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NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins

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Peroxidase/H_2O_2-mediated radical coupling of 4-hydroxycinnamaldehydes produces 8–O–4-, 8–5-, and 8–8-coupled dehydrodimers as has been documented earlier, as well as the 5–5-coupled dehydrodimer. The 8–5-dehydrodimer is however produced kinetically in its cyclic phenylcoumaran form at neutral pH. Synthetic polymers produced from mixtures of hydroxycinnamaldehydes and normal monolignols provide the next level of complexity. Spectral data from dimers, oligomers, and synthetic polymers have allowed a more substantive assignment of aldehyde components in lignins isolated from a CAD-deficient pine mutant and an antisense-CAD-downregulated transgenic tobacco. CAD-deficient pine lignin shows enhanced levels of the typical benzaldehyde and cinnamaldehyde end-groups, along with evidence for two types of 8–O–4-coupled coniferaldehyde units. The CAD-downregulated tobacco also has higher levels of hydroxycinnamaldehyde and hydroxybenzaldehyde (mainly syringaldehyde) incorporation, but the analogous two types of 8–O–4-coupled products are the dominant features. 8–8-Coupled units are also clearly evident. There is clear evidence for coupling of hydroxycinnamaldehydes to each other and then incorporation into the lignin, as well as for the incorporation of hydroxycinnamaldehyde monomers into the growing lignin polymer. Coniferaldehyde and sinapaldehyde (as well as vanillin and syringaldehyde) co-polymerize with the traditional monolignols into lignins and do so at enhanced levels when CAD-deficiency has an impact on the normal monolignol production. The implication is that, particularly in angiosperms, the aldehydes behave like the traditional monolignols and should probably be regarded as authentic lignin monomers in normal and CAD-deficient plants.

Introduction

Aldehydes are well-known components in lignins,[1–10] and are responsible for the characteristic phloroglucinol staining of lignified tissues.[11] Relatively recent studies on plants deficient in enzymes in the monolignol biosynthetic pathways have renewed interest in the possible incorporation of 4-hydroxycinnamaldehydes **1** (Fig. 1†) and their derived hydroxybenzaldehydes **2** into lignins, perhaps replacing some of the normal monolignol **3** component.

It has long been recognized that coniferaldehyde **1G** is a viable substrate for free-radical coupling reactions analogous to those that occur with the standard monolignols **3**.[5,12] It is possible to make coniferaldehyde synthetic lignins (DHPs) for example.[5,13] Connors *et al.*[12] prepared and purified some of the coniferaldehyde dehydrodimers **5**, but insufficient NMR data for current structural studies were available.

Whether aldehydes 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 plants deficient in cinnamyl alcohol dehydrogenase (CAD; E.C. 1.1.1.195) are examined. In gymnosperms, in which the lignins are predominantly composed of guaiacyl units **4G**, CAD catalyses the final biosynthetic step from coniferaldehyde **1G** to coniferyl alcohol **3G**. CAD has also been detected and purified from various angiosperms,[14–19] where there are several isoform groups, named CAD1, CAD2, CAD1P, and CAD2P. These isozymes differ in amino acid sequence, molecular weight, and substrate specificity,[20] and each group has isozymes that have different sub-units.[16,21] The most studied, CAD2, appears to be the main CAD enzyme in the lignin biosynthetic pathway. It was believed that the same CAD2 existed in both gymnosperms and angiosperms, and that CAD in gymnosperms was more specific for coniferaldehyde **1G** whereas CAD in angiosperms had equal activities towards both

† The IUPAC name for guaiacyl is 4-hydroxy-3-methoxyphenyl, syringyl is 4-hydroxy-3,5-dimethoxyphenyl, sinapaldehyde is 3,5-dimethoxy-4-hydroxycinnamaldehyde, coniferaldehyde is 4-hydroxy-3-methoxycinnamaldehyde and coumaran is 2,3-dihydrobenzofuran.

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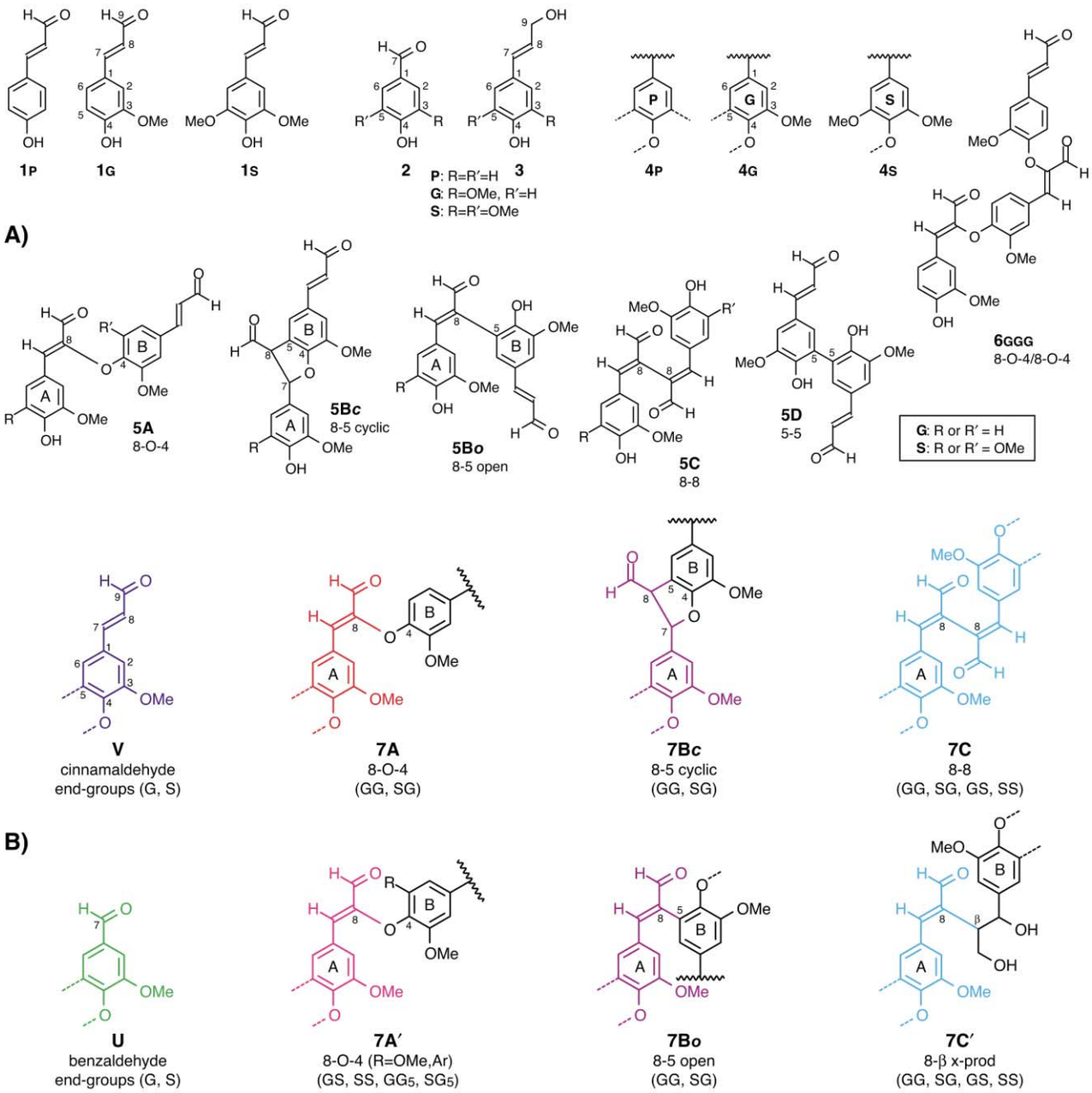

Fig. 1 A) Structures of monomeric, dimeric, oligomeric and polymeric units. The numbering system follows the convention for lignins. The A- and B-rings are arbitrarily defined to allow the units to be discussed in the text. B) Units in lignins with undefined groups attached *via* possible coupling reactions at their 4–O- and 5-positions. Units **U** and **V**, as well as **A** (for 8–O–4), **B** (for 8–5), **C** (for 8–8), and **D** (for 5–5) are taken from the unit labels established in the recent review "Solution-state NMR of Lignins".[40] Smaller qualifiers are: G = guaiacyl, S = syringyl, P = *p*-hydroxyphenyl, ***c*** = cyclic (phenylcoumaran) form, ***o*** = open (non-cyclic) form; in dimeric units, the "A-ring" unit is first —thus **7ASG**, for example, is from sinapaldehyde coupled at its 8-position to a guaiacyl unit coupled at its 4–O-position. †

coniferaldehyde **1G** and sinapaldehyde **1S**.[22,23] However, Chiang and co-workers have recently discovered a sinapyl alcohol dehydrogenase (SAD) from aspen which has 60 times greater enzymatic efficiency for sinapaldehyde **1S** than the known CAD enzyme.[24] We will continue to use CAD here as the generic term for cinnamyl alcohol dehydrogenase.

Although it has been suggested from flux studies using suspension-cultured *Pinus taeda* that CAD should not be rate limiting,[25] down-regulation of CAD in a variety of mutants and transgenics clearly leads to an accumulation of hydroxycinnamaldehydes **1** (at the apparent expense of the monolignols **3**) and an apparent buildup of their content in resultant lignins,[1–5,26–31] although confirming the association of these aldehydes with the polymeric lignin component has sometimes been difficult;[29,32] much of the aldehyde component can remain as low molecular mass extractable compounds whose location is uncertain.

After a tobacco-CAD enzyme was purified and characterized and the gene obtained,[16] tobacco CAD-downregulated transgenic plants were produced.[3,28,33] The lignin levels of CAD-downregulated transgenics were similar to those in the normal tobacco, but the xylem had a red–brown coloration, a common phenotype of CAD-deficiency. Lignin isolated from tobacco CAD-downregulated transgenic plants with 8% residual CAD activity was preliminarily characterized by NMR methods; the appearance of aldehyde peaks corresponding to new aldehydic structures in the lignin has been noted.[27] More detailed NMR studies, coupled with data derived from synthetic model compounds, allowed details of the cross-coupling propensities of coniferaldehyde and sinapaldehyde to be elucidated *in vivo*.[34,35] The CAD enzyme in loblolly pine was also purified and characterized,[17] and a CAD-deficient mutant pine was discovered.[36] This mutant had a similar Klason lignin level to normal pine, despite CAD levels being less than 1% of those in the normal

pine. The CAD-downregulated pine mutant also had red–brown colored xylem (see later in Fig. 4), and lignins were more easily extractable in alkaline solution than from the control. This pine mutant lignin was characterized by NMR studies[26] prior to the NMR study of the tobacco CAD-downregulated transgenics. Significantly enhanced DHCA (dihydroconiferyl alcohol) levels were strikingly revealed; DHCA seemed to partially offset the reduced coniferyl alcohol levels in the mutant's lignin. The original claims in that initial paper relating to this component were challenged,[37] but have since been validated as more diverse data has been accumulated.[35,38–40] The paper dealing mainly with the elevated DHCA levels also began to discern the nature of the more minor aldehyde components.[26] However, the aldehyde region in that pine still needed detailed structural elucidation to clarify the aldehyde coupling and cross-coupling reactions involved. Minor aldehyde peaks in the spectra were originally assigned speculatively, but were definitively identified later.[27,35,40] One of the main deductions from the initial studies[26,41] is that plants with curtailed abilities to produce traditional monolignols appeared to be able to utilize other phenolics at their disposal to produce polymeric "lignin" components. This concept was controversial,[42,43] but has garnered increasing support with each new lignin-biosynthetic-pathway mutant or transgenic that has been structurally examined.

Here we examine by NMR methods the dimerization and oligomerization of coniferaldehyde **1G** and sinapaldehyde **1S**, and the copolymerization of coniferaldehyde **1G** with coniferyl alcohol **3G** to produce synthetic lignins (DHPs). Spectral data from these model reactions are used to provide a more substantive elucidation of the structures of aldehyde moieties in lignins from CAD-deficient plants as illustrated using a gymnosperm (the mutant pine) and an angiosperm (an antisense-CAD transgenic tobacco). The result offers considerable new understanding of how hydroxycinnamaldehydes and hydroxybenzaldehydes are incorporated into various structures during lignification.

Results and discussion

NMR data on aldehyde components were gathered from several sources in order to provide the necessary database for assigning the various structures in isolated lignins. It is important to emphasize, as pointed out in a recent review on "Solution-state NMR of Lignins",[40] that ^{13}C-NMR data from "sufficiently good" model compounds will be the same as the corresponding data for such units in lignins (in the same solvent). This is because ^{13}C-NMR chemical shifts are only influenced by through-bond, and not through-space interactions except in the severe case of steric compression. Conversely, ^{1}H-NMR chemical shifts can be strongly influenced by through-space as well as through-bond interactions. Proton chemical shifts therefore need not be the same in simple models as they are in the polymer. For most lignin units, the degree of agreement has been remarkably good, implying that the shape of the molecule in the models is similar in the polymer.[40,44] However 1D proton shifts should be augmented with 2D correlation data to validate structural assignments.

Simple monomers (Tables 1 and 2) already provide the required data for modeling hydroxycinnamaldehyde and hydroxybenzaldehyde end-groups. The dehydrodimers provide better end-group models (the "B-ring" in dehydrodimers **5A**–**5B**, as well as **5D**, Fig. 1) for the 4–O- and/or 5-linkages modeled. The dehydrodimers also begin to provide the requisite detail of particularly the 8-linked aldehydes. In many ways, these are the most important models, since lignification is principally concerned with monomers reacting predominantly at their 8- or β-positions with the 4–O- or 5-positions of the growing lignin polymer. The 8–8-linked dehydrodimer **5C** is a model only for homocoupling reactions, although it might be expected to adequately model the cross-coupled unit **7C**′ (*e.g.* from the 8–β-cross-coupling of coniferaldehyde and coniferyl alcohol radicals). As will be discussed below, the actual course of the dehydrodimerization and oligomerization reactions was better studied by examining crude reaction products (using 2D NMR) of [9-^{13}C]-labeled coniferaldehyde or sinapaldehyde. The next level of detail comes from spectra of *in vitro* dehydropolymerization reactions. Finally, with the NMR data from all of these model reactions, spectra from the two CAD-deficient plants, a gymnosperm and an angiosperm, can be more fully interpreted. The result provides a better understanding of the incorporation of both hydroxycinnamaldehydes and hydroxybenzaldehydes into guaiacyl and guaiacyl–syringyl lignins, and some insight into the nature of the CAD-deficiency.

Hydroxycinnamaldehyde and hydroxybenzaldehyde end-groups

The only aldehyde structures in lignins that had been observed in NMR spectra until recent studies on mutants and transgenics were the hydroxybenzaldehyde **U** and hydroxycinnamaldehyde **V** end-groups.[6,10,45,46] These are therefore considered to be the traditional aldehyde groups in lignins. Hydroxycinnamaldehyde end-groups **V** (Fig. 1B) can arise from the incorporation of an hydroxycinnamaldehyde monomer **1** into lignin by coupling at its 4–O- or 5-positions (generally with a monolignol), or remain (as the "B-moieties") following homo-coupling of hydroxycinnamaldehydes. Hydroxybenzaldehyde units (producing benzaldehyde end-groups **U** in Fig. 1B) result from hydroxybenzaldehydes **2**.[47] It is not known whether they result directly from incorporation of hydroxybenzaldehyde monomers **2** (vanillin and syringaldehyde) into lignin, or are produced post-lignification from hydroxycinnamaldehyde **V** (Fig. 1B) units in lignin; the former is strongly implicated in the current work, but is not necessarily exclusive.

The chemical shifts of several key peaks of hydroxycinnamaldehyde **1** and hydroxybenzaldehyde **2** monomers and dehydrodimers are important to identify aldehyde end-group structures in lignins (Tables 1 and 2). Coniferaldehyde **1G** and sinapaldehyde **1S** have coincident 9-carbon peaks at ~194 ppm; the 7-proton chemical shifts are also similar (7.57 and 7.55 ppm). Since sinapaldehyde is symmetrical, the 2- and 6-carbons are coincident at 107.4 ppm, but coniferaldehyde has different chemical shifts for carbons 2 (112.0 ppm) and 6 (124.7 ppm). As seen in Tables 1 and 2, these coniferaldehyde chemical shifts are similar in 4–O–β and 5–β/4–O–α (phenylcoumaran) etherified models for cross-coupled coniferaldehyde units in dimeric models, and in the coniferaldehyde dehydrodimers **5AGG**, **5BGG**, and **5DGG** (the underline represents the terminal, or "B-unit" of the dehydrodimer, see Tables 1 and 2). We don't have similarly etherified models for sinapaldehyde **1S**, but suspect that the crucial 9, 7, 2 and 6 shifts (that are seen in diagnostic ^{13}C–^{1}H correlations, see later) also do not vary significantly. Vanillin **2G** and syringaldehyde **2S** also have coincident 7-carbon peaks at 191.1 ppm, but syringaldehyde has its 2/6 proton peak at 7.15 ppm, whereas vanillin shows marginally different proton peaks for protons 2 and 6 (7.41 and 7.44 ppm). Vanillin end-units **UG** have more diagnostic shifts when 5-substituted (in 5–5, 5–O–4, or presumably 5–β structures), so these types of units can potentially be differentiated. However, the lack of separation between syringyl vs guaiacyl hydroxycinnamaldehyde or hydroxybenzaldehyde carbon shifts makes it clear that 1D ^{13}C-NMR spectra will not be sufficient for distinguishing between these components; 2D NMR methods exploiting other (correlatable) differences are required.

Hydroxycinnamaldehyde dimers (and pathways from quinone methide intermediates)

Structures **5** (Fig. 1A) resulted from homo-coupling of hydroxycinnamaldehydes. The coniferaldehyde dehydrodimers except structures **5D**, **5BcGG** (see below) and the trimer **6GGG**

Table 1 ^{1}H-NMR data for (un-acetylated) hydroxybenzaldehyde and hydroxycinnamaldehyde monomers (acetone-d_6)

		δ_H							
Model[a]	DB#[b]	**7**	**8**	**9**	**2**	**5**	**6**	OMe	ArOH
1P, *p*-Coumaraldehyde	152	7.58 (d, 15.8)	6.62 (dd, 15.8, 7.7)	9.64 (d, 7.7)	6.94 (m)	7.61 (H-3,5, m)	6.94 (m)	—	9.00 (br s)
1P–(4-OMe)	215	7.60	6.65	9.66	7.02	7.68	7.02	—	—
1G, Coniferaldehyde	144	7.57 (d, 15.8)	6.67 (dd, 15.8, 7.8)	9.64 (d, 7.8)	7.38 (d, 2.0)	6.92 (d, 8.2)	7.21 (dd, 8.2, 2.0)	3.93 (s)	8.23 (br s)
1G–(4–O–β)*e*	3011	7.58	6.79	9.65	7.38	7.11	7.27	3.90	—
1G–(4–O–β)*t*	3010	7.59	6.70	9.66	7.40	7.15	7.25	3.91	—
1G–(5–β/4–O–α)	2021	7.59	6.65	9.63	7.29	—	7.32	3.91	—
1G–(5–5)	3033	7.59	6.68	9.65	7.36	—	7.27	3.95	?[c]
1S, Sinapaldehyde	153	7.55 (d, 15.8)	6.69 (dd, 15.8, 7.7)	9.63 (d, 7.7)	7.08 (s)		7.08 (s)	3.90 (s)	8.00 (br s)
2P, *p*-OH-Benzaldehyde	14	9.84 (s)			7.79 (d, 8.7)	7.00 (d, 8.7)	7.79 (d, 8.7)		9.40 (br s)
2P–(4–OMe)	203	9.86	—	—	7.85	7.08	7.85	—	—
2G Vanillin	15	9.81 (s)	—	—	7.46 (d, 1.8)	7.00 (d, 8.6)	7.43 (m)	3.91 (s)	8.30 (br s)
2G–(4–O–α)	258	9.81	—	—	7.46	7.15	7.39	3.97	—
2G–(5–β/4–O–α)	3061	9.82	—	—	7.42	—	7.53	3.92	—
2G–(4–O–Me/5–5)	71	9.96	—	—	7.58	—	7.44	4.01	—
2G–(4–O–5/5–O–4)	2049	9.86	—	—	7.40	—	6.88	3.90	—
2G–(5–O–4)	2049	9.74	—	—	7.26	—	7.01	3.94	?[c]
2G–(5–5/4–O–β)	3062	10.06	—	—	7.65	—	7.74	4.03	—
2G–(5–5/4–O–α)	3062	10.02	—	—	7.56	—	7.70	3.91	—
2S, Syringaldehyde	41	9.81 (s)	—	—	7.21 (s)	—	7.21 (s)	3.90 (s)	8.23 (br s)
2S–(4–O–β)	228	9.90	—	—	7.25	—	7.25	3.95	—
5AGG {**1G**–(4–O–8)}	3031	7.58 (d, 16.0)	6.70 (dd, 16.0, 7.7)	9.65 (d, 7.7)	7.49 (d, 2.0)	6.82 (d, 8.3)	7.15 (dd, 8.3, 2.0)	3.99 (s)	—
5B*c*GG {**1G**–(5–8/4–O–7)}	3059	7.61 (d, 15.8)	6.70 (dd, 15.8, 7.7)	9.64 (d, 7.7)	7.38 (s)	—	7.43 (s)	3.92 (s)	—
5B*o*GG {**1G**–(5–8)}	3030	7.57 (d, 15.8)	6.66 (dd, 15.8, 7.8)	9.61 (d, 7.8)	7.44 (d, 2.0)	—	7.01 (d, 2.0)	3.96 (s)	—
5DGG {**1G**–(5–5)}	3033	7.59 (d, 15.8)	6.68 (dd, 15.8, 7.8)	9.63 (d, 7.8)	7.36 (d, 2.0)	—	7.27 (d, 2.0)	3.95 (s)	—
5AGG (8–O–4)	3031	7.32 (s)	—	9.52 (s)	7.58 (d, 2.0)	6.88 (d, 8.3)	7.33 (dd, 8.3, 2.0)	3.75 (s)	8.32 (br s)
5B*c*GG (8–5/7–O–4)	3059	6.20 (d, 6.5)	4.50 (d, 6.5)	9.94 (s)	7.06 (d, 1.8)	6.84 (d, 8.2)	6.90 (dd, 8.2, 1.8)	3.82 (s)	8.29 (br s)
5B*o*GG (8–5 open)	3030	7.57 (s)	—	9.69 (s)	6.87 (d, 2.0)	6.77 (d, 8.3)	6.98 (dd, 8.3, 2.0)	3.47 (s)	8.43 (br s)
5CGG (8–8)	3032	7.78 (s)	—	9.66 (s)	7.28 (d, 2.1)	6.82 (d, 8.3)	7.21 (dd, 8.3, 2.0)	3.70 (s)	8.29 (br s)
5CSS (8–8)	3060	7.79 (s)	—	9.67 (s)	7.02 (s)	—	7.02 (s)	3.72 (s)	7.94 (br s)
6GGG {**5AGG**–(8–O–4)}	3058	7.30 (s)	—	9.48 (s)	7.52 (d, 2.0)	6.85 (d, 8.3)	7.30 (dd, 8.3, 2.0)	3.69 (s)	8.37 (br s)
6GGG {(4–O–8)–**5AGG**}	3058	7.33 (s)	—	9.53 (s)	7.67 (d, 2.0)	6.79 (d, 8.3)	7.28 (dd, 8.3, 2.0)	3.83 (s)	—
6GGG {**1G**–(4–O–8)+}	3058	7.58 (d, 15.9)	6.70 (dd, 15.9, 7.7)	9.65 (d, 7.7)	7.48 (d, 2.0)	6.82 (d, 8.3)	7.15 (dd, 8.3, 2.0)	3.98 (s)	—

[a] See Fig. 1 for compound numbers and **P**, **G**, **S** designation. In dimers or trimers, ring designations are left to right as drawn; the underlined unit is the one with the data shown—*e.g.* **6GGG** is data for the first (free-phenolic) moiety of trimer **6**. [b] Data are from, or have been entered into, the "NMR Database of Lignin and Cell Wall Model Compounds."[66] Compound sources are given there. [c] Data should exist but are not seen and are not reported in the Database.

were previously isolated by Connors *et al.*,[12] but insufficient NMR data were provided. Quinone methide intermediates have important roles in determining the final products of hydroxycinnamaldehyde coupling, just as they are important intermediates in the biosynthesis and degradation of lignin.[48–52] When coniferaldehyde **1G** 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.[48] Addition of water to quinone methides is involved when there are no favorable internal trapping mechanisms, or there is no acidic 8-proton; water therefore adds to the quinone methide β–O–4-coupling product between two hydroxycinnamyl alcohols **3** or an hydroxycinnamyl alcohol and a lignin dimer or higher oligomer. In the case of the hydroxycinnamaldehydes **1**, however, the quinone methide has new options. The resultant 8-proton is particularly acidic (even more acidic than in ferulate analogs) because of the aldehyde group; elimination of the 8-proton allows re-aromatization. From coniferaldehyde **1G** therefore, the 8–O–4-dimer produced is **5AGG** (Fig. 1A),[12] without addition of water at the 7-position of the quinone methide intermediate—the addition of water to the quinone methide cannot compete with the faster 8-proton elimination. An 8–O–4/8–O–4-trimer **6GGG** was also isolated here, confirming that 8–O–4-coupling to another monomer or to a preformed dimer can occur. The 8–8-dimer **5C** similarly regains 7,8-unsaturation by 8-proton elimination from the two quinone methide moieties in the same manner as the 8–O–4-dimer. The products are both analogous to those produced by ferulate where the intermediate quinone methides also have acidic 8-protons.[53]

Table 2 ^{13}C-NMR data for (un-acetylated) hydroxybenzaldehyde and hydroxycinnamaldehyde monomers (acetone-d_6)

		δ_C									
Model[a]	DB#[b]	**1**	**2**	**3**	**5**	**5**	**6**	**7**	**8**	**9**	OMe
1P, *p*-Coumaraldehyde	152	126.7	131.5	116.8	161.2	116.8	131.5	153.6	127.0	193.8	—
1P–(4–OMe)	215	127.9	131.3	115.4	163.1	115.4	131.3	153.2	127.4	193.8	—
1G, Coniferaldehyde	144	127.5	111.7	150.8	148.8	116.2	124.7	153.9	127.0	193.8	56.4
1G–(4–O–β)*e*	3011	130.2	112.9	152.1	151.2	118.5	123.9	153.5	128.5	194.1	56.4
1G–(4–O–β)*t*	3010	129.9	112.6	151.7	151.7	117.9	123.8	153.3	128.2	193.6	56.4
1G–(5–β/4–O–α)	2021	131.2	113.6	145.7	152.4	129.0	119.6	154.1	127.1	193.8	56.5
1G–(5–5)	3033	126.1	110.0	149.4	126.1	149.6	127.1	154.4	126.8	193.9	56.5
1S, Sinapaldehyde	153	126.2	107.4	149.0	140.3	149.0	107.4	154.2	127.3	193.7	56.7
2P, *p*-OH-Benzaldehyde	14	130.3	132.8	116.6	163.8	116.6	132.8	191.0	—	—	—
2P–(4–OMe)	203	131.1	132.4	115.2	165.4	115.2	132.4	191.1	—	—	56.0
2G, Vanillin	15	130.6	110.8	148.9	153.5	115.9	127.0	191.1	—	—	56.3
2G–(4–O–α)	258	132.1	111.4	151.6	153.4	115.9	126.0	191.3	—	—	56.3
2G–(5–β/4–O–α)	3061	132.4	113.4	145.7	154.9	131.2	121.4	190.9	—	—	56.4
2G–(4–O–Me/5–5)	71	133.2	111.9	153.0	154.3	133.0	127.2	191.5	—	—	56.4
2G–(4–O–5/5–O–4)	2049	134.8	108.0	155.0	138.3	153.7	109.9	191.7	—	—	56.9
2G–(5–O–4)	2049	128.7	108.4	149.7	143.0	147.2	110.2	191.0	—	—	56.8
2G–(5–5/4–O–β)	3062	134.4	111.7	154.2	152.5	133.3	125.3	191.7	—	—	56.6
2G–(5–5/4–O–α)	3062	134.6	111.9	154.4	152.8	133.7	125.2	191.7	—	—	56.3
2S, Syringaldehyde	41	129.0	107.7	148.9	142.9	148.9	107.7	191.1	—	—	56.6
2S–(4–O–β)	228	133.3	107.2	154.4	141.8	154.4	107.2	191.7	—	—	56.6
5AGG {**1G**–(4–O–8)}	3031	130.2	112.6	150.4	148.9	115.3	123.6	153.2	128.3	193.9	56.5
5B*c*GG {**1G**–(5–8)}	3059	129.7	114.3	146.0	151.8	125.8	119.5	153.5	127.7	193.8	56.5
5B*o*GG {**1G**–(5–8)}	3030	127.4	110.6	149.3	148.6	136.3	126.4	153.8	127.3	194.0	56.6
5DGG {**1G**–(5–5)}	3033	126.1	110.0	149.4	126.1	149.6	127.1	154.4	126.8	193.9	56.5
5AGG (8–O–4)	3031	125.3	114.0	148.5	150.6	116.3	126.8	138.2	147.7	187.5	56.0
5B*c*GG (8–5/7–O–4)	3059	132.2	110.7	147.9	148.6	115.9	119.8	85.2	62.9	197.6	56.3
5B*o*GG (8–5 open)	3030	127.4	113.6	148.1	150.2	116.0	126.9	151.4	123.2	193.4	55.6
5CGG (8–8)	3032	127.5	113.7	148.4	150.5	116.2	126.3	152.8	134.4	192.7	56.1
5CSS (8–8)	3060	126.1	109.1	148.7	140.1	148.7	109.1	153.0	134.8	192.7	56.5
6GGG {**5AGG**–(8–O–4)}	3058	125.2	114.0	148.5	150.6	116.3	126.8	138.3	147.5	187.4	55.9
6GGG {(4–O–8)–**5AGG**}	3058	128.0	114.9	149.9	148.5	115.2	125.6	137.2	148.5	187.7	56.1
6GGG {**1G**–(4–O–8)+}	3058	130.4	112.6	150.4	148.7	115.4	123.6	153.1	128.4	193.9	56.5

[a] See Fig. 1 for compound numbers and **P**, **G**, **S** designation. In dimers or trimers, ring designations are left to right as drawn; the underlined unit is the one with the data shown—*e.g.* **6GGG** is data for the first (free-phenolic) moiety of trimer **6**. [b] Data is from, or has been entered into, the "NMR Database of Lignin and Cell Wall Model Compounds."[66] Compound sources are given there.

Useful insight into the relative amounts and the nature of the dimers was gained from small-scale (5–10 mg) experiments using synthesized [9-^{13}C]-labeled coniferaldehyde or sinapaldehyde. Assignments for well-characterized peaks 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 HMQC and HMBC experiments where the aldehyde carbonyl region could be selectively and quickly acquired.

The 8–5-coupled dehydrodimer isolated by Connors *et al.* was the non-cyclic dimer **5B*o*GG**.[12] It has therefore since been considered that the 8-proton elimination (pathway a, Fig. 2) was faster than the internal trapping of the quinone methide (pathway b). We have discovered by running NMR spectra of the crude reaction products from [9-^{13}C]-labeled coniferaldehyde that, in biomimetic peroxidase/H_2O_2 reactions at neutral pH, the 8–5-cyclic phenylcoumaran **5B*c*** is the kinetic product, not the open 8–5-dimer **5B*o***. This cyclic phenylcoumaran product **5B*c*** is analogous to those formed from coniferyl alcohol or ferulate.[53] Rearomatization of the B-moiety is therefore faster than 8-proton elimination of the A-moiety, so the result is the intramolecular nucleophilic quinone methide trapping reaction.

Proof of the structural assignment for the phenylcoumaran 8–5-product **5B*c*** is shown in Fig. 3. Fig. 3A is a partial HMBC spectrum, showing just the aldehyde carbonyl carbon region, of crude products (dimers **5** plus possibly oligomers **7**) from a peroxidase/H_2O_2 reaction of coniferaldehyde **1G**. It shows nicely resolved aldehyde ^{13}C resonances (also seen from the data in Table 2) with diagnostic ^{13}C–^{1}H long-range correlations whereby the various dehydrodimeric units **7** and **V** were readily assigned. Unit **7B*c*GG** shows clear correlations from the aldehyde carbonyl carbon at 197.6 ppm with two aliphatic protons at ~6.3 and ~4.5 ppm, a diagnostic fingerprint for the phenylcoumaran structure (and agreeing with the data for the **5B*c*GG** dimer in Tables 1 and 2). Fig. 3B shows a more complete HMBC spectrum of the crude product in which all of the correlations for **5B*c*/7B*c*** are highlighted. Extensive long-range correlations between proton A7 and carbons A9, B4, A1, B5, A6, A2, and A8 proves the cyclic nature of the product; such long-range correlations are over either 2- or 3-bonds so the correlations between proton A7 and carbons B4 and B5 are diagnostic for the 5-membered phenylcoumaran ring. It is therefore well established that, under the dehydrogenation conditions used here, it is the cyclic form of the 8–5-dehydrodimer **5B*c*** that forms from coniferaldehyde.

This cyclic phenylcoumaran structure is, however, not as stable as other dimers. Attempted isolation of **5B*c*** from the mixture by using routine TLC produced mainly the ring-opened elimination product, dimer **5B*o***. Isolation by flash column did provide a product rich in the cyclic product for NMR, but certainly not free of other components. Ensuring that the crude coniferaldehyde dehydrodimers were not excessively dried helped maintain the phenylcoumaran structure **5B*c*** and it could be successfully isolated in substantial portions even by TLC. Acetylation of the dehydrodimer mixture also converted the phenylcoumaran **5B*c*** to the (acetate of) acyclic dimer **5B*o*** by the mechanism assumed (again *via* the quinone methide) in Fig. 2 (also see Fig. 4J where the cyclic component in Fig. 4E has disappeared). HMBC spectra show the disappearance of the easily recognizable correlations of the phenylcoumaran (Fig. 3) after acetylation (not shown). Even dissolving the crude mixture in acetone–water for the spectrum in Fig. 4F appears to have destroyed some of the cyclic component (although this is

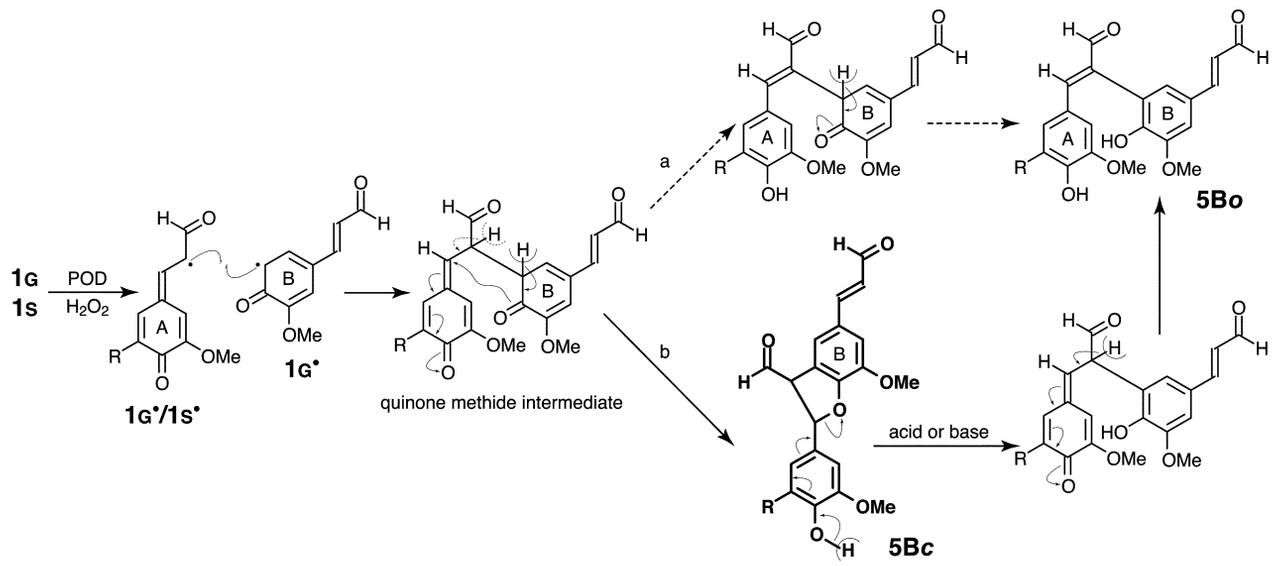

Fig. 2 Pathways to 8–5-coupled dehydrodimers **5B**. Pathway a is the previously accepted pathway, with 8-proton elimination being faster than re-aromatization of the B-ring and the internal phenolate trapping of the quinone methide, producing the acyclic product **5B*o***. However, the cyclic phenylcoumaran 8–5-dimer **5B*c***, is the kinetic product observed here at neutral pHs from coniferaldehyde *via* peroxidase/H_2O_2 indicating that rearomatization and quinone methide trapping is faster than 8-proton elimination pathway b. Dehydrodimer **5B*c*** however 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-dehydrodimer **5B*o***. Analogous ring-opening is likely to occur under acidic conditions. Only if the dehydrodimer is etherified before isomerizing will the phenylcoumaran structure be preserved in the polymer. There is some evidence from the spectra in Fig. 4 that the cyclic form may exist in isolated (and presumably *in situ*) lignins.

from a different reaction than Fig. 4E). The point to be made here is that the course of the coupling and rearomatization reactions favors formation of the phenylcoumaran structure (as the kinetic product), whereas subsequent reactivity causes the structure to open (to give the thermodynamically more stable product). It is assumed that if the cyclic phenylcoumaran product becomes etherified as such during further lignification reactions, the cyclic form would then be stable. There is tentative evidence for the cyclic coniferaldehyde-8–5-structure in plant isolates (see discussion below under Lignin Spectra).

Unlike with coniferyl alcohol where the overwhelmingly major products are produced by coupling reactions at the β-position, coniferaldehyde (like its ferulate analog) will undergo 5-coupling reactions. Thus the 5–5-dehydrodimer **5D** is observed in the product mixtures (~13% of the purified products). Presumably cross-coupling reactions between coniferaldehyde **1G** and guaiacyl units **4G** can also produce 5–5-units. As is normal with such units, they are integrated into the polymer *via* further coupling reactions. Coupling is still possible at the 8-position to form 8–O–4-ethers. 4–O-Coupling is also possible, either from the cinnamaldehyde directly or following 8–O–4-ether formation, to produce dibenzodioxocins,[40,54] for example. It is not yet known to what extent 5-coupled coniferaldehyde units retain their double bond and therefore show up as structure **V** in lignin spectra.

Dehydrogenation polymers (DHPs)

The next step in modeling lignins is the introduction of aldehydes into polymeric systems, dehydrogenation polymers (DHPs), by methods which somewhat mimic lignification. Even with the slow addition of monomers, the polymerization is largely a bulk polymerization with too many dimerization events rather than the cross-coupling of monomers with the growing polymer.[40,47,55] Nevertheless, all the correct coupling and cross-coupling products that are found in lignins are usually produced, just in different ratios.[40,56] To gather the required NMR data, it is not usually necessary to resort to more time-consuming methods to enhance cross-coupling reactions such as diffusion of monomers through dialysis membranes.[55,57] Simple *in vitro* DHP methods were used here to produce polymers to allow acquisition of the 1D ^{13}C-NMR spectra shown in Figs. 4G and K (as well as for 2D experiments, not shown).

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. 4. The direction of the shifts upon adding water (necessary for the solution of unacetylated lignins) is illustrated with the crude dehydrodimeric products (Figs. 4E–F) and the copolymer DHP (Fig. 4G). Dotted assignment lines in Fig. 4 have been authenticated by further 2D correlation experiments. Once the models and the lignins are acetylated, the data coincide much more closely (right-hand plots in Fig. 4). The aldehyde-carbon regions of spectra from lignin isolates are also shown in Fig. 4.

In synthetic cross-coupling reactions, as seen in the DHP example, Fig. 4G, that copolymerizes [9-^{13}C]-coniferaldehyde **1G** (10%) with coniferyl alcohol **3G** (90%), one feature of the synthetic lignification is notable: most of the coniferaldehyde appears *not* to have coupled at its 8-position. The dominant aldehyde end-group peak **V** 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 entirely reflected in the isolated lignins, but similar details could be observed in the CAD-deficient pine lignin. The discrepancy likely results from the considerably more "bulk" nature of synthetic lignification. With excess coniferyl alcohol in the system, the likely cross-coupling reaction is between coniferaldehyde **1G** and coniferyl alcohol **3G** monomer radicals. As has been seen countless times, coniferyl alcohol overwhelmingly couples at its β-position in cross-coupling reactions. Although coniferaldehyde may also couple at its 8-position, as seen from the (unauthenticated) **7C′** peak, it more commonly couples at the 4–O- or 5-position. As a result, most coniferaldehyde remained as end-groups as evidenced by the large **V** peak in the synthetic lignin, Figs. 4G and K.

A new product remains unauthenticated at present but is thought to be the 8–β′-cross-product **7C′** (Fig. 1). If so, the minor change in its chemical shift indicates that, like the 8–8-coupled dimer **7C**, 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 which is

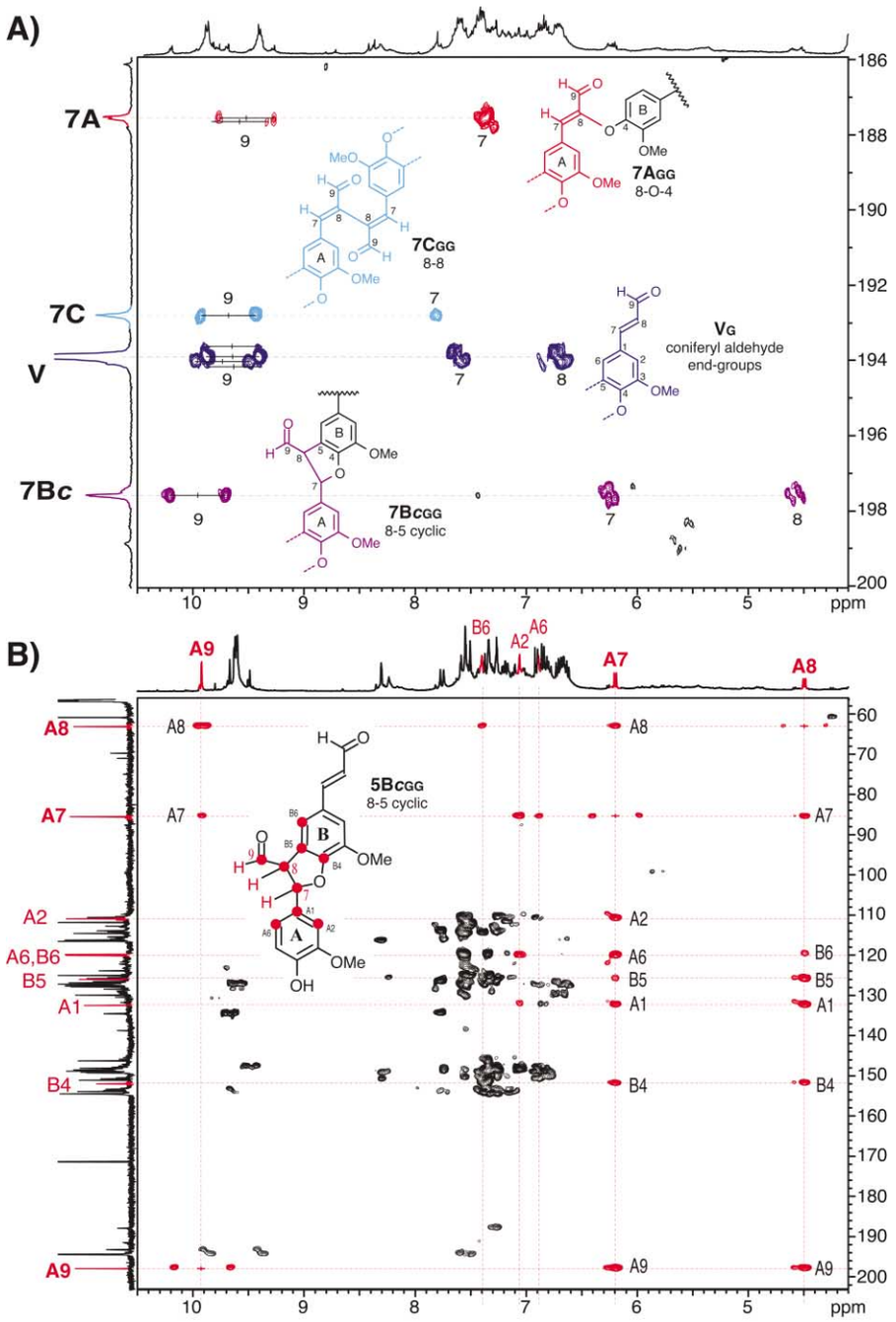

Fig. 3 A) NMR evidence for the dimer/oligomer assignments of aldehyde ^{13}C NMR peaks to structures **7A**–**C** and **V** (Fig. 1), and proof that the 8–5-dimer is in the cyclic phenylcoumaran form following peroxidase-mediated radical coupling at neutral pH. The sample is the crude dimer fraction formed using [9-^{13}C]-labeled coniferaldehyde. 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- **7A** and 8–8-units **7C** are clearly unsaturated as shown by their correlation with single 7-protons. End-units **V** (which also derive from the B-moieties of dehydrodimers **5A** and **5B**, and from **5D**) show correlations to both unsaturated sidechain protons (7 and 8). The 5–5-dimer **5D** has the same correlations as the end-units **V**. The coincident chemical shifts of both carbons and protons make it virtually impossible to differentiate the 5–5-structure from other end-units. The 8–5-unit **7B*c*** shows typical correlations of the (cyclic) phenylcoumaran structure. B) Further proof for the cyclic form of the 8–5-coupled dehydrodimer in the crude mixture of dehydrodimers from the extensive full sidechain HMBC correlations. The correlations between proton A7 and carbons B4 and B5 in particular prove the cyclic phenylcoumaran structure.

internally trapped by the phenolic-OH, 8-proton elimination is faster here than the internal trapping by the primary alcohol.

The copolymer synthetic lignin (Figs. 4G and K) also contains 8–O–4-coupled aldehyde dehydrodimeric units **7A**, as well as the 8–O–4-cross-product **7A′** that was not seen in the coniferaldehyde-only reactions (Figs. 4F and J). Units **7A′** are from cross-coupling of an hydroxycinnamaldehyde radical with a radical from a preformed lignin oligomer (B-moiety) which is likely to be 5–5-or 4–O–5-structures in this DHP (as described in the following section on lignins).

Lignin spectra

CAD-downregulated tobacco transgenic. Well-dispersed aldehyde peaks at 180–200 ppm in the ^{13}C-NMR spectra show considerable differences between lignins from the CAD-deficient transgenic (Figs. 4I and M) and the control (not shown) as has been well documented.[27,35,40] The control lignin had the normal cinnamaldehyde **V** and benzaldehyde **U** end-group structures, but peaks labeled **7C**, **7A**, and **7A′** (Figs. 4I and M) were new in the transgenic's lignin. The basic assignments for the acetylated

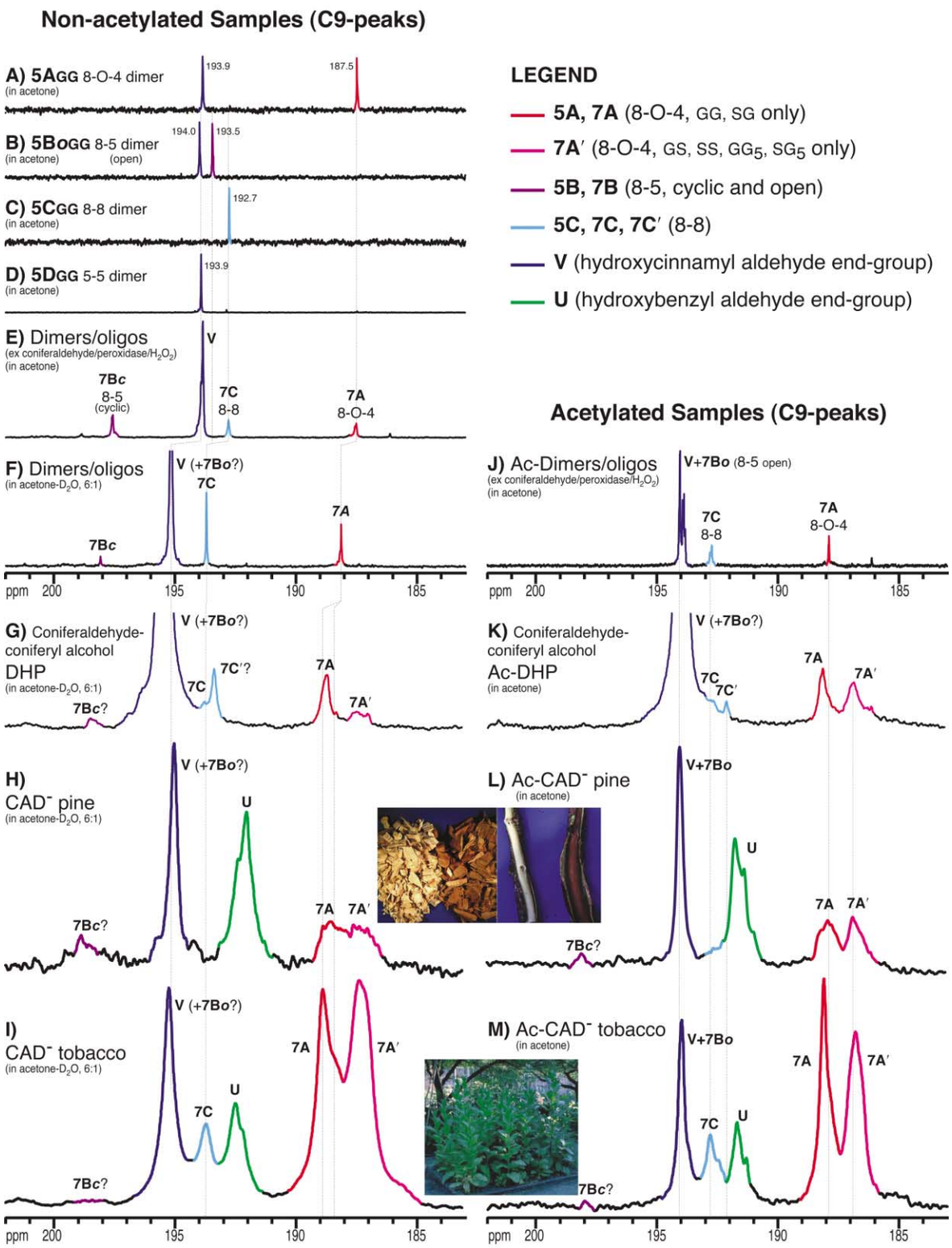

Fig. 4 Aldehyde regions (180–200 ppm) of ^{13}C-NMR spectra of synthetic dehydrodimers, oligomers and lignins, and isolated lignins from a CAD-deficient transgenic tobacco and a mutant pine. Left: aldehyde sub-regions of ^{13}C-NMR spectra of: A)-D) various coniferaldehyde dimers (relating to structures in Fig. 1) run in acetone-d_6; crude mixtures of dimers resulting from low-extent oligomerization of [9-^{13}C]coniferaldehyde with horseradish peroxidase and H_2O_2 in E) acetone-d_6 and F) acetone-d_6–D_2O, 6 : 1 (note: this was from a different reaction than the one above it); G) a synthetic lignin (DHP) prepared from [9-^{13}C]coniferaldehyde (10%) and coniferyl alcohol (90%) in 6 : 1 acetone-d_6–D_2O; H) an isolated lignin from a CAD-deficient pine mutant; and I) an isolated lignin from uniformly ^{13}C-enriched (~13%) antisense-CAD-downregulated tobacco. Note that radical coupling of coniferaldehyde *via* peroxidase/H_2O_2 clearly produces the cyclic (phenylcoumaran) 8–5-dimer corresponding to structure **5B*c*/7B*c***. We do not currently have a model compound for the 8-β′-cross-product **7C**′ seen in the copolymer DHP in G, so this peak remains unauthenticated. If it is the 8-β′-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 9-OH (as occurs in 8–8-coupling of coniferyl alcohol to pinoresinol). Substantial solvent and matrix-dependent shifts are noted for the unacetylated dimers, oligomers, and 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 authenticated by further correlation experiments (not shown) on the lignin samples. 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 are verified (and further authenticated by diagnostic correlations in various 2D NMR experiments). Note that the cyclic phenylcoumaran form of the 8–5-dimer **5B*c*** converts to the opened product **5B*o*** upon acetylation (see Fig. 2) except, presumably, when it becomes etherified during lignification—traces appear to remain in the DHP and the lignins.

(Fig. 4M) and unacetylated (Fig. 4I) lignins were readily made by comparison with model and DHP data in Fig. 4, and *via* HMBC spectra with characteristics analogous to those in Fig. 3A for the DHP. The major types of units are distinguishable, with long-range correlations yielding information about their nature. Unfortunately, the 8–5-units have overlapping or ambiguous correlations in all spectra and cannot therefore be unambiguously identified here.

Cinnamaldehyde end-groups, V. These end-groups can arise from two sources. They may arise from preliminary dehydrodimerization reactions of coniferaldehyde or sinapaldehyde, forming dehydrodimers **5A**, **5B** or **5D** which retain the unsaturated cinnamaldehyde sidechain. In the case of **5A** and **5B**, these end-group units are now etherified and will not therefore be able to undergo further coupling reactions at their 8-positions. Dimer **5D** is unique in that it can still undergo 8-coupling reactions, so end-groups **V** would only result from dimer **5D** if the subsequent polymerization reaction was cross-coupling with a monolignol to form a dibenzodioxocin (etherified) structure. Alternatively, end-groups **V** result as the B-rings from cross-coupling reactions of hydroxycinnamaldehydes **1** at their 4–O- or 5-positions with monolignols (at their β-positions), or possibly with a preformed lignin oligomer at its 4–O- or 5-positions. Not all of these possibilities can be distinguished with the NMR data available. The long-range correlations in dark blue starting from the aldehyde carbonyl carbon in units **V** identify proton-7 at ~ 7.6 ppm, Fig. 5A. This proton correlates with carbons in the aromatic ring, allowing a distinction between syringyl and guaiacyl units. The observations of a strong S2/6 carbon correlation (at 107.4 ppm) and the guaiacyl G2 (111.7 ppm) and G6 (124.7 ppm) carbon correlations indicate that both coniferaldehyde and sinapaldehyde have been incorporated into this transgenic's lignin in end-group units **V**.

Benzaldehyde end-groups, U. As noted above from spectra of the monomers, the 2- and 6-protons correlating to the aldehyde carbonyl, carbon-7, in benzaldehydes **U**, are resolved with the syringyl protons having lower chemical shifts. It is readily apparent from the HMBC spectrum in Fig. 5A that most of the benzaldehydes **U** in the CAD-deficient tobacco lignin arise from syringaldehyde. The huge HMBC correlation (centered at 191.7/7.21 ppm) between the aldehyde carbon and the S2/6 proton of syringaldehyde units **U**s was enhanced in the transgenic over the normal tobacco lignin (not shown). The green contours labeled **U**s show expected correlations to S2/6 protons at ~108 ppm and no obvious guaiacyl G2 or G6 correlations, confirming the syringyl nature of those aldehydes. (Strictly, 5–O–4-linked vanillin units will have resonances masked by the syringyl units and appear in the green colored peaks, so these may not be resolved.) A smaller guaiacyl benzaldehyde unit **U**G is apparent with its correlation at ~191.1/7.56 ppm; the proton to ring-carbon correlations overlap with those of the G2 and G6 carbons in unit **V** described above (and colored dark blue). It appears that sinapaldehyde **1S** has been rather substantially converted to syringaldehyde **2S** and incorporated into this lignin fraction, whereas coniferaldehyde **1G** has mainly been incorporated as such and less effectively converted to, or incorporated as, its vanillin **2G** counterpart. That vanillin units will incorporate into lignins if available is evidenced by its significant incorporation in the pine lignin, Fig. 5B (see below).

8–8-Coupled units, 7C. Sinapaldehyde **1S**, like sinapyl alcohol **3S**, favors 8–8-coupling, in part because there are fewer options than for coniferaldehyde **1G** (which has an additional 5-position available for radical coupling). From the HMBC spectrum (Fig. 5A), only 8–8-sinapaldehyde structures were detected in the CAD-deficient transgenic tobacco lignin. The cyan-colored correlation labeled **7C** between the 9-carbon and 7-proton was weak but the correlation between the 2/6-carbon and 7-proton was as pronounced as others. We could detect no traces of coniferaldehyde 8–8-structures (Fig. 4A, cyan crosses) as detected in synthetic lignins (Fig. 4K).

8–O–4-Coupled units, 7A and 7A′. Two significant 8–O–4-peaks **7A** and **7A′** at 188.1 ppm and 186.8 ppm in the ^{13}C-NMR were the primary new features identified in the transgenic tobacco plant (Figs. 4M and 5A). When the B-ring is a normal guaiacyl unit (arising from dehydrodimerization of coniferaldehyde, or from cross-coupling of either coniferaldehyde or sinapaldehyde with a lignin guaiacyl unit) the carbon is at ~188.1 ppm, with the correlated 7-proton centered at 7.28 ppm, as in the red contour labeled **7A** in Fig. 5A. When the B-ring is a syringyl unit, or possibly a 5-substituted guaiacyl unit, as we hope to establish later, the carbon is at ~186.8 ppm, with the correlated 7-proton centered around 6.7 ppm, as in the magenta and dark red contours labeled **7A′** in Fig. 5A. As has been communicated more fully elsewhere,[34] the 8–O–4-syringyl component **7A′**, is derived from coupling reactions involving both coniferaldehyde **1G** (magenta G2 and G6 carbon correlations at 115.2 and 124.0 ppm) and sinapaldehyde **1S** (dark red S2/6 carbon correlations at 108.1 ppm). The clear implication is that both coniferaldehyde **1G** and sinapaldehyde **1S** will cross-couple with syringyl units **4S** in the growing polymer to form 8–O–4-structures. On the other hand, the 8–O–4-guaiacyl component **7A** derives only from a coupling reaction involving sinapaldehyde (see red S2/6-carbon correlations at 108.1 ppm) and not coniferaldehyde (see the red crosses where correlations would be present, as seen later in the CAD-deficient pine of Fig. 5B). Here the implication is that sinapaldehyde **1S** but not coniferaldehyde **1G** will cross-couple with guaiacyl units **4G** in the growing polymer. And as noted previously,[34,35] this apparent selectivity is explained by nothing more than simple chemical cross-coupling propensities—coniferaldehyde will not readily cross-couple with guaiacyl units *in vitro* either. It will however dehydrodimerize to **5AGG**. Presumably this is the source of the **7A** peaks in the crude coniferaldehyde dimers (Fig. 4J) and in the copolymer DHP (Fig. 4K). The absence of coniferaldehyde-8–O–4-guaiacyl peaks **7A** in this tobacco lignin however suggests that dehydrodimerization of coniferaldehyde is not a significant reaction occurring during lignification, and that most of the aldehydes incorporated into 8–O–4-structures are therefore the result of endwise cross-coupling reactions of a monomer with the growing polymer. Such reactions of monolignols are the foundations of lignification and indicate that the aldehyde monomers are incorporating into these lignins analogously to the traditional monolignols and are therefore likely to be true "lignin" monomers.

CAD-deficient pine mutant. Despite having a residual CAD activity of less than 1% of normal levels, incorporation of hydroxycinnamaldehydes into the CAD-deficient pine mutant's lignin was relatively low compared to the CAD-downregulated transgenic tobacco lignin. Most of the aldehydes remained as simple extractable, non-polymeric components.[36,58] The major unanticipated incorporation of dihydroconiferyl alcohol (DHCA)[26] and its guaiacylpropane-1,3-diol (GPD) derivative[59] complicates this analysis.

Aldehyde levels in the mutant pine's soluble lignin fraction were enhanced over the control.[26] The increase was mainly due to increased hydroxycinnamaldehyde **V** and hydroxybenzaldehyde **U** end-group units. Smaller peaks labeled **7A** and **7A′**, analogous to those found in tobacco were also evident, Figs. 4H, 4L and 5B; here they appear enhanced over those in the spectra originally published[26] as a result of more extensive EDTA-washing (and possible fractionation).

Cinnamaldehyde end-groups, V. Typical hydroxycinnamaldehyde end-groups **V** were observed to be present due to the peak at 194.0 ppm in the ^{13}C NMR spectra of acetylated lignin (Figs. 4L, 5B). In this case, pine being a softwood, the units do not derive from sinapaldehyde (as seen by the absence of S2/6 carbon correlations in Fig. 5B, and as noted in the tobacco spectrum of Fig. 5A). The guaiacyl correlations are more complex (and overlap with the benzaldehyde **U** correlations) but

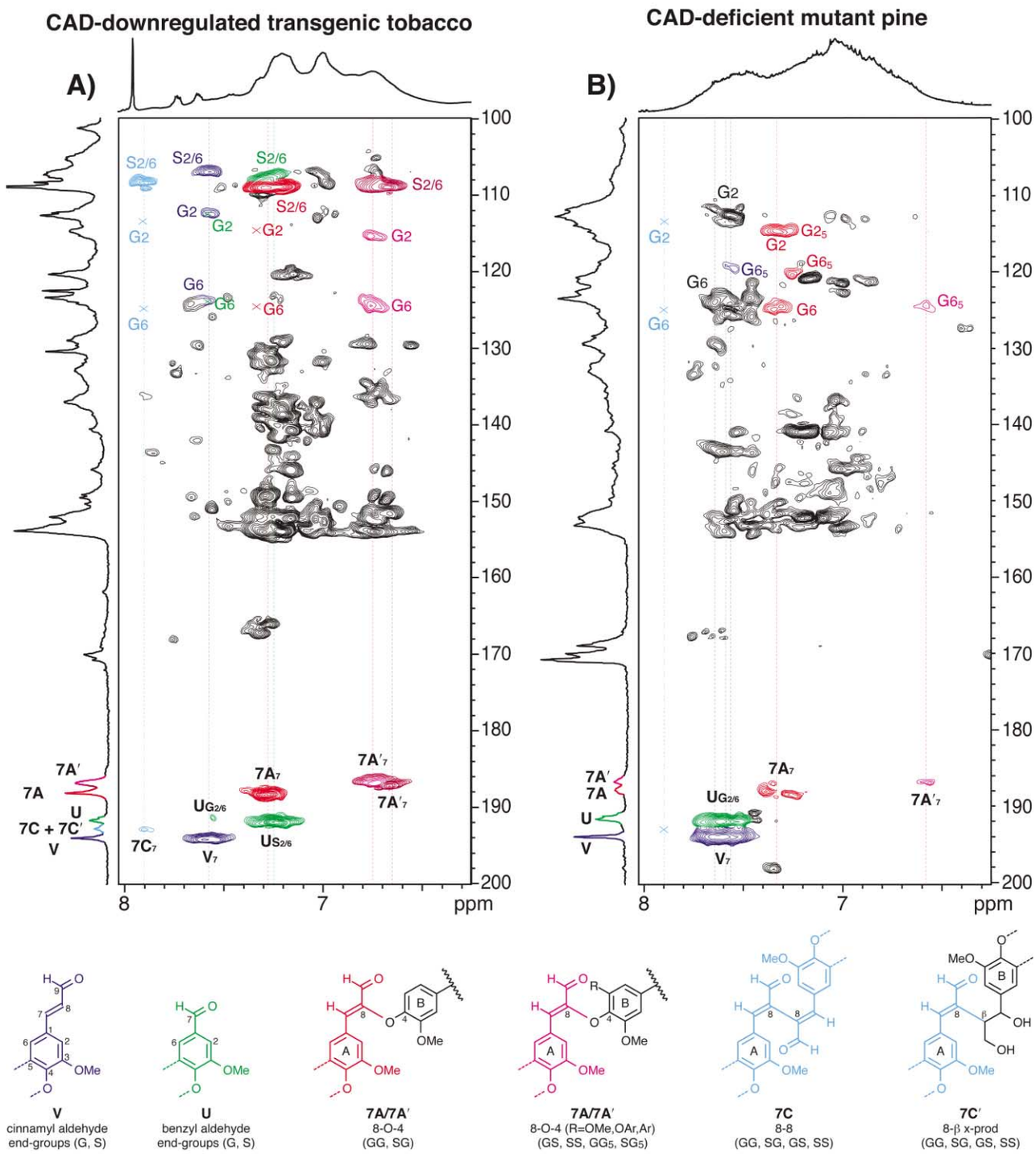

Fig. 5 Partial HMBC spectra of lignins from the CAD-deficient A) tobacco transgenic and B) mutant pine, allowing the syringyl/guaiacyl nature of the various dehydrodimers and cross-products to be assigned. See the text for details. Subscripts 5 indicate a 5-linkage on the unit. G2 and G6 assignments for the **U**G and **V** units in the pine lignin, Fig. 5B, overlap too severely to allow accurate color-coding of these peaks.

appear to be assignable mainly to G2 and G6 carbons in both 4–O-etherified and 5–β/4–O–α- (phenylcoumaran) structures as indicated in Fig. 5B (from the data in Tables 1 and 2).

*Benzaldehyde end-groups, **U**.* The benzaldehyde **U** aldehydic 7-carbon was at 191.7 ppm, and correlates strongly with the ring 2/6 protons over a broad range centered at about 7.6 ppm (and therefore overlapping in the proton dimension with **V**-units). These can be mainly attributed to vanillin units **U**G by examination of the HMBC spectrum, Fig. 5B. Thus vanillin **2**G contributes substantially to the aldehyde component in this CAD-deficient pine lignin, whereas it had only a minor contribution in the tobacco lignin (Fig. 5A).

*8–8-Coupled Units, **7C**.* There were no 8–8-structure peaks visible in the mutant pine's lignin spectra. This reinforces the idea expressed above that the main 8–8-products in the tobacco lignin derive from sinapaldehyde coupling, and that coniferaldehyde is less involved in 8–8-coupling reactions. Analogously enhanced 8–8-coupling is seen with sinapyl alcohol over coniferyl alcohol in normal hardwood *vs*. softwood lignins.[60] However, the analogous ferulates undergo extensive 8–8-homocoupling.[53] The 8–8-dehydrodimer of coniferaldehyde represents only about 1% of the dimer fraction in the model coupling reactions. The absence of 8–8-coupled products in the pine lignin can therefore be taken as an indication that a similarly low 8–8-coupling propensity occurs *in vivo* or, as in the tobacco, the aldehydes are incorporated mainly by cross-coupling rather that homo-dehydrodimerization reactions.

8–O–4-Coupled units, 7A and 7A′. The important but weak carbon peaks at 187.9 and 186.8 ppm (Figs. 4L and 5B) appear analogous to those from 8–O–4-structures in the transgenic tobacco. Indeed peak **7A** at 187.9 ppm has strong but disperse correlations (red, Fig. 5B) suggesting the presence of coniferaldehyde-8–O–4-guaiacyl structures. The correlations suggest that the unit appears in both 4–O-etherified and 5-linked units (see the correlation labeled $G6_5$). We noted above and previously,[34] however, that coniferaldehyde did not readily cross-couple with guaiacyl units in lignins. Either that is in fact what is happening here when there are fewer options (including no possibility of the favorable coupling reactions with syringyl units), or these units all arise from dehydrodimers **5A** which become incorporated into the lignin by further coupling reactions. Unfortunately that issue remains unresolved here as the NMR data do not allow cross-coupling to be distinguished from dehydrodimerization in these 8–O–4-structures **7A**. The peak labeled **7A′** at 186.8 ppm is more complex to interpret. In the tobacco spectrum it was noted that this was derived from hydroxycinnamaldehydes 8–O–4-coupled with syringyl units **4S**. Obviously, there are no syringyl units in this pine, or in the synthetic DHP which also displays a **7A′** peak. The weak correlations (in magenta) suggest a guaiacyl ring (*i.e.* derived from coniferaldehyde **1G**) as expected, but reveal nothing of the 8-coupled unit. Its elucidation and identification has been developed beyond the scope of this study and remains a focus of current research, but it is perhaps obvious to state here that 5-linked guaiacyl "B-rings" (see structure for **7A′** in Fig. 5) may sufficiently mimic syringyl units in their NMR behavior. GPD and DHCA were massively incorporated into this lignin.[26] These monomers do not have unsaturated side chains, so are limited to coupling at the aromatic ring 4–O- and 5- positions, and are expected to be mostly involved in the terminal units in lignin. These 5–5- or 4–O–5-dehydrodimers of homo- or cross-coupled GPD and DHCA are possible candidates for the syringyl-like 8–O–4-cross-coupled structures in the pine mutant's lignin; certainly the elevated 5–5-level is the explanation for the elevated dibenzodioxocin levels.[40] Peak **7A′** is therefore simply assigned here as arising *via* coniferaldehyde **1G** coupling 8–O–4 to a 5-substituted guaiacyl end-unit in the growing polymer, and provides further evidence for endwise coupling reactions incorporating coniferaldehyde into this polymer, albeit on a far smaller scale than was evident in the angiosperm, tobacco.

Implications

The phenolic polymers isolated from the mutant pine and the transgenic tobacco contain substantial aldehyde components, copolymerized by radical coupling reactions that typify lignification. The polymers contain cross-coupling products of coniferaldehyde or sinapaldehyde with lignin oligomers (*e.g.* 8–O–4-structures **7A** and **7A′**; Figs. 4 and 5) as well as possible homo-coupling products derived from dehydrodimers **5A** and **5C**. The hydroxycinnamaldehydes therefore appear to be true components that are polymerized by radical coupling mechanisms into phenylpropanoid polymers that may function as lignins. Obviously this is not the same polymer that would be produced (and can be isolated) when the plant does not have a CAD-deficiency. It appears, as originally proposed,[26,41] that the plants utilize more of the hydroxycinnamaldehyde precursors of the normal monolignols as monomers when the plant's ability to complete the biosynthesis of monolignols is impeded. Further evidence has recently come from the identification of molecular marker compounds of hydroxycinnamaldehyde-8–O–4-coupled units in transgenic poplar lignins.[32] 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 and still the major components of the transgenic and mutant lignins are the traditional monolignols. However, in the gymnosperm, it appears from the build-up of extractable hydroxycinnamaldehyde, hydroxybenzaldehyde, and DHCA monomers in the plant stems that producing the polymer from these components is not straightforward. In synthetic polymer systems too, coniferaldehyde is not as readily incorporated as coniferyl alcohol. Nevertheless, the hydroxycinnamaldehydes **1**, and their derived hydroxybenzaldehydes **2**, become a significant part of the polymer fraction.

There has been debate about whether these phenylpropanoid polymers derived from monomers other than the three traditional monolignols **3** are in fact lignin, and whether they are functioning as lignin in the plant.[42,43] The important first question answered from the observations reported here 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 aldehydes may be expected in the lignifying zone even in the absence of CAD-deficiency. The high levels of aldehydes in lignins from CAD-deficient mutants and transgenics and the incorporation of monomers by homo- and hetero-coupling reactions into polymeric fractions suggests 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,[43,61] it is logical that they will be incorporated into the polymer, depending on their abilities to form radicals and their cross-coupling propensities under the conditions of lignification. Many monomers (*e.g.* ferulates, acylated monolignols, 5-hydroxyconiferyl alcohol) other than the traditional monolignols have been shown to be components of lignins, so there is ample precedence for the incorporation of non-traditional monomers into lignins.[35,40,43] There is also particularly good evidence from studies on COMT-deficient angiosperms that substitution of a traditional monolignol with a novel phenolic component can be accommodated in lignification—5-hydroxyconiferyl alcohol is readily incorporated in the place of sinapyl alcohol into such lignins.[35,62] Exclusion of, for example, the hydroxycinnamaldehydes from lignin monomer definitions would result in the troubling implication that CAD-deficient plants contain little lignin since most of the lignin molecules probably contain incorporated aldehyde units; this is likely even in normal plants.

Conclusions

Hydroxycinnamaldehyde monomers appear to be incorporated reasonably 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 CAD-deficiencies have elevated levels of aldehydes with bonding patterns discernable from NMR that provide evidence suggesting the incorporation of monomeric hydroxycinnamaldehydes (*e.g.* 8–O–4-cross-coupled structures) by endwise coupling onto the phenolic end of the growing polymer. Work is still required to determine whether the plant is really producing a modified lignin by the incorporation of 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-polysaccharide portion and it is logical to classify them broadly as lignins. It has already been shown that CAD-deficient angiosperms are more easily delignified[63] (attributable to the greater phenolic content and greater solubility of the lignin oligomers in the base) and may be more digestible,[64] even though it has been shown that aldehyde components *per se* reduce digestibility of the cell walls that incorporate

them.[65] Processing problems or advantages of such modified plants are likely to be significant issues in the near future.

Experimental

General

All chemicals, enzymes and solvents were purchased from Aldrich and Sigma (Milwaukee, WI, USA) unless otherwise noted. Flash column chromatography was performed on an ISCO/Biotage system using FLASH 40 pre-packed silica-gel cartridges (Biotage) with the peaks detected using a UA-6 UV/VIS detector (ISCO. Inc). Preparative TLC was carried out on pre-coated 2.0 mm Sil-G-200/UV_{254} plates (Macherey-Nagel).

NMR methods

A Bruker DRX-360 fitted with a 5 mm ^{1}H/broadband gradient probe with inverse (^{1}H-detected) geometry was used for all NMR experiments. Acetylated lignins or model compounds were dissolved in 0.5 mL acetone-d_6; unacetylated lignins were dissolved in acetone-d_6 (0.42 mL) and deuterium oxide (D_2O, 0.07 mL). The central acetone solvent peak was used as the internal reference (δ_C 29.80, δ_H 2.04). 1D NMR (^{1}H, ^{13}C and DEPT-135) and 2D NMR (HMQC and HMBC) experiments used standard Bruker pulse programs. HMQC (inv4gstp) spectra were used to elucidate structures of model compounds. The one-bond coupling constant ($^1J_{CH}$) was set at 145 Hz for HMQC experiments; HMBC (inv4lplrnd) spectra were optimized for aldehyde correlations in both lignin samples and model compounds—170.5 Hz for the one-bond $^{13}C_9$–$^1H_9$ coupling constant ($^1J_{CH}$) and 80 ms for the long-range coupling delay ($0.5/^nJ_{CH}$) corresponding to a coupling constant of 6.25 Hz. Spectra for the dimeric models and lignins were recorded and compared using the same plotted regions.

Model compounds

Dehydrodimers **5** from coniferaldehyde (**1G**, 4-hydroxy-3-methoxycinnamaldehyde) and sinapaldehyde (**1S**, 3,5-dimethoxy-4-hydroxycinnamaldehyde) were synthesized and purified by method of Connor *et al.*,[12] which uses horseradish peroxidase (type II, 150–200 units per mg solid) and H_2O_2. The synthesized dehydrodimers were used for full NMR spectral assignments. All other models reported in Tables 1 and 2 are from data collected over many years in the DFRC and USFPL laboratories, as documented by their entries in the NMR database.[66]

Coniferaldehyde dehydrodimers (5, Fig. 1)

Coniferaldehyde **1G** (1.1 g, 6.15 mmol) was dissolved in acetone : water (100 mL : 1.5 L). Horseradish peroxidase (11 mg; EC 1.11.1.7, 158 purpurogallin units per mg solid, type II) in water was added to the solution which was then stirred. Hydrogen peroxide (1%, 10.45 mL, 3.1 mmol) was added dropwise into the reaction solution over 30 min (pH was checked frequently and maintained at 5–6 by controlling the addition of peroxide) while the solution was stirred. The yellow solution changed to red during the addition. The solution was stirred for an additional 1 h. The mixture was extracted with EtOAc and washed with water. The EtOAc layer was NOT dried (as would be typical) to avoid damaging the cyclic phenylcoumaran 8–5-structures. Mild evaporation gave a red solid. Pre-separation of the crude products was performed by flash column chromatography ($CHCl_3$: EtOAc, 1 : 1, v/v), and each fraction was further purified on preparative TLC plates using combination of various solvent systems such as $CHCl_3$: MeOH (99 : 1, v/v) and EtOAc : hexane (2 : 1, v/v) until relatively pure compounds were obtained. Yields of isolated products were variable and low. From 1.1 g of coniferaldehyde, 8–8 **5C** (2 mg), 8–O–4 **5A** (25 mg), 5–5 **5D** (25 mg), 8–5 (open) **5B*o*** (39 mg), and 8–5 (cyclic) **5B*c*** (97 mg) coniferaldehyde dehydrodimers and small amounts of an 8–O–4-trimer **6** (2 mg) were obtained.

3-(4-Hydroxy-3-methoxy-phenyl)-2-[2-methoxy-4-(3-oxo-propenyl)phenoxy]propenal (coniferaldehyde 8–O–4-dehydrodimer) **5AGG**. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

2-(4-Hydroxy-3-methoxyphenyl)-7-methoxy-5-(3-oxo-propenyl)-2,3-dihydrobenzofuran-3-carbaldehyde (coniferaldehyde cyclic 8–5-dehydrodimer) 5B*c*GG. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

2-[2-Hydroxy-3-methoxy-5-(3-oxopropenyl)phenyl]-3-[4-hydroxy-3-methoxyphenyl)propenal (coniferaldehyde open 8–5-dehydrodimer) 5B*o*GG. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

2,3-Bis(4-hydroxy-3-methoxybenzylidene)succinaldehyde (coniferaldehyde 8–8-dehydrodimer) 5CGG. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

3-[6,2′-Dihydroxy-5,3′-dimethoxy-5′-(3-oxopropenyl)-biphenyl-3-yl]propenal (coniferaldehyde 5–5-dehydrodimer) 5DGG. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

3-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxy-4-{2-[2-methoxy-4-(3-oxopropenyl)phenoxy]-3-oxopropenyl}phenoxy)-propenal (coniferaldehyde 8–O–4/8–O–4-dehydrotrimer) 6GGG. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

Sinapaldehyde dehydrodimers (Fig. 2)

Method I. The method of synthesizing coniferaldehyde dehydrodimers was also applied to sinapaldehyde. Sinapaldehyde (1 g, 4.81 mmol), acetone : water (20 mL : 980 mL), horseradish peroxidase (5 mg) and hydrogen peroxide (1%, 20 mL) were used. Only the 8–8-dehydrodimer **5CSS** was isolated.

Method II. Sinapaldehyde (530 mg, 2.59 mmol) was dissolved in EtOAc (30 mL). Silver(I) oxide (720.7 mg, 3.11 mmol) was added and the reaction mixture stirred overnight at room temperature. The mixture was filtered through a fine sintered glass filter to remove Ag, and evaporated to give a red solid. Separation of the crude products was performed on preparative TLC plates ($CHCl_3$: EtOAc, 1 : 1, v/v), until relatively pure compounds were obtained. Only the 8–8-dehydrodimer **5CSS** was obtained as in method I.

2,3-Bis(4-hydroxy-3,5-dimethoxybenzylidene)succinaldehyde (sinapaldehyde 8–8-dehydrodimer) 5CSS.. ^{1}H-NMR, Table 1; ^{13}C-NMR Table 2.

Synthesis of [9-^{13}C]coniferaldehyde

The labeled coniferaldehyde was prepared from vanillin *via* ethyl ferulate and coniferyl alcohol as follows. Acetylated ethyl [9-^{13}C]ferulate was prepared as described previously,[67] except that an acetate protecting group was used (*i.e.* acetylated vanillin was the starting material for the Wittig–Horner reaction with triethyl [1-^{13}C]phosphonoacetate (99 atom% ^{13}C). The acetylated ethyl [9-^{13}C]-ferulate was obtained as a pale yellow solid in 93% yield. NMR δ_H 1.27 (3H, t, J = 7.1 Hz, CH_3CH_2), 2.24 (3H, s, Ac Me), 3.89 (3H, s, OMe), 4.20 (2H, qd, J = 7.1, 3.0 Hz, CH_3CH_2), 6.55 (1H, dd, J = 16.0, 2.4 Hz, 8-H), 7.09 (1H, d, J = 8.1 Hz, 5-H), 7.23 (1H, dd, J = 8.1, 1.8 Hz, 6-H), 7.41 (1H, d, J = 1.8 Hz, 2-H), 7.64 (1H, dd, J = 16.0, 6.8 Hz, 7-H). Note: protons (CH_3CH_2, 7-H, and 8-H) are long-range coupled to the labeled 9-^{13}C with coupling constants of 3.0, 6.8, and 2.4 Hz). [9-^{13}C]Coniferyl alcohol was prepared using diisobutylaluminium hydride (DIBAL-H, 10 eq.), as described previously,[68] to give [9-^{13}C]**3G** as a yellow–white solid in essentially quantitative yield; concomitant phenolic deacetylation occurs.[69] NMR δ_H 3.85 (3H, s, OMe), 4.17 (2H, dtd, J = 140.4,

5.5, 1.7 Hz, 9-H), 6.20 (1H, dtd, J = 15.9, 5.5, 4.1 Hz, 8-H), 6.48 (1H, ddt, J = 15.9, 7.1, 1.7 Hz, 7-H), 6.76 (1H, d, J = 8.1 Hz, 5-H), 6.84 (1H, dd, J = 8.1, 1.9 Hz, 6-H), 7.04 (1H, d, J = 1.9 Hz, 2-H).

The aldehyde was prepared by oxidation of the alcohol using dichloro-5,6-dicyanobenzoquinone (DDQ).[70] [9-^{13}C]Coniferyl alcohol **3G** (774 mg, 4.28 mmol) was dissolved in THF (50 mL). DDQ (1.167 g, 5.14 mmol) was added and stirred overnight. The resulting solution was dried on an evaporator. The product was separated from DDQ and the trace of starting material by flash column chromatography ($CHCl_3$–EtOAc, 1 : 1). [9-^{13}C]-Coniferaldehyde [9-^{13}C]**1G** (208 mg, 1.16 mmol, 27%) was obtained as a brown solid after drying. NMR δ_H 3.90 (3H, s, OMe), 6.64 (1H, ddd, J = 15.9, 7.8,1.3 Hz, 8-H), 6.91 (1H, d, J = 8.3 Hz, 5-H), 7.17 (1H, dd, J = 8.3, 2.0 Hz, 6-H), 7.33 (1H, d, J = 2.0 Hz, 2-H), 7.52 (1H, dd, J = 16.0, 9.2 Hz, 7-H), 9.61 (1H, dd, J = 170.6, 7.8 Hz, 9-H).

Synthesis of [9-^{13}C]sinapaldehyde

[9-^{13}C]Sinapaldehyde was synthesized by the same methods as for [9-^{13}C]coniferaldehyde. Acetylated ethyl [9-^{13}C]sinapate was obtained as yellow crystals in essentially quantitative yield from acetylated syringaldehyde. NMR δ_H 1.28 (3H, t, J = 7.1 Hz, CH_3CH$_2$), 2.25 (3H, s, Ac Me), 3.86 (6H, s, OMe), 4.20 (2H, qd, J = 7.1, 3.2 Hz, CH$_3$CH_2), 6.55 (1H, dd, J = 16.0, 2.4 Hz, 8-H), 7.05 (2H, s, 2-, 6-H), 7.61 (1H, dd, J = 16.0, 6.7 Hz, 7-H). [9-^{13}C]Sinapyl alcohol [9-^{13}C]**3S** was obtained as a yellow–white solid in essentially quantitative yield following DIBAL-H reduction. NMR δ_H 3.81 (6H, s, OMe), 4.24 (2H, br d, J = 141.5 Hz, 9-H), 6.26 (1H, dtd, J = 15.8, 5.6, 4.1 Hz, 8-H), 6.48 (1H, ddt, J = 15.8, 6.8, 1.6 Hz, 7-H), 6.71 (2H, s, 2-,6-H), 7.36 (1H, s, 4-OH). DDQ oxidation produced [9-^{13}C]sinapaldehyde **1S** as a brown solid in 56% yield. NMR δ_H 3.88 (6H, s, OMe), 6.67 (1H, ddd, J = 15.8, 7.8,1.3 Hz, 8-H), 7.06 (2H, s, 2-,6-H), 7.53 (1H, dd, J = 15.8, 9.4 Hz, 7-H), 7.90 (1H, s, OH), 9.63 (1H, dd, J = 170.35, 7.7 Hz, 9-H).

Preparation of dehydration polymers (DHPs)

Three different solutions were prepared for the DHP synthesis. (*I*) Coniferaldehyde (34.1 mg) and coniferyl alcohol (306.0 mg) were dissolved in acetone (5 mL) and the solution was added to phosphate buffer (140 mL, 10 mM, pH = 6.5). (*II*) 30% H_2O_2 (225 μL) was diluted with phosphate buffer (140 mL). (*III*) Horse radish peroxidase (4 mg) was dissolved in phosphate buffer (70 mL). Solutions (*I*) and (*II*) were added to the solution (*III*) using a peristaltic pump at a flow rate of 6 mL h^{-1}. The resultant solution was stirred for a further 3 days. Then the DHP mixture was transferred into a plastic centrifuge bottle and centrifuged for 20 min at 1000*g*. The precipitates were carefully collected and washed with water using a 6 μm nylon membrane filter and freeze-dried. The resultant DHP (94.1 mg) was obtained as a red–brown powder.

Plant materials and lignin isolation

Antisense CAD transgenic tobacco. Production of ^{13}C-enriched antisense CAD tobacco plants and the lignin isolation have been described previously.[27] The tobacco transgenic had a cell wall that was 15% Klason lignin, and the final yield of soluble lignin was 17%. Acetylation was achieved using acetic anhydride and pyridine. The acetylated lignins were extracted into freshly distilled ethyl acetate and washed with aqueous EDTA to remove trace metal contaminants prior to NMR study.[40]

CAD-*null* mutant loblolly pine (*Pinus teada* L.). Identification and characterization of heterozygous parent and the homozygous daughter trees have been previously described.[36,39] Cell wall Klason lignin levels were 31% w/w for the normal pine and 32% for the CAD mutant pine. Lignins were isolated as previously described;[26] the final yield of lignin was 17% after removal of water-soluble components and metal ions by washing with aqueous EDTA (6 mM, pH 8). Acetylation of samples of each of the lignins was achieved using acetic anhydride and pyridine. The acetylated lignins were extracted into freshly distilled ethyl acetate and washed extensively with aqueous EDTA (6 mM, pH 8) to remove trace metal contaminants prior to NMR study for better resolution and signal-to-noise levels. The aldehyde region of the spectra is more highly resolved than in previously published spectra of this mutant.[26,35,40]

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