Chapter 1

Lignin and Lignan Biosynthesis: Distinctions and Reconciliations

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Before 1996, the framework within which lignin biosynthesis was understood at the molecular level had not fundamentally changed for 4 decades. During the same period nothing at all had been explicitly proposed about the mechanistic basis for lignan formation *in vivo*. The associated deficit in plant biochemistry was not minor: lignins and lignans together account for roughly 30% of the organic carbon in vascular plants. On the other hand, the biochemical transformations in phenylpropanoid metabolism leading, *via* the shikimate-chorismate pathway through phenylalanine or tyrosine, to the so-called monolignols (namely, the monomeric lignin/lignan precursors) came to be reasonably well documented. Indeed, attention has more recently been drawn to the identification of *de facto* rate-limiting steps in the various metabolic segments of the pathway as potential control-points for biotechnological manipulation. The most curious characteristic usually attributed to the lignin/lignan biosynthetic pathway is that the monolignol-derived radical coupling processes leading to lignans are regio- and stereospecific, whereas those resulting in lignin macromolecules ostensibly are not. Now that the molecular basis for the dehydrogenative dimerization of monolignols to lignans has been unraveled, however, it appears likely that an analogous mechanism may be operative in the dehydrogenative polymerization of monolignols to lignins. The investigation of this possibility has indeed become a central concern in the field of lignin biosynthesis.

It has been almost three decades (*1*) since a comprehensive attempt had been made to summarize and evaluate contending views about lignin biosynthesis. Accordingly, the present volume draws together the current, yet conceptually vastly differing, ideas about how lignin composition and structure are established in living plants. It differs from all prior contributions, not only in introducing fresh evidence to support a compelling new paradigm that seeks to understand how lignin structure is established *in vivo*, but also in including lignan biosynthesis for comparative purposes. Indeed it appears that the first committed step in lignan biosynthesis bears an important relationship to the manner in which the configurations of lignins are specified *in vivo*.

Lewis and Sarkanen; Lignin and Lignan Biosynthesis
ACS Symposium Series; American Chemical Society: Washington, DC, 1998.

Collectively, lignins and lignans are *the* major metabolic products of phenylpropanoid metabolism in vascular plants. For woody plants, they typically account for more than 20% of the weight of angiosperms and over 25% of that of the gymnosperms. Together, they constitute some of the metabolically most expensive products generated by plants (*2*), and are derived from the shikimate-chorismate pathway (Figure 1) (*3, 4*) which produces the aromatic amino acids, phenylalanine and tyrosine (Figure 2) (*5*). As shown in Figure 3, extension of the phenylpropanoid pathway in vascular plants, from phenylalanine onwards, ultimately leads to both the polymeric lignins as well as the dimeric/oligomeric lignans. The lignins fulfill essential functions by providing structural reinforcement to (woody) plant tissues, thereby allowing all vascular plants to stand upright. Moreover, this cell-wall reinforcing process provides both the corresponding vasculature which is necessary for water conduction, as well as in assisting the means for re-orientating stems and branches in response to changes in mechanical stresses and light levels. The lignans, on the other hand, are a ubiquitous group of closely related *non-structural* phenolic metabolites, which are primarily dimeric although higher oligomers exist. They play substantive roles in plant defense, through their potent biocidal (*6-9*) and antioxidant (*6, 10-13*) properties, and many also have important functions in medicine (*14-16*) and health maintenance (*17-22*).

From an evolutionary perspective, perhaps the most significant aspect of the lignins and lignans lies in the fact that, in their absence, vascular plants would *not* readily survive. Indeed, the continued existence of *all* terrestrial animal forms is in one way or another dependent on vascular plants and, hence, on the lignin/lignan biosynthetic pathway. Moreover, it is the differential expression of this pathway that is largely responsible for much of plant biodiversity: the variable deposition of these substances, in terms of their amount and specific composition, can dramatically alter the woody textures of plants, as well as affect other properties, such as heartwood color, durability and rot resistance, and even their (aromatic) fragrance.

Recognition of these factors has prompted the study of the lignin and lignan biosynthetic pathways, in order to develop biotechnological strategies directed to the rational re-engineering of woody and non-woody plants with respect to their lignin/lignan contents and composition. The goals of such studies are manifold and include: enhancing the quality (texture, color, durability, *etc.*) of specific woody plants for lumber and fine furniture applications; either lowering the lignin contents of specific woody plants, or rendering them more susceptible to chemical/ biochemical delignification protocols for pulp and paper manufacture; lowering lignin contents in domestic livestock feedstocks, in order to reduce waste disposal difficulties; re-engineering more rigid (structurally reinforced) plants for agricultural purposes that would be better able to survive in harsher climates; providing intermediate chemicals for further processing (*e.g.* into new 'bio'polymers); improving health protection by increasing levels of cancer-preventing lignans in staple dietary foodstuffs; expanding sources of important (lignan-derived) pharmaceuticals (*e.g.* podophyllotoxin); exploiting the antioxidant properties of lignans; *etc.*

This overview is intended as a brief commentary upon the salient accomplishments in the field over the last six decades. It begins with the confusion attending the ascertainment of the fundamental constitution of the lignins and lignans, and then proceeds towards a clarification of the characteristic biochemical transformations that are responsible for their biosynthesis.

Brief Historical Development of Ideas

Phenolic substances account for *ca.* 30-40% of all organic carbon (*23*) in vascular plants, of which the lignins are the predominant metabolites. Yet a determination of the actual mechanisms for the formation of lignin polymers *in vitro* and *in vivo* has, for several decades, been thwarted by the persistence of a profoundly misguided paradigm. In the early 1930's, a tremendous controversy materialized over the

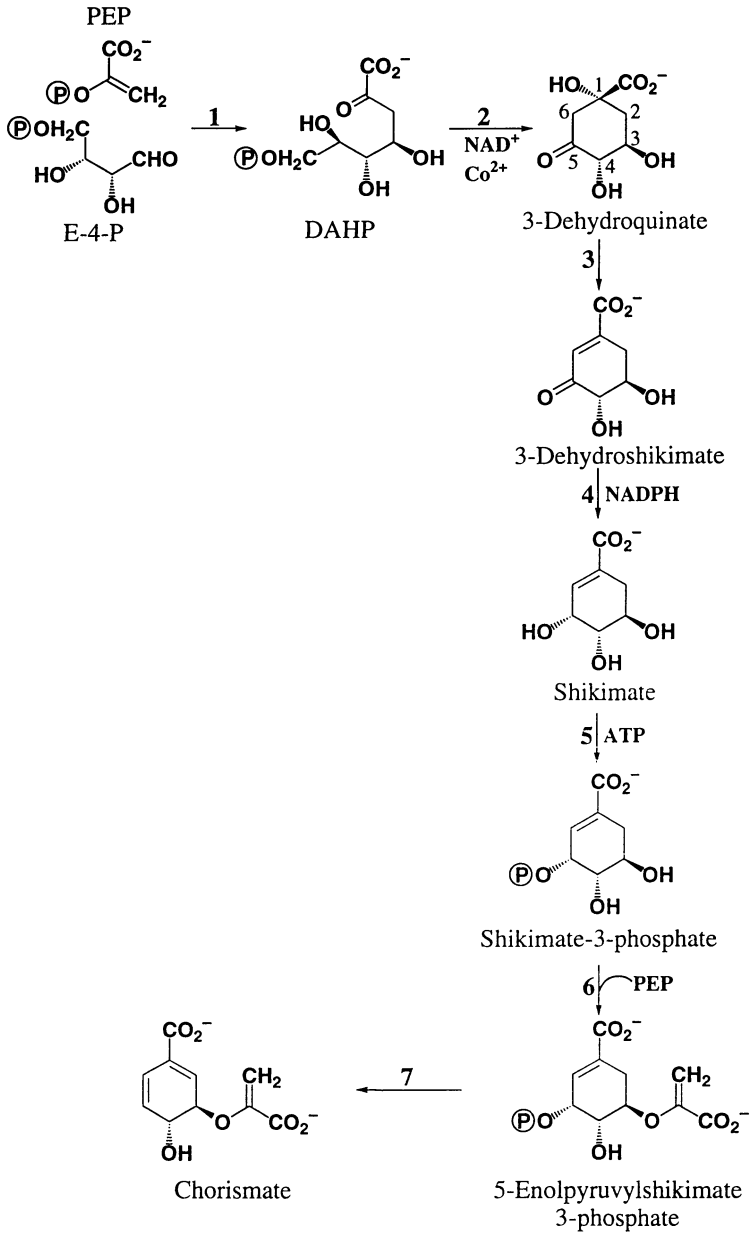


Figure 1. The shikimate-chorismate pathway: **1,** 3-deoxy-D-arabinose heptulosonic acid-7-phosphate (DAHP) synthase; **2,** dehydroquinate synthase; **3,** 3-dehydroquinate dehydratase; **4,** 3-dehydroshikimate reductase; **5,** shikimate kinase; **6,** 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase; **7,** chorismate synthase.

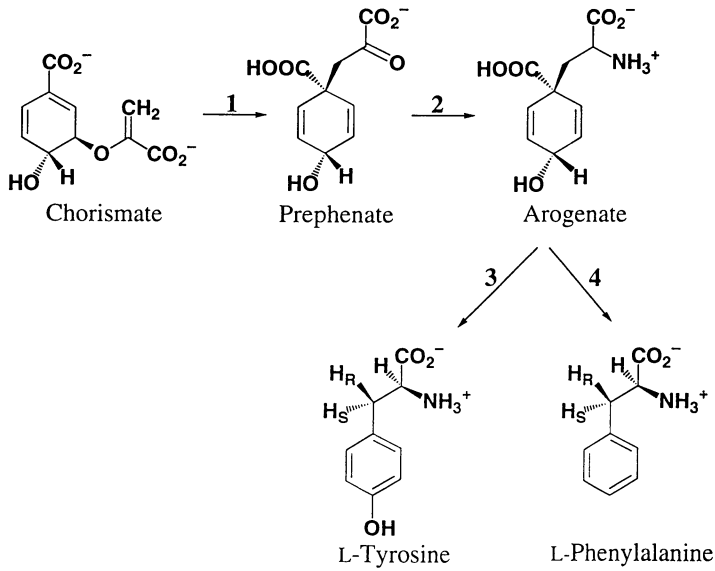


Figure 2. Biosynthetic pathway from chorismate to phenylalanine and tyrosine via arogenate: **1,** chorismate mutase; **2,** prephenate amino transferase; **3,** arogenate dehydrogenase; **4,** arogenate dehydratase.

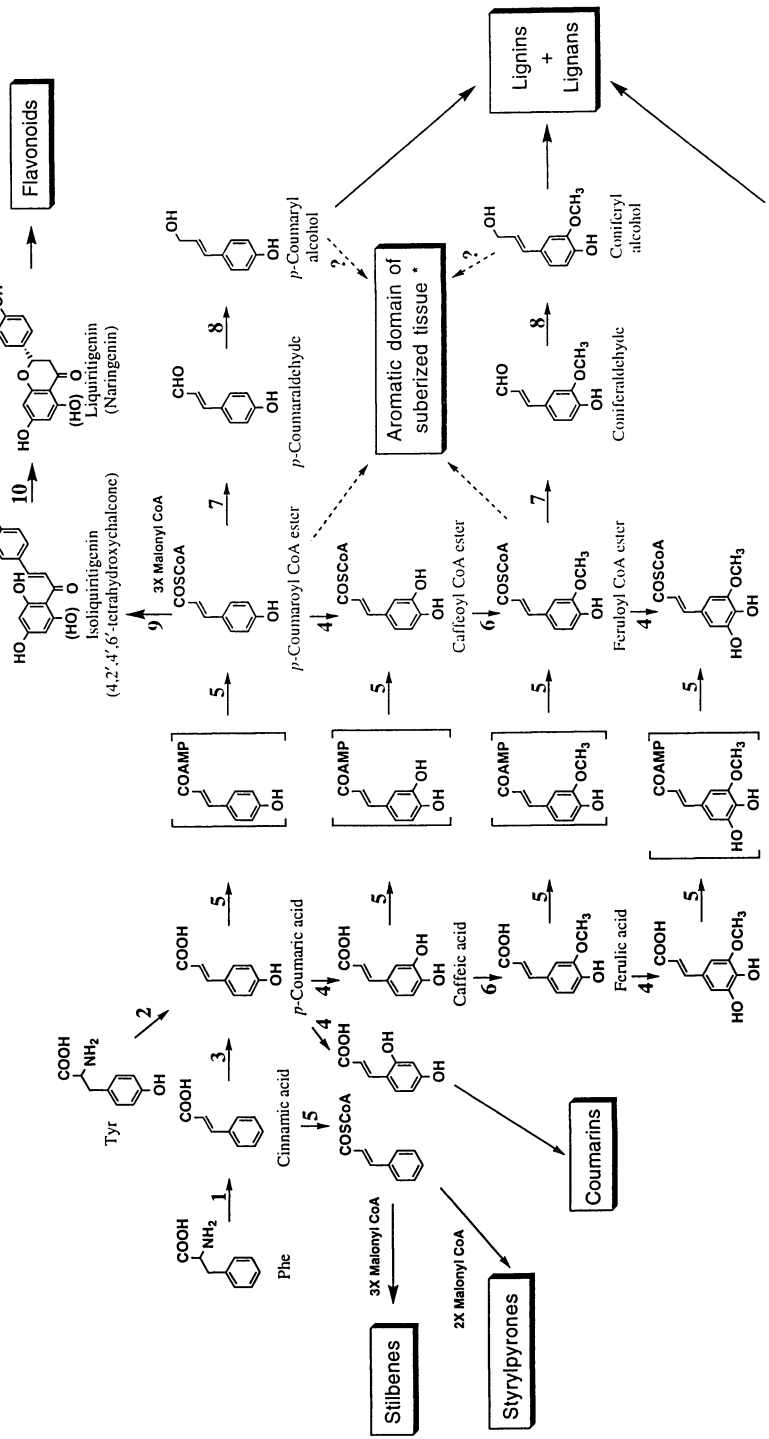
chemical nature of lignins: various proponents described it as either a polysaccharide-derived polymer, arising from degradation of hemicelluloses, cellulose or pectins (*24*), and others proposed that even the terpenoids were products of altered lignin metabolism (*25*). Yet, much earlier, Ferdinand Tiemann (*26*) and Peter Klason (*27*) had speculated that lignin was derived from *E*-coniferyl alcohol **1** (Figure 4), and in 1933 Holgar Erdtman suggested that the monolignol (a term coined later) was converted into lignin *via* a dehydrogenative polymerization process (*28*). It was not until the 1950's that radiotracer experiments had clearly established *E*-coniferyl alcohol **1** and related monolignols to be, in fact, lignin precursors (*29*). Further studies revealed the details of the biosynthetic pathway between phenylalanine and the monolignols to involve the sequence of transformations shown in Figure 3 (see ref. (*30*) for a review).

Karl Freudenberg, Takayoshi Higuchi and others then attempted to characterize the enzymology involved in the phenolic coupling reactions that give rise to lignification (*31-35*). Curiously, they studied the dehydrogenative polymerization of *E*-coniferyl alcohol **1** using crude mushroom (laccase) (*32*) and horseradish (peroxidase) (*31, 35*) extracts, even though the enzymes employed did not originate from lignifying tissues! Thus, the enzymology responsible for the formation of an important natural product—Nature's second most abundant biopolymer, no less—relied upon enzymes from sources that had no connection with the biosynthetic pathway itself. Evidence for the involvement of comparable processes *in vivo* was thought to be provided by the observation that these crude enzyme preparations readily converted coniferyl alcohol, in the presence of O_2 and H_2O_2 as respective co-substrates, into products which *at that time* were assumed more or less to represent lignin. That is, according to these investigators, the only enzymatic control of lignin assembly involved free-radical generation from the monolignols with subsequent coupling occurring non-enzymatically. This supposition represented a departure from *all* other known biochemical processes, since no explicit control of the final configuration of the product was envisaged.

The (unresolved) problem is that, *in vitro*, the initial dehydrogenative coupling products from *E*-coniferyl alcohol **1** are primarily racemic (±)-dehydrodiconiferyl alcohols **2**, (±)-pinoresinols **3** and (±)-*erythro/threo* 8–*O*–4′-coniferyl alcohol ethers **4** in ratios of approximately 6:3:2 (see Figure 4 for structures). These products are formed through non-specific bimolecular coupling between free radical species generated by one-electron oxidation of the monolignol. On the other hand, lignin biopolymers possess frequencies of interunit linkages which differ markedly from the proportions of the different dimers produced *in vitro*. That is, natural lignins mainly embody 8–*O*–4′ (≥50%) and dehydrodiconiferyl alcohol (~10%) substructures, together with a variety of other linkages present in relatively low abundance. This has been established not only through degradative analyses (*36*), but also by specific *in situ* carbon-13 labeling, which was initially applied in order to study lignin biosynthesis (*37, 38*). There is, therefore, a marked contrast between lignin structures *in vivo* and those of the so-called monolignol dehydropolymerisates produced *in vitro*.

The early 1930's also saw the classification of an abundant group of dimeric phenylpropanoid compounds, linked through 8–8′ bonds, as lignan(e)s, a term which was used by R. D. Haworth in 1936 (*25*). Otto Gottlieb subsequently introduced the term neolignan to encompass all non 8–8′ linkages (*39, 40*), and further modified this definition to encompass the products from allylphenol coupling (*41*). However, throughout the present volume the term lignan is used to describe *all* possible coupling products as long as the linkage type is specified (8–8′, 8–5′, 8–*O*–4′ *etc.*, Figure 4). Importantly, lignans are often found in plants in optically pure form, although the particular antipode present can and does vary with the species (*42-45*). This contrasts with the racemic linkages which are believed to be incorporated into lignin biopolymers.

Erdtman (*46*) and various other investigators also studied monolignol coupling, but with the goal of attempting to determine how regiospecific or stereoselective control could be engendered to give optically active 8–8′ linked lignan products.


Flavonoids
Lignins + Lignans
Aromatic domain of suberized tissue *
Coumarins
Stilbenes
Styrylpyrones
3X Malonyl CoA
2X Malonyl CoA
Tyr
Phe
Cinnamic acid
p-Coumaric acid
Caffeic acid
Ferulic acid
5-Hydroxyferulic acid
Isoliquiritigenin (4,2',4',6'-tetrahydroxychalcone)
Liquiritigenin (Naringenin)
p-Coumaroyl CoA ester
Caffeoyl CoA ester
Feruloyl CoA ester
5-Hydroxyferuloyl CoA ester
p-Coumaraldehyde
Coniferaldehyde
p-Coumaryl alcohol
Coniferyl alcohol

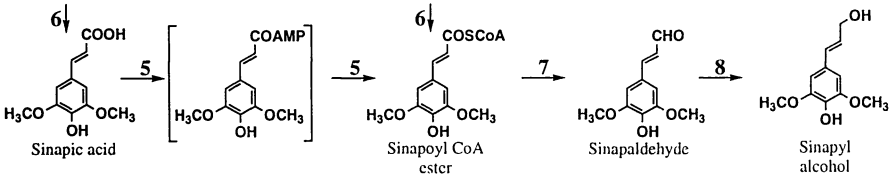


Figure 3. Main elements of the phenylpropanoid pathway: lignins and lignans are monolignol derived. **1,** phenylalanine ammonia-lyase; **2,** tyrosine ammonia-lyase (mainly in grasses); **3,** cinnamate-4-hydroxylase; **4,** hydroxylases; **5,** CoA ligases involving AMP and CoA ligation, respectively; **6,** *O*-methyltransferases; **7,** cinnamoyl-CoA:NADP oxidoreductases; **8,** cinnamyl alcohol dehydrogenases; **9,** chalcone synthase; **10,** chalcone isomerase. [Note: Conversions from *p*-coumaric acid to sinapic acid and corresponding CoA esters are boxed since dual pathways *appear* to be in effect; *: may also involve *p*-coumaryl and feruloyl tyramines, as well as *small* amounts of monolignols].

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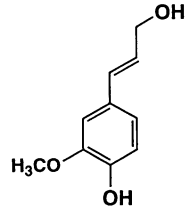
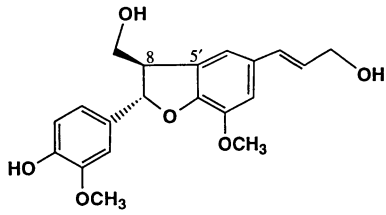
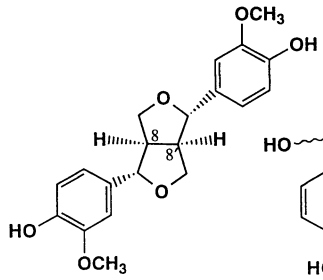


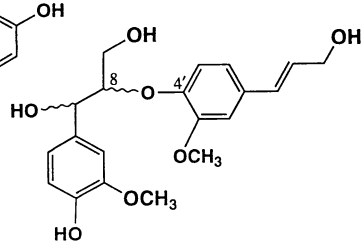
1: Coniferyl alcohol



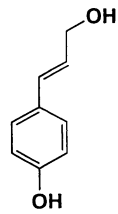
2: 8,5′-Coupling



3: 8,8′-Coupling



4: 8-*O*-4′-Coupling



5: *p*-Coumaryl alcohol

Figure 4. *E*-Coniferyl alcohol **1** and the major racemic products (**2-4**) obtained by non-specific free-radical coupling, and *E*-*p*-coumaryl alcohol **5**.

Such studies were unsuccessful, since all attempts again engendered formation of the various well-known racemic products resulting from linkages between the different possible coupling sites present in the substrate.

Accordingly, none of the enzyme-catalyzed phenoxy radical coupling reactions *in vitro* leading to either lignans or lignins has satisfactorily duplicated those encountered *in vivo*. Given this fact, it is unclear why the non-specific dehydrogenative coupling hypothesis, which has *no* counterpart elsewhere in biochemistry, has not been substantially challenged, or at least seriously reconsidered. From a broader perspective, however, recent years have witnessed a growing interest in not only delineating how the coupling reactions leading to lignins and lignans actually occur, but also how the configurations with respect to lignin monomer composition are determined, and how the carbon (*i.e.* monomeric substrate) is allocated to the pathway. Each of these questions is now surveyed in some detail below.

Phenylpropanoid Metabolic Flux: Its Modulation and Control

In 'simple' aquatic plants, such as algae, biosynthesis of the aromatic amino acids, phenylalanine and tyrosine, is primarily directed to protein formation. The shikimate-chorismate-derived pathway to these essential proteinaceous building blocks is shown in Figures 1 and 2, and forges the link between glycolysis, the pentose phosphate pathway and aromatic amino acid metabolism. Evolution of vascular plants, on the other hand, significantly exploited the carbon flux into phenylalanine/tyrosine biosynthesis, *via* the shikimate-chorismate pathway, but not for protein biosynthesis. Instead, the phenylalanine (tyrosine) so formed was conscripted into phenylpropanoid metabolism, ultimately affording lignins, lignans and related metabolites. Altogether, some 30-40% of all organic carbon in vascular plants is 'stored' in this manner. Yet, curiously, as in the enzymatic control of phenylpropanoid phenoxy radical coupling, how carbon flux is *differentially* targeted into phenylpropanoid metabolism and its distinct biochemical branches has received little detailed attention.

For example, the shikimate-chorismate 'upstream' biosynthetic segment of phenylpropanoid metabolism has been almost completely ignored, whether in how it serves (tissue-specifically) to regulate the overall phenylpropanoid pathway or functions as a means for the storage of metabolites ultimately targeted to the phenylpropanoid pathway. Accordingly, it is appropriate that the second chapter (by Carol Bonner and Roy Jensen) in the present volume be devoted to a summary of current knowledge about the regulation and control of this metabolic segment. Significantly, however, metabolic control analysis (*47-50*) has not yet been carried out on the pathway between erythrose-4-phosphate and phosphoenol pyruvate to prephenic acid *in planta*, an essential matter if regulation of this segment of the pathway is to be fully understood.

Beyond prephenic acid, the next metabolic segment involves formation of the aromatic amino acids, phenylalanine (Phe) and tyrosine (Tyr), and their subsequent deamination to afford cinnamic and *p*-coumaric acids. This is treated as a distinct segment since it is here where both carbon and nitrogen metabolism directly interface. Thus, as can be seen from Figure 2, nitrogen is introduced at the point of prephenic acid transamination to afford arogenic (Agn) acid, which can then be converted into either phenylalanine (or, to a much lesser extent, tyrosine) in tissues undergoing active phenylpropanoid metabolism. Originally, it was thought that prephenic acid was the precursor of (*p*-hydroxy)phenylpyruvic acid(s), with the latter being transaminated to generate phenylalanine (tyrosine). However, pioneering work by Jensen *et al.* (*5*) identified arogenate as the pivotal precursor of the two aromatic amino acids. Indeed, all subsequent detailed enzymological studies to date have identified the arogenate pathway alone as leading to the aromatic amino acids in vascular plants; thus, the (*p*-hydroxy)phenylpyruvate pathway has not been validated.

Given that nitrogen is only introduced into the pathway just prior to the formation of Agn, Phe and Tyr, it is remarkable that, when phenylalanine and

tyrosine are conscripted for phenylpropanoid metabolism rather than for protein synthesis, the nitrogen is immediately removed (Figure 5). The enzymes involved, phenylalanine and tyrosine ammonia lyases, were discovered by Jane Koukol and Eric Conn (*51*) and Arthur Neish (*52*) in 1961, respectively, and the former is probably the most extensively investigated in all 'secondary' metabolism. Interestingly, whether the phenylpropanoid pathway is induced or not, none of the three aromatic amino acids (arogenate, phenylalanine or tyrosine) build up to any appreciable extent, suggesting that one of the preceding steps may be 'rate-limiting' and thus a plausible candidate for a control point in the pathway. Additionally, since an equimolar amount of ammonium ion (and hence nitrogen) is liberated for every mole of phenylpropanoid product formed, then an efficient nitrogen recycling process has to be in effect or otherwise active phenylpropanoid metabolism would be nitrogen limited. In view of the 1989 assertion that such a nitrogen recycling process must therefore be operative (*2*), some considerable satisfaction attended the demonstration several years later (*23, 53, 54*) that this was achieved *via* ammonium ion reassimilation through the glutamine synthase/glutamine 2-oxoglutarate amino transferase pathway (Figure 5). In this way, regeneration of the amino donor, L-glutamate, was able to occur, the latter undergoing subsequent transamination to afford arogenate and ensuring that the overall flux of carbon into the phenylpropanoid pathway continued without any further demand on nitrogen from the plant. Clearly, any disruption of this cycle would disrupt phenylpropanoid metabolism. However, regulation of this point in the pathway would only be useful if the modulation of carbon flux (Figure 3) could be *selectively* achieved into the lignin and/or lignan pathways, respectively.

Beyond the deamination step, the cinnamic (*p*-coumaric) acid(s) formed can be metabolized *in planta* into the monolignols (and hence lignins and lignans), as well as a plethora of phenylpropanoid (acetate) derived-products, such as the suberins, flavonoids, coumarins, and styrylpyrones. It is important to note, however, that formation of a particular metabolite normally can only occur in either a tissue-specific, temporally specific, or, in some instances, species-specific manner. For example, administration of the phenylalanine ammonia lyase inhibitor, AOPP, to developing *Zinnia elegans* tracheids in cell culture resulted in the complete inhibition of lignin biosynthesis but apparently did not affect any other branchpoint pathways (*55*). Importantly, in this case, the overall architecture of the *Zinnia* tracheids was fully established during the deposition of cellulose, hemicellulose (including pectins) and structural proteins. Clearly, therefore, any *selective* targeting of this part of the pathway in a lignin specific manner (*i.e.* by targeting the appropriate tissue) could be envisaged to have a major impact on the carbon flux into *lignin* formation.

The steps beyond cinnamic and *p*-coumaric acids leading to the monolignols have received most attention in regard to their being potential regulatory steps, even though no metabolic control analysis has yet been described. Instead, the molecular approach has primarily been devoted to attempts at determining the effects of differentially expressing genes encoding putative regulatory enzymes in the *lignin* biosynthetic pathway (Figure 3) (*56-62*). As can be seen, there are essentially only four types of transformations beyond cinnamic acid, namely aromatic hydroxylations (*63-65*), *O*-methylations (*66-68*), CoA ligations and (consecutive) NADPH dependent reductions (*69*). It cannot be overemphasized, however, that the pathway as shown is deceptively simple. It has many branching points, such as to the lignins and lignans, which bifurcate at *only* the monolignol level or, in the case of the flavonoids, where the 'point of departure' from the pathway is formation of the *p*-coumaryl CoA esters. To restate what was said earlier, vascular plant species not only display various segments of the pathway to different extents, but particular tissues/cell types are only involved in *specific segments* of the pathway (*e.g.* flavonoids). Accordingly, it is bewildering that so many multifarious contributions to the literature have variously referred to each of the specific enzymatic steps between phenylalanine and the monolignols as being rate limiting or potentially regulatory for lignin. Put another way, all gene expression/activation studies to date have focused on putatively

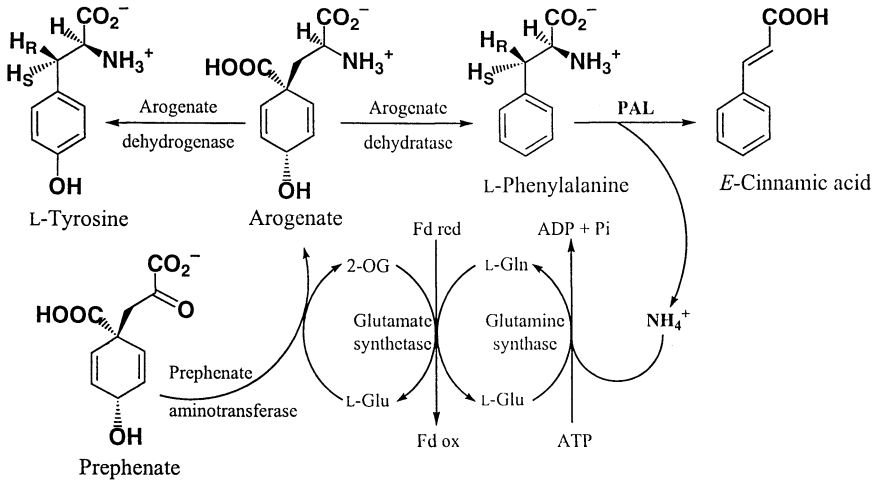


Figure 5. Proposed nitrogen cycling metabolism during active phenylpropanoid metabolism.

regulatory steps, but without any definitive data establishing that they are, indeed, rate-limiting or inevitably lignin-specific.

Considering the pathway itself, the first aromatic hydroxylation step leading to *p*-coumaric acid is catalyzed by a cytochrome P-450 NADPH-dependent oxidase (*63, 64*), as is the later conversion of ferulic acid to 5-hydroxyferulic acid (*65*). On the other hand, the step converting *p*-coumaric acid into caffeic acid is still uncertain. Originally described as a non-specific phenolase [see refs. (*70-72*)], it is now considered but not yet proven to be a distinct kind of oxidase, perhaps of a cytochrome P-450 type (*73*). Beyond the hydroxylation steps, the order in which the corresponding CoA esters are formed and *O*-methylation reactions occur is currently somewhat unclear. This is because a growing body of evidence indicates that different *O*-methyltransferases are capable of preferentially methylating, in some cases, the free hydroxycinnamic acids (*74-75*) and, in others, the corresponding CoA esters (*76-78*). The physiological significance of this differential *O*-methylation capability is as yet unknown, except that it may point either to redundancy in the pathway, or to distinct metabolite targeting of specific cell types and tissues, or to the involvement of selective metabolic pathway targeting (*e.g.* to lignins or lignans). Beyond the *O*-methylation and CoA ligation steps, the remaining transformations involve consecutive NADPH-dependent reductions, catalyzed by cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (*69*), respectively. Curiously, both steps have often been described as lignin-specific *and* rate-limiting, since they catalyze the last two transformations leading to monolignols (*59, 79*). However, this may be an oversimplification, given that monolignols, such as coniferyl alcohol, can be deployed for both lignin and lignan biosynthesis. Moreover, it is as yet unclear why these processes are so often described as rate-limiting, since lignifying cells undergo the coordinated induction of the entire shikimate/chorismate and phenylpropanoid pathways in a tissue-specific manner.

For the foregoing reasons, there has long been a need to characterize the pool levels of different metabolites in the phenylpropanoid pathway under substrate saturating conditions, in order to attempt to identify what, if any, are the rate-limiting steps. In this regard, the only work of this type carried out to date has employed *Pinus taeda* cell suspension cultures, which can be induced to undergo active phenylpropanoid metabolism when grown in a solution containing 8% sucrose. Over a 96 h period, these cells respond by generating an extra-cellular monolignol-derived dehydropolymerisate, whose formation can be inhibited by addition of 20 mM KI, an H_2O_2 scavenger (*80*). Under these conditions, however, no extracellular lignin-like material is formed, but instead essentially only the monolignols, *p*-coumaryl and coniferyl alcohols, are secreted into the medium. Time course analyses for the build-up of each possible metabolic product from phenylalanine onward to the monolignols has been recently determined (A. M. Anterola *et al.*, Washington State University, unpublished obervations, 1997). These experiments, however, revealed that only *p*-coumaric, cinnamic and caffeic acids build-up during the induction of the pathway, *i.e.* none of the other intermediates preceding the steps that had been claimed to be rate-limiting in the pathway (*i.e.* the CoA esters, aldehydes or monolignols) were observed to accumulate significantly beyond the pre-existing pool levels. This indicates that *none* of the latter steps function in a rate-controlling manner. Moreover, given that the monolignols can have more than one metabolic fate, it is important that care be taken in interpreting the results of any attempts to enhance or suppress CAD or CCR enzyme levels in the pathway.

Monolignol Transport into the Cell Wall

The next issue that also remains unresolved is the nature of the intermediates being transported from the cytoplasm into the lignifying cell wall. Lignification occurs in a spatially and temporally well-defined manner (*81*), whereby, for example, *p*-coumaryl and coniferyl alcohols are *differentially* targeted to specific (initiation) sites in the lignifying cell wall (see Chapter 22). However, there is still some controversy as to

whether it is the free-monolignols, or their glucosides, that are transported across the plasma membrane into the cell wall. The formation of monolignol glucosides in the cytoplasm is said to involve the action of the corresponding glucosyltransferase(s), and it is proposed that these metabolites are then transported into the cell wall where a β-glucosidase catalyzes the regeneration of the monolignols (*1*). Stefan Marcinowski and Hans Grisebach attempted sometime later to determine whether monolignol glucosides were obligatory intermediates for lignification (*82*). However, their results suggested that lignin was predominantly formed by polymerization of the monolignols being transported directly rather than as their glucoside derivatives. These observations could be interpreted as indicating either that the monolignol glucosides are storage metabolites, or that they are involved in some distinct (*e.g.* tissue-specific or lignan-specific) metabolic subsector. Indeed, it is puzzling that monolignol glucosides are apparently only found in very few plant species. Thus, the question has not yet been resolved as to whether the monolignol glucosides are required for transportation into lignifying plant cell walls or not, but ongoing work in the Ellis and Savidge laboratories is beginning to bring a substantial measure of clarification to this important subject (*83, 84*).

Oxidases and Peroxidases Involved in Lignification

For almost five decades, profound uncertainty has enveloped the enzymology of *tissue specific* bimolecular phenoxy radical coupling leading to the lignins, lignans, suberins and other phenolic natural products. Using lignin formation as an example, peroxidases (*32, 80, 85, 86*), peroxidases and laccases (*32, 87, 88*), laccases (*31, 89-91*), (poly)phenol oxidases (*92*), coniferyl alcohol oxidase (*93*), and even cytochrome oxidases (*94*) have all been implicated. This has occurred primarily because of the large variety of oxidative enzymes with broad substrate specificity that exists in plants, to which the assignment of physiological function has unfortunately all too often been arbitrary. Some of the confusion may have stemmed from the fact that all of these enzymes are present in numerous isoenzyme forms, and all are capable of converting lignin precursors (monolignols) such as *E*-coniferyl alcohol **1** into corresponding dimers like (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols and (±)-guaiacylglycerol 8–*O*–4′-coniferyl alcohol ethers *via* bimolecular radical coupling processes in open solution (Figure 4). As mentioned earlier, further oxidative coupling then gives rise to the lignin polymers. It is doubtful, however, that the formation of such an important and ubiquitous class of macromolecules would be catalyzed in a haphazard manner by such a wide range of enzymes *in vivo*.

Perhaps in response to this uncertainty about the actual enzyme(s) involved, more recent attempts to establish a precise physiological function for particular peroxidase or laccase isoenzymes have taken a different approach. Transgenic plants were obtained in which genes encoding supposed lignin-forming peroxidase (*95-97*) and laccase (*98*) isoenzymes had been introduced in either sense and/or antisense orientations. Although each study was carefully executed, the results obtained, for example, in tobacco (*Nicotiana tabacum*) and yellow poplar (*Liriodendron tulipifera*), by targeting peroxidase and laccase, respectively, were equivocal. With the peroxidase sense/antisense experiments, although interesting changes in growth and development were noted, the effect on lignin formation was relatively minor. Fritig *et al.* have since suggested that these 'lignin-specific' peroxidases may only facilitate hypersensitive plant responses but not lignification proper (*99*). Somewhat comparable (*i.e.* small) effects on lignin synthesis in yellow poplar were obtained with attempts to suppress laccase gene expression. In a similar manner, studies with antisense transgenic tomato plants, targeting a putative suberin-specific peroxidase isoenzyme, had little effect on suberization (*100*).

Thus, no unequivocal proof has ever been obtained showing that any particular laccase or peroxidase isoenzyme has either regulated dehydrogenative monolignol coupling or had an exclusive role in lignification or suberization. This should not be taken to imply, however, that peroxidase(s) do not play a critical role in lignin

formation; indeed, compelling evidence for the role of an H_2O_2-dependent peroxidase in 'lignin' biosynthesis has been achieved using *Pinus taeda* cell suspension cultures (*80*). Thus, when the H_2O_2 scavenger, KI, was added to the *P. taeda* culture medium, lignification in the *Pinus taeda* cell walls was *totally* suppressed, as was the formation of an extracellular 'lignin-like' precipitate. However, even though no lignin synthesis occurred, the cells were still able to biosynthesize the monolignols, *E*-coniferyl **1** and *E*-*p*-coumaryl **5** alcohols, *de novo* (see Figure 4 for structures). [This was shown to be the case by the secretion of both monolignols into the culture medium in radiolabeled form, following [U-^{14}C]-Phe administration and its subsequent metabolism.] This observation was important from two perspectives: first, these findings gave strong support in favor of a role for peroxidase in lignin biosynthesis and, second, a system was *at last* available to study monolignol formation *in vivo* without subsequent polymerization. This study did not, however, yield any insight into how the phenoxy radical coupling processes themselves are controlled *in vivo*.

Dehydrogenative Polymerization of Monolignols to Lignins

As already mentioned, it has long been believed that lignins are assembled by the coupling of radicals produced through the single-electron oxidation of monolignols and corresponding phenolic monomer residues in the oligomeric and polymeric components which result. In native lignin macromolecules, roughly half of the linkages are of the 8–*O*–4′ alkyl aryl ether type in lignified plant cell walls (*36*). Since dehydrogenative polymerization of monolignols *in vitro* does not yield the same relative proportions of interunit linkages as observed in native biopolymers, certain facets of the macromolecular assembly processes *in vivo* differ significantly from the dehydrogenative polymerization of monolignols in open solution.

It has not been possible accurately to account for the ratios of interunit linkages among dehydrodimers formed *in vitro* simply on the basis of unpaired electron densities on the atomic centers of the interacting monolignol radicals. In ESR spectra, the hyperfine coupling constants to adjacent protons suggest that the unpaired electron spin densities at C5 and C8 in coniferyl alcohol-derived radicals are very similar to one another (*101*). Moreover, molecular orbital calculations tend to indicate that the unpaired electron density on the (ESR-silent) 4–*O* is appreciably lower than on C5 and C8 (T. J. Elder, Auburn University, personal communication, 1997). These findings are in accord with the observation that 8–5′ and 8–8′ linked dilignols predominate over 8–*O*–4′ linked dimers in product mixtures resulting from the enzyme-catalyzed dehydrodimerization of coniferyl alcohol *in vitro* (*102-104*). In contrast, the heats of formation deduced from molecular orbital calculations indicate that the stabilities of the σ-complexes directly resulting from 8–5′, 8–8′ and 8–*O*–4′ coupling of coniferyl alcohol-derived radicals are all very similar (*105*). On the other hand, the calculated energies of the σ-complexes formed directly from 5–5′ and 4–*O*–5′ coupling are substantially higher (*105*).

The literature provides no relevant basis for understanding what occurs during dehydrogenative coupling between dilignols and monolignols as the first step towards the formation of macromolecular lignin chains. It has been reported that 4–*O*–8″ linkages predominate during the enzyme-catalyzed dehydrogenative coupling of coniferyl alcohol with 8–5′, 8–8′ and 8–*O*–4′ linked dilignols *in vitro* (*106, 107*). Unfortunately, no attempt has been made to determine unpaired electron densities on atomic centers in guaiacyl radicals where there is no conjugation with a C7–C8 double bond. These values, and the estimated heats of formation for the σ-complexes directly arising from the various possible coupling modes, could shed some light upon the unexpected absence of 4–*O*–5″ linkages in the trimers dehydrogenatively formed from coniferyl alcohol and the 8–5′, 8–8′ and 8–*O*–4′ linked dilignols.

Noncovalent interactions play an important role in certain phenoxy radical coupling processes investigated *in vitro*, and presumably contribute to the analogous events which occur in lignifying plant cell walls. For example, as far as the 8–8′ coupling mode is concerned, the dehydrodimerization of *E*- and *Z*-isoeugenol and

E-2,6-dimethoxy-4-propenylphenol has led exclusively to *threo* products (*108*). Such effects are likely to be much more generally significant than hitherto recognized; thus the frequencies of the different interunit linkages formed between (mono-, oligo- and poly-) lignol radicals *in vitro* will presumably depend to varying extents upon (i) the unpaired electron densities on the atomic centers about to become coupled, (ii) noncovalent interactions between the approaching radicals, and (iii) the energies of the σ-complexes directly resulting from each coupling mode.

Apart from those operative *in vitro*, a further factor will play an important, and perhaps decisive, role in establishing the modes of coupling between lignol radicals in lignifying plant cell walls. This effect arises from pronounced noncovalent interactions between the radicals and pre-existent macromolecules occupying particular locations within the plant cell wall matrix. When such effects become dominant, complete regio- and sterospecific phenoxy-radical coupling occurs, as in the combination of two coniferyl alcohol-derived free radicals to give (+)-pinoresinol exclusively under the control of a dirigent protein (*109*). One of the particular challenges with which the field of lignin biosynthesis must now contend involves the question of whether macromolecular lignin configurations are established in a comparably specific way.

Determinants of Lignin Configuration

Monolignols appear to be incorporated into native lignins in the same order as they are formed in their biosynthetic pathway (*110*), *viz. p*-coumaryl, coniferyl and sinapyl alcohol. Since these monomeric precursors differ according to whether methoxyl groups are present at C3 and C5 in the aromatic ring, the frequencies of interunit linkages to these positions will decrease in this order also. The effect is reinforced by accompanying changes in the unpaired electron density on the atomic centers in the corresponding series of monolignol-derived radicals, which at the 4-*O* more than doubles according to Austin Model 1 semi-empirical molecular orbital calculations (*101*).

The frequencies of interunit linkages in dehydropolymerisates formed from coniferyl alcohol *in vitro* vary substantially according to the conditions employed. Thus, when the monolignol is added all at once ('Zulaufverfahren' or mixing method) to the enzyme-containing solution, initial coupling to dimers is followed by further polymerization which progressively lowers the reactivity of the radical species; termination eventually occurs before the (so-called 'bulk') dehydropolymerisate components have had the opportunity of attaining high molecular weights. On the other hand, when the monolignol is added gradually ('Zutropfverfahren' or drop method) to the same dehydropolymerizing medium, dimers are likewise produced initially, but they quickly undergo further polymerization into higher molecular weight components; these then couple preferentially with the radicals being formed in low concentration from the monolignol that continues to be slowly introduced into the system. The (so-called 'end-wise') dehydropolymerisate species resulting from Zutropfverfahren embody a much higher proportion of 8–*O*–4′ linkages than those produced by Zulaufverfahren, as would be expected from the differences observed between monomer-monomer (*102-104*) and monomer-dimer (*106, 107*) dehydrogenative coupling frequencies. It has been suggested that lignins *in vivo* may be composites of 'bulk' and 'end-wise' dehydropolymerisate domains (*111*), the configurations of which represent the consequences of local fluctuations in monolignol-derived radical concentrations during lignification.

The primary structures of macromolecular lignin chains (*viz.* the sequences of interunit linkages and monomer residues), given the variability in composition among different morphological regions of plant cell walls, have been traditionally held to be random. Yet the secondary structures of lignins appear to be quite well-defined by comparison. From Raman spectral analyses with polarized incident laser beams, the aromatic rings of the lignin observed in *Picea marina* tracheid cross-sections appear to be parallel to the cell-wall lumen surface (*112*). It has been proposed that this is an

outcome of the plant cell-wall matrix becoming gradually more hydrophobic during the course of lignification. As a consequence, water is claimed to be progressively displaced from what was originally a swollen polysaccharide gel, resulting in anisotropic shrinkage which tends to orient the lignin aromatic rings into directions parallel to the cell-wall surface because the effect is ostensibly greater in the radial direction (*110*). Disregarding the question of whether such a sequence of events could be consistent with the thermodynamics of lignification processes, it is worth pointing out that, when the mechanical integrity of the cell wall is preserved, shrinkage is necessarily greater in the tangential than radial direction.

Partial clarification of this matter could have been forthcoming from computer simulations of lignin structures that use simplified space-filling structures to depict monomer residues interconnected by the six most commonly occurring linkages in softwood lignins (*113, 114*). When the structures were built inside thin lamellar boundaries representing domains in the polysaccharide matrix of the secondary wall, a degree of spontaneous alignment was obtained with respect to the microfibrillar direction owing to the elongated shapes of the spaces within which the lignin representation was assembled. Analogous simulation of lignification in less restricted spaces representing the middle lamella region engendered an entirely random orientation of the monomer residues and, interestingly, a more highly crosslinked structure than inside the lamellar boundaries used to invoke the secondary wall (*113, 114*).

The fact that the monomolecular film thicknesses of lignin derivatives from woody tissues are independent of sample molecular weight has become a truism of almost classical proportions in lignin chemistry (*115*). This observation together with the molecular dimensions apparent in electron micrographs (*116*) indicates that lignin derivatives consist of disk-like macromolecular components cleaved from lamellar parent structures which are about 2 nm thick. Potassium permanganate staining of lignifying *Pinus radiata* cell walls has revealed a plausible cause for this feature of macromolecular lignin configuration (*81*). Lignin deposition in the secondary wall takes place much more rapidly in directions that are aligned with, rather than perpendicular to, the cellulose microfibrillar axes. A particularly important aspect of the process lies in the observation that the lignin domains maintain a more or less uniform density as they expand. This is most clearly evident in the middle lamella region where only after neighboring lignin domains make contact do the intervening spaces become filled in (*81*). Obviously there must be strong nonbonded attractive interactions between the polymeric lignin chains because a crosslink density of 0.052 (*117*) involving tetrafunctional branch points (*118*) in the macromolecular structure cannot account for such effects.

Each of the individual lignin domains has developed from an initiation site which was presumably incorporated into the cell wall before the onset of lignification (*81*). The nature of these initiation sites largely determines the mechanism of macromolecular lignin assembly *in vivo*, and the literature bears witness to more than one conceivable alternative in this regard. The 8–8′ linkage has been detected in ryegrass between lignin monomer residues and ferulate moieties that are ester-linked to α-L-arabinofuranose units in arabinoxylan (*119*). The possibility thus presented itself that ferulate-polysaccharide esters could act as initiation sites for lignification (*119*). On the other hand, with polyclonal antibodies, proline-rich protein epitopes have been spatially and temporally correlated with lignin formation in developing cell walls of the maize coleoptile (*120*) and in secondary walls of differentiating protoxylem elements in the soybean hypocotyl (*121*). It has accordingly been suggested that proline-rich proteins may act as a scaffold for initiating lignification at their tyrosine residues (*121*). There is no doubt that the identity of the initiation sites and their function for lignification in plant cell walls has emerged as a subject of central importance in the field of lignin biosynthesis.

Biosynthesis of Lignans

The lignans constitute a very widespread group of phenylpropanoid natural products, being found in all plant parts, including (woody) stems, rhizomes, roots, seeds, oils, exuded resins, flowers, leaves and bark tissues (*122*); their amounts differ between tissues and species. Around the turn of the last century, investigations were initiated to determine the structures of some of the most common lignans (originally called lignanes) (*25*). It was quickly concluded that they were a series of *dimeric* substances linked through 8–8′ bonds. This initial classification, unfortunately, failed to recognize that other lignan skeletal types (*e.g.* 8–5′; 8–3′; 8–1′; 5–5′; *etc.*) were also present in many plant species/tissues (*39, 40*), and that lignan(e)s can also have much higher molecular weights. It is now known that lignans encompass a wide range of structural motifs (and molecular sizes) (*39-41, 123*), rather than being restricted to the simple 8–8′ linked dilignols as previously thought. A few examples are given for illustrative purposes in Figure 6.

Frequently, although not always, lignans are found in optically active form, where the particular antipode observed can vary with plant species. For example, (+)-pinoresinol is present in *Forsythia* species (*45*), whereas the (–)-antipode occurs in *Daphne tangutica* (*44*) and (+)-sesamin occurs in *Calocedrus formosana* (*42*) with the (–)-form accumulating in a *Zanthoxylum* species (*43*). In most cases, however, lignan structures cannot arise solely from phenolic coupling, since other *post-coupling* modifications are frequently evident, such as oxidations, reductions, skeletal rearrangements, carbon-carbon bond cleavage reactions, demethylations, oligomeric assemblies and so on. In addition to these structural permutations, the deposition of lignans can also often be very extensive, as exemplified by cases such as western red cedar (*Thuja plicata*) whose heartwood can consist of up to 20% (w/w) of these components (*124*). In such instances, both the lignins and lignans coexist in large amounts in the mature tissues.

The distinction between lignans (dimers and oligomers) and macromolecular lignins has long been a contentious issue as far as their biosynthesis is concerned. This is partly because there has been no clear demarcation between the biochemical pathways, and partly because of their structural similarities. Accordingly, several years ago establishing the precise difference between lignan and lignin biosynthesis appeared to be a particularly worthwhile goal.

The initial focus addressed formation of the most common lignan types, namely those embodying the 8–8′ linkage. For experimental convenience, attention was directed towards *Forsythia* species where the lignan biosynthetic pathway has now been established to follow the scheme shown in Figure 7 (*103, 109, 125-135*). As can be seen, the entry point involves stereoselective coupling of two *E*-coniferyl alcohol molecules to afford (+)-pinoresinol (*109*). In an analogous manner, coupling of two *E*-coniferyl alcohol molecules also occurs in developing flax (*Linum usitatissimum*) seed, but here the corresponding (–)-antipode is formed (J. Ford *et al.*, Washington State University, unpublished results, 1997). Thus, the prevalent coupling mode is, not unexpectedly, dependent upon the plant species. The pinoresinol formed serves as the entry point into complex biochemical pathways leading to lignans such as sesaminol (*Sesamun indicum*) (*136*), plicatic acid (*Thuja plicata*) and podophyllotoxin (Z. Q. Xia *et al.*, Washington State University, unpublished results, 1997).

In the case of *Forsythia* species, the protein responsible for this stereoselective process, the discovery of which incidentally marked the first example of regio- and stereospecific control of phenoxy radical coupling, has been purified to apparent homogeneity (*109*), its gene cloned and the recombinant protein expressed in a functional recombinant form as described in Chapter 22. Interestingly, the dirigent protein which controls this transformation lacks oxidative catalytic capacity by itself, but in the presence of an appropriate oxidase, such as laccase, is able to confer absolute specificity to the coupling reaction. The mechanism envisaged involves the coupling of free-radicals, derived from two molecules of *E*-coniferyl alcohol, where

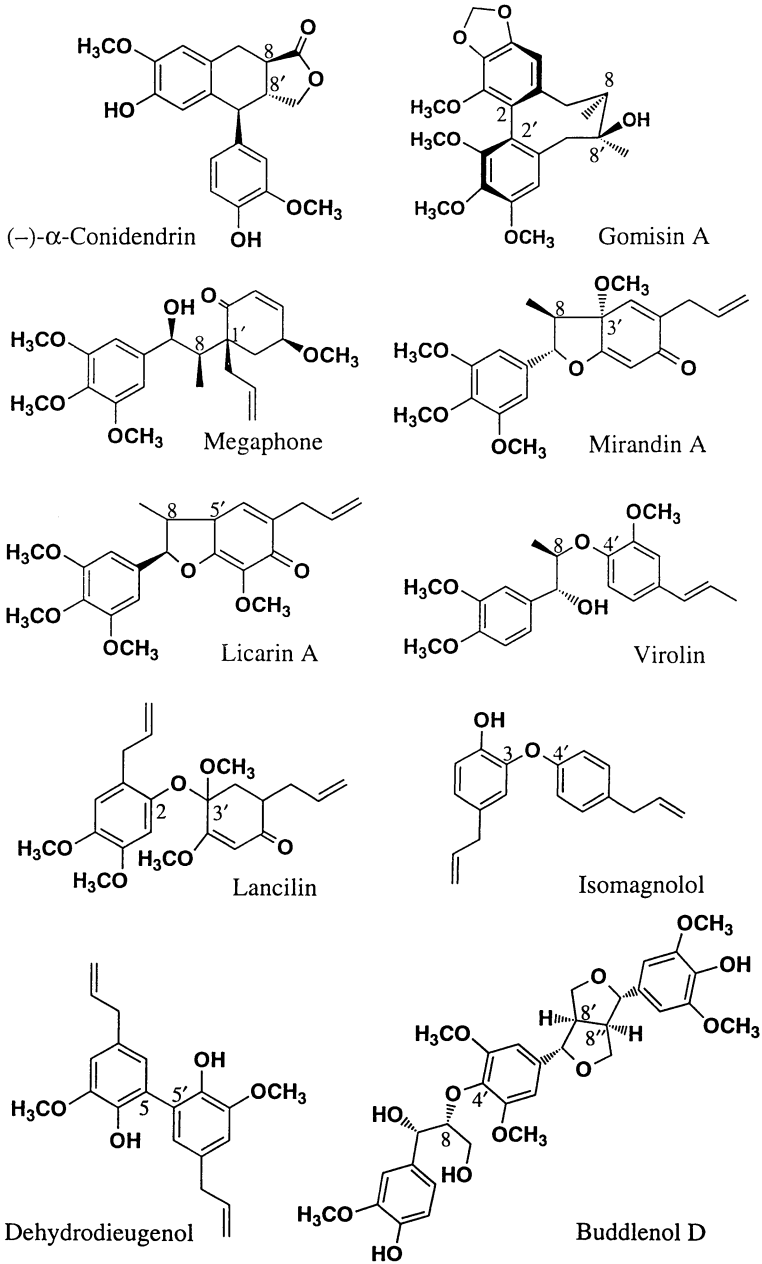


Figure 6. Examples of various lignans encompassing differing structural motifs (interunit linkages).

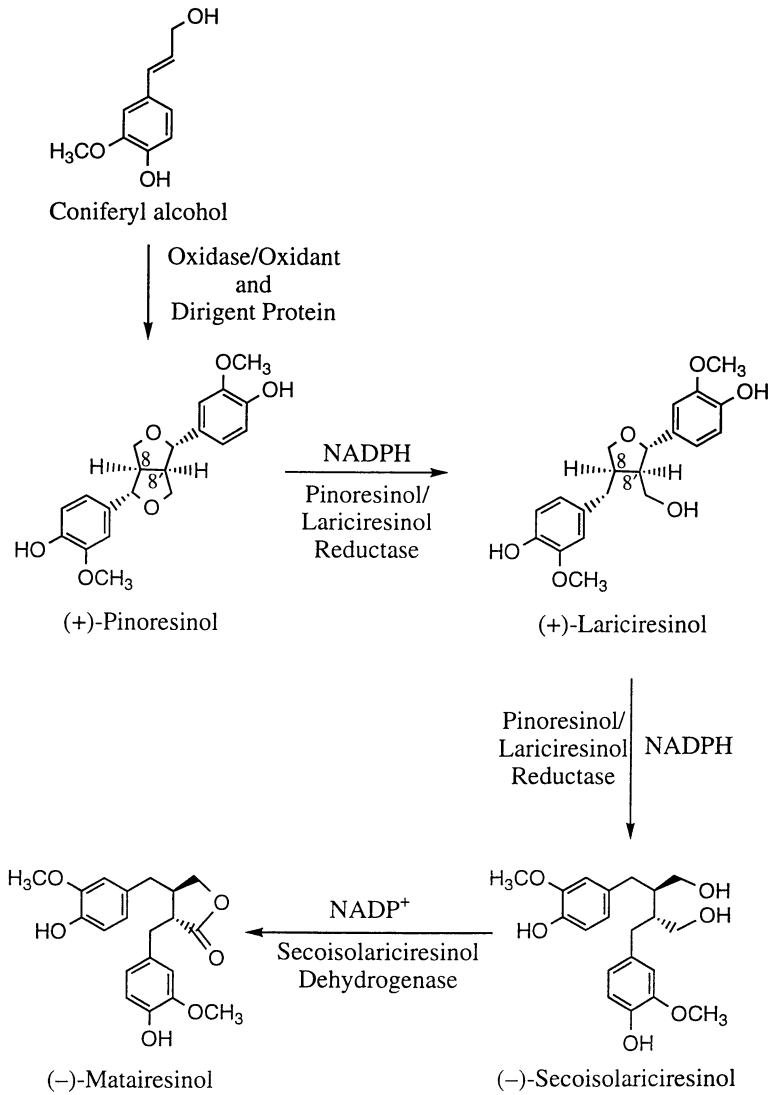


Figure 7. Lignan biosynthetic pathway in *Forsythia intermedia*.

the oxidase catalyzes the single electron oxidation step and the dirigent protein specifies the mode of coupling.

In *F. intermedia*, beyond (+)-pinoresinol, two sequential highly enantiospecific NADPH dependent reductions are catalyzed by a single reductase, namely, (+)-pinoresinol/(+)-lariciresinol reductase, to afford (–)-secoisolariciresinol (*via* (+)-lariciresinol) (*132, 135*). In this plant species, the (–)-secoisolariciresinol so formed can then undergo enantiospecific dehydrogenation to afford (–)-matairesinol (*125, 127*).

Given that other plants may possess the opposite antipodes, it is worth emphasizing that, in every case examined thus far, all biochemical processes involving coupling and post-coupling modifications in lignans are under explicit control: the biochemical processes associated with 8–8′ linked lignan formation involve highly controlled stereoselective and/or enantiospecific transformations. Furthermore, it is now evident that a new class of proteins exists that is capable of engendering the *distinct* specific coupling modes that account for the different lignan skeletal forms present in various plant species.

Another abundant lignan type is the one containing the 8–5′ linkage motif, as illustrated by dehydrodiconiferyl alcohol, its dihydro derivatives, and other metabolic variants. These substances are formed, as reported previously (*137*), by reduction of the allylic side chain of dehydrodiconiferyl alcohol and/or demethylation reactions, at least in *Pinus taeda* (Pinaceae). In other woody plants such as *Cryptomeria japonica* (Taxodiaceae) (*138*), the lignans present apparently also undergo further transformation, *i.e.* they contain dehydrodiconiferyl alcohol analogs which have not only undergone reduction of the allylic side-chain and the phenylcoumaran ring, but are also modified by acylation. A tentative biosynthetic pathway, which is being studied by Lewis *et al.*, is shown in Figure 8.

Thus, in summary, the various biochemical transformations associated with the lignan pathways are now yielding to systematic inquiry, and are revealing that the phenoxy radical coupling reactions and subsequent post-coupling modifications are under explicit biochemical control.

Relationship between Lignan and Lignin Biosynthetic Pathways

No discussion about the products of dehydrogenative monolignol coupling and any subsequent transformations would be complete without some attention being given to the contrasting distinctions between the lignan and lignin biosynthetic pathways. It is becoming increasingly evident that both pathways are *fully* independent, and that there is considerable subtlety involved in the temporal, spatial and tissue-specific expression of each. Accordingly, although many of the details await fuller documentation at the enzymatic and gene (expression) level, it is timely to compare and contrast what is now known about each metabolic system.

The lignans are initially typically formed as optically active dimers that can then undergo various modifications including the formation of higher molecular weight oligomers, which are in danger of being mistaken for lignin macromolecules. An evaluation of gene sequence(s) for the dirigent (monolignol coupling) protein from *Forsythia* revealed that it has no homology to any other protein (gene) of known function (*139*). Just as interestingly, the NADPH-dependent pinoresinol/lariciresinol reductase displays considerable sequence homology to that of isoflavonoid reductases (~62% similarity and ~42% identity), the isoflavonoids being a group of compounds involved in plant defense (as phytoalexins). Indeed, it was pointed out earlier (*140*) that the gene(s) encoding lignan reductases presumably predate the isoflavonoid reductases, given that the latter are only observed in few plant species (*e.g.* legumes) whereas the lignan reductases (based on chemotaxonomic considerations) are widespread throughout the ferns, gymnosperms and angiosperms.

In accordance with gene sequence comparisons and the biological properties of specific lignans (*e.g.* as antioxidants, fungicides, cytotoxins, antivirals, antifeedants, *etc.*), it seems evident that the *primary* role of the lignans, irrespective of their molecular size, is in plant defense, *i.e.* as part of the arsenal of compounds used to

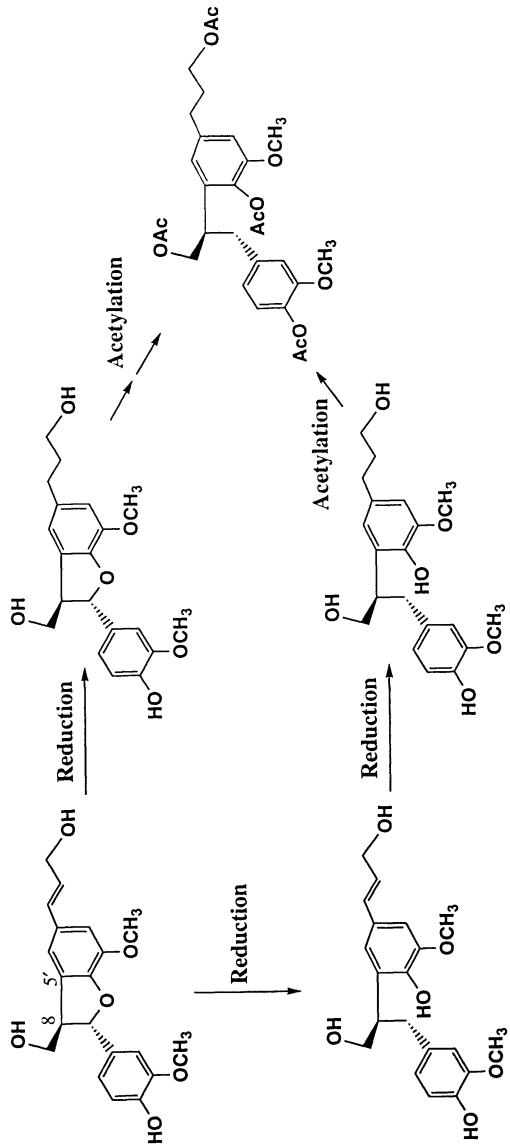


Figure 8. Proposed biosynthetic pathway to lignans present in *Cryptomeria japonica*.

ensure plant survival over extended periods. The lignans can be biosynthesized constitutively in variable amounts ranging from relatively low levels in flower petals to extensive deposition in heartwood tissues, such as cedar and redwood. In the latter case, deposition of the majority of the lignans occurs only after lignification is complete *via* extrusion into the sapwood from specialized conducting cells, such as the ray parenchyma. This deposition can continue in massive quantities, as exemplified by the *ca.* 20% (w/w) lignans found in western red cedar. Moreover, since these substances not only are present as simple dimers, but also can exist as insoluble oligomers, they have frequently been misidentified as 'secondary' or 'abnormal' lignins, even though they are formed through quite distinct biochemical pathways (*141*). Indeed, it cannot be emphasized enough that, even though their solubilization may require conditions normally associated with lignin removal, they are only present as non-structural infusions. Thus a vista is beginning to emerge where the lignan biosynthetic pathway, involving characteristic coupling processes and precise post-coupling modifications, is entirely distinct from the lignin-generating machinery.

It is, therefore, pertinent next to compare and contrast current understanding about lignin biosynthesis with what is now being observed in lignan formation. As described earlier, the prevailing dogma has insisted that lignin biosynthesis occurs in a manner whereby, following monolignol-derived free-radical generation, no direct control of macromolecular assembly is exercised at the enzymatic level. Moreover, it is most unfortunate that the initial studies which spawned this idea employed crude enzyme preparations (*e.g.* mushroom laccase) which were not even involved in lignin biosynthesis in the first place. This early working hypothesis did not, however, explain the preponderance of 8–*O*–4′ linkages in lignin biopolymers. Nor did it readily account for the heterogeneity of lignins within plant cell walls (*vide supra*).

The most important aspect of the entire lignification process lies in the fact that the individual monolignols are transported into the cell wall after deposition of all of the structural carbohydrates and proteins, *i.e.* when the overall architecture has already been established. Lignin formation itself then appears to be initiated at distinct (initiation) sites in the lignifying cell wall (*81*). These sites are temporally and spatially correlated with the deposition of particular proline-rich proteins (*120, 121*), the primary stuctures of which could conceivably encode in some explicit manner (yet undemonstrated) *lignin* primary structure. Indeed, some preliminary analysis suggests that there could possibly be a correlation between the dirigent proteins controlling lignan assembly and the proline-rich proteins associated with lignification. In the latter case, it would appear that macromolecular lignin assembly occurs *via* an end-wise dehydrogenative polymerization process where the monolignol-derived free-radicals are coupled to the growing polymer chain. If correct, the involvement of the putative proteinaceous template would provide a mechanism for the positioning of the individual monomers in such a way as to confer a specific sequence of interunit linkages upon the first macromolecular lignin chain to be synthesized, which would thereafter undergo direct replication as biosynthesis continues [(*142*) and Chapters 15 and 22].

Indeed, this *new* paradigm, together with the ideas clarifying the relationship with the lignan biosynthetic pathways, brings order into what was once believed to be a 'chaotic' biochemical series of events. It can account for the preponderance of the lignans in their various forms, lignan deposition in various tissues and cell-types, and the 'infusion' process deploying *non-structural* oligomeric lignans which have often been erroneously described as 'Brauns native lignins' or 'secondary lignins' in heartwood. Moreover, these ideas persuasively explain why heartwood had been thought to contain oligomeric components ostensibly formed *via* acidolytic degradation of lignin macromolecules in the aging tissues (*111*). It has now become more likely that they are distinct biochemical entities. Lastly, and perhaps most importantly, the new working hypothesis provides a plausible means for controlling lignin biopolymer assembly in sapwood in a biologically well-defined way. This is because the involvement of the proline-rich proteins *or other compatible polypeptides*

affords a plausible mechanism for controlling macromolecular lignin assembly during monolignol transport from the plasma membrane into the lignifying cell wall.

Accordingly, a recent claim that macromolecular lignin assembly will utilize other precursors, if the proper monolignols are not available, was quite unexpected (*143*). This was based upon an analysis of a supposed mutant of loblolly pine (*Pinus taeda*) whose 'lignin' was purported to contain 30% (w/w) dihydroconiferyl alcohol substructures (*143*). It was proposed that this plant was unable to biosynthesize coniferyl alcohol fully, but instead generated dihydroconiferyl alcohol *via* some unknown biosynthetic route. Careful evaluation of the reported data revealed that only about 17% of this 'lignin' had been extracted from the tissues for analysis, and that the total contribution to the overall plant lignin from the dihydroconiferyl alcohol unit was < 5-6%. These data are much more in accord with the consequences of the infusion process resulting in the deployment of insoluble lignan oligomers, in which are incorporated the reduction products of dehydrodiconiferyl alcohol, as previously reported (*80, 137,* and Chapter 25). Therefore, there appears to be no biochemical justification for identifying these substances as 'abnormal' lignins. This, in the absence of substantive biochemical data supporting such an assertion, does little to advance the field. On the contrary, it returns to a primitive working hypothesis that far predates the Freudenberg era!

Acknowledgments

The authors thank the United States Department of Agriculture (9603622), the National Science Foundation (MCB-9631980), the U.S. Department of Energy (DE-FG03-97ER20259), the U.S. National Aeronautics and Space Administration (NAG100164), McIntire-Stennis for generous support of these studies, as well as the Lewis B. and Dorothy Cullman and G. Thomas Hargrove Center for Land Plant Adaptation Studies. Acknowledgment for support of this work is also made to the Vincent Johnson Lignin Research Fund at the University of Minnesota, and the Minnesota Agricultural Experiment Station. [Paper No. 974436805 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station, funded through Minnesota Agricultural Experiment Station project No. 43-68, supported by Hatch Funds.]

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