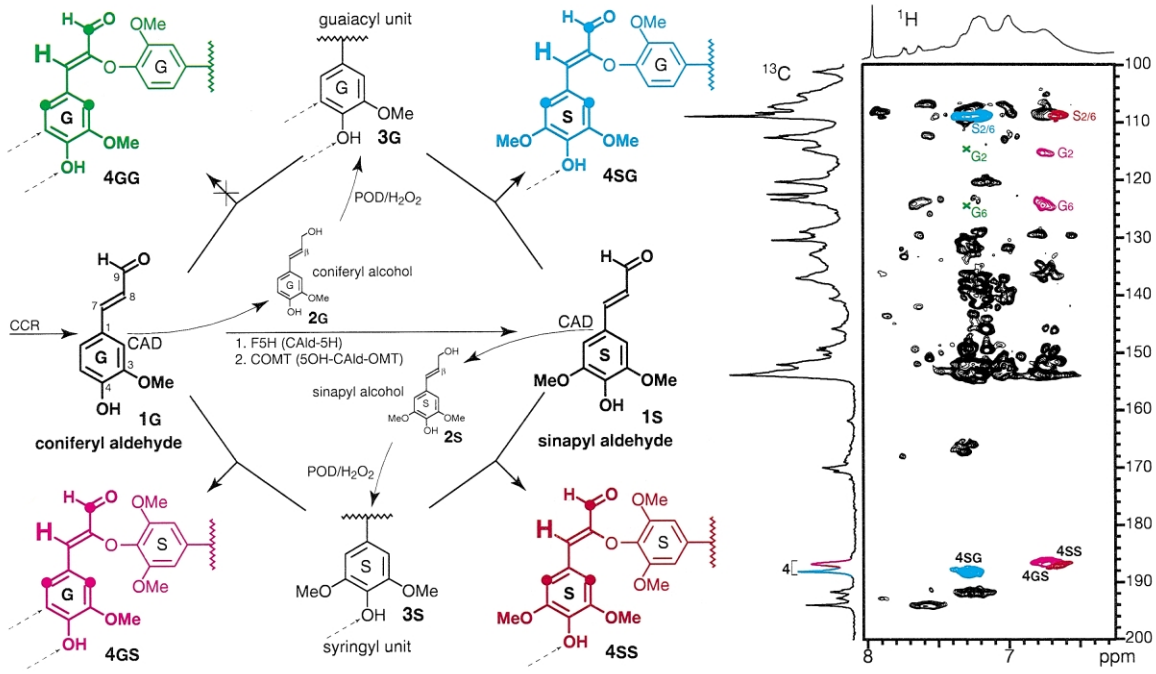

Fig. 2. Cross-coupling of hydroxycinnamyl aldehydes **1**G and **1**S with guaiacyl and syringyl lignin units **3**G and **3**S can potentially produce four 8–O–4-cross-coupled structures **4**. Only three of the four cross-products (**4**GS, **4**SG and **4**SS but not **4**GG) are significantly represented, as determined by analysis of the gradient-HMBC partial spectrum to the right. The HMBC section shows, in a lignin isolated from CAD-deficient tobacco stems, correlations from the aldehyde carbonyl carbons (185–195 ppm) to the H7 protons within three-bonds in structures **4** that have been further incorporated into lignin at positions indicated by the dashed arrows. These H7 protons diagnostically correlate with the 2- and 6-carbons of the cinnamyl aldehyde ring; the G2 and G6 carbons for guaiacyl units, and the higher-field equivalent S2/6 (syringyl) carbons are identified and colored correspondingly. Colored dots on the structures indicate the carbons correlated by $^{3}J_{C-H}$ to the H7-protons (which are shown in a larger font size) shown in the HMBC spectrum. The aldehydes at ~188.1 ppm, with their corresponding H7s at ~7.3 ppm result from hydroxycinnamyl aldehydes **1** 8–O–4-coupled to guaiacyl units **3**G in lignin; correlations from H7 indicate, however, that sinapyl aldehyde **1**S, but not coniferyl aldehyde **1**G, couples with guaiacyl units **3**G — the green X's show where correlations are present in a model for **4**GG. The aldehydes at ~186.7 ppm, with their corresponding H7s at ~6.7 ppm result from hydroxycinnamyl aldehydes **1** 8–O–4-coupled to syringyl units **3**S in lignin; correlations from H7 indicate that sinapyl aldehyde **1**S and coniferyl aldehyde **1**G each couple with syringyl units **3**S. S = syringyl, G = guaiacyl, CCR = cinnamoyl-CoA reductase, CAD = cinnamyl alcohol dehydrogenase, F5H = ferulate 5-hydroxylase which has recently also been termed CAld5H = coniferyl aldehyde 5-hydroxylase, to reflect the preferred in vivo substrate (Osakabe et al., 1999; Humphreys et al., 1999), COMT = caffeoyl *O*-methyl transferase (for which the preferred substrate appears to be 5-hydroxyconiferyl aldehyde, Li et al., 2000), POD = peroxidase. Convention for compound numbering: e.g. **4**GS = a guaiacyl aldehyde (coniferyl aldehyde **1**G) coupled 8–O–4 to a syringyl lignin unit (**3**S).

lignols to cross-couple with (free-phenolic) guaiacyl and syringyl units in lignins (Syrjanen and Brunow, 1998, 2000). With the availability of a ^{13}C-enriched lignin from a CAD-deficient tobacco transgenic (Ralph et al., 1998) and a series of model compounds (Kim et al., 2000), NMR methods can be used to ascertain hydroxycinnamyl aldehyde in vivo cross-coupling propensities. Data revealing such details of plant lignification are rarely obtained.

Radical cross-coupling of monolignols **2**, Fig. 2, with the growing lignin oligomer/polymer **3** is the major reaction occurring during lignification. Thus, the hydroxycinnamyl alcohol **2** (primarily at its β-position) couples with phenolic units **3** (at the 4–O- or 5-position for guaiacyl units **3**G, and almost exclusively at the 4–O-position for syringyl units **3**S) to form a chain-extended oligomer/polymer, also with the general formula **3**. Fig. 2 (right) shows a selected region of a long-range ^{13}C–^{1}H correlation (gradient-HMBC) spectrum from a CAD-deficient tobacco lignin, with peaks in the ^{13}C-projection and the resultant contours in the HMBC spectrum colored to match structures on the left for easy identification. In fact, the aldehyde structures in the lignins likely have been further incorporated into the polymer by primarily 4–O-coupling with the next monolignol, so they are not strictly the phenolic compounds **4** shown. Available coupling sites are indicated in Fig. 2 by the dashed arrows.

As the caption to Fig. 2 describes, correlation of the aldehyde carbonyl carbons in cross-products **4** to the H7-protons three-bonds away identifies the type of lignin units involved. Protons H7 resonate at ~7.3 ppm for hydroxycinnamyl aldehydes (either **1**G or **1**S) coupled 8–O–4 to guaiacyl units **3**G (compounds **4**GG and **4**SG, δ_C = 188.1 ppm), whereas they resonate considerably upfield, at ~6.7 ppm when coupled 8–O–4 to syringyl units **3**S (compounds **4**GS and **4**SS, δ_C = 186.8 ppm). Correlations from these H7 protons into the ring

identify the hydroxycinnamyl aldehyde involved in the coupling; three-bond correlations identify equivalent S2/6 carbons derived from sinapyl aldehyde **1**S units at higher field than the non-equivalent G2 and G6 carbons from units derived from coniferyl aldehyde **1**G. Therefore, for 8–O–4 cross-coupled units **4** in lignins, the G/S nature of both the hydroxycinnamyl aldehyde component (coupled 8-) and the lignin unit (coupled 4–O-) are diagnostically revealed. Other aromatic protons in the complex lignin polymer resonate in the H7 regions so correlations that are not of interest here will result; an absence of correlations is therefore more diagnostic, revealing the absence of a component.

Of the four possible aldehyde 8–O–4 incorporation products **4**, Fig. 2, only three can be detected in the antisense-CAD tobacco lignin by NMR methods. Product **4**GG is conspicuously absent. The data imply that sinapyl aldehyde **1**S cross-couples 8–O–4 with both guaiacyl and syringyl units (to form cross-coupled structures **4**SG and **4**SS), but that coniferyl aldehyde **1**G cross-couples 8–O–4 *only* with syringyl units **3**S and not with guaiacyl units **3**G. Thus, cross-coupling product **4**GS is readily detected, whereas **4**GG cannot be detected even when spectra are viewed at close to the baseplane noise level. This cross-coupling propensity is presumably the reason why 8–O–4-coupled structures are not found to be prominent in CAD-deficient softwood lignins (Ralph et al., 1997).

Attempting to prepare compounds modeling **4**GG by biomimetically cross-coupling coniferaldehyde **1**G (at the eight-position) with coniferyl alcohol **2**G or with a simple guaiacyl model 1-(4-hydroxy-3-methoxy)-ethanol (at the 4–O-position), also failed to produce 8–O–4-coupled cross-products. Apparently the same factors that promote cross-coupling in vitro apply in vivo, suggesting that these radical coupling reactions and those involved in "normal" lignification are likely to be under simple chemical control in the plant (Sederoff et al., 1999). In the cross-products, the chemical shifts match those in model compounds synthesized by traditional means (Kim et al., 2000). Also available from the HMBC spectrum in Fig. 2 is some information regarding the incorporation of the hydroxycinnamyl aldehydes **1** and their derived hydroxybenzyl aldehydes at ring 4–O-positions, as described elsewhere (Kim et al., 2000).

Examination of the spectra also provides significant information regarding the incorporation of hydroxycinnamyl aldehydes into "bulk" vs. "endwise" polymers (Sarkanen, 1971). "Bulk" lignins are characterized by frequent dimerization reactions, whereas endwise polymerization, the normal process occurring during lignification in the secondary cell wall, is from cross-coupling of monomers with the growing polymer. Dimerization of coniferaldehyde produces the 8–O–4-coupled dimer (analogous to **4**GG, Fig. 2), along with the other anticipated 8–8- and 8–5-coupled dimers (Connors et al., 1970). The absence of signals for coniferaldehyde coupled with G-units (of which the coniferaldehyde dimer **4**GG is an example), is evidence that coniferaldehyde does not dimerize at an appreciable level and therefore is not significantly involved in "bulk" lignification.

In summary, in 8–O–4-coupling reactions, coniferyl aldehyde cross-couples only with syringyl units, whereas sinapyl aldehyde cross-couples with both guaiacyl and syringyl units. These observations require no departure from the existing theory of radical coupling of phenols into polymeric lignins since they appear to reflect simple chemical coupling propensities.

3. CAD-deficient pine: incorporation of dihydroconiferyl alcohol and guaiacylpropane-1,3-diol

Preliminary observations (Ralph et al., 1997) on the novel lignins from a CAD-deficient pine (*Pinus taeda*) mutant are being strengthened as more diverse evidence accumulates (Dimmel et al., 1999; Ralph et al., 1999a; Lapierre et al., 2000). The plant, despite a reduction in CAD levels to $< 1\%$ of normal levels (MacKay et al., 1997), appeared to be producing normal levels of lignin (Klason lignin values were almost identical). A possible interpretation is that the CAD-deficiency is not sufficient to change the system and that there is still sufficient flux through the residual enzyme levels that normal lignification can proceed. The lignin in the CAD-deficient pine mutant however was highly colored and stained strongly with phloroglucinol, suggesting a higher aldehyde component. From NMR studies on isolated lignins, aldehydes and dihydroconiferyl alcohol were substantial components of that lignin (Ralph et al., 1997, 1999c).

New aldehyde components were misidentified originally (Ralph et al., 1997), but corrected in subsequent publications (Ralph et al., 1998, 1999b,c). A book chapter on NMR details how the incorrect assignment was made (Ralph et al., 1999c). The aldehyde components all stem from incorporation into the lignin of more significant quantities of coniferyl aldehyde (and its product vanillin) than in normal pine. The low aldehyde levels seen in normal lignins could possibly result from post-lignification oxidation, but the levels seen in this and other CAD-deficient plants suggest that incorporation of hydroxycinnamyl (and hydroxybenzyl) aldehydes into lignins is a normal process that becomes more significant when the pathway to the final monolignols is downregulated.

The CAD-deficient pine lignin had another feature that may or may not be attributable directly to the CAD-deficiency. Since this was a natural mutant other unknown enzymes may have also been impacted by the mutation(s); however, this is unlikely because the genetic determinant of the modified wood colocalized with the coding

sequence for the enzyme CAD (MacKay et al., 1997). Substantial levels of an unexpected unit derived from dihydroconiferyl alcohol (DHCA, **5**, Fig. 3) were identified in the mutant's lignin (Ralph et al., 1997, 1999c; Sederoff et al., 1999). DHCA is found at lower levels in normal plant lignins (Lundquist and Stern, 1989; Fukagawa et al., 1991; Brunow et al., 1998; Ralph et al., 1999c) but its derivation is not completely clear. Claims that the unit could only arise as a modified metabolic product *following* dimerization of traditional lignin monomers (Gang et al., 1998; Lewis et al., 1998) were incorrect; there is ample evidence that DHCA is formed as a monomer in the CAD-deficient pine and that the monomer is incorporated into the lignin via radical coupling processes (Ralph et al., 1997, 1999c; Sederoff et al., 1999; Lapierre et al., 2000). NMR showed that about half of the DHCA units in the soluble lignin fractions were involved in 5–5-coupled structures (Ralph et al., 1997, 1999c); coniferyl alcohol has never been shown to 5-5-dimerize. We were able to dissolve the (dioxane-water insoluble) residual lignin in acetyl or propionyl bromide. These reagents acylate and α-brominate the lignin, and cause some other structural changes, but in no way (other than acylation) affect the saturated aryl propanol sidechain. HMQC and COSY NMR experiments then unambiguously determined that DHCA was a component of that residual lignin although quantification was not possible (Ralph et al., 1999c).

More recently, guaiacylpropane-1,3-diols (GPDs) were also identified in lignins isolated from the CAD-deficient pine mutant, Fig. 3. This helped to unravel the nature of weak correlations often seen in pine lignin ^{13}C–^{1}H correlation NMR spectra; smaller amounts of GPDs are now recognized to be present in lignins from normal pines and presumably other softwoods. The structures in the complex lignin polymers are readily proven by NMR of isolated lignins.

Fig. 3a shows a subplot of the sidechain region from a 2D HMQC-TOCSY NMR experiment on an acetylated lignin isolated from the mutant pine, highlighting the new (acetylated) guaiacylpropane-1,3-diols **6** (red) along with the DHCA **5** units (green). Data from a model compound, 1,3-diacetoxy-1-(4–O-benzyl-3-methoxyphenyl)-propane (Ralph et al., 1999b), are at the center of the yellow circles and obviously match well.

As with other novel units found in lignins from transgenic or mutant plants, traces of the same components can be found in lignins from control plants. Fig. 3b shows the α-C/H region of an HSQC spectrum of the mutant's lignin, where the β-aryl ether units (blue) and the strong new GPD unit (red) appear most cleanly. Fig. 3c shows the same region in lignin from a

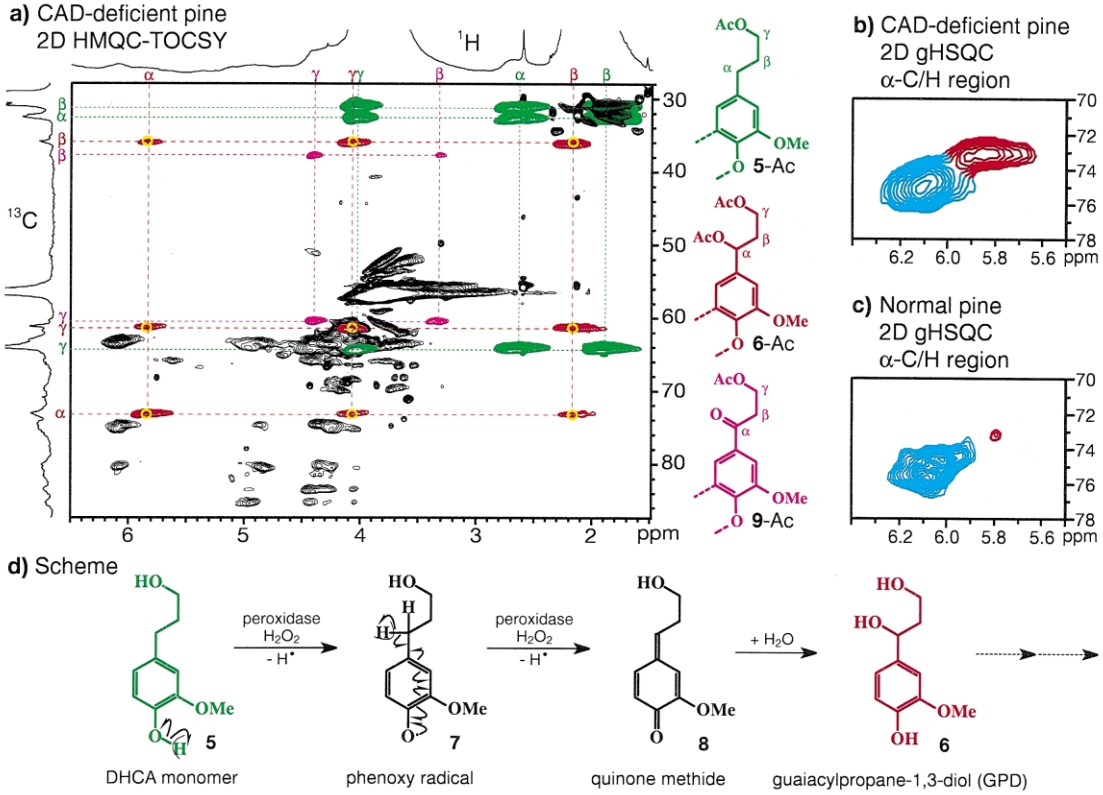

Fig. 3. NMR spectra showing new GPD **6** structures (red) and derived benzylic ketone analogs **9** (magenta), along with DHCA **5** units (green); data from acetylated model **6**–Ac are at the centers of the yellow circles; some β-aryl ether units are also shown (blue) in the HSQC spectra in b and c. The mechanism for producing GPD **6** from DHCA is shown in d.

normal pine control. Although the GPD peak is weak, it is diagnostic and, with other correlations evident (not shown), well authenticated.

GPD monomeric units derive from DHCA monomer **5** via the action of peroxidase and hydrogen peroxide, Fig. 3d. The mechanism, via a vinylogous quinone methide **8**, involves two H-radical abstractions. Abstraction from a benzylic CH_2 to produce quinone methides from phenoxy radicals has been noted previously (Zanarotti, 1982). When DHCA is subjected to peroxidase-H_2O_2, monomeric GPD **6** as well as the range of homo- and crossed-dimers involving DHCA and GPD are found (Peng and Ralph, unpublished).

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

In summary, another previously unidentified unit present in small quantities in normal lignins is a major component of the hydroxyphenylpropanoid polymeric component of a pine mutant deficient in CAD. Those units, guaiacylpropane-1,3-diols, arise from conversion of dihydroconiferyl alcohol monomers by radical reactions and also incorporate into lignins as monomers. Like DHCA however, GPD **6**, which does not possess an unsaturated sidechain, is limited to coupling on the ring 4–O-, 5-, and possibly 1-positions, necessarily forming terminal units in lignin (except via 5–O–4-coupling). High MW polymers cannot be created without the incorporation of authentic hydroxycinnamyl alcohols (or other cinnamyl derivatives) as well. The CAD-deficient pine lignin is more soluble in alkali and has reduced molecular weight (Dimmel et al., 1999).

4. 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, Fig. 1. If COMT is downregulated, 5-hydroxyconiferyl aldehyde might build up and might be expected to be reduced to 5-hydroxyconiferyl alcohol if the next enzyme, CAD, is sufficiently non-specific. In fact, as illustrated in Fig. 1b, it appears that 5-hydroxyconiferyl 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).

NMR provides beautiful evidence that benzodioxane structures are produced in lignins which incorporate 5-hydroxyconiferyl alcohol. Fig. 4b shows the sidechain region of an HMQC spectrum from an (acetylated) isolated lignin from a COMT-deficient poplar (*Populus tremula* × *Populus alba*) described recently (Jouanin et al., 2000). 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 antisense suppression (Van Doorsselaere et al., 1995; Lapierre et al., 1999). As with the dibenzodioxocins **D** (Karhunen et al., 1995a,b), 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 δ_C/δ_H of 76.8/4.98 (α), and 75.9/4.39 (β) are diagnostic for the benzodioxanes **H**; the γ-correlations overlap with those in other lignin units. Anticipated shifts occur following acetylation; in the unacetylated lignins (not shown), the correlations are centered at δ_C/δ_H of 76.5/4.87 (α), and 78.9/4.06 (β). As seen in Fig. 4b and d, the sidechain correlations are consistent with those in a model compound for the *trans*-benzodioxane **10**–Ac, synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit (Ralph et al., 2000). The α-proton shift deviated the most presumably since this is an acetylated (and therefore originally free-phenolic) model, rather than a phenol-etherified structure which would correspond to most of the units in the lignin polymer. Lignins from COMT antisense poplars also contain benzodioxane units (Ralph et al., 2000).

A reasonable quantification of this unit can be done by measuring volume integrals in the 2D spectra (Zhang et al., 2000), particularly if the similar Cα–Hα correlations are used. The ratios in the transgenic (and in the wild-type control in parentheses) are 53% (88%) aliphatic-sidechain β–O–4-units **A**, 13% (3%) phenylcoumarans **B**, 5% (7%) resinols **C**, 6% (0%) dibenzodioxocins **D**, 5% (2%) coniferyl alcohol endgroups **X**, and 18% (0%) of the new benzodioxane units **H**. The 5-hydroxyconiferyl alcohol-derived benzodioxane **H** units are therefore the second most abundant interunit type in this 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 β-ether frequency (normal β-ether **A** plus dibenzodioxocins **D** plus benzodioxanes **H**) in the transgenic is around 77%, 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, β–1-structures). Although the data are limited at present, it appears that 5-hydroxyconiferyl alcohol may quantitatively make up for the sinapyl alcohol deficiency.

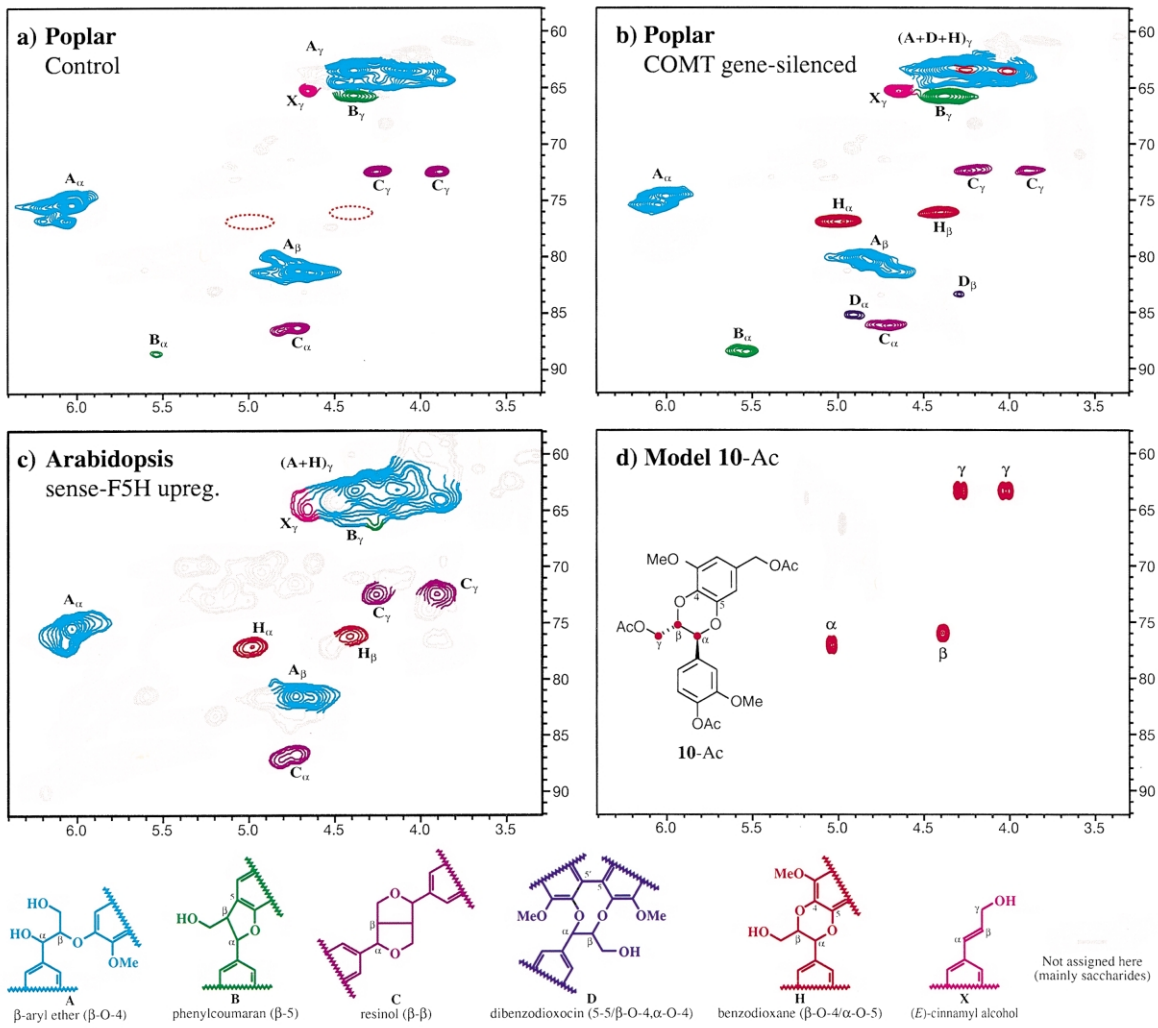

Fig. 4. Partial spectra from gradient HMQC NMR experiments highlighting new peaks for benzodioxane units **H**. Lignins were from (a) a control poplar, (b) a COMT-downregulated transgenic, (c) an F5H-upregulated Arabidopsis, and (d) similar correlations from a benzodioxane model **10**–Ac. The major unit coding is the same as that used in Ralph et al., 1999c and other references; it is suggested that **H** be reserved for 5-hydroxyguaiacyl benzodioxane units in lignin NMR spectra.

The incorporation of 5-hydroxyconiferyl alcohol into lignins has been documented several times (Hwang and Sakakibara, 1981; Lapierre et al., 1988; Jacquet, 1997; Suzuki et al., 1997). Benzodioxanes have been proposed to be in lignins previously (Hwang and Sakakibara, 1981; Jacquet, 1997; Jouanin et al., 2000), and analogous structures occur in lignans (Ishikawa et al., 1995; Su et al., 1997), but until now the occurrence of benzodioxanes in lignins has not been observed directly. The likely mechanism for formation of the novel benzodioxane units is shown in Fig. 5, as described in the caption.

There is another sample in which benzodioxanes are evident, an interesting variant in the COMT-deficiency class. Arabidopsis transgenics with upregulated F5H have previously been shown to have only a minor guaiacyl component (Meyer et al., 1998; Marita et al., 1999). Contours previously unidentified in the 2D NMR spectra of their lignins (Marita et al., 1999) now obviously result from benzodioxane structures, Fig. 4c. 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-hydroxy units (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 spectrum in Fig. 4c.

As further evidence accumulates from degradative and NMR methods that coniferyl and sinapyl alcohols couple with the new terminus created by incorporation of 5-hydroxyconiferyl alcohol monomers into the growing polymer (e.g. Jouanin et al., 2000), it becomes clear that 5-hydroxyconiferyl alcohol can be used as a

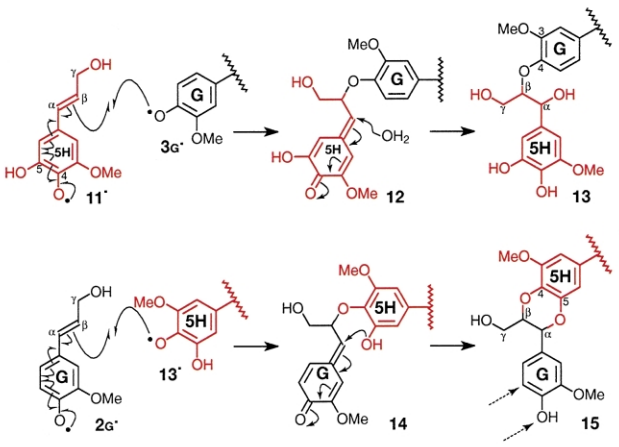

Fig. 5. Production of benzodioxanes **15** in lignins via incorporation of 5-hydroxyconiferyl alcohol **11** into a guaiacyl lignin. Only the pathways producing β-ether units are shown, but β–5-(phenylcoumaran) units are also possible. Cross-coupling with syringyl units is also possible but must be less pronounced in lignins which have a low syringyl content due to strong COMT downregulation. Cross-coupling of 5-hydroxyconiferyl alcohol **11**, via its radical **11·**, with a guaiacyl lignin unit **3**G, via its radical **3**G**·**, produces a quinone methide intermediate **12** which re-aromatizes by water addition to give the β-ether structure **13** (possessing a 5-hydroxyguaiacyl end-unit). This unit is capable of further incorporation into the lignin polymer via radical coupling reactions of radical **13·**. Reaction with the monolignol coniferyl alcohol **2**G, via its radical **2**G**·**, produces a quinone methide intermediate **14**. This time, however, quinone methide **14** can be internally trapped by the 5-OH phenol, forming a new 5–O–α-bond, and creating the benzodioxane ring system in **15**. The presence of structures **15** in COMT-deficient transgenic plants is diagnostically revealed by NMR, units **H** in Fig. 4.

lignin monomer by plants to offset 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, and no biochemical evidence..." (Lewis, 1999) needs to be revisited. In fact, plants and other organisms have long used loosely ordered chemistry to enhance their viability and resistance, as eloquently stated in a letter to Science (Denton, 1998).

5. Conclusions

Recent studies have shown that manipulating specific lignin-biosynthetic-pathway genes produces profound alterations in plant lignins. Although the lignins in mutant and transgenic plants may appear to be strikingly different from normal lignins, they often represent merely broad compositional shifts; most of the novel units that have been found to date appear to be (often previously unidentified) minor units in lignins from 'normal' plants. A salient observation is that the process of lignification appears to be flexible enough to readily incorporate phenolic phenylpropanoids other than the traditional monolignols. The incorporation of hydroxycinnamyl aldehyde monomers as well as 5-hydroxyconiferyl alcohol 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' flexible approach toward lignification, i.e. polymerizing monolignol precursors and derivatives along with the traditional monolignols, is a testament to their flexible strategy; in a single generation, these plants have circumvented genetic obstacles to remain viable. The recognition that novel units can incorporate into lignin provides significantly expanded opportunities for engineering the composition and consequent properties of lignin for improved utilization of valuable plant resources.

6. Experimental

6.1. Plant materials and lignin isolation

Isolation of the lignins for which NMR spectra are reported here were all previously described. All isolations follow the basic scheme: solvent extraction to remove major extractives and protein, ball milling, crude cellulases digestion to remove the bulk of the polysaccharides, dioxane–water (96:4) extraction to remove soluble lignin fractions, and some clean up of this lignin fraction by EDTA washing to remove some saccharide impurities and metal contaminants which can otherwise reduce the quality of the NMR data. All of the isolated lignins described here were acetylated and dissolved in acetone-d_6 (~60–100 mg in 0.4 ml) for NMR. In some cases, the proportion of the lignin that could be extracted and solubilized for NMR studies was as low as 17%; in poplar samples it is more typically 65%. Criticisms that these lignins are therefore not representative, or are contaminated by non-lignin extractives are slowly being dispelled as the results of more diverse studies, primarily using degradative methods, emerge. It is nevertheless important that this potential sample limitation be known, so the yields are reproduced here. Our comments on what constitutes a lignin for this study are delineated in the second paragraph of Section 1.

The sense-F5H-upregulated (C4H-promoted) Arabidopsis lignin (Fig. 4c) was obtained in 27.5% yield (Marita et al., 1999). The CAD-deficient (antisense downregulated) tobacco lignin (Fig. 2) was isolated in 24% yield (Ralph et al., 1998). Lignin from the CAD-deficient loblolly pine mutant (Fig. 3) was isolated in 17% yield (Ralph et al., 1997). Poplar lignins in Fig. 4a, b were isolated in 65% yields (Ralph et al., 2000).

6.2. NMR experiments

NMR spectra were acquired on a Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The central acetone solvent peak was used as internal reference (δ_C 29.80, δ_H 2.04). We used the standard Bruker implementation (inv4gstp) of the gradient-selected inverse (^{1}H-detected) HMQC experiment for the spectra in Fig. 4, the standard (inv4gslplrnd) gradient-selected inverse-detected HMBC experiment with a 100 ms long-range coupling delay for the spectrum in Fig. 2, and a (non-gradient) HMQC-TOCSY (invbmltp) with a 100 ms mixing time for the spectrum in Fig. 3a. These experiments and their applications to lignins are detailed in a recent book chapter on "Solution-state NMR of Lignins" (Ralph et al., 1999c).

Acknowledgements

Partial funding for the Dairy Forage Research Center authors was from USDA-NRI (Plant Growth and Development No. 96-35304-3864; Improved Utilization of Wood and Wood Fiber No. 97-02208 and No. 99-02351) and the Division of Energy Biosciences, US Department of Energy 2000 awards (No. DE-AI02-00ER15067); for NCSU was by NIH (GM45344-07), DOE (DE-FG05-92ER20085), and the NCSU Forest Biotechnology Industrial Research Consortium; for French and Belgium 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, US Department of Energy (No. DE-FG02-94ER20138).

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