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Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids

John Ralph[1,2,*], Knut Lundquist[3], Gösta Brunow[4], Fachuang Lu[1,2], Hoon Kim[1,2], Paul F. Schatz[1], Jane M. Marita[1], Ronald D. Hatfield[1], Sally A. Ralph[5], Jørgen Holst Christensen[6] & Wout Boerjan[6]

[1]*U.S. Dairy Forage Research Center, USDA-Agricultural Research Service, 1925 Linden Drive West, Madison, WI 53706, U.S.A.;* [2]*Department of Forestry, U. Wisconsin-Madison, Madison, WI 53706, U.S.A.;* [3]*Department of Organic Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden;* [4]*Department of Chemistry, University of Helsinki, Helsinki, Finland;* [5]*USDA-Forest Service, One Gifford-Pinchot Drive, Madison, WI 53705, U.S.A.;* [6]*Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Universiteit Gent, Technologiepark 927, 9052 Gent, Belgium; *Author for correspondence (Tel: (608) 264-5407; Fax: (608) 264-5147; E-mail: jralph@wisc.edu)*

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Abstract

Lignins are complex natural polymers resulting from oxidative coupling of, primarily, 4-hydroxyphenylpropanoids. An understanding of their nature is evolving as a result of detailed structural studies, recently aided by the availability of lignin-biosynthetic-pathway mutants and transgenics. The currently accepted theory is that the lignin polymer is formed by combinatorial-like phenolic coupling reactions, via radicals generated by peroxidase-H_2O_2, under simple chemical control where monolignols react endwise with the growing polymer. As a result, the actual structure of the lignin macromolecule is not absolutely defined or determined. The "randomness" of linkage generation (which is not truly statistically random but governed, as is any chemical reaction, by the supply of reactants, the matrix, etc.) and the astronomical number of possible isomers of even a simple polymer structure, suggest a low probability of two lignin macromolecules being identical. A recent challenge to the currently accepted theory of chemically controlled lignification, attempting to bring lignin into line with more organized biopolymers such as proteins, is logically inconsistent with the most basic details of lignin structure. Lignins may derive in part from monomers and conjugates other than the three primary monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols). The plasticity of the combinatorial polymerization reactions allows monomer substitution and significant variations in final structure which, in many cases, the plant appears to tolerate. As such, lignification is seen as a marvelously evolved process allowing plants considerable flexibility in dealing with various environmental stresses, and conferring on them a striking ability to remain viable even when humans or nature alter "required" lignin-biosynthetic-pathway genes/enzymes. The malleability offers significant opportunities to engineer the structures of lignins beyond the limits explored to date.

Abbreviations: 4CL – 4-coumarate:CoA ligase; C3H – *p*-coumarate 3-hydroxylase; HCT – *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; CCoAOMT – caffeoyl-CoA *O*-methyltransferase; CCR – cinnamoyl-CoA reductase; F5H – ferulate 5-hydroxylase; CAld5H – coniferaldehyde 5-hydroxylase; COMT – caffeic acid *O*-methyltransferase; AldOMT – (5-hydroxyconifer)aldehyde *O*-methyltransferase; CAD – cinnamyl alcohol dehydrogenase; NMR – nuclear magnetic resonance (spectroscopy); DFRC – derivatization followed by reductive cleavage; TIZ – tosylation, iodination, zinc (a DFRC method); DHP – dehydrogenation polymer.

Introduction

Crucial to the evolution of land plants, the lignin polymer is increasingly well understood while at the same time being increasingly misunderstood and misrepresented. This article does not aim to examine all that is known about lignin and lignification, for which there are already significant reviews (Gould, 1966; Harkin, 1967; Freudenberg and Neish, 1968; Sarkanen and Ludwig, 1971; Harkin, 1973; Adler, 1977; Higuchi, 1980; Higuchi and Nakatsubo, 1980; Monties and Lapierre, 1981; Kondo et al., 1987; Monties, 1989; Higuchi, 1990; Sakakibara, 1991; Sederoff and Chang, 1991; Lin and Dence, 1992; Boudet et al., 1995; Whetten and Sederoff, 1995; Baucher et al., 1998; Lewis and Sarkanen, 1998; Brunow et al., 1999; Lewis et al., 1999; Boudet, 2000; Donaldson, 2001; Humphreys and Chapple, 2002; Baucher et al., 2003; Boerjan et al., 2003; Higuchi, 2003), but seeks to inspect some of the thornier issues of lignin structure and lignin biosynthesis. The starting point considered here is the polymerization step itself, the step in which peroxidase is highly implicated; biosynthesis of lignin monomers, the monolignols, has already been well reviewed (Sederoff and Chang, 1991; Whetten and Sederoff, 1995; Lewis et al., 1999; Dixon et al., 2001; Boerjan et al., 2003; Higuchi, 2003).

It would be useful to begin with a definition of lignin, or more accurately lignins, since lignins represent a class of polymers with considerable diversity. However, the nature of lignins creates problems for this endeavor. The most recent book on "Lignin and Lignan Biosynthesis" (Lewis and Sarkanen, 1998) fails to clarify the picture (Lewis et al., 1998a). The ideal definitions for chemical entities are structural definitions. Thus, ethanol is unambiguously defined by its chemical formula, CH_3–CH_2–OH. Definitions of derivation are problematic. For example, ethanol could be "a product of the natural fermentation of sugars" or "an industrial product from the hydration of ethylene." Similarly, cellulose is better described as "a regular anhydropolymer of glucose" (with more descriptive allusions to β-(1>4)-linked units, etc.) than as "a polymer derived from glucose by the action of glucosyltransferases." Functional or property-based definitions may be useful in describing the roles of lignins in plants, but tell us nothing useful about their structure. The definition of a compound (or class of compounds) aims initially to discriminate between the compound and other compounds (or other classes of compounds). The definition should include information that makes it possible to unambiguously identify the compound by appropriate experiments. The problem with lignins is that they are not regular structures like cellulose or any of the proteins. They have a variability, in both composition and structure, and an irregularity that defies exact description. Structural examinations of the polymer are crucial, yet only a few groups are engaged in studies aimed at teasing out structural details. An attempt at a complete structural characterization can be found in a recent review (Brunow et al., 1999); it required half a page, but remains probably the most concise comprehensive definition to date, and is cited verbatim here.

> "This description is not a chemical definition of protolignins, but summarizes the main structural features based on knowledge available today. Protolignins are biopolymers consisting of phenylpropane units with an oxygen atom at the *p*-position (as OH or O–C) and with none, one or two methoxyl groups in the *o*-positions to this oxygen atom. These *o*-positions may alternatively be C-substituted or O-substituted with other substituents than methoxyl. Only a few of the aromatic units are substituted in other ring positions. A few percent of the building blocks in protolignins are not phenylpropane units. The side chain is missing or shortened, or the unit is replaced by a quinoid group. The phenylpropane units are attached to one another by a series of characteristic linkages (β–O–4, β–5, β–β, etc.) or, alternatively, exist as members of a series of characteristic end groups (e.g. cinnamaldehyde units). Practically all the types of structural elements detected in protolignins have been demonstrated to be formed on oxidation of the *p*-hydroxycinnamyl alcohols in vitro (Freudenberg and Neish, 1968; Adler, 1977). The structural elements in protolignins are not linked to one another in any particular order. Protolignins are not optically active. The polymer is branched and cross-linking occurs. In addition, the following facts should be noted: (1) there are strong indications of the occurrence of linkages between protolignin and carbohydrates, (2) some types of protolignins are esterified with phenolic acids (grass lignins with *p*-coumaric acid and certain other lignins, such as aspen lignin, with *p*-hydroxybenzoic acid), and (3) scattered observations suggest that there are some units, for example, dihydroconiferyl alcohol units, that cannot be thought to have been produced on oxidation of *p*-hydroxycinnamyl alcohols."

Most researchers utilize definitions that describe the polymers in general terms and the monomers

from which they are derived. For example, Higuchi states "*Lignins are aromatic polymers of methoxylated phenylpropanoids connected by both ether and carbon-carbon linkages, and classified...*" (Higuchi, 2003). Better would be to stipulate 4-hydroxyphenylpropanoids; nonetheless even such a useful, simple, and yet broad definition leaves aspects of lignins and lignification unrepresented, as will be seen below. Notions of molecular mass, function, lignin purity, and genetics also enter some definitions (Rouhi, 2001). The lignin polymer is often described as a cross-linked network. In fact, the degree of cross-linking is very low – the number of intermonomer linkages is only slightly larger than the number of units.

As has been the case historically with a vast array of natural products, early structural studies lead to an insightful hypothesis of lignins' mode of formation, a mechanism for the process of lignification. Aspects of lignin structure and lignin synthesis are therefore intimately related. After a brief review of the role of peroxidases in lignification, the current theory of lignin biosynthesis as a way toward understanding the key features of the lignin structure and its implications will be examined.

The role of peroxidase in lignification

Classical peroxidases (class III) are monomeric, heme-containing glycoproteins that are found exclusively in the secretory pathways. These enzymes can dehydrogenate cinnamyl alcohols, and many other phenolic substrates, at the expense of H_2O_2. Most peroxidases studied so far can efficiently oxidize coniferyl alcohol but are less efficient in oxidizing sinapyl alcohol; examples of peroxidases with high reactivity to sinapyl alcohol or the analog syringaldazine have been reported (Christensen et al., 1998; Tsutsumi et al., 1998; Sasaki et al., 2004), suggesting the existence of monolignol-specific peroxidase isoenzymes.

That peroxidases are involved in the polymerization of lignin is generally accepted. However, despite the fact that peroxidases are among the most intensively studied plant enzymes, the ascription of particular functions to specific isoenzymes has been extremely difficult. This is primarily due to the low substrate specificities for most peroxidases, the high gene redundancy and high sequence conservation among gene family members, the ability of peroxidases to alter phytohormone levels, and the high reactivity of the formed radicals (Welinder, 1992; Lagrimini et al., 1997b; Christensen et al., 2001; Tognolli et al., 2002).

The main arguments that peroxidases participate in lignin polymerization are the ability of peroxidases to dehydrogenate monolignols generating a lignin-like dehydrogenation polymer (DHP) (Freudenberg and Neish, 1968), that lignin is polymerized *in planta* after addition of H_2O_2 to tissue sections (Müsel et al., 1997), the co-localization of specific peroxidases with lignifying tissues, and the induction of peroxidase expression by biotic or abiotic treatments that also induce lignification (Ros Barceló et al., 1989; Mäder, 1992; Sato et al., 1993; Otter and Polle, 1997; Christensen et al., 1998; Quiroga et al., 2000; Christensen et al., 2001; Egea et al., 2001; Li et al., 2003b). Transgenic plants over-expressing or down-regulating specific peroxidases have been extensively studied (Lagrimini et al., 1993; Sherf et al., 1993; Kawaoka et al., 1994; McIntyre et al., 1996; Kristensen et al., 1997; Lagrimini et al., 1997a; Huh et al., 1998; Ray et al., 1998; Amaya et al., 1999; Christensen et al., 2000b; Elfstrand et al., 2001; Elfstrand et al., 2002; Kawaoka et al., 2003) and in a few cases lignin-related phenotypes have been observed (Lagrimini et al., 1997b; El Mansouri et al., 1999; Talas-Oğraş et al., 2001; Li et al., 2003b). From one study it has been shown that specific peroxidases are responsible for lignin polymerization after wounding as seen by reduced deposition of lignin-like material in peroxidase-down-regulated plants (Lagrimini et al., 1997b).

In most transgenic plants alterations in growth or development were observed, making interpretations in relation to developmentally regulated lignification difficult. Two recent studies, however, report a reduced lignin content as a consequence of a reduction in peroxidase expression (Talas-Oğraş et al., 2001; Li et al., 2003b). These plants may potentially constitute the final proof for the participation of peroxidase in developmentally-regulated lignin polymerization, provided they do not display pleiotropic phenotypes.

Lignification: (bio)synthesis of the lignin polymer

Lignification, in analogy with other polymerization processes, is the means by which lignin macromolecules grow. Thus the primary reactions of concern are those in which the macromolecule is extended by the coupling of a new monomer to the growing polymer. Branching reactions are also important. They can oc-

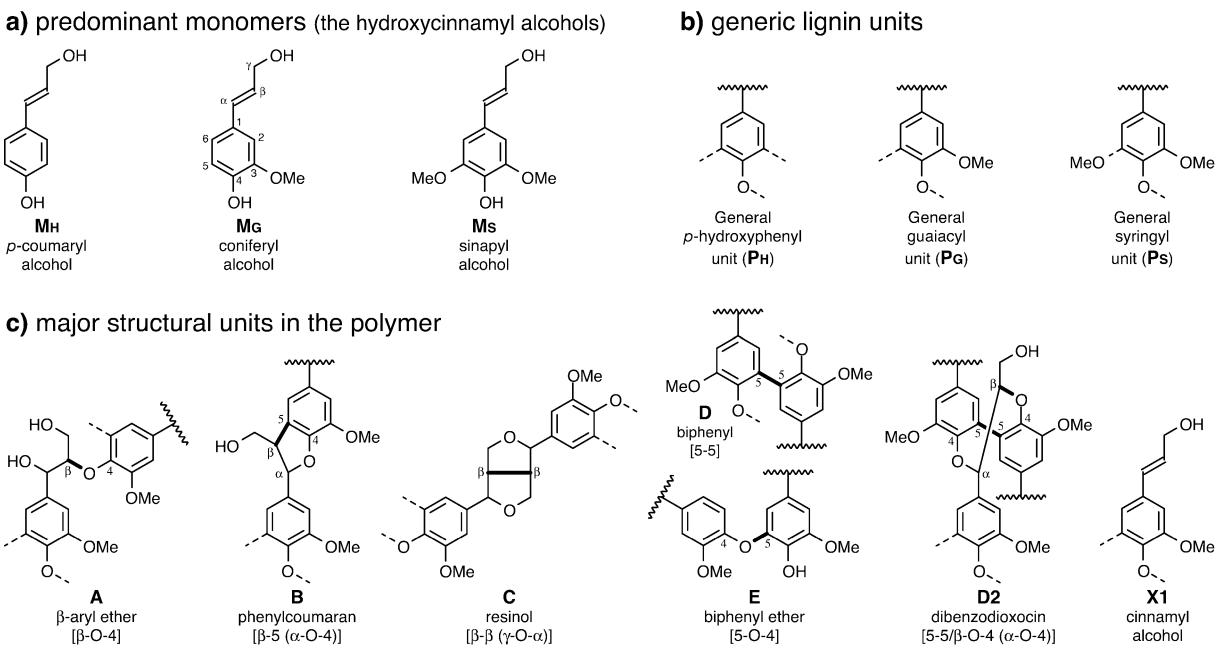

Figure 1. (a) Primary lignin monomers **M**, the monolignols. (b) Lignin polymer **P** units are denoted based on the methoxyl substitution on the aromatic ring as generic **PH**, **PG**, and **PS** units. (c) Major structural units in the polymer; the bolded bonds are the ones formed in the radical coupling reactions. The numbering follows that established in a recent review (Boerjan et al., 2003).

cur when two sequential reactions are possible at the growing end, i.e. the phenolic end, of the polymer.

The primary monomers for lignification are the three *p*-hydroxycinnamyl alcohols: *p*-coumaryl **MH**, coniferyl **MG**, and sinapyl **MS** alcohols, Figure 1a, monolignols **M** differing in their degree of methoxylation. In the polymer **P**, these monolignols produce *p*-hydroxyphenyl **PH**, guaiacyl **PG** and syringyl **PS** units, Figure 1b. Due to the capability of electron-delocalized radicals to couple at various sites, a variety of structural units are found in the resulting polymer, Figure 1c. Interesting details regarding the biosynthetic pathways to these monolignols, and significant revisions, continue to appear but the pathway is relatively well characterized (Baucher et al., 1998; Li et al., 2000; Dixon et al., 2001; Li et al., 2001; Humphreys and Chapple, 2002; Baucher et al., 2003; Boerjan et al., 2003). The enzymes are known, as are their genes. Erdtman was the first to recognize that major lignin structural features were consistent with a process of radical coupling of phenols (Erdtman, 1933). Simple chemical dehydrodimerization reactions of coniferyl alcohol, using either the peroxidase-H_2O_2 system that is implicated *in vivo*, or other chemical single-electron oxidants including various Fe, Mn, Cu, and Ag salts, produce three dehydrodimeric coupling products in comparable amounts, Figure 2a. Most reviews and texts also include other coupling modes for the monolignols, but these are in fact not observed; 5–5- and 4–O–5-coupled structures in lignins do not arise from monolignol-monolignol coupling reactions. The products involve at least one of the monolignols coupling at its favored β-position. Evidence for further coupling reactions between the monolignol and the initially formed dimer became available from well-characterized oligomers (Freudenberg and Neish, 1968; Adler, 1977). The notion that lignification was a process involving "uncontrolled" radical coupling reactions was therefore born, although Erdtman suspected that lignification might be found to be more highly controlled (Erdtman, 1957), as noted in the following quote.

> "The question whether lignins are formed by random polymerization of phenylpropane derivatives or whether there is some degree of order cannot be answered at present. Much can be said in favor of the first view, but, even if we accept the dehydrogenation concept, this does not mean that lignins must necessarily be completely irregular. The reactivity in various positions of the primary radicals is certainly different and this will cause some degree of order. It is probably a great oversimpli-

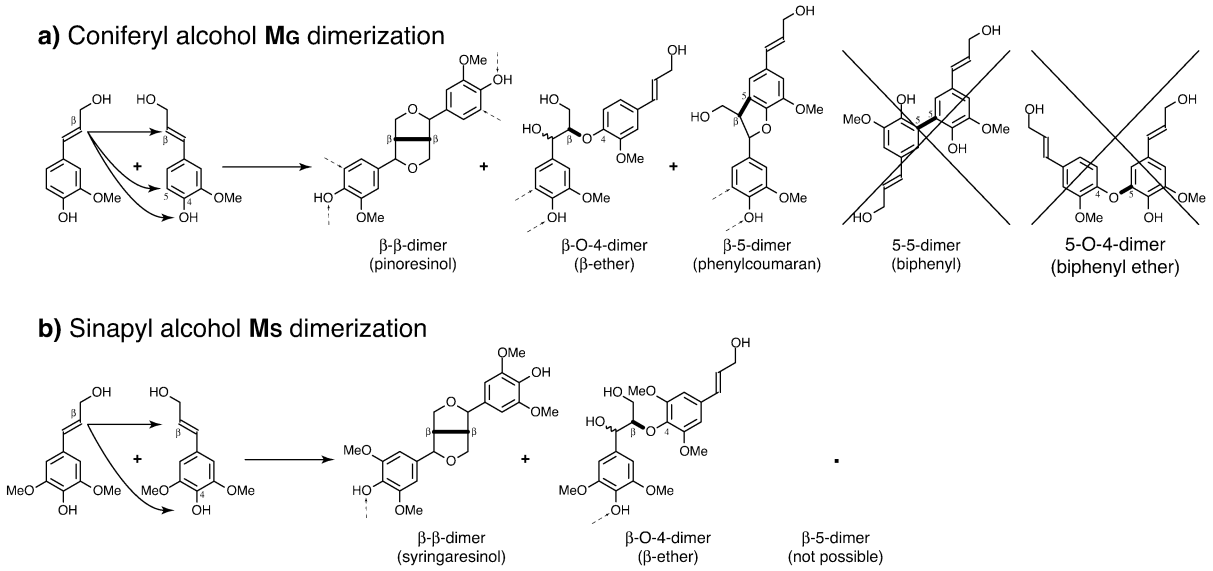

Figure 2. Dehydrodimerization of monolignols. (*a*) Dehydrodimerization of coniferyl alcohol **MG** produces only three dimers, in each of which at least one of the coniferyl alcohols is coupled at its β-position. The 5–5- and 5–O–4-dehydrodimers shown in most texts (and crossed out in this figure) do not actually arise in any significant way from monolignol dehydrodimerization reactions. The new bond formed by the coupling reaction is bolded. (*b*) Sinapyl alcohol **Ms** analogously dehydrodimerizes to only two products.

fication to assume that the biological formation of lignin can be imitated by crude enzyme extracts or by ferric ions."

Noting the ability of the hydroxycinnamyl alcohol radicals to potentially couple at several positions (4–O-, 5-, β-, as well as 1-), in a process that was only under simple chemical control (i.e. not controlled by proteins or enzymes), termed "random" coupling (Harkin, 1967). The process is not, or course, truly statistically random, since chemical processes resulting in more than one product will have their outcomes influenced by the nature of the reactants, the nature of stabilizing post-coupling reactions, the matrix and solvent, physical conditions (e.g. temperature, pressure), etc. The process is essentially "combinatorial" i.e. all of the various possible coupling modes are available, but are not equally probable (Sederoff et al., 1999; Boerjan et al., 2003).

Synthetic lignins, so-called dehydrogenation polymers (DHPs), can be prepared *in vitro* from monolignols oxidized by peroxidase-H_2O_2. Such synthetic lignins are valuable for exploring and understanding coupling and post-coupling reactions, and as models for spectroscopic and reactivity studies. However, they differ from native or isolated lignins; in particular they have a lower β–O–4-ether content (Nimz and Lüdemann, 1974; Gagnaire and Robert, 1978; Brunow and Lundquist, 1980; Brunow and Wallin, 1981; Nimz et al., 1981; Robert and Brunow, 1984; Faix, 1986; Lewis et al., 1987; Faix and Beinhoff, 1988). The primary reason is that dehydrodimerization reactions are over-represented. Dehydrodimerization of coniferyl alcohol yields three primary products, whereas sinapyl alcohol yields two, Figure 2. Coupling at the β-position is favored for coniferyl and even more strongly for sinapyl alcohol; the coupling products always result from coupling of at least one of the monolignols at its β-position. In coupling reactions, including in a biomimetic peroxidase-H_2O_2 system, the β-ether dimer is typically produced in less than one third of the yield in the coniferyl alcohol case (Tanahashi et al., 1976; Katayama and Fukuzumi, 1978), and at only about the 9% level for sinapyl alcohol (Tanahashi et al., 1976). Monolignol radicals preferentially couple with like monolignol radicals (when available) rather than cross-couple with dimers or higher oligomers (Syrjanen and Brunow, 2000). Dehydrodimerization reactions are therefore over-represented in synthetic lignins even when attempts are made to introduce the monolignol slowly (Freudenberg, 1956; Sarkanen, 1971; Terashima et al., 1995). Consequently, the β-ether frequency is low, considerably lower than in typical lignins. Limiting the diffusion rates (and therefore monolignol radical concentrations) to favor cross-coupling reactions reveals that β-ethers are strongly favored in cross-

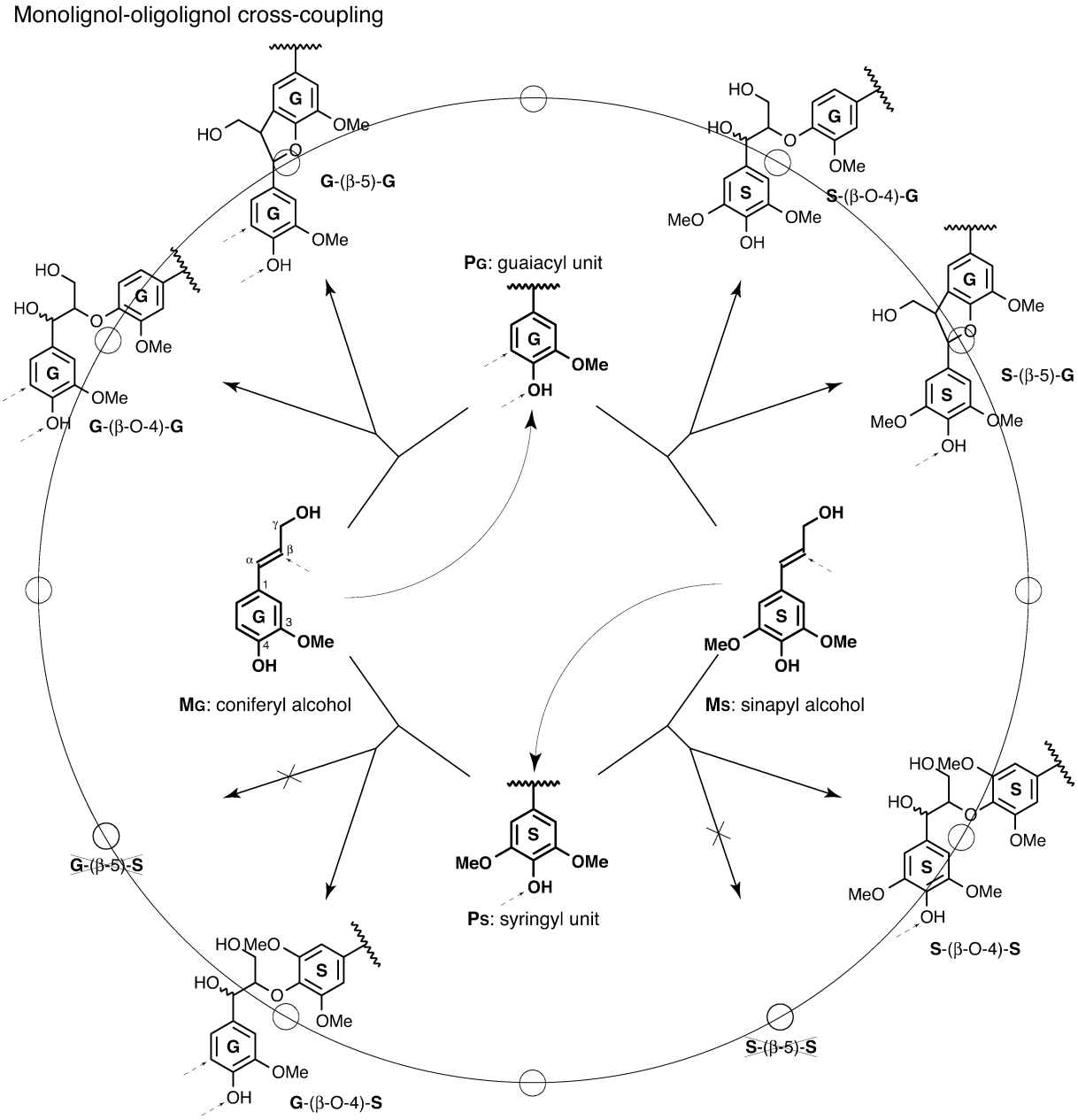

Figure 3. Lignification differs substantially from dimerization of monolignols. Cross-coupling of the monolignol coniferyl **M**G or sinapyl alcohol **M**s with a **G** polymer unit **P**G gives only two main products, the β–O–4- and β–5-products (upper portion of figure). Already this explains why there are more β-ethers formed during lignification than in lignin dimerization, where there are three products (Figure 2a). Cross-coupling between a monolignol (either coniferyl or sinapyl alcohol) and an **S** unit **P**s in the polymer has essentially only one outcome – the β–O–4 unit (lower portion of figure), explaining why high **S** lignins have elevated β-ether levels. A shorthand for naming units is introduced; for example, **G**-(β-O-4)-**S** units arise from coupling of a coniferyl alcohol monomer **M**G at its favored β-position with a syringyl phenolic end **P**s of the growing polymer (at its 4–O-position). Sites of further coupling reactions during lignification are indicated by dashed arrows.

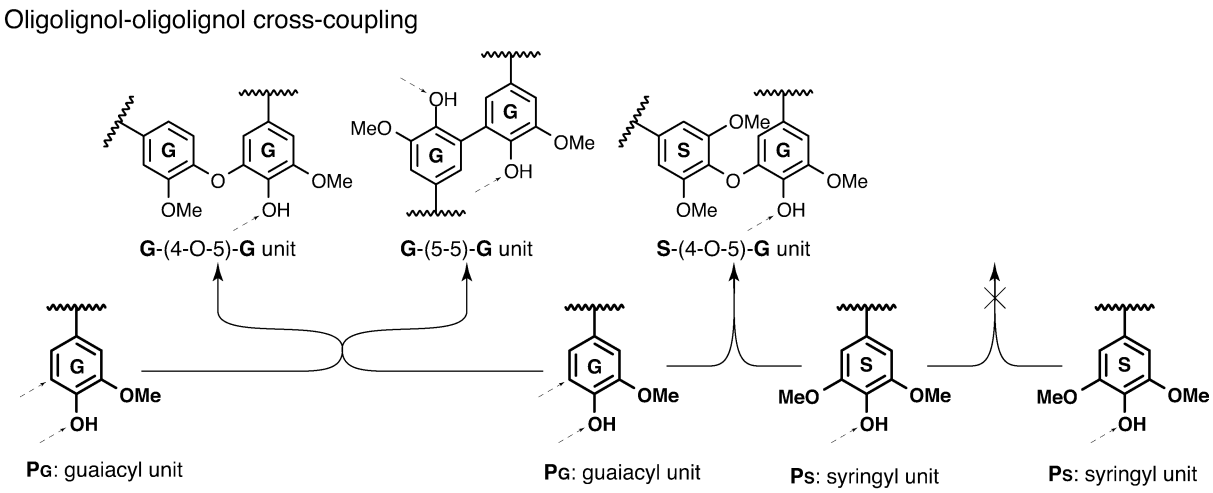

Figure 4. Coupling of preformed oligomers is the source of most of the 5–5- and 4–O–5-units. Neither 5–5- nor 5–O–4-units can be formed between **S** units; cross-coupling of **G** and **S** units can furnish 5–O–4-linked structures. Sites of further coupling reactions during lignification are indicated by dashed arrows.

coupling reactions. In model reactions carried out via diffusion of coniferyl alcohol through dialysis tubing where coniferyl alcohol reacted with a monomeric lignin model, apocynol, the ratio of β–O–4 product to β–5 was 10:1 and in experiments with a guaiacyl dimer, only the β–O–4 trimer was found (Syrjanen and Brunow, 2000). In native softwood lignin, β–O–4 bonds outnumber β–5 bonds roughly 4 to 1. This means that the regioselectivity of cross-coupling *in vitro* may be even higher than that *in vivo*. It also means that regiospecific formation of β–O–4 bonds in lignins can be achieved by simply controlling the supply of monolignol radicals and no other agents are necessary to "explain the mystery of specificity of radical precursor coupling in lignin biosynthesis" (Davin and Lewis, 2000). This feature was recognized early on as evidenced by eloquent passages in various treatises (Sarkanen and Ludwig, 1971; Adler, 1977), and has been emphasized repeatedly, e.g. in (Lundquist, 1973) and (Syrjanen and Brunow, 1998).

Recently, another explanation for the differences between lignin and DHP has been put forward; in coupling reactions, both reactants (monomers, oligomers or the polymer) have to be dehydrogenated either by peroxidases or by other means. For the oxidation of polymeric lignin, it is difficult to imagine that the polymer can be accommodated in the narrow substrate channel of peroxidases, and it was therefore suggested that oxidized phenylpropanoids could act as mediating- or shuttle-substrates to dehydrogenate the polymer (Takahama and Oniki, 1996; Takahama et al., 1996; Takahama and Oniki, 1997; Christensen et al., 2000a; Hatfield and Vermerris, 2001; Ralph et al., 2004). Such transfer happens swiftly from coniferyl alcohol to sinapyl alcohol (Takahama, 1995; Takahama and Oniki, 1997; Fournand et al., 2003; Sasaki et al., 2004), but recently the transfer to polymeric lignin was shown to be inefficient (Sasaki et al., 2004). The same authors isolated a peroxidase isoenzyme (CWPO-C) that, in contrast to horseradish peroxidase (HRP), was able to oxidize polymeric lignin directly. The DHP generated with this peroxidase had a higher proportion of β–O–4 linkages, as determined by thioacidolysis, and a higher molecular weight than a DHP generated with HRP, probably due to a lower proportion of dehydrodimerization (Aoyama et al., 2002; Sasaki et al., 2004). The fact that this enzyme also can oxidize ferrocytochrome c suggests an electron transport chain from the active site to the surface of the molecule. These findings suggest that the differences between DHP and lignin may be explained, at least in part, by the specific activity of the peroxidase.

The key feature of lignification, at least in the secondary wall, involves coupling reactions between a monolignol and the growing lignin polymer, Figure 3, or between two lignin oligomers, Figure 4. The significant differences between this process and dehydrodimerization are hopefully clear from comparison of Figures 2 vs. 3. Lignin polymer extension by a monolignol principally involves cross-coupling reactions between the monomer and the polymer. Simplist-

ically, Figure 3 already shows how the frequency of β-ether units in lignins is far greater than it is in dehydrodimerization reactions, Figure 2. Ignoring coupling at the 1-position for now, essentially there are only two possible courses for coupling of an hydroxycinnamyl alcohol at its favored β-position with the guaiacyl phenolic **PG** (at its 4–O- or 5-positions) (*vs* three possibilities for dehydrodimerization, Figure 2), and only one (*vs* two) for coupling with a syringyl unit **PS** (at its 4–O-position). The dominant β–β-coupling mode observed in dehydrodimerization (Figure 2) is simply not possible in cross-coupling reactions — the oligomer cannot couple at its β-position. Also, it becomes clear why high-syringyl lignins have higher β-ether contents — there is essentially only one pathway available for either monolignol to couple with a syringyl unit **PS** and that is β–O–4-coupling.

In the current theory for lignification, the actual polymerization step is controlled only by normal chemical concerns involved in any chemical reaction: coupling and cross-coupling propensities, reactant concentrations, the matrix and conditions. This has several ramifications. The first is that, due to the "chance" nature of the combinatorial-like coupling reactions, there is no fixed sequence of units in a lignin polymer. Although there are statistical probabilities for the nature of the "next" unit resulting from cross-coupling of a specific monolignol with the polymer, which may change with the course of the polymerization, the sequence of β–5-, β–O–4-, and other linkages is not fixed. This is the "random" connotation that frequents the literature. Secondly, again subject to chemical coupling propensities and the availability of reactants at the lignifying zone, there is the potential to introduce the three monomers into the lignin also in a combinatorial sense. Ignoring for now the less-abundant *p*-coumaryl alcohol monolignol **MH**, the phenolic end of a growing lignin chain, whether it is guaiacyl or syringyl, can be extended by coupling with either a coniferyl **MG** or sinapyl **MS** alcohol, if available, leading to considerable structural heterogeneity (Lapierre et al., 1982; Terashima et al., 1986; Tollier et al., 1986; Hori and Meshitsuka, 2000). Although the four possible coupling products are seen in β-ether structures released by incomplete thioacidolysis (Ralph and Grabber, 1996), some are not favorable processes; for example it has been difficult to couple sinapyl alcohol to a guaiacyl dimer *in vitro* (Syrjanen and Brunow, 1998; Landucci and Ralph, 2001). Thirdly, being solely a chemical reaction, the units formed are racemic; they have chiral carbons (two for each new unit formed) but there is no preference for one enantiomer over the other. Special means are required to generate chiral molecules in synthetic chemical reactions, as opposed to reactions catalyzed by enzymes or proteins. Since lignification occurs within a chiral matrix (all polysaccharides and proteins are chiral), the potential for optical induction exists, but extensive evidence to date is that lignins are not optically active. If that is true, then any biological mechanism for defining a structural sequence in a lignin polymer is highly unlikely. An implication of these "random" events and the racemic nature of the polymer is that, as a lignin molecule becomes larger, the chance that it will have exactly the same structure as another molecule of lignin diminishes rapidly, quickly approaching astronomical improbability. These concepts are covered in more depth below.

Another previously unappreciated implication of the solely chemical nature of the polymerization coupling reactions is that various phenolic compounds finding themselves at the site of lignification may, subject to simple chemical arguments such as the ability to form radicals (via peroxidase-H_2O_2, or via radical transfer reactions) and (cross-)coupling propensities, become incorporated into the polymer. In fact, at least in some plants, there may be few lignin molecules of any significant size that are purely derived from the three primary monolignols. As will be illustrated below, the flexibility of the lignin polymerization system may allow plants to produce polymers with sufficiently matched properties to adequately perform the normal lignin functions, allowing the plant to remain viable, even when monolignol availability to the plant cell wall becomes severely compromised, for example by the loss of a crucial enzyme for monolignol biosynthesis. We shall also reveal that there are several other phenolics that are routinely used as lignification "monomers" by certain classes of plants. Lignin heterogeneity may therefore be even broader than generally portrayed.

Lignin structure

With a polymer as complex and as heterogeneous as lignin, structural analysis is anything but straightforward. Yet it is the detailed analysis of lignin structure that provides insight back to the biosynthetic pathways that are involved in lignification. As we shall see, many mysteries remain. This section will be segmented under various headings in an attempt to

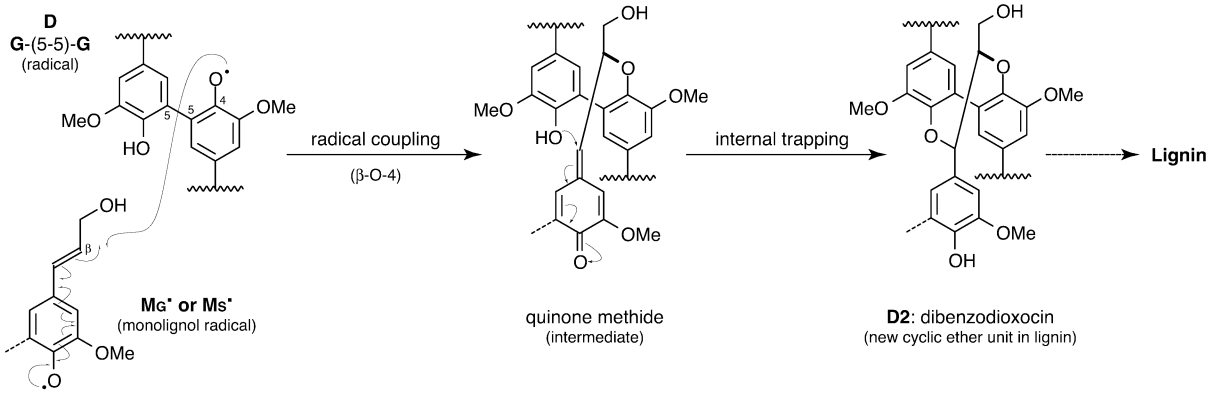

Figure 5. The formation of dibenzodioxocin units **D2** in lignins. Following 5–5-coupling of two phenolic end-units (see Figure 4), the next endwise coupling reaction with a monolignol (reacting at its favored β-position, and coupling to the only available position, the 4–O-position, of one of the phenolic moieties) produces the normal quinone methide intermediate (see later in Figure 6). This quinone methide is internally trapped by the other phenol in the 5–5-moiety, producing an 8-membered ring, a dibenzodioxocin structure. Such structures are strikingly prevalent in high-guaiacyl lignins (see later in Figure 10).

limit the confusion over aspects of structure. A few caveats follow. Although it is possible to infer certain details from degradative procedures on the whole plant material (preferably after removal of all non-cell wall components), many of the structural observations come from spectroscopic analyses, principally NMR, which require isolation of lignin fractions. The isolation process does not allow the entire lignin fraction to be obtained, and its degree of representation may be of concern (Björkman, 1957; Obst and Kirk, 1988; Lundquist, 1992b). Moreover, it typically involves a severe milling step (ball-milling) which breaks bonds. The structural alterations have been rather well studied and principally involve reducing the degree of polymerization (creating new free-phenolic endgroups as well as new side-chain endgroups), sidechain oxidation, and sidechain degradation primarily in the endgroups (Chang et al., 1975; Lundquist et al., 1977; Schmidt Michael et al., 1997; Lapierre and Lundquist, 1999; Ikeda et al., 2002). As far as can be ascertained, interconversion of structural unit types has not been observed. It is not possible to isolate substantial fractions of a plant's lignin in a pure form (without considerable structural alteration). Small amounts of polysaccharides (attached to the lignin) are present in milled wood lignins (Lundquist, 1992b) but do not normally interfere with the structural analysis. Indeed a recent method allows for NMR characterization of lignins following full solubilization of the entire cell wall component after ball-milling (Lu and Ralph, 2003). In principle, the entire lignin can therefore now be examined, but the caution regarding structural changes from ball-milling remains. Tannins and other polyphenolics may be difficult to exclude from isolated lignins (Sarkanen and Hergert, 1971; Hergert, 1977), although they have not been evident in any recent NMR studies to the extent that has been suggested (Lewis, 1999).

Predominant units

As has been well reviewed, lignins are characterized by certain prominent inter-unit linkages, Figure 1c. It must be emphasized that these linkages derive primarily from monomer-oligomer or oligomer-oligomer coupling reactions, and less importantly from monomer-monomer couplings. The main linkages are β–O–4 **A** and β–5 **B** (mainly from monomer-oligomer couplings, Figure 3), 5–5 **D** and 4–O–5 **E** (from oligomer-oligomer couplings, Figure 4), and β–β **C**, and β–1 couplings. More will be said about the latter two below. Units involved in 5–5-linkages have recently undergone structural reevaluation. Addition of a monolignol (at its favored β-position) to a 5–5-unit **D** (at one of the 4–O-positions), followed by internal trapping of the quinone methide by the other phenol, results in an unanticipated 8-membered ring, Figure 5 (Karhunen et al., 1995a, b; Kukkola et al., 2001). Such dibenzodioxocin units **D2** are beautifully resolved in 2D NMR spectra (see later in Figure 10) (Karhunen et al., 1995b; Ralph et al., 1999c; Marita et al., 2001; Ralph et al., 2001a; Ralph et al., 2001c; Marita et al., 2003b; Marita et al., 2003a). The majority of 5–5-units are now recognized as being involved in such dibenzodioxocin structures **D2** (Ralph et al.,

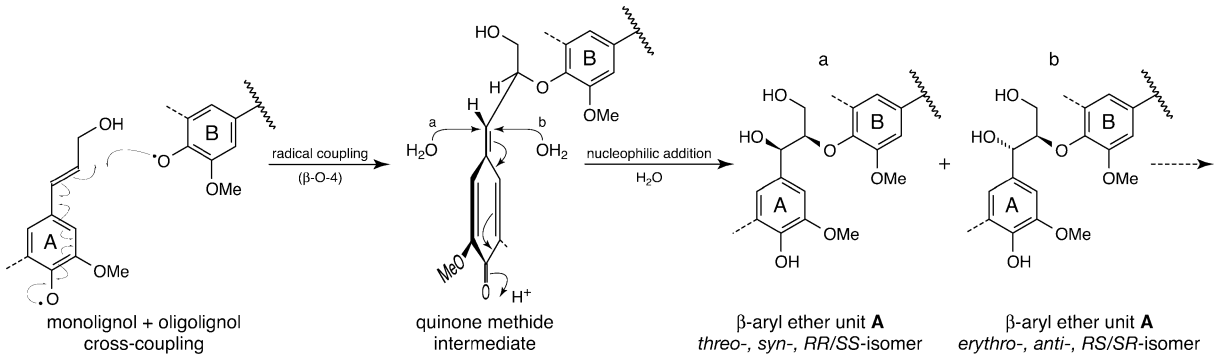

Figure 6. β-Ether isomers. Any coupling reaction of a hydroxycinnamyl alcohol at its favored β-position produces a quinone methide intermediate. In the case of β–O–4-coupling, these quinone methides add water to form the final β-ether products, arylglycerol-β-aryl ethers. Water can add from either face of the planar quinone methide, producing two possible isomers, the *threo-* (or *syn-*, or *RR/SS-*) and the *erythro-* (or *anti-* or *RS/SR-*) isomers. The resultant stereochemical preference is dictated by the guaiacyl/syringyl nature of each ring, but is most influenced by the nature of the B-ring. Thus β-guaiacyl ethers are formed in essentially 50:50 *erythro:threo* ratios, whereas β-syringyl ethers are about 75:25. The ratios depend somewhat on conditions, but all indications are that the kinetic ratios *in vivo* are essentially the same as those observed *in vitro*. Thermodynamically, i.e. the distribution if equilibration is allowed, the *erythro:threo* ratios are on the order of 50:50 and 55:45, respectively, from model studies; the ratios observed therefore do not reflect equilibration (Brunow et al., 1993).

1999c; Argyropoulos et al., 2002). The importance of both 5–5- and 4–O–5-structures **D** and **E** are that they represent branch-points in the lignin polymer.

The only bonds that can be readily and diagnostically broken are the β–O–4-ethers **A**. Their cleavage forms the basis of a variety of lignin analytical methods such as acidolysis (Lundquist, 1992a), thioacidolysis (Rolando et al., 1992), and the related DFRC and TIZ methods (Lu and Ralph, 1997; Katahira et al., 2003), as well as being the key to lignin degradation during chemical pulping.

Stereochemistry is kinetically determined.

An important aspect of lignification is the post-coupling re-aromatization reactions that occur. The β-ether units **A** derive from β–O–4-coupling (of a monolignol with an oligomer), followed by addition of water to the intermediary quinone methide, Figure 6. The resultant β-ether structure has two distinct, chemically different, isomeric forms, commonly referred to as *erythro-* and *threo*-isomers. Each of these isomers has different physical properties and dictates different spatial constraints on the resultant polymer. The isomers also naturally differ in their chemical reactivity; for example, etherified *erythro*-isomers are cleaved more rapidly in alkaline pulping (Obst, 1983). So what controls the ratio of the two isomers during lignification? Evidence suggests that the products are kinetically controlled. That is, the isomer distribution results from the chemical propensity of the water nucleophile to add to one face of the quinone methide versus the other. The alternative, thermodynamic control, occurs when the products are allowed to chemically equilibrate such that the isomer ratio reflects the relative stability of the two isomers. Isomer ratios in lignins differ from the thermodynamic equilibrium ratios, suggesting that the isomers formed during lignification do not subsequently interconvert (equilibrate). *In vivo* and *in vitro*, β–O–4-guaiacyl ethers are produced essentially in a 50:50 *erythro*:*threo* ratio which is about the same as the equilibrium distribution (Brunow et al., 1993). However, β–O–4-syringyl ethers are produced in a 75:25 *erythro:threo* ratio, *vs* the equilibrium distribution of ~55:45 (Brunow et al., 1993; Bardet et al., 1998). The isomer ratios therefore correlate well with syringyl:guaiacyl ratios in dicots (Akiyama et al., 2003). As further evidence for kinetic control of isomer ratios during lignification, β–β-structures **C** (resinols) in lignins, both natural and synthetic, are essentially in a single form whereas the equilibrium ratio of pinoresinol to *epi*-pinoresinol is ~1:1 (Lundquist and Stomberg, 1988). The observed isomer ratios of the various units in lignins are entirely consistent with coupling reactions under kinetic chemical control.

β–β-Unit conundrums

Until recently, it seemed logical that the minor resinol (β–β-coupled) units **C**, arose from direct monolignol-monolignol coupling to create a β–β-dehydrodimer, which became a starting point for the growth of the lignin polymer (Figure 7, pathway a). Indeed, monolignols (and especially sinapyl alcohol **MS**) fa-

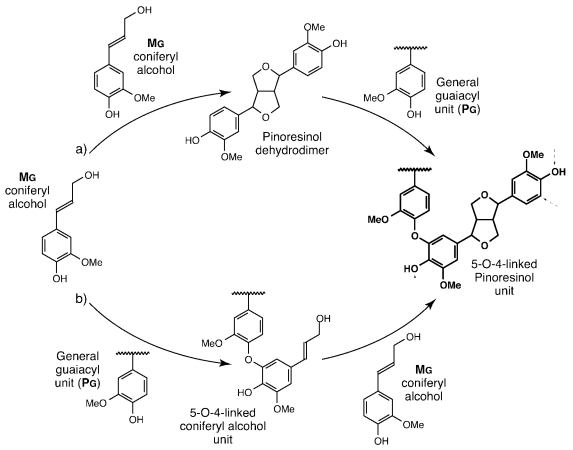

Figure 7. Two possible pathways by which pinoresinols can form in 5–O–4-structures that will not release pinoresinol dimers via degradative methods. a) Coniferyl alcohol dehydrodimerization produces pinoresinol as usual. 5–O–4-Cross-coupling of the pinoresinol with a guaiacyl oligomer produces the 5–O–4-linked structure. b) Cross-coupling of coniferyl alcohol directly at its 5-position (rather than its favored β-position) with a guaiacyl oligomer (at its 4–O-position) produces a 4–O–5-structure retaining a (free-phenolic) hydroxycinnamyl alcohol unit. This attached hydroxycinnamyl alcohol unit can cross-couple at its β-position with a new monolignol at its β-position to generate the pinoresinol unit within the growing oligomer chain. There is little evidence that 5-coupling of coniferyl alcohol (pathway b) is a prominent reaction, but there is also no compelling reason why pinoresinol units in spruce would favor 5-coupling with the growing polymer rather than undergoing endwise chain extension by reaction with fresh monolignols. To be released as pinoresinol units by acidolytic methods (acidolysis, thioacidolysis, DFRC), the pinoresinol unit must be simply etherified 4-O-β at both phenolic ends, or etherified at one end and un-attached (free phenolic) at the other. It is therefore possible to understand why they are not highly releasable (only 4-O-β-bonds are cleaved in these acidolytic methods), particularly as they should also be found in 5–β-linked (phenylcoumaran) units. Why released thioacidolysis trimers appear to be heavily involved in 5–O–4-linked structures, however, remains somewhat mysterious.

vor β–β-coupling. However, in softwoods (which are derived predominantly from coniferyl alcohol **MG**), there is a puzzling observation; pinoresinol is not significantly released from β-ether cleaving reactions. Thus acidolysis of spruce did not release detectable pinoresinol (Lundquist, 1970); thioacidolysis released only traces of the same dimeric products as were produced from pinoresinol (Lapierre et al., 1991; Jacquet et al., 1997; Önnerud and Gellerstedt, 2003) and the DFRC degradation also released only traces of such products (Lu, 2004, unpublished), Figure 8. DFRC of pine released similarly low levels (Lu, 2004, unpublished); the product originally attributed to β–β-dimers from pinoresinol in early work (Peng et al., 1998) was in fact a secoisolariciresinol product — see below. When thioacidolysis trimers (following Raney Ni desulfurization) were examined, most of the β–β-products appeared to be 5–O–4-linked, Figure 7, (Önnerud and Gellerstedt, 2003), explaining why β–β-dimers were released in such low quantities. This group suggested, as Lundquist had earlier (Lundquist, 1992c), that pinoresinol units might derive not from monolignol-monolignol coupling reactions (Figure 7, pathway a), but from the 5-coupling of a monolignol to the growing oligomer, leading to an in-chain resinol formation, pathway b (Zhang et al., 2003). At this point, evidence for 5-coupling of monolignols is not compelling and there is currently no way to distinguish this route from one in which preformed pinoresinol dimers couple at their 5-positions with other lignin oligomers (pathway a).

What is the derivation of the β–β-dimers reported in pine DFRC products (Peng et al., 1998)? They originate from reduced units in the lignins, secoisolariciresinol units, Figure 8 that must have been ether linked. Such units release the DFRC dimers **Dseco** observed, and also give rise to distinct acidolysis (Lundquist and Stomberg, 1988) and thioacidolysis (Lapierre et al., 1991) products **Tseco** attributed to the same structures. An NMR investigation showed that secoisolariciresinol units are present in isolated spruce lignins at about half the level of pinoresinol units (Zhang et al., 2003). The level of **Dseco** releasable by DFRC is about 4 times the level of releasable pinoresinol units **Dpr**, however (Figure 8). The appearance of secoisolariciresinol structures in lignins is problematic. How do they get there? They must arise from post-β–β-coupling reactions, possibly directly from reductive trapping of the quinone methide intermediates (Zhang et al., 2003), or via reductase transformations on pinoresinol as have been well demonstrated in lignans (Katayama et al., 1993). But why are they in the lignin polymer? The released products are not optically active (Ralph et al., 1999a), whereas the lignan products from softwoods are (Sakakibara et al., 1987), so they appear not to be lignan derived. To date, their appearance in lignins has been well authenticated, but the method by which secoisolariciresinol finds itself in the lignifying zone for incorporation into lignins is unknown.

A very recent finding was unanticipated. In spruce isolated lignin it is possible to detect a β–β-coupled α–O–α-tetrahydrofuran that must result from addition of water to the intermediary quinone methide (Zhang and Gellerstedt, 2004). A similar structure is found in Kenaf when trapping of the quinone methide by the

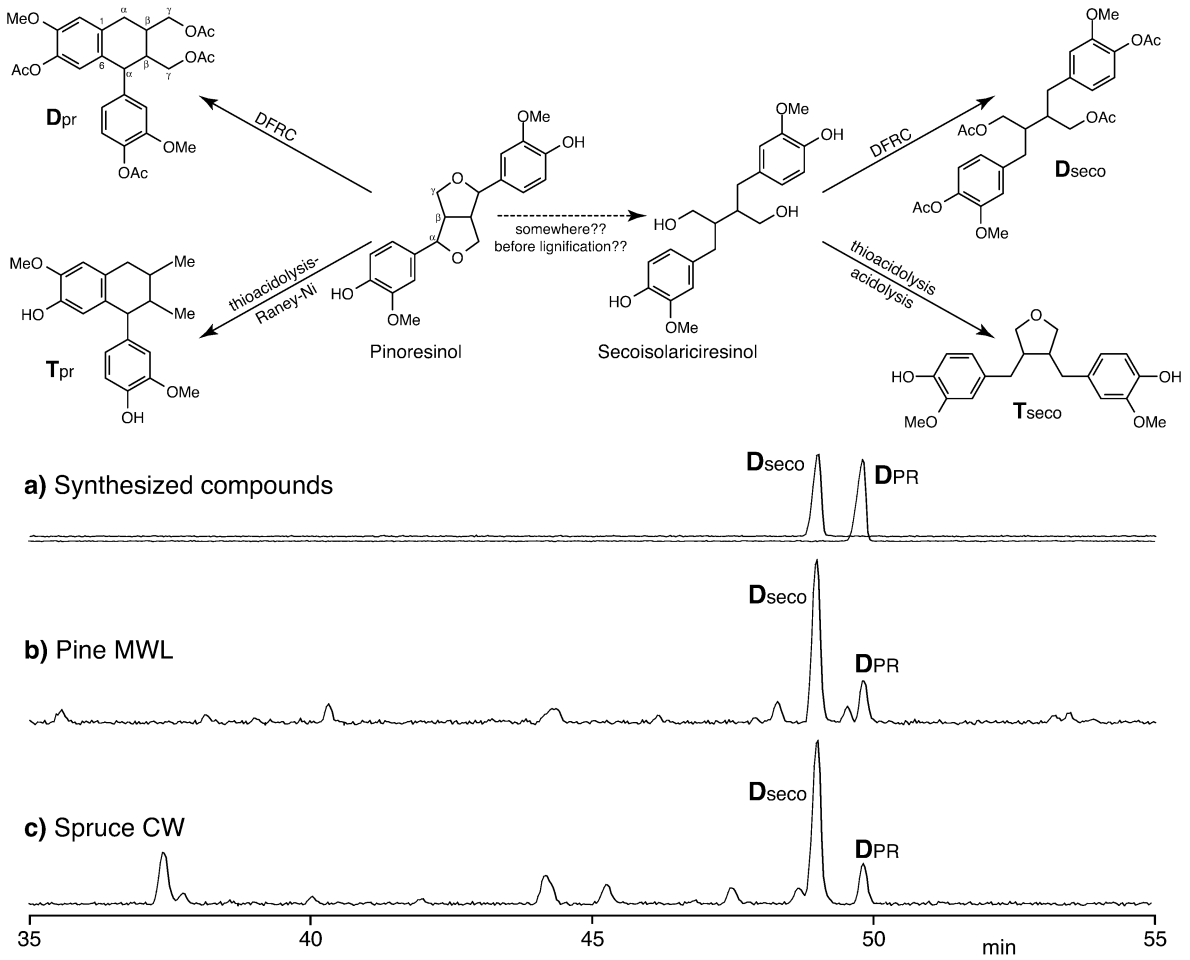

Figure 8. Products from thioacidolysis/Raney-Ni and DFRC of β–β-linked guaiacyl dimers. Pinoresinol (and its 4-O-β-etherified structures in lignins) produces α–6-cyclized products, mainly **T**pr and **D**pr. Interestingly, such structures (and the acidolysis product, pinoresinol) are released only at extremely low levels from spruce or pine, suggesting that they are present in other than 4–O–β-etherified structures. Secoisolariciresinol (and its 4–O–β-etherified structures in lignins) releases the tetrahydrofuran structure **T**seco (lower right) by thioacidolysis (and acidolysis) and acetylated secoisolariciresinol **D**seco (top right) by DFRC, in high yields. The DFRC product **D**seco was initially attributed erroneously to pinoresinol structures in lignins (Peng et al., 1998). It is not known how secoisolariciresinol units come to be in lignins. Bottom Figure: Gas chromatogram, following pre-cleanup by thin layer chromatography to enrich this β–β-fraction, of DFRC products from a) authentic synthetic compounds, b) pine lignin c) spruce cell walls, showing that the major pinoresinol product **D**pr is released at only about 25% of the level of the secoisolariciresinol product **D**seco.

γ-OH is prevented due to its natural acylation (Lu and Ralph, 2002). Analogs have also been found in ferulate coupling in grasses and cereal grains (Ralph et al., 2000; Grabber et al., 2002; Ralph et al., 2004). However, β–β-structures other than the resinols **C** were not expected monolignol coupling products since it has always been assumed that internal trapping of the quinone methide by the γ-OH would be rapid and complete. It suggests that lignification may be consistent with low pH conditions where the protonation-dependent addition of water is more rapid; the paucity of acyclic α-aryl ethers in lignins has been noted to be consistent with low-pH conditions (Brunow et al., 1989).

β–1-structures

The β–1-coupling mode is less often discussed, in part because it is more complex (Lundquist and Miksche, 1965; Harkin, 1967; Lundquist et al., 1967; Sarkanen and Ludwig, 1971). β–1-Coupling appears to be seen only in the coupling of a monolignol with a pre-formed β–O–4-phenolic end-unit, Figure 9. Its presence in lignins is evidenced by the acidolysis

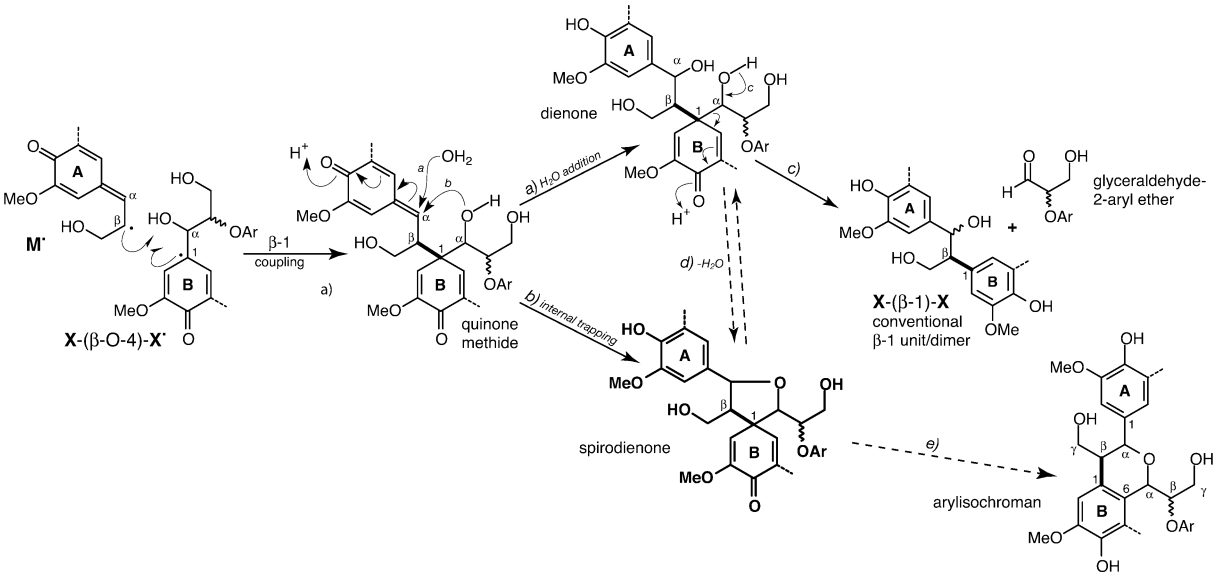

Figure 9. β–1-Cross-coupling mechanisms. Cross-coupling of a monolignol (at its favored β-position) with a preformed β-ether unit produces the usual quinone methide intermediate. The quinone methide may be trapped by water (as in the β-ether case, pathway a) to form a dienone; alternatively, the intermediate may be logically (in hindsight) internally trapped by the α-OH to form a spirodienone (pathway b); the dienone may also dehydrate to the spirodienone (pathway d). Spirodienone structures appear to be stable in lignins and are now readily detected by NMR (see Figure 10). The dienone or spirodienone structures may generate the conventional β–1-unit (pathway c). As seen in the DFRC products, acidolytic reactions can produce the arylisochroman rearrangement products (pathway e). Arylisochromans can be found at low levels in isolated lignins. Whether the conventional β–1-product X–(β–1)–X (X = G or S), or arylisochromans, are present in the native lignins as such (from rearrangement during or post lignification) or if they are released from dienone structures during lignin isolation is unknown.

stilbene product (not shown) from dehydration and release of formaldehyde from X–(β–1)–X (X = G or S, Figure 9) (Lundquist, 1970, 1992a), and by the detection of the co-produced glyceraldehyde-2-aryl ethers in lignin (Lundquist et al., 1967; Matsumoto et al., 1981), Figure 9. β–1-Dimers are anomalously prominent in dimer fractions from acidolysis (Lundquist, 1992a), thioacidolysis (Lapierre et al., 1991), or DFRC (Peng et al., 1998). The conventionally described X–(β–1)–X unit has been observed in isolated lignins by NMR, usually at low levels (Lundquist, 1987; Ede et al., 1990; Ede and Brunow, 1992; Kilpeläinen et al., 1994a; Kilpeläinen et al., 1994b; Ede et al., 1996; Zhang and Gellerstedt, 2001). The occurrence of dienone structures was suggested as an explanation for the discrepancy between degradation and NMR spectral studies (Lundquist, 1987).

All combinations of β–1-coupling are observed. In birch lignin β–1-structures of the syringyl-syringyl type predominate and there are about equal amounts of guaiacyl-guaiacyl and syringyl-guaiacyl (or guaiacyl-syringyl) β–1-structures (Li et al., 1996). The number of syringyl-guaiacyl β–1-structures is about the same as the number of guaiacyl-syringyl β–1-structures. The combinatorial nature of lignification seems to be well demonstrated with this unit type.

Formation of β–1-structures in lignins likely produces dienones, Figure 9 (Lundquist and Miksche, 1965; Lundquist et al., 1967; Brunow and Lundquist, 1991). Mild acidolysis of methylated wood produced β–1 dimers with an unmethylated B-ring; the monomethylated β–1 dimers were almost exclusively methylated in the A-ring and the B-ring was free, suggesting that it is not actually in the form of a phenolic-OH in lignin (Gellerstedt and Zhang, 1991). In addition to the dienones postulated from water addition to the quinone methide (Lundquist and Miksche, 1965; Lundquist et al., 1967), spirodienones were proposed as logical intermediates (Brunow et al., 1995). NMR evidence for spirodienones was found in poplar lignins (Ämmälahti et al., 1998). Sinapate can form isolable analogs upon oxidative coupling (Setälä et al., 1999). Further compelling NMR evidence for spirodienones in spruce and aspen lignins has now been reported (Zhang and Gellerstedt, 2001). We demonstrate here that high-syringyl lignins contain such units (Figures 10c, 10d1), with remarkable amounts of the syringyl spirodienones in Kenaf bast fiber lignins, Fig-

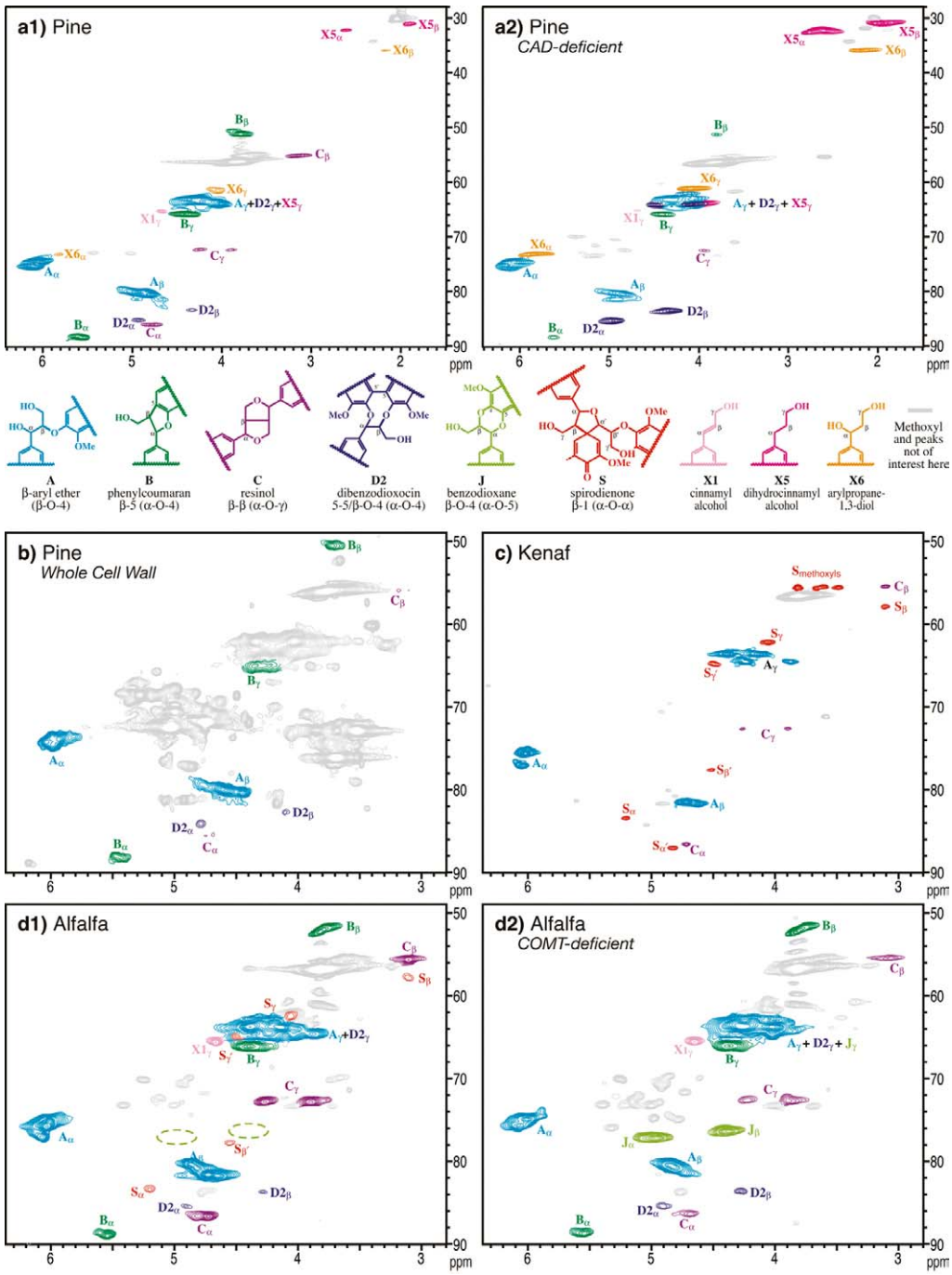

Figure 10. HSQC NMR spectra illustrating normal and novel structures in acetylated lignins (in acetone-d_6 unless otherwise noted). a1) Pine milled wood lignin; note that dihydrocinnamyl **X5** and arylpropane-1,3-diol **X6** units are present in normal pine lignins, along with the conventional units **A**-**D2**. a2) CAD-deficient pine milled wood lignin showing the high level of dibenzodioxocins **D2**, and structural units **X5** and **X6** from the high levels of dihydroconiferyl alcohol **M5G** (Figure 11) and the derived guaiacylpropane-1,3-diol **M6G** incorporated into the polymer. b) Pine whole cell walls (acetylated, in $CDCl_3$) using a recent solubilization method (Lu and Ralph, 2003); note that at least some correlations from the prominent units are well separated even in the whole cell wall mixture containing predominant polysaccharides. c) High-resolution spectrum of the highly syringyl-rich Kenaf bast fiber lignin, highlighting peaks from spirodienone β–1-structures **S** (Zhang et al., 2004). d1) Alfalfa lignin, in which spirodienones **S** are also present. d2) COMT-deficient alfalfa lignin showing prominent benzodioxane units **J** (not present in the wild type, Figure d1) from the significant incorporation of 5-hydroxyconiferyl alcohol **M5H** into the polymer (Marita et al., 2003b). Note that the parent structures in the lignins are shown; the correlations labeled in the spectra are from acetylated analogs.

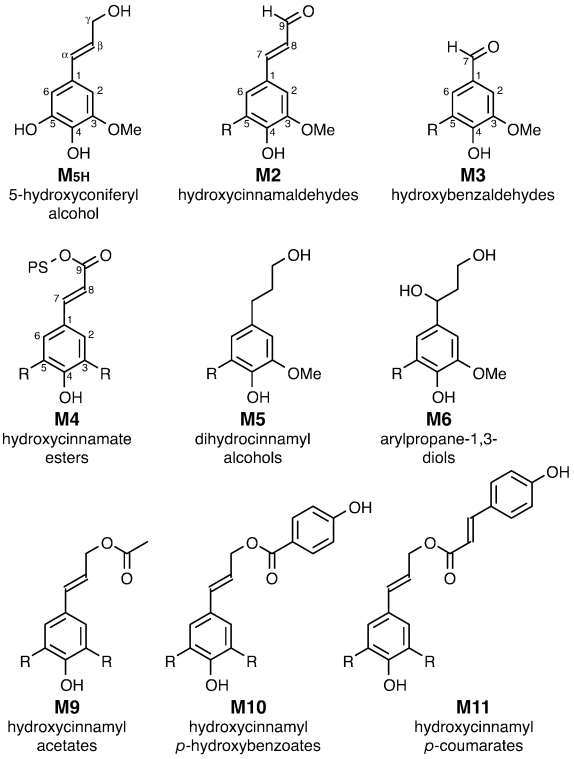

Figure 11. Phenolics that might, and in some cases must, be considered lignin "monomers." See text for details. PS = polysaccharide. Monomer designation is from a recent review on Lignin Biosynthesis (Boerjan et al., 2003).

ure 10c (Zhang et al., 2004). The isolation of arylisochromans following DFRC degradation, and the observation of trace amounts in isolated lignins, also suggests that dienone β–1-coupling products are formed during lignification (Ralph et al., 1998a). Compounds assigned as having β–6-linkages resulting from degradative procedures (Sudo and Sakakibara, 1974; Yasuda and Sakakibara, 1976, 1977; Lapierre et al., 1991) should be carefully re-examined to determine if they are in fact their β–1/α–6 arylisochroman isomers (and therefore also result from β–1-coupled units).

Other natural lignin "monomers"

A feature of lignins that is often ignored is the presence of units derived from phenolics other than the three primary monolignols, Figure 11. Many lignins have various groups acylating the γ-hydroxyl. For example, kenaf bast fiber lignins are over 50% γ-acetylated (Ralph, 1996). Evidence has recently been presented (Lu and Ralph, 2002), and more evidence will be forthcoming, that these acetates arise not from acetylation of the lignin polymer, but from lignification using pre-acetylated lignin monomers **M9**. In other words, monolignol conjugates, beyond the monolignol biosynthetic pathway, are being used as "monomers" for the lignification in kenaf, at strikingly high levels. Grass lignins have γ-*p*-coumarate substituents on up to ~10% of their units; a mature maize isolated lignin had a 17% content by weight (Ralph et al., 1994b). Early NMR evidence suggested that these were produced from pre-*p*-coumaroylated monolignols **M11**, since the *p*-coumarates were found indiscriminately on all types of lignin units (Ralph et al., 1994b). Similarly, various poplar/aspen, palm, and willow varieties have γ-*p*-hydroxybenzoate substituents (Smith, 1955; Nakano et al., 1961; Landucci et al., 1992; Sun et al., 1999; Meyermans et al., 2000; Li and Lundquist, 2001; Lu and Ralph, 2003). Cross-coupling products of sinapyl alcohol **MS** and sinapyl *p*-hydroxybenzoate **M10** have recently been found in lignifying poplar xylem (Lu et al., 2004; Morreel et al., 2004). NMR evidence for cross-products of **M10S** in poplar, aspen, and palms, and the *p*-coumarate analogs **M11S** in corn, is just becoming available (Lu and Ralph, 2004, unpublished). Natural lignins therefore appear to result, in part (and sometimes substantially) from polymerization of "monomers" other than the three primary monolignols **MH**, **MG** and **MS** (Sarkanen, 1971; Ralph et al., 2001c; Boerjan et al., 2003).

The situation is perhaps even more complex. Evidence suggests that phenolics exported to the lignifying zone during lignification may be incorporated into lignins, subject to their chemical coupling propensities and other factors. This is hardly surprising if the polymerization process is only under simple chemical control. The derivation of these phenolics, shown in Figure 11, is more of a mystery. Reduced monomers such as dihydroconiferyl alcohol **M5G** are incorporated into most softwood lignins (Sakakibara, 1980; Lundquist and Stern, 1989) and possibly in hardwoods (Fukagawa et al., 1991). Dihydroconiferyl alcohol **M5G**, in the presence of peroxidase and hydrogen peroxide (which generate the radicals necessary for lignification), produces the monomer guaiacyl propane-1,3-diol **M6G**; derived units **X6** can also now be found in most softwood lignin samples (Ralph et al., 1999b). A CAD-deficient pine had substantial amounts of dihydroconiferyl alcohol **X5** and guaiacylpropane-1,3-diol **X6** units in its polymer, Figure 10a2 (Ralph et al., 1997; Sederoff et al., 1999; Lapierre et al., 2000). As pointed out in the original article (Ralph

et al., 1997), and later independently verified (Lapierre et al., 2000), the dihydroconiferyl alcohol monomer **M5G** underwent substantial homo-coupling but was also found to be cross-coupled with the normal monolignol, coniferyl alcohol. The resultant lignin was rich in dibenzodioxocins **D2** (Figure 10a2) resulting from initial 5–5-dehydrodimerization of dihydroconiferyl alcohol or its derived guaiacylpropane-1,3-diol (Ralph et al., 1999c).

Hydroxycinnamaldehydes **M2** have always been considered to be part of lignin and are the basis of the famous phloroglucinol lignin staining method (Adler et al., 1948). Studies on plants deficient in the CAD, the enzyme that produces the monolignol from its hydroxycinnamaldehyde precursor, confirm that hydroxycinnamaldehyde monomers **M2** will incorporate into lignins, and particularly intimately into the syringyl-guaiacyl lignins in angiosperms (Ralph et al., 2001c; Kim et al., 2002; Lapierre et al., 2004). Derived hydroxybenzaldehydes **M3** may also incorporate as monomers (Kim et al., 2003).

More difficult to recognize as part of the lignin polymer is the incorporation, by radical coupling, of esters and amides of *p*-hydroxycinnamates, ferulate in particular. Ferulates **M4G**, acylating arabinoxylan polysaccharides in grasses (but accounting for less than 1% of the cell wall mass), are involved in radical coupling reactions that cross-link cell wall polysaccharides by dehydrodimerization and dehydrotrimerization (Ralph et al., 1994a; Ralph et al., 1998b; Bunzel et al., 2003; Rouau et al., 2003; Ralph et al., 2004). It is now well established that cross-coupling of the ferulates or the dehydrodiferulates with lignin monomers (and perhaps oligomers) is a mechanism for cross-linking lignins and polysaccharides in grasses (Ralph et al., 1995; Ralph et al., 2004). The ferulates therefore enter the lignin by the same types of radical coupling reactions that typify lignification, and become integral to the structure, being only partially releasable by known cleavage methods.

More controversial are the feruloyl amides (not shown). As pointed out in a tobacco study, these are associated with a wounding response, but the ferulate moieties can nevertheless be demonstrated, by NMR and thioacidolysis methods, to be incorporated into the polymer (Ralph et al., 1998c). Terminal pendant groups, like tyramine units and *p*-coumarates in grass lignins, are mobile; they therefore have long relaxation times causing the resonances to appear much sharper than the remaining resonances from the polymer. The sharp tyramine resonances in 1D ^{13}C NMR spectra have been misinterpreted as implying low molecular mass artifacts (Anterola and Lewis, 2002). The ferulate moieties, also part of the incorporated tyramine ferulate units, produce broad and disperse resonances (and are in fact almost impossible to find in the 1D ^{13}C NMR spectrum), due to their tight incorporation into the polymer by coupling at a variety of sites. Finding these ferulates, and providing evidence for their coupling, requires more sophisticated NMR experiments (Ralph et al., 1998c). As the ferulates are part of the polymer, obviously so too are the tyramine units that have been shown to be attached to those ferulates.

Lignification is a process to produce cell wall polymers in the plant via coupling reactions of phenolics. Although the three primary monolignols are the major phenolics utilized in "normal" lignification, there is a range of other phenolic compounds that may be incorporated, sometimes at significant levels, in normal and lignification-perturbed plants. Structural studies of lignins continue to expand this concept. The challenge in the future will be to understand the originating pathways and their interactions, and the mechanisms of transport to the wall.

New insights from mutants and transgenics

Schreiber is most recently quoted as noting that "*if you want to understand something, it is very useful if you can perturb it*" (Schreiber, 1999). Modern biotechnologies provide precise new tools for examining the impacts of down- and up-regulating each step in the monolignol biosynthetic pathway, and combinations of such steps. Nature herself has explored some of these genetic events, and natural mutants may also be particularly revealing. These approaches have been reviewed recently (Baucher et al., 2003; Boerjan et al., 2003), and will not be dealt with in depth here, but it is worth visiting relevant topics that have been misrepresented or misunderstood. A greatly simplified version of the pathway showing the enzymes of interest in the discussion that follows is given in Figure 12. The full pathway can be found elsewhere (Boerjan et al., 2003).

CAD-deficient pine revisited

The first mutant discovered to have seriously deviant lignin structural anomalies was probably bm3 maize to be discussed in the next section on COMT-deficient plants. Garnering the most attention, however, has

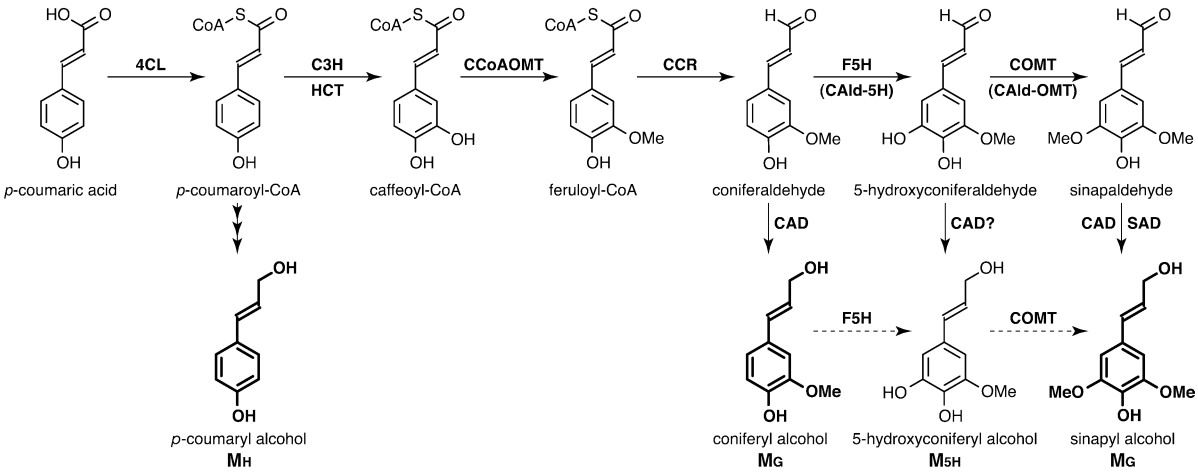

Figure 12. Simplified monolignol biosynthetic pathway. Note that, at least in Arabidopsis, the C3H/HCT conversion involves more steps (Boerjan et al., 2003). Traditional names for the enzymes involved are: 4CL = 4-coumarate:CoA ligase; C3H = *p*-coumarate 3-hydroxylase; HCT = *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; CCoAOMT = caffeoyl-CoA O-methyltransferase; CCR = cinnamoyl-CoA reductase; F5H = ferulate 5-hydroxylase; CAld5H = coniferaldehyde 5-hydroxylase; COMT = caffeic acid O-methyltransferase; AldOMT = (5-hydroxyconifer)aldehyde O-methyltransferase; CAD = cinnamyl alcohol dehydrogenase. As noted here and in a recent review (Boerjan et al., 2003), many of these enzymes are no longer thought to function on the substrates for which they were originally named.

been the initial structural studies on the massively different lignins in a mutant pine (Ralph et al., 1997), shown to be deficient in the enzyme CAD (Mackay et al., 1995). Although coniferaldehyde build-up was anticipated and evidenced, the incorporation of coniferaldehyde into the lignins was not nearly to the extent expected by model studies (Higuchi et al., 1994). In fact the coniferaldehyde and derived vanillin contents were only marginally greater. It was not until later studies on angiosperms that the explanation became evident. Coniferaldehyde will not cross-couple with normal guaiacyl units, either *in vivo* or *in vitro*, and therefore cannot enter into the endwise polymerization process that typifies lignification (Kim et al., 2000, 2003; Ralph et al., 2001c). It is relegated to homo- and crossed dehydrodimerization reactions, resulting in terminal cinnamaldehyde groups. Coniferaldehyde will β–O–4-cross-couple efficiently with syringyl units, and thus incorporates well into angiosperm lignins (Kim et al., 2000, 2002; Ralph et al., 2001c; Lapierre et al., 2004). Recently, the small incorporation of coniferaldehyde into the interior of the lignin polymer in the pine has been linked to its coupling with "syringyl-like" groups, i.e. 5-substituted guaiacyl units such as in 5–O–4- or possibly 5–5-units (Kim et al., 2003).

But it was the massive incorporation of dihydroconiferyl alcohol **M5G** that was more striking. To this date, the origin of this monomer in the CAD-deficient pine remains unknown. But it was clear from the original study that it was being incorporated as a monomer (Ralph et al., 1997). Much of it was present as 5–5-linked structures, which were later shown to be largely involved in dibenzodioxocin structures, Figure 10a2 (Ralph et al., 1999c). Insinuations that dihydroconiferyl alcohol units had to arise from post-coupling reactions (Lewis et al., 1998b; Lewis, 1999), as in lignan analogs, can be dismissed (Ralph et al., 1997, 1999c; Sederoff et al., 1999). Coniferyl alcohol invariably couples at its β-position and does not 5–5-couple – see Figure 2a. More recent establishment of the incorporation of dihydroconiferyl alcohol, as a monomer, into the pine's lignin has been from independent sources and methods (Lapierre et al., 2000; MacKay et al., 2001), and from the observation that guaiacylpropane-1,3-diol units **X6** also occur at high levels in this pine's lignin, Figure 10a2 (Ralph et al., 1999b); guaiacylpropane-1,3-diol **M6G** itself derives directly from dihydroconiferyl alcohol **M5G** under the peroxidase-H_2O_2 conditions (Ralph et al., 1999b). Both dihydroconiferyl alcohol **X5** and guaiacylpropane-1,3-diol **X6** units are now routinely found in softwood lignins.

Preliminary results from this study led to the suggestion that plants might produce functional polymers that allow them to remain viable by conscripting available phenolics for free-radical-mediated polymerization in the cell wall (Ralph, 1997). The notion

has been increasingly supported by studies on various transgenics in which monomer substitution is clearly demonstrated (Ralph et al., 2001c; Boerjan et al., 2003).

CAD-deficient angiosperms

It is now well established, from studies on a variety of CAD-deficient angiosperms, that coniferaldehyde and sinapaldehyde can incorporate integrally into angiosperm lignins. In addition to revealing NMR experiments (Kim et al., 2000, 2003; Ralph et al., 2001c), a set of thioacidolysis marker compounds have been identified that arise from hydroxycinnamaldehyde-β–O–4-coupled units in the polymer (Kim et al., 2002). The marker levels increase with the degree of CAD deficiency, and the markers can be noted in thioacidolysis products at lower CAD-deficiency levels than are required to cause phenotypic changes in the plants (Lapierre et al., 2004). Further details of the incorporation of the coniferaldehyde vs sinapaldehyde and of their derived benzaldehyde analogs have been reviewed (Ralph et al., 2001c; Kim et al., 2003).

F5H-deficient angiosperms

Huge syringyl:guaiacyl compositional shifts result from manipulating the hydroxylase F5H, which now appears to operate at the coniferaldehyde level and has therefore been named CAld5H (Humphreys et al., 1999; Osakabe et al., 1999). The earliest study was in Arabidopsis where an F5H-deficient mutant had no syringyl component to its lignin (Meyer et al., 1998; Marita et al., 1999). More strikingly, when F5H was sense-upregulated in this mutant line, the guaiacyl content fell to less than 3%, indicating the massive compositional flexibility possible in Arabidopsis (Marita et al., 1999). It was later discovered that the subsequent COMT activity (see below) could apparently not keep pace with the enhanced production of 5-hydroxyconiferaldehyde, since the lignin showed the clear signatures of 5-hydroxyconiferyl alcohol incorporation (Ralph et al., 2001c). Aspen does not seem to suffer from the COMT-limitation noted in Arabidopsis; no increase in 5-hydroxyguaiacyl markers could be detected in F5H-overexpressed plants. In aspen, F5H-upregulation in normal lines or those also down-regulated in 4CL significantly enhanced the lignin S:G ratio (Li et al., 2003a). Such changes are considered particularly valuable for the chemical pulping of wood; higher syringyl lignins have higher β-ether contents and are therefore more readily cleaved under pulping conditions. Most recently, F5H-upregulation in poplar, using the Arabidopsis C4H promoter, resulted in impressive S:G ratios, up to ~13:1, far beyond anything that has been reported in natural plants (Huntley et al., 2003). To date, manipulation of F5H has not been tested in forage grasses or legumes. There has been a suggestion that S:G increases also result in improved digestibility, as recently reviewed (Grabber et al., 2004), although Grabber's work has clearly shown that such compositional differences *per se* have no direct link to digestibility (Grabber et al., 1997).

Manipulation of F5H levels provides compelling evidence that plants have considerable malleability in the levels of the primary monolignols that can be tolerated. Although normal plants of any given species have settled to rather narrow S:G ranges, clearly plants are capable of sustaining considerable variation, with guaiacyl contents ranging from ~3 to ~100%.

COMT-deficient angiosperms

The most striking structural changes in the lignin polymer noted to date have been in COMT-deficient plants (Lapierre et al., 1988, 1999, 2001; Van Doorsselaere et al., 1995; Marita et al., 2001, 2003a, b; Ralph et al., 2001c; Lu et al., 2003). COMT is the enzyme responsible for methylation of 5-hydroxyguaiacyl substrates produced by F5H and therefore operates primarily also at the aldehyde level (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000). COMT-deficiency limits a cell's ability to produce the sinapyl alcohol monolignol **MS**, and consequently syringyl lignin. However, the preceding hydroxylation step utilizing F5H is not reversible. Hence the 5-hydroxyguaiacyl intermediate, presumably 5-hydroxyconiferaldehyde (Figure 12), builds up; there is apparently no serious product inhibition to back-up the monolignol synthesis to the guaiacyl level. The cell's CAD enzymes are evidently able to reduce 5-hydroxyconiferaldehyde to 5-hydroxyconiferyl alcohol, for it is primarily 5-hydroxyconiferyl alcohol **M5H** that is exported to the cell wall and becomes incorporated into lignins (Hwang and Sakakibara, 1981; Lapierre et al., 1988; Ralph et al., 2001c).

Although such *o*-diphenols (catechols) can and will undergo oxidations producing quinones which can undergo subsequent transformations, such reactions are not apparent in the cell wall. The lignins incorporate the 5-hydroxyconiferyl alcohol **M5H** as

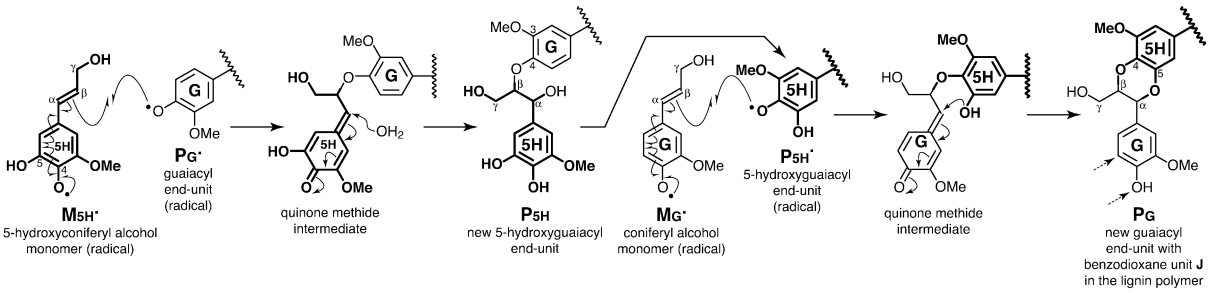

Figure 13. Mechanism of 5-hydroxyconiferyl alcohol **M5H** incorporation into a guaiacyl lignin, producing novel benzodioxane units **J** in the polymer. Only the pathways producing β-ether units are shown, but β–5- (phenylcoumaran) units are also possible. Cross-coupling with syringyl units is also possible but is less pronounced in lignins which have a low syringyl content due to strong COMT downregulation. Cross-coupling of 5-hydroxyconiferyl alcohol **M5H**, via its radical **M5H•**, with a guaiacyl lignin unit **PG**, via its radical **PG•**, produces a quinone methide intermediate which re-aromatizes by water addition to give the β-ether structure possessing a 5-hydroxyguaiacyl end-unit **P5H**. This unit is capable of further incorporation into the lignin polymer via radical coupling reactions of radical **P5H•**. Reaction with the monolignol coniferyl alcohol **MG**, via its radical **MG•**, produces a quinone methide intermediate again. This time, however, the quinone methide can be internally trapped by the 5-OH, forming a new 5–O–α-bond, and creating the benzodioxane ring system. The presence of benzodioxane structures **J** in COMT-deficient transgenic plants is diagnostically revealed by NMR (Figure 10d2).

they do the other monolignols (Ralph et al., 2001c). In other words, the 5-hydroxyconiferyl alcohol **M5H** couples at its favored β-position with the phenolic end of the growing polymer, presumably either syringyl **PS** or guaiacyl **PG** units, primarily at the 4–O-position, Figure 13. The newly created free-phenolic 5-hydroxyguaiacyl endgroup on **P5H** is now available for coupling with a new monomer. NMR and DFRC evidence is clear that coniferyl alcohol **MG**, sinapyl alcohol **MS**, or another 5-hydroxyconiferyl alcohol **M5H** will couple (at their β-positions) with this group (Lapierre et al., 2001; Marita et al., 2001, 2003b; Ralph et al., 2001c; Lu et al., 2003). It is therefore logical that 5-hydroxyconiferyl alcohol **M5H** must be considered to be an authentic monolignol in these plants, even though it is not one of the three primary monolignols. Since there are two possible phenolic-OH groups that could be involved in the coupling reaction, proof that coupling was to the 4–O-position was accomplished via the thioacidolysis product, and via model reactions and NMR (Lapierre et al., 2001). The notable difference between the incorporation of 5-hydroxyconiferyl alcohol **M5H** and the primary monolignols comes after the radical coupling step. The intermediate quinone methide can now be internally trapped by the other phenol, the 5-OH that is not normally present. Such trapping results in cyclic benzodioxane structures **J** that are not seen in normal lignin units derived from the primary monolignols (Ralph et al., 2001a). Indeed, as 5-hydroxyconiferyl alcohol **M5H** substitutes for sinapyl alcohol **MS** in plants with increasing COMT-deficiency, the structures of the lignins become more dominated by the presence of benzodioxane units **J**. Chains of such structures are evident in NMR spectra of lignins from plants with severe COMT down-regulation (Marita et al., 2003b). Today, with sensitive NMR methods, traces of these benzodioxanes can be observed even in normal plants (Li et al., 2003a), implying that the monolignol pathway may not fully complete its sinapyl alcohol production even under normal circumstances.

The structures of the resultant lignins deviate significantly from their normal wild-type counterparts. Structural models created from the analytical data illustrate some striking differences (see later in Figure 14). Obviously, the properties of the lignin polymer must also change, since properties are dependent on structure. The benzodioxane rings lend a rigidity and linearity to the polymer. Moreover, despite their being β-ethers, benzodioxanes are inefficiently cleaved under alkaline pulping conditions, unlike their syringyl counterparts (Ralph et al., 2001b; Lu and Ralph, 2004). So pulping of COMT-deficient plants is significantly poorer (Lapierre et al., 1999; Pilate et al., 2002). However, the digestibility of such plants appears to be improved (Guo et al., 2001). We surmise that this improvement is due to the nature of the new quinone methide intermediates. The conventional intermediates typically add water (Figures 6, 13), but may also be trapped by polysaccharide hydroxyls or uronate carboxyls resulting in cross-linking between polysaccharides and lignins. Even low-level cross-linking has been shown to have considerable negative impact on cell wall digestibility (Grabber et al., 1998). Since quinone methides involving 4–O-coupling of 5-hydroxyguaiacyl units are efficiently in-

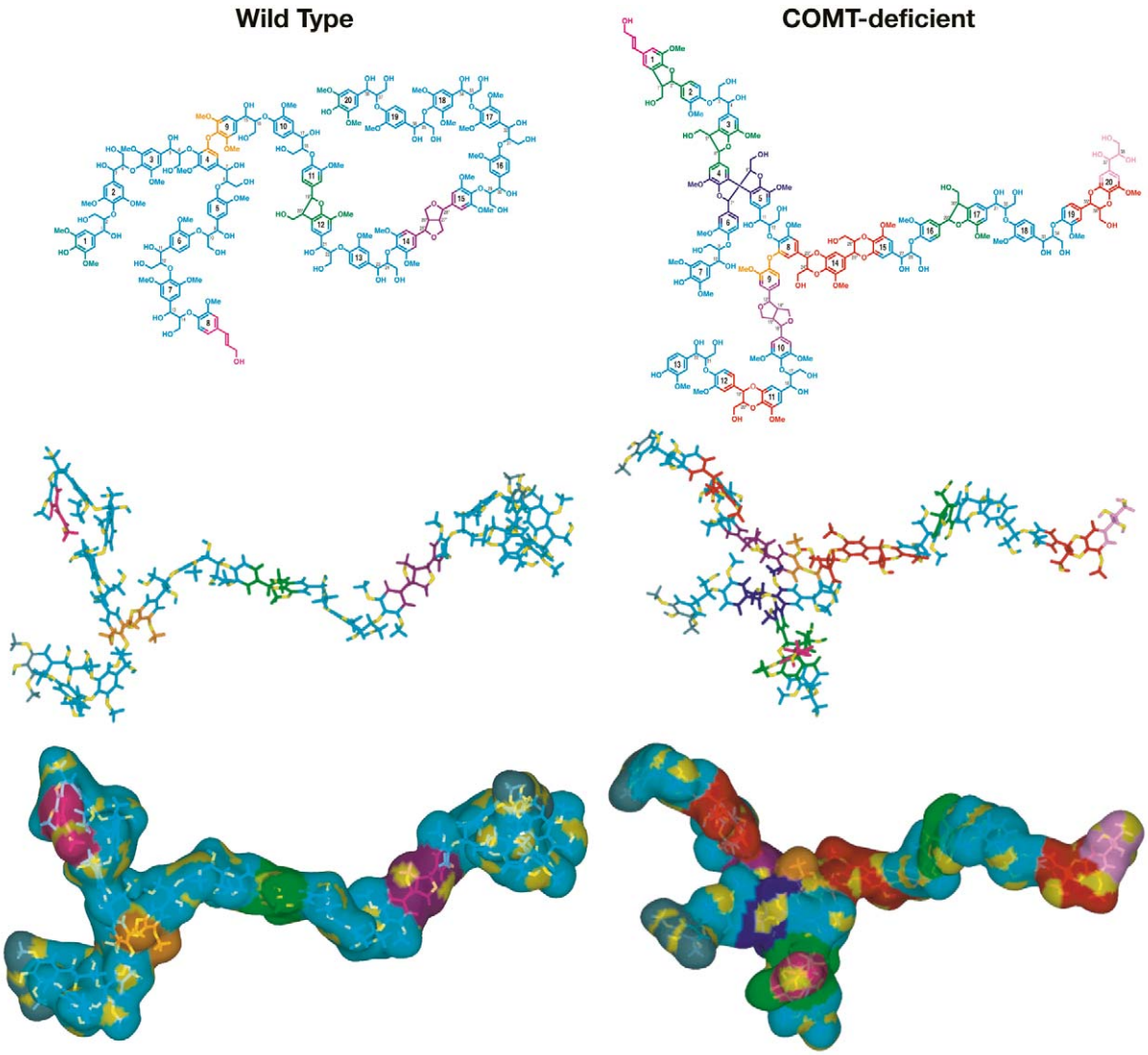

Figure 14. Poplar lignin models. These models, derived from 20 monomeric units, were created to accompany a recent review (Boerjan et al., 2003). There is also a web page from which 3D and other files may be obtained along with descriptions of the data from which they were constructed, and compositional data of the models (Ralph, 2001). Color coding across all model structures are similar. This way it is readily seen, for example, that β-ether units **A** (cyan) are prevalent in the wild-type lignins, but less prevalent in the COMT-deficient lignin. Since it is only these units that efficiently cleave by thioacidolysis, DFRC, or in pulping reactions, it is clear why the COMT-deficient lignin is less easily degraded. The new benzodioxane units **J** in the COMT-deficient lignin are colored red. The branch points (4–O–5-units **E**, orange; dibenzodioxocin units **D2**, dark blue) are also easily identified in the models. The structures represent only one of many millions of isomers. For the model for milled wood lignin from the wild type poplar, containing 20 phenylpropanoid units, there are 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 3 pairs of centers are fixed (asterisked centers; e.g. *trans*-ring junctions in phenylcoumarans). We therefore insinuate that there are 2^{35} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{34} (17,179,869,184) real viable isomers for the structure shown. For the model for milled wood lignin from the COMT-deficient poplar, also containing 20 phenylpropanoid units, there are again 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 10 pairs of centers are fixed (asterisked centers; e.g. *trans*-ring junctions in phenylcoumarans, *trans*-ring junctures in the benzodioxanes) in this case; the significant incorporation of 5-hydroxyconiferyl alcohol leads to a high proportion of benzodioxane structures which are mostly in the *trans*-ring form. We therefore calculate that there are 2^{28} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{27} (134,217,728) real viable isomers for the structure shown. Clearly, their racemic nature alone confers incredible complexity and variability on polymeric lignins. The structures have been minimized in place, but it must be emphasized that each unit is probably just a local minimum – full optimization over all conformational space in a molecule of this size is impractical. They are ONLY MODELS!

ternally trapped by the 5-OH, unlike the syringyl counterparts which they displace, they are not available for such cross-linking reactions with other components of the cell wall.

Despite the massive alterations in lignin structure caused by utilizing 5-hydroxyconiferyl alcohol **M5H** in place of the conventional sinapyl alcohol **MS**, plant viability and growth appears to be uncompromised. With the additional example of hydroxycinnamaldehyde **M2** (Figure 11) incorporation into CAD-deficient angiosperms (above), it logically appears that phenolics that incorporate well into the lignification scheme by entering into the predominant endwise coupling reactions with the growing polymer chain (mainly forming β-ethers) are the best surrogate monomers to be tolerated in lignification.

C3H-deficient angiosperms

One significant enzyme on the monolignol biosynthetic pathway had not, until recently, been examined. C3H is the crucial 3-hydroxylase allowing guaiacyl and syringyl units to be derived from *p*-coumaryl intermediates, Figure 12. Although grasses and softwoods, particularly compression wood, derive in small measure from *p*-coumaryl alcohol **MH**, lignins are overwhelmingly derived from coniferyl **MG** and sinapyl **MS** alcohols. An Arabidopsis mutant deficient in C3H proves the key nature of this step in monolignol biosynthesis (Franke et al., 2002). A particularly stunted plant, the mutant produces lignins that are derived from *p*-coumaryl alcohol but are totally devoid of the normal guaiacyl and syringyl units. Although far from healthy, such plants again illustrate the incredible metabolic plasticity of the lignification process. Other plant species downregulated in C3H are only just becoming available for detailed structural studies.

Implications of studies on monolignol-biosynthetic-pathway mutants and transgenics

One benefit of the apparent plasticity of the lignification process, and its ability to integrate compatible phenolics, is that it provides unparalleled opportunities to engineer lignin. To date, up- and down-regulating genes in the monolignol pathway has been explored to determine if the resultant plants are better substrates for important natural and industrial processes ranging from ruminant digestibility to chemical pulping. But perhaps we have only begun to scratch the surface of the engineering that is possible. The actual process of polymerization involves oxidative coupling of phenols. We have already seen evidence for phenols other than the three primary monolignols (e.g. acylated hydroxycinnamyl alcohols, hydroxycinnamaldehydes, 5-hydroxyconiferyl alcohol, dihydroconiferyl alcohol, guaiacylpropane-1,3-diol) being incorporated into the process. Some alterations have resulted in strongly colored woods, notably in CAD down-regulated plants (Cherney et al., 1991; Halpin et al., 1994; Higuchi et al., 1994; Baucher et al., 1996; Jouanin et al., 2000), which may find niche aesthetic applications. Obviously, the plant has requirements of its polymer, but the scope of the engineering that is possible may extend well beyond the tinkering that has been done to date on the monolignol pathway. A better understanding of how biochemical pathways interact is now required.

Models for oligomeric lignins; structural complexity

It is possible to construct hypothetical models for lignins based on the available analytical data. The most popular models were from Adler (Adler, 1977) and Nimz (Nimz, 1974), which are now considered to have too many α-ethers and obviously don't incorporate the latest findings such as the prevalent dibenzodioxocin 5–5-structures **D2**. The latest details are accommodated in a newer softwood lignin model (Brunow, 2001). It must be emphasized that these are simple structural models, for a chunk of hypothetical lignin polymer with a given number of units, derived from the available data. In no way should these models be construed as implying any particular sequence of monomeric units.

Models for poplar lignins were recently developed to illustrate two concepts, Figure 14 (Boerjan et al., 2003). The first are the structural differences caused by partial sinapyl alcohol substitution by 5-hydroxyconiferyl alcohol accompanying a reduction in COMT (see caption to Figure 14). Secondly, we wanted to stress a concept that has not been fully appreciated, and has been erroneously challenged (Lewis, 1999). Lignin, unlike most other natural polymers, is a racemic polymer. Proteins, incorporate only L-amino acids. There is only a single stereoisomer of the resultant protein. Polysaccharides are assemblies of optically pure (usually D-) monosaccharides. Again, there is only a single stereoisomer of the result-

ant polysaccharide molecule. Lignin monomers do not have any optical activity – unlike the protein and polysaccharide monomers, they possess no chiral centers. Optical centers are however created in each coupling reaction involving the sidechain β-position, two per event. The result is that the number of isomers of any "randomly" formed lignin structure increases with its degree of polymerization, quickly becoming astronomical. Thus a β-ether dimer, for example, has 4 optical isomers and half that number (i.e. 2) of "real" chemically distinct isomers. A β-ether trimer has 8 chemically distinct isomers, that can be resolved in high-resolution proton NMR spectra (Ralph, 1993). A β-ether tetramer has 32 isomers. And so it progresses geometrically. A pure random β-ether 110-mer was noted to have about the same number of isomers as there are atoms in our galaxy (Ralph, 1993). To begin considering the complexity of actual lignins, the other structures that can be formed (β–5, β–1, β–β, etc.) must be figured in, along with the potential to incorporate different monomers. It is clear that the number of ways to put together a chunk of lignin becomes astronomical. There are some constraints. For example, β–5 (Li et al., 1997) and β–β-units (Lundquist and Stomberg, 1988) form specific quinone methide isomers. Thus two of the optical centers are constrained relative to each other, reducing the number of possible isomers in structures containing them by a factor of 2. And some combinations of units are unlikely. Nevertheless, the complexity becomes overwhelming.

The poplar lignin models in Figure 14, conforming as closely as possible to available data, illustrate the concept of a racemic polymer quite well. The wild-type poplar model, containing only 20 monomeric units, has over 17 billion possible isomers. As an illustration of the effect of isomer constraints for some unit types, the COMT-deficient lignin model with the same number of units and the same number of optical centers, but containing more constrained β–5, dibenzodioxocin, and benzodioxane units, has a mere 134 million isomers!

The striking realization that hits one contemplating these notions is that it becomes unlikely that there are two lignin molecules over a given degree of polymerization (DP) that are structurally identical in all the plants on this entire planet!! It would be satisfying to calculate the DP necessary to make that assertion, but the exercise is too complex for these authors. Regardless, the implications of the fact that lignins are racemic polymers needs to be more widely appreciated.

Challenges to the accepted view of lignification

Are there any observations that are inconsistent with the currently accepted theory of lignification? We find none! There are certainly aspects of lignification that remain unknown and, as with any scientific hypothesis or theory, it is not absolute and can be modified or completely discarded to accommodate new observations.

However, there have been recent objections to the current theory. The most prominently articulated proposal stems from the belief that lignin polymer assembly by non-enzymatic coupling reactions under simple chemical control "represented a departure from all other known biochemical processes, since no explicit control of the final configuration of the product was envisaged," (Lewis, 1999), notions also expressed elsewhere (Davin et al., 1997; Lewis et al., 1998a, b; Davin and Lewis, 2000). Extrapolating observations from the new class of "dirigent" proteins that regio- and stereospecifically couple monolignol radicals to make (optically active) lignans, Lewis's group suggested that they had discovered a mechanism by which lignification could be brought into line with the synthesis of regular polymers like proteins (Lewis and Davin, 1998; Gang et al., 1999).

The new supposition was that arrays of dirigent proteins, later revised to arrays of dirigent protein sites, could orchestrate the synthesis of a lignin polymer with absolute structural control (Lewis and Davin, 1998; Gang et al., 1999). Support for the dirigent protein array model is the observation that an antibody binds to epitopes that appear to coincide with lignifying regions (Gang et al., 1999). The dirigent gene appears to be highly expressed in the late stages of wood development (Hertzberg et al., 2001), and the protein has a signal peptide that is anticipated to deliver it to the cell wall. However, the arguments against the earlier-developed theory are unfounded. For example, the opinion that control of coupling by dirigent proteins is required to explain the proclaimed "anomaly" of high β-ether frequencies (Lewis and Davin, 1998; Lewis, 1999), i.e. that β-ether frequencies are higher in lignins than in *in vitro* coupling products, is simply incorrect. As detailed above, straightforward chemical cross-coupling reactions compellingly affirm that nothing more than control of monolignol (radical) supply is required to explain high β-ether frequencies; the selectivity for preferential β-ether formation has been convincingly demonstrated *in vitro* (Syrjanen and Brunow, 1998, 2000, 2001).

What observations refute any notion of absolute structural control? The following three examples should suffice.

Lignins are not optically active

There are no racemic proteins. Enzymes and proteins, including the dirigent proteins, are optically active, have optically active binding sites, and produce optically active products. As reviewed (Ralph et al., 1999a; Akiyama et al., 2000), in a variety of studies over decades, using a variety of methods, fragments released from the lignin structure (under conditions which have been proven to retain optical activity) have never been found to be optically active. And entire isolated polymer fractions have no detectable optical activity.

Arguments followed that there might be two sets of dirigents, equally represented, encoding opposite antipodes (Lewis and Davin, 1998; Gang et al., 1999). Applying Occam's razor that it is the aim of science to present the facts of nature in the simplest and most economical conceptual formulations (Thorburn, 1918), this proposal must be dismissed. The number of possible coupling reactions required to create the bonds in a hardwood lignin is around 50, suggesting that at least this many dirigents, specific with respect to the substrate monomers or oligomers and their modes of coupling, would be required. If another set is needed to encode the mirror images, around 100 dirigent proteins/sites are required. This ignores the likely inability of the dirigent protein, or any protein containing the array of dirigent sites, to actually interact with two growing lignin polymers to effect the 5–5- and 4–O–5-couplings which are rather prevalent mechanisms involved in combining growing polymer chains and creating branching (see Figure 4). The hypothesis that lignin primary chains might replicate by template polymerization (Guan et al., 1997; Sarkanen, 1998), where one chain dictates the exact mirror opposite chain in the next assembled molecule (Lewis and Davin, 1998), is equally problematic. There simply isn't enough room in the lignifying wall, replete with already laid down polysaccharides, to allow such an assembly; examination of various cell wall models illustrates this concept (Carpita and Gibeaut, 1993; Terashima et al., 1993; Jurasek, 1996, 1998a, b). Experiments aimed at template replication using a homopolymer model as the template would quickly prove or refute structural replication. The provided lignin "template" is likely nothing more than an assembly surface onto which monomers may absorb and react; other polymers, unrelated to lignin, could have the same effect of "engendering polymer formation."

The malleability of lignification

As noted above, massive changes in the syringyl:guaiacyl composition of lignins result from blocking sinapyl alcohol production or by efficiently diverting coniferyl alcohol production toward sinapyl alcohol, by manipulating a single gene/enzyme such as F5H. How can a biochemical assembly that is programmed to synthesize a defined structure, incorporating coniferyl alcohol and sinapyl alcohol monomers in a specific order with a specified bonding pattern, tolerate an imposed absence of monomers required to make that polymer? The observations from mutants and transgenics cannot be accommodated by a model requiring the exact stipulation of lignins' primary structure. Plants also respond rapidly to gravitropic stress; gymnosperms produce compression wood lignins with higher *p*-coumaryl alcohol **MH** levels, for example (Timell, 1982; Fukushima and Terashima, 1991).

Beyond the primary monolignol compositional malleability, it was stipulated that monomer substitution could not be accommodated in any controlled biopolymer assembly process (Lewis, 1999). However, evidence continues to mount that plants are creating functional "lignin" polymers from available phenolics when synthesis of their primary monomers is downregulated. The most compelling example is the clear substitution of 5-hydroxyconiferyl alcohol for sinapyl alcohol in angiosperms that are deficient in the enzyme COMT (see above). Obviously, the resulting polymer, containing novel benzodioxane units, has a markedly different structure, and consequent properties, than the normal lignin created with sinapyl alcohol. And yet these highly modified lignin polymers appear to have the properties required to allow the plant to remain viable. Monomer substitution is unquestionably occurring in these plants. This is not the first example. In the more highly controlled biosynthesis of xyloglucan polysaccharides, L-galactose is substituted for L-fucose in fucose-deficient *mur-1* mutants of Arabidopsis (Zablackis et al., 1996). Apparently the plant was able to conscript the structurally similar sugar to produce its analog polymer when fucose synthesis was thwarted. Lignification's solely chemical and combinatorial nature precludes it from being termed an

assembly — that term presupposes that lignins are biosynthesized with full structural control.

The problem of lignin-unit stereochemistry

A compelling argument that lignin structure *per se* is not under absolute control comes from examining the stereochemistries of lignin units. Enzymes are proficient at adding nucleophiles such as water stereospecifically, i.e. to one face of the molecule resulting in a single isomer (which should also be optically active). Yet all indications are that the quinone methide intermediates formed directly after β–O–4-coupling simply add water in an uncontrolled (chemical) fashion, Figure 6. Thus, as documented above, either *in vivo* or *in vitro*, β–O–4-guaiacyl ethers are produced essentially in a 50:50 *erythro*:*threo* ratio, whereas β–O–4-syringyl ethers are produced in a 75:25 ratio, the ratios depending somewhat on pH (Brunow et al., 1993). Furthermore, although the *erythro*- and *threo*-isomers have two enantiomers each, they are formed in equal amounts, i.e. there is no optical activity (Akiyama et al., 2000). Acylated syringyl monolignols have less *erythro*-selectivity, *in vivo* results appearing to match those *in vitro* again (Lu and Ralph, 2003, unpublished). If the primary structure of lignins is to be absolutely specified, why would this feature of the polymer, in its most abundant β-ether units, remain uncontrolled? It cannot be argued that the ratios observed are simply as a result of eventual thermodynamic equilibrium. Indeed, the ratios are clearly kinetically controlled, since the equilibrium *erythro:threo* ratios are 50-55:50-45. Similarly, the diastereomer distribution of other types of lignin structures appears to be kinetically controlled (Lundquist et al., 2003). The logical conclusion is that the plant is not actively controlling isomer distribution and that it is simply under chemical control.

Is lignification a controlled event?

Let there be no mistake, lignification is carefully controlled! The supply of monomers to the lignifying zone is carefully orchestrated by the cell. *p*-Coumaryl, coniferyl, and sinapyl alcohols, as well as their conjugates (e.g. sinapyl *p*-coumarate in grasses), are metered out to the wall with obvious temporal and spatial control (Terashima et al., 1993). The concentration of radicals appears to be carefully limited, through supply of the cofactor, H_2O_2, required for peroxidase-assisted oxidations (dehydrogenations), and possibly by radical quenching agents such as ascorbate (Christensen et al., 2000a; Sakihama et al., 2002). Nucleation sites for starting the lignification process seem to be carefully laid out (prior to lignification); for example, ferulates in grasses have been implicated as nucleation sites (Ralph et al., 1995). Harris' group revealed that ferulates are present in gymnosperm cell walls as well (Carnachan and Harris, 2000). Ferulates should therefore not be ruled out as explanations for the beautiful pictures showing apparent nucleation behavior in softwood walls that are beginning to lignify (Donaldson, 1994, 2001; Terashima et al., 2004). The binding of peroxidases to specific epitopes within the cell wall is potentially a means to control lignin deposition in space within the cell wall. A peroxidase isolated from zucchini binding to pectin exemplifies this concept (Carpin et al., 2001). The cell maintains careful control over the supply of monolignols and the conditions for polymerization. These controls however must not be confused with control over the assembly of the lignin polymer. All evidence is that the actual structure of a lignin polymeric unit is controlled only by the chemical reactions that are involved, the supply of available monomers, radical-generating capacity, and the conditions in the wall. To date, no evidence for any control of the lignin primary structure by an organized assembly process is compelling. Statements that "there is now little doubt that the biochemical control of lignification is implemented at the levels of both monomer deposition and linkage specification" (Gang et al., 1999), or the proclaimed evidence for "full biochemical control of lignin assembly, i.e. via monomer transport, oxidation, and directed polymerization" (Patten et al., 2003) are simply unfounded.

Conclusions

Lignins as natural plant polymers are becoming increasingly well characterized. Various aspects of lignin deposition, such as the transport of monomers and the control of monomer supply, need considerably more work. As for the process of lignification, the last step in producing the polymer from the supplied monomers via peroxidase-assisted oxidation, the existing model for chemically-controlled combinatorial coupling reactions involving various monomers and the growing polymer accommodates the known facts very well. New unifying hypotheses and theories remain welcome, but must be subjected to normal scientific debate and testing. Lignin structural studies

will continue to play a key role in understanding the nature of this enigmatic class of polymers.

Rather than being labeled as abominations of nature due to their combinatorial synthesis, lignins should be seen as marvelously developed polymers crucial to sustaining plant functions. They allow water transport, strengthen the wall, and provide various defense mechanisms. These features require a resilience and indeed a metabolic plasticity to react to stresses of various kinds. A hard-wired polymer would simply reduce the flexibility to respond. Even an event as serious as completely knocking out a gene (either by natural or artificial methods) crucial to the synthesis of a lignin monomer may not prevent a plant from responding by making a differently structured polymer and remaining viable. Such is the case with F5H and COMT, which are crucial for making sinapyl alcohol, and for which complete knockout mutants exist (in Arabidopsis, for example). Lignification is therefore seen as a remarkably evolved solution allowing plants considerable flexibility in dealing with various environmental stresses, and conferring on them a striking ability to remain viable even when humans or nature alter "required" lignin-biosynthetic-pathway genes/enzymes. The malleability provides significant opportunities to engineer the structures of lignins beyond the limits explored to date. Also we thank Richard Dixon, Catherine Lapierre, Lise Touanin, Liming Zhang, Richard Helm, Stéphane Quideau, Junpeug Peng, Alain Boudet, Ronald Sederoff, Vincent Chiang and many others whose nationals from published studies contributed to this manuscript.

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