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Perspective

Lignin Monomers from beyond the Canonical Monolignol Biosynthetic Pathway: Another Brick in the Wall

José C. del Río,* Jorge Rencoret, Ana Gutiérrez, Thomas Elder, Hoon Kim, and John Ralph

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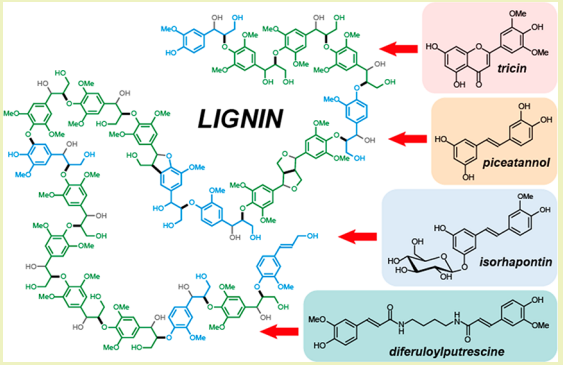
ABSTRACT: Lignin is conventionally defined as being formed by the oxidative polymerization of three main monolignols, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, that are derived from the general phenylpropanoid biosynthetic pathway. Many other phenolic compounds that are also derived from the phenylpropanoid pathway are also known to perform as genuine lignin monomers in many plants, as is the case of the monolignol ester conjugates, phenolic compounds arising from the truncated biosynthesis of monolignols, or ferulate esters. Recent investigations, however, have indicated that phenolic compounds arising from beyond the canonical phenylpropanoid pathway, namely flavonoids, hydroxystilbenes, and hydroxycinnamic amides, may also behave as authentic lignin monomers and are incorporated into the lignin in some plants, further challenging the traditional definition of lignin. This is the case of the flavone tricin that is incorporated into the lignin of grasses and other monocots, the hydroxystilbene piceatannol (together with resveratrol and isorhapontigenin, at lower levels) that has been found in the lignins of palm fruit shells, their respective *O*-glucosides (astringin, piceid, and isorhapontin) that are present in the lignin of Norway spruce bark, or the ferulic amides feruloyltyramine, incorporated into the lignin of tobacco and potato tubers, and diferuloylputrescine, which appears to be incorporated into maize kernel lignin. These valuable compounds are potentially available in high amounts and at low cost and may be obtained from the waste products from the processing of agricultural or forest biomass.



KEYWORDS: *Tricin, Piceatannol, Isorhapontin, Feruloyltyramine, Diferuloylputrescine*

INTRODUCTION

Lignin is an aromatic polymer characteristic of the cell walls of terrestrial plants, where it provides rigidity and mechanical support to the plant, helps the transport of water and solutes, and plays a role in protecting the plant against pathogens. Lignin comprises around 15−40% of the plant biomass and is typically underutilized in selective conversion processes aimed at obtaining carbohydrates, mostly for the production of cellulose during pulping for pulp and paper or via pretreatments to subsequently saccharify and ferment its derived sugar monomers to various biofuels; lignin is a low-value waste product that is mostly burned for co-generation of heat and power. However, the lignin polymer has an aromatic/phenolic skeleton that makes it an attractive raw material for producing chemicals, materials, and fuels that are currently obtained from fossil resources.[1−3] Lignin is available in high amounts from lignocellulosic residues from the processing of agricultural or forest biomass, and represents a sustainable source to obtain valuable products. The conversion of low-value lignin side streams into high-value products will provide additional revenue streams for both papermaking and biofuel industries. The valorization of the lignin polymer from different lignocellulosic substrates is therefore crucial for adding value to these lignocellulosic materials.

Applications for lignin have been widely addressed and include the production of power, biofuels, and syngas products, the formation of macromolecules such as plastics, polymeric foams, membranes, and carbon fibers, and the formation of low-molecular-weight phenolic compounds.[2−10] However, the structural complexity, heterogeneity, and variability of the lignin polymer hinder the development of efficient conversion technologies for these lignocellulosic materials. Indeed, and despite decades of study on lignin structure, new structural features are still being revealed, and several phenolic compounds, including also phenolics from biosynthetic pathways other than the general phenylpropanoid pathway, are continually being revealed in the lignin of several plants, thus

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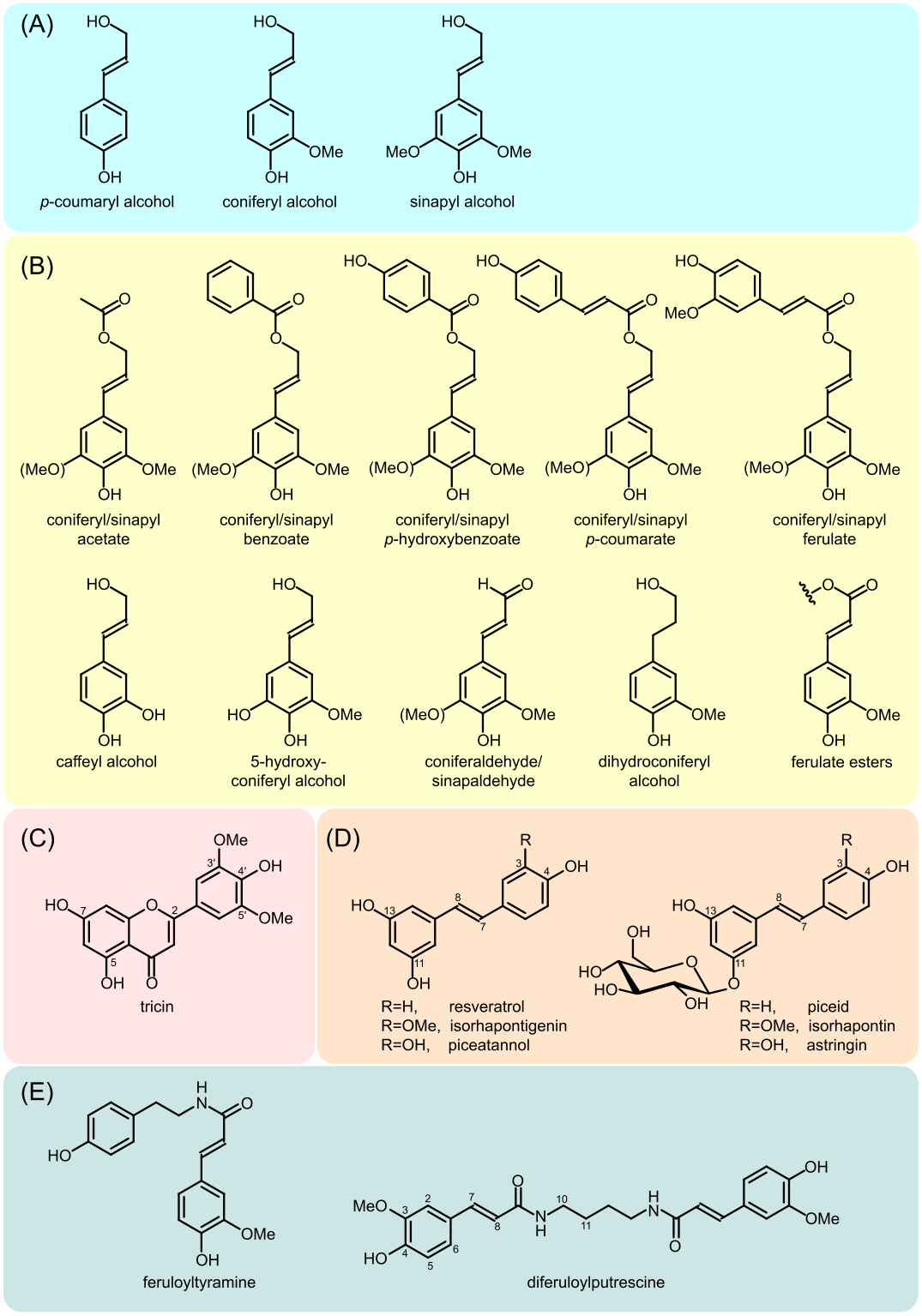

Figure 1. (A) Structures of the three canonical monolignols, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. (B) "Non-conventional" lignin monomers also derived from the monolignol biosynthetic pathway. Lignin monomers acylated at the γ-OH with acetate, benzoate, *p*-hydroxybenzoate, *p*-coumarate, and ferulate; caffeyl alcohol; 5-hydroxyconiferyl alcohol; hydroxycinnamaldehydes; dihydroconiferyl alcohol; ferulate esters. (C) Phenolic compounds derived from flavonoids (tricin), (D) hydroxystilbenes (resveratrol, isorhapontigenin, and piceatannol, and their respective *O*-glucosides piceid, isorhapontin, and astringin), and (E) hydroxycinnamamides (feruloyltyramine and diferuloylputrescine) biosynthetic pathways, that have been found to act as true lignin monomers in several plants.

expanding the traditional definition of lignin. Enhanced understanding of the biosynthesis, composition, and structure of lignins is essential for the full valorization of the lignocellulosic biomass. In this paper, we review the recent discoveries of phenolic compounds arising from beyond the general phenylpropanoid pathway, namely flavonoids, hydroxystilbenes, and

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hydroxycinnamic amides, that have been found to behave as authentic lignin monomers in some plants and that appear to be integrally incorporated into the lignin polymer. To the extent that they can be released by viable processes, these novel lignin monomers increase the range of valuable products that may be obtained from the lignin polymer, thus greatly enhancing the value of certain lignocellulosic materials.

LIGNIN BIOSYNTHESIS AND STRUCTURE

Lignin is produced by the oxidative radical polymerization of three major *p*-hydroxycinnamyl alcohols (the so-called monolignols, Figure 1A), *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, that are at the origin of the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units following incorporation into the lignin polymer.[11,12] The monolignols derive from the general phenylpropanoid biosynthetic pathway. Their biosynthesis starts with the deamination of phenylalanine (or tyrosine) and, briefly, involves sequential hydroxylation reactions of the aromatic ring, followed by phenolic *O*-methylation and the reduction of the carboxylic acid group in the side chain to an aldehyde and finally to an alcohol, by various enzymes.[11−19] The enzymes implicated in biosynthesis of monolignols have been widely studied during the past decades and include phenylalanine/tyrosine ammonia lyase (PTAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), ferulate 5-hydroxylase (F5H), *p*-coumarate 3-hydroxylase (C3H), *p*-hydroxycinnamoyl-CoA:quinate/shikimate hydroxycinnamoyltransferase (HCT), caffeoyl shikimate esterase (CSE), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), caffeoyl-CoA reductase (CCR), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD); in some cases the primary substrate is no longer represented in the classical name, but the names and abbreviations have been customarily retained. Once synthesized, the monolignols are delivered to the cell wall, where they are oxidized to form radicals in a reaction mediated by peroxidases and/or laccases and then polymerized in a combinatorial fashion by free-radical coupling mechanisms in an end-wise manner, generating a series of substructures with a small variety of interunit linkages within the polymer.[11,12] No protein involvement is found during the polymerization process, which is a purely chemically controlled process, as evidenced by the lack of optical activity of the lignin and its degradation products.[20] The most predominant interunit linkages in the lignin polymer are *β*-*O*-4 alkyl aryl ethers that comprise around 50−80% of total linkages in native lignins; other linkages/substructures are *β*-5/phenylcoumarans, *β*-*β*/resinols, 5-5/dibenzodioxocins, 5-*O*-4/biphenyl ethers, and *β*-1/spirodienones. This mechanism of lignin biosynthesis results in a lignin polymer that, in contrast to most other biopolymers, lacks any regular and ordered repeating units, making it particularly recalcitrant and difficult to depolymerize. The composition and structure of the lignin significantly vary among taxa and cell type, as well as the environmental conditions and maturity stage.[21−26] In general terms, hardwoods are composed of S and G units, softwoods are made up of G units and small amounts of H units, and grasses include all three units, although H units have often been overascribed due to limitations in analytical techniques and their interpretation.[27] However, besides the three canonical monolignols, several other phenolic compounds are also known to behave as true lignin monomers in many—likely all—plants, thus increasing the complexity of the lignin polymer.

"NON-CONVENTIONAL" LIGNIN MONOMERS DERIVED FROM THE PHENYLPROPANOID PATHWAY

It is now increasingly recognized that many other phenolic compounds (Figure 1B), also originating from the general phenylpropanoid biosynthetic pathway, also behave as true lignin monomers in many plants, participating in radical coupling reactions and being incorporated into the lignin polymer. This is the case of monolignol ester conjugates (with acetates, *p*-coumarates, *p*-hydroxybenzoates, or the newly found ferulate and benzoate analogs) that are found in a variety of plants.[28−35] Monolignol acetates are ubiquitous in angiosperms and are found in high amounts (sometimes reaching up to 80% acetylation degree) in the lignins of several plants, such as kenaf (*Hibiscus cannabinus*), sisal (*Agave sisalana*), and abaca (*Musa textilis*), as well as in some hardwoods, such as hornbeam (*Carpinus betulus*) that has a degree of monolignol acetylation of up to 45%.[28,29,36−38] Monolignol *p*-hydroxybenzoates are widely found in palms, poplar, willow, and aspen;[32,39−43] they have also recently been found at particularly high levels in the lignin of the seagrass *Posidonia oceanica*, which has *p*-hydroxybenzoylation in both G (73%) and S (61%) lignin units.[44] Monolignol *p*-coumarates are typical components of grass lignins and have also been found in significant amounts in other monocots, such as curaua (*Ananas erectifolius*) and abaca.[29−31,45,46] Monolignol ferulates have been recently described, although at low levels, in different plants.[33] Monolignol benzoates have also been recently reported in the lignins of some palms, such as date (*Phoenix dactylifera*) and macaúba (*Acrocomia aculeata*) palms,[34,42] and were recently found in trace levels in aspen in a study revealing that co-downregulation of *C3H* and *C4H* genes elevated the level markedly.[35] The incorporation of these monolignol ester conjugates into the lignin polymer generates characteristic and diagnostic structures, as is the case of the tetrahydrofuran structures produced from the *β*-*β* coupling of *γ*-acylated monolignols, which demonstrates that they behave as true lignin monomers.[28−32,37,43,47]

Besides the above monolignol ester conjugates that may be regarded as monomers from extensions of the monolignol biosynthetic pathway, products resulting from pathway truncation, or from incomplete conversion during some pathway steps, may also enter lignification and therefore behave as lignin monomers. As an example of pathway truncation, hydroxycinnamaldehydes, the immediate precursors of monolignols, have been found in the lignins of various mutant and transgenic plants deficient in CAD,[48−53] but are also present at low levels in most lignins. Phenolic compounds derived from the incomplete methylation of aromatic 3- and 5-hydroxy intermediates on the pathway that nevertheless continue full side-chain transformation to the alcohol, such as the caffeyl and 5-hydroxyconiferyl alcohols, have been found incorporated into the lignins of OMT-downregulated transgenics,[54−57] and in some plant seed coats.[58−60] Lignins derived exclusively from caffeyl alcohol (so-called C-lignin) were discovered in seed coats of vanilla orchid (*Vanilla planifolia*) and in some members of the Cactaceae, Euphorbiaceae, and Cleomaceae families; this C-lignin has an unusual structure, being essentially a homopolymer of caffeyl alcohol linked through *β*-*O*-4-coupling and producing chains of benzodioxane structures.[58−60] Lignins composed of 5-hydroxyguaiacyl units derived from 5-hydroxyconiferyl alcohol, and also forming benzodioxane chains, were found in the seeds of three species of Escobaria.[59] The benzodioxane structures

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produced from the incorporation of caffeyl and 5-hydroxyconiferyl alcohols provided evidence for their participation in radical coupling reactions during lignification.[58−60]

Monomers that derive from the phenylpropanoid pathway in some way but do not fit into the above categories are also common. Dihydroconiferyl alcohol has been described in gymnosperm lignins and is an abundant component in a CAD-deficient pine mutant.[61−63] Guaiacylpropane-1,3-diol is also found in gymnosperm lignins and has been shown to derive from dihydroconiferyl alcohol.[62] Polysaccharide hydroxycinnamate esters (particularly ferulates) are incorporated into the lignin of grasses.[64,65] Ferulates mostly acylate the arabinosyl residues of (glucurono)arabinoxylans and participate in oxidative coupling reactions forming ferulate dehydrodimers, and higher dehydro-oligomers, through different linkages (i.e., 8-*O*-4, 4-*O*-5, 8-8, 5-5, and 8-5 linkages); in addition, ferulates and dehydrodiferulates can also cross-couple with monolignols or the growing lignin chain, producing a lignin−hydroxycinnamate−carbohydrate complex.[31] On the other hand, ferulic acid itself also appears to behave as an authentic lignin monomer, incorporated at low levels into the lignin structure in CCR-deficient plants; incorporation of ferulic acid into the lignin polymer causes the formation of acetals that reveal its incorporation through radical coupling reactions.[66,67]

Overall, it is therefore evident that all the phenolic compounds mentioned above, which are still derived in some way from the general phenylpropanoid biosynthetic pathway, are integrally incorporated into lignins by radical coupling reactions that typify lignification, and logically they need to be considered authentic lignin monomers.

LIGNIN MONOMERS FROM BEYOND THE MONOLIGNOL BIOSYNTHETIC PATHWAY

Recent investigations from our groups have demonstrated that other phenolic compounds arising from outside the classical monolignol biosynthetic pathway, namely the flavonoid, hydroxystilbene, or hydroxycinnamamide biosynthetic pathways (Figure 1C−E), also behave as authentic lignin monomers, participating in radical coupling reactions with monolignols and/or lignin oligomers to become incorporated into the lignin in several plants.[68−75] The characteristic that all these "novel" lignin monomers have in common is that they are metabolic hybrids, as they are derived from a combination of the phenylpropanoid biosynthetic pathway and other biosynthetic pathways, such as the acetate/malonate-derived polyketide biosynthetic pathway (in the case of flavonoids and hydroxystilbenes) or the amino acid biosynthetic pathway (in the case of the hydroxycinnamamides).

Lignin Monomers from the Flavonoid and Hydroxystilbene Biosynthetic Pathways. Flavonoids and hydroxystilbenes share a common biosynthetic origin, as they are produced from a *p*-coumaroyl-CoA unit and three malonyl-CoA units, added sequentially by a polyketide synthase and producing a tetraketide intermediate. Depending on the activity of the polyketide synthase, chalcone synthase (CHS) or stilbene synthase (STS), subsequent folding and cyclization of the generated tetraketide intermediate result in the production of either a chalcone or a stilbene.[76] Flavonoids include different classes of compounds, such as flavones, flavanones, flavonols, isoflavones, anthocyanins, chalcones, aurones, xanthones, and flavanols.[77,78] Simple hydroxystilbenes, however, have a more restricted number of compounds that, by definition, consist of two phenolic moieties linked by an ethylene bridge and, similarly to monolignols, are particularly prone to participating in oxidative coupling reactions to produce dimers and higher oligomers.[78−81] Flavonoids and hydroxystilbenes are known to participate in oxidative radical coupling reactions with monolignols to produce the so-called flavonolignans and stilbenolignans that occur in many plants,[82−84] which reveals their compatibility with lignification. Recent investigations have demonstrated that some members of the flavonoids (such as the flavone tricin) and hydroxystilbenes (such as resveratrol, isorhapontigenin, and piceatannol), and including their respective *O*-glucosides (piceid, isorhapontin, and astringin), behave as authentic lignin monomers in some plants and become incorporated into the lignin by radical coupling reactions with monolignols and the growing lignin polymer.[42,68,69,71,75]

Tricin. The flavone tricin (Figure 1C) was the first phenolic compound derived from outside the canonical monolignol biosynthetic pathway that was recognized as a true lignin monomer participating in cross-coupling reactions with monolignols and being integrally incorporated into the lignin polymer.[68] Tricin is a secondary metabolite widely present in many plants, principally grasses, where it can occur in extractable form as free tricin, or forming tricin-*O*-glycosides, flavonolignans, and flavonolignan glycosides.[85] But more importantly, a significant fraction of tricin was also discovered to be incorporated into the lignin polymer in many plants. Tricin was first discovered in the lignin from wheat straw, and its structure was fully established by detailed NMR analyses that also revealed that the 4′-OH of ring-B was not free but linked to the lignin polymer through 4′-*O*-*β* ether bonds (as shown in Figure 2).[68] Biomimetic radical coupling reactions of tricin with the three canonical monolignols confirmed that tricin can cross-

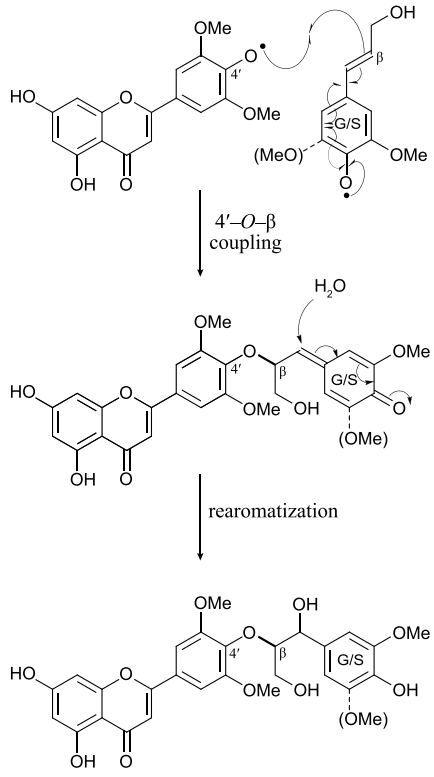

Figure 2. Mode of incorporation of tricin into the lignin polymer through 4′-*O*-*β*-linkages.

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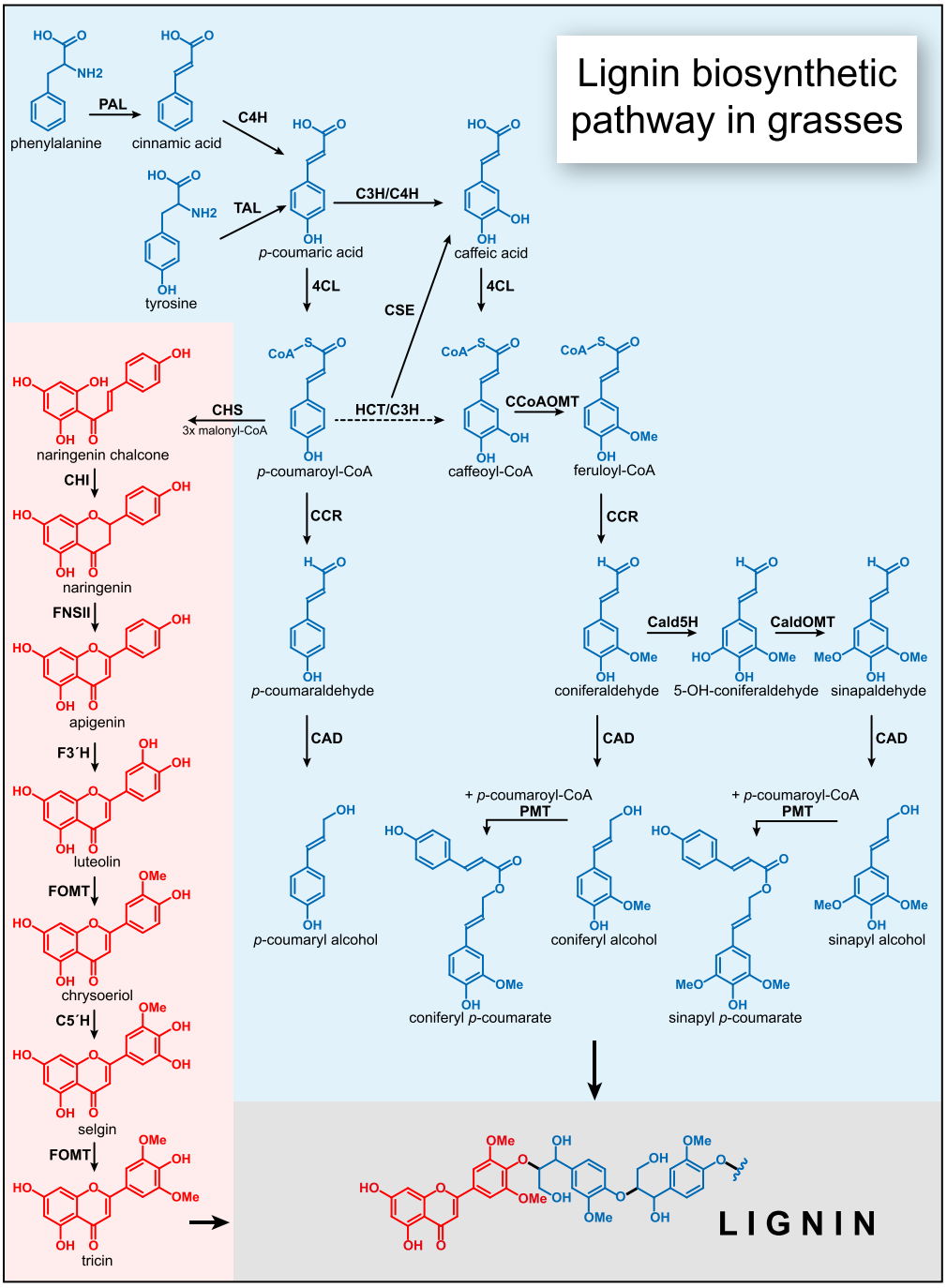

Figure 3. Lignin biosynthetic pathway in grasses showing the interconnections of the monolignol and the flavonoid biosynthetic pathways. PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase; C3H, *p*-coumaroyl ester 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; Cald5H (= F5H, ferulate 5-hydroxylase), coniferaldehyde 5-hydroxylase; CaldOMT (= COMT, caffeate *O*-methyltransferase), 5-hydroxyconiferaldehyde *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; PMT, *p*-coumaroyl-CoA:monolignol transferase; CHS, chalcone synthase; CHI, chalcone isomerase; FNSII, flavone synthase II; F3′H, flavonoid 3′-hydroxylase; FOMT, flavonoid *O*-methyltransferase; C5′H, crysoeriol 5′-hydroxylase.

couple with monolignols (or the growing lignin chain) exclusively through 4′-*O*-*β*-ether linkages following the mechanism illustrated in Figure 2; no other types of cross-coupled products were formed, nor were homodimeric coupling products seen.[71] These results confirmed those already observed in native lignins, and corroborated the contention that tricin can only cross-couple with monolignols through its phenylpropanoid moiety by 4′-*O*-*β* linkages. As tricin cannot undergo radical dehydrodimerization and its only possible mode of incorporation into the lignin is via 4′-*O*-*β*-coupling, tricin can thus only appear at the starting end of the lignin chain, and this suggests a potential role as a nucleation site for lignification in grasses (and in other monocots).[71]

The analysis of the phenolic metabolites present in the lignifying zone of maize revealed the occurrence of a wide array of tricin-containing flavonolignans resulting from the coupling

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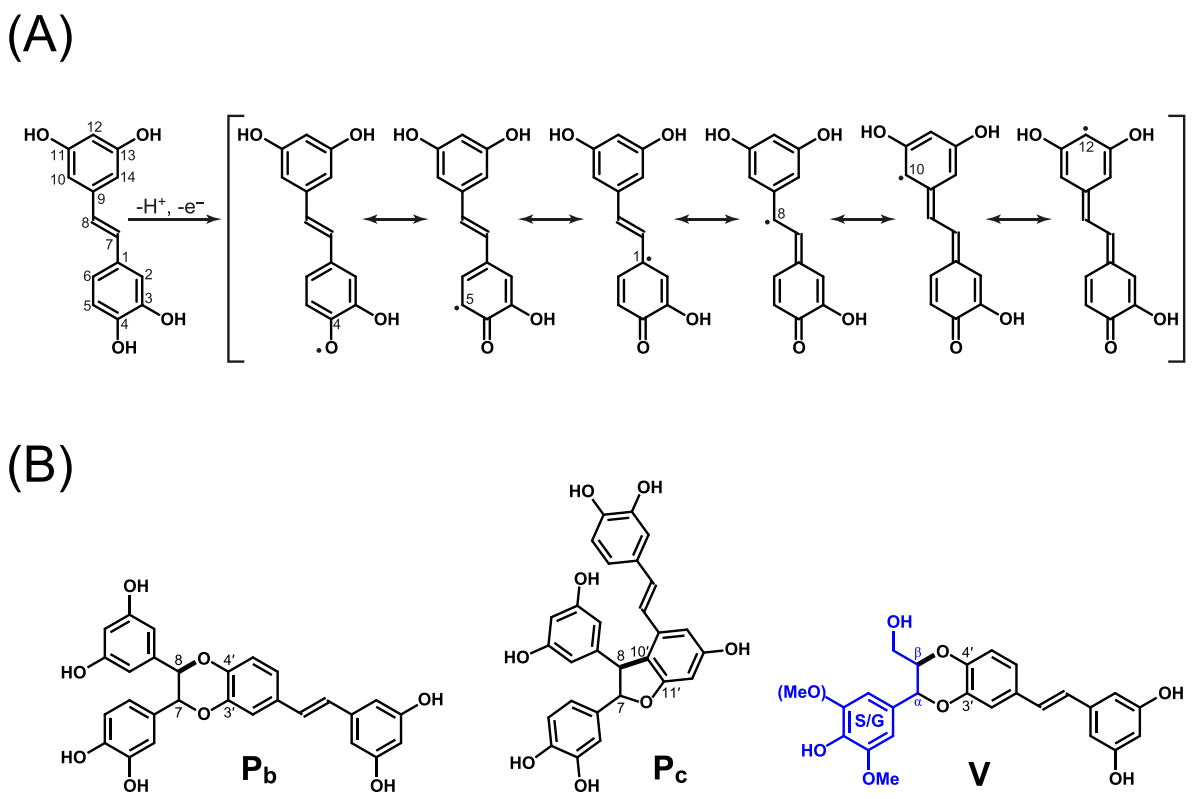

Figure 4. (A) Oxidative radicalization resulting from one-electron oxidation of piceatannol stabilized by delocalization at the 4-*O*-, 5-, 1-, 8-, 10-, and 12-positions. (B) Modes of incorporation of piceatannol into the lignin polymer of palm fruit endocarps: $\mathbf{P_b}$, benzodioxane structure formed via 8-*O*-4′-type radical coupling of two piceatannol units followed by internal trapping of the quinone methide intermediate by the 3′-hydroxyl group; $\mathbf{P_c}$, phenylcoumaran structure formed by the radical coupling of two piceatannol units followed by a subsequent 11′-*O*-7 bonding during re-aromatization of the quinone methide intermediate; **V**, benzodioxane structure formed by cross-coupling of a monolignol (at its *β*-position) and the catechol moiety of piceatannol (at its *O*-4′-position), followed by internal trapping of the quinone methide intermediate by the 3′-hydroxyl group in the piceatannol unit.

of all three monolignols with tricin, forming the respective tricin-4′-*O*-(*β*-arylglyceryl) ethers, along with the products arising from the cross-coupling of tricin with the different *γ*-acylated monolignols (the coniferyl and sinapyl acetate and *p*-coumarate conjugates).[72] These dimers are similar to the flavonolignans *threo*/*erythro* tricin-4′-*O*-*β*-guaiacylglycerol ethers, also known as salcolins A and B, that have been described in several plants.[86−88] Trimeric compounds arising from the coupling of tricin-(4′-*O*-*β*)-monolignols with a further 4-*O*-*β* or 5-*β* linkage to another monolignol (including acylated monolignols) were also identified among the maize metabolites. In all cases, these tricin-containing metabolites were found to be fully racemic,[72] further evidencing the combinatorial nature of the cross-coupling reactions between tricin and monolignols. It was, therefore, evident that the dimeric compounds resulting from the coupling of tricin with monolignols (and with their acetate and *p*-coumarate conjugates) should not be termed flavonolignans (which should be optically active); hence, these compounds should be considered to be oligomers that are intended for the lignin polymer, and the more appropriate term "flavonolignols", or specifically "tricin-oligolignols", was coined for them.[72]

Following the discovery of tricin in the lignin from wheat straw, several other studies started to report the presence of tricin in the lignins of other grasses, including giant reed (*Arundo donax*),[89] bamboo (*Phyllostachys pubescens*),[90] carex (*Carex meyeriana*),[91] barley (*Hordeum vulgare*),[92] rice (*Oryza sativa*),[93] maize (*Zea mays*),[71] and sugar cane (*Saccharum* sp.),[94] suggesting that tricin is a ubiquitous component in the lignin of grasses. Moreover, tricin was also found in the lignin of coconut (*Cocos nucifera*) coir,[41] the first report of tricin in the lignin of a plant out of the Poaceae family, expanding the range of plants with tricin incorporated into their lignins to other monocots. All these studies revealed that tricin occurs widely among monocot lignins, being particularly widespread and abundant in the lignin from grasses. A further survey of more than 50 plants from different origins (including angiosperm monocots and eudicots, and gymnosperms) expanded the range of species that have tricin incorporated into their lignins and indicated that it was widely distributed in all lignins from species of the Poaceae family, being particularly abundant in oat (*Avena sativa*) and wheat (*Triticum durum*) straws, as well as in Brachypodium (*Brachypodium distachyon*).[73] The survey indicated that tricin also occurred in the lignins of other monocots, and that besides coconut, tricin was present in the lignins of the vanilla plant (*Vanilla planifolia* and *V. phalaenopsis*) and in boat orchid (*Cymbidium nonna*) from the Orchidaceae;[73] minor amounts of tricin were also found in the lignin from curaua (*Ananas erectifolius*) from the Bromeliaceae. In general terms, tricin was absent in the lignins from eudicotyledons, although minor amounts were detected in the lignin of alfalfa (*Medicago sativa*) from the Fabaceae.[73,95] No traces of tricin were observed in the lignins from gymnosperms. In addition, all these studies revealed that tricin was only present in the lignins of the aerial parts of the plants, suggesting a possible role as a UV

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protection agent; in addition, tricin can confer antimicrobial and antioxidant properties to the plant.[96,97]

All these reports demonstrated that tricin is an important component in the lignins of all grasses, and several studies have addressed the biosynthetic pathways of tricin and its interconnection with the monolignol biosynthetic pathway, leading to its incorporation into the lignin polymer, which is summarized in Figure 3. The biosynthesis of flavones is controlled by CHS that converts *p*-coumaroyl-CoA into naringenin chalcone. The resulting naringenin chalcone is then isomerized by chalcone isomerase (CHI) to the flavanone naringenin, which is the common precursor for the biosynthesis of all major classes of flavonoids. The biosynthetic pathway leading from naringenin to tricin in grasses has only been recently elucidated.[98,99] These investigations indicated that naringenin is converted into apigenin by a flavone synthase II (FNSII), and subsequent sequential hydroxylations and *O*-methylations at the B-ring produce the respective luteolin, chrysoeriol, selgin, and finally the flavone tricin. It has also been recently demonstrated in several grasses (maize, rice, and sorghum) that the COMT involved in the synthesis of sinapaldehyde and sinapyl alcohol in the monolignol biosynthetic pathway also participates in the biosynthesis of tricin *in planta*.[99−102] The interconnection of the monolignol and the flavonoid biosynthetic pathways in the biosynthesis of grass lignins was fully evidenced in a CHS-deficient mutant in maize, that prevented the formation of naringenin chalcone, and tricin in the last step, and therefore, the subsequent incorporation of tricin into the lignin polymer.[103]

Although tricin is the only flavonoid that has been found, to date, incorporated into the lignin in some wild plants, it is likely that other flavonoids may also occur in other lignins. The occurrence of several other flavonoids from different families, such as taxifolin, quercetin, eriodictyol, dihydrotricin, apigenin, luteolin, and selgin, which are known to cross-couple with monolignols, forming different flavonolignans,[82,83] is an indication that these phenolic compounds might also be compatible with lignification. It is then probable that, in the coming years, we may see the discovery of other flavonoids incorporated into the lignin in some plants.

Piceatannol, Resveratrol, and Isorhapontigenin. Hydroxystilbenes are in another class of polyphenolic compounds that have been recently reported to behave as authentic lignin monomers in some plants. The hydroxystilbenes piceatannol, resveratrol, and isorhapontigenin have been found incorporated into the lignin of palm fruit endocarps.[42,69] The first evidence for the occurrence of hydroxystilbenes incorporated into the lignin polymer came from the release of significant amounts of piceatannol from lignin preparations isolated from several palm fruit (macaúba, carnauba, and coconut) endocarps by the so-called "derivatization followed by reductive cleavage" (DFRC) degradation method.[69] DFRC selectively cleaves *β*-*O*-4 bonds in lignins, releasing the corresponding lignin monomers involved in those linkages.[104,105] This indicates that piceatannol (and to a lower extent resveratrol and isorhapontigenin) is an important component in the lignins of palm fruit endocarps, and that at least a part of them are present in the lignin polymer as *β*-ether-linked structures, the ones cleaved by DFRC. However, and unlike the flavone tricin, which has only one possible mode of coupling with monolignols through 4′-*O*-*β* linkages, piceatannol can couple and cross-couple with other piceatannols as well as with monolignols (and oligolignols) by a variety of pathways, generating condensed structures that are not amenable to DFRC. It is therefore likely that the real amounts of piceatannol and other hydroxystilbenes incorporated into these lignins could be significantly higher than those released by DFRC degradation. Similarly to monolignols, hydroxystilbenes can be oxidized by peroxidases to form radicals that are stabilized by resonance, as shown for piceatannol in Figure 4. These radicals can participate in radical coupling reactions with other piceatannols, as well as with monolignols and the growing lignin chain, to become incorporated into the lignin polymer through different linkages and producing different structures in the polymer. Important information regarding the different forms of incorporation of piceatannol into the lignin was provided by NMR analyses and *in vitro* biomimetic synthesis that identified the different linkages and structures involving piceatannol in the lignin polymer, as depicted in Figure 4. These structures included a dehydrodimerization product of the coupling of two piceatannol units involving 8-*O*-4′/3′-*O*-7 linkages and producing a benzodioxane structure ($\mathbf{P_b}$) similar to the stilbene dimer cassigarol E,[106−108] another dehydrodimerization product of two piceatannol units involving 8-10′/11′-*O*-7 linkages and producing a phenylcoumaran structure ($\mathbf{P_c}$) similar to the stilbene dimer scirpusin B,[109−111] and a cross-coupling product of a piceatannol unit and a monolignol involving *β*-*O*-4′/3′-*O*-*α* linkages and producing a benzodioxane structure ($\mathbf{V}$) similar to the stilbenolignan aiphanol.[112] *In vitro* biomimetic dehydrodimerization/oligomerization of piceatannol produced the same types of structures ($\mathbf{P_b}$, $\mathbf{P_c}$, and $\mathbf{V}$) observed in the lignins of palm fruit endocarps. Likewise, biomimetic cross-coupling reactions between piceatannol and monolignols (coniferyl and sinapyl alcohols) corroborated that the benzodioxane structures $\mathbf{V}$ can be easily formed during the radical reaction.[69] The occurrence of all of these homo- and cross-coupled structures in the lignins of palm fruit endocarps conclusively demonstrates that the hydroxystilbene piceatannol performs as an authentic lignin monomer, participating in radical coupling reactions during lignification and being incorporated into the lignin. A further study of the thermodynamics of these reactions using density functional theory (DFT) calculations confirmed that the energetics of the coupling and cross-coupling reactions are comparable with those of the monolignol coupling, proving again the compatibility of piceatannol with the lignification process.[113]

The incorporation of hydroxystilbenes into the lignins of palm fruit endocarps was suggested to have a potential role in seed protection. The incorporation of piceatannol (and other hydroxystilbenes) augments the lignin by including other phenolic compounds present in the cell wall into the lignin polymer, which indeed may form more condensed structures, such as the phenylcoumarans and benzodioxanes shown above, likely contributing to endocarp hardening. Hydroxystilbenes can also provide additional antioxidant, antifungal, and antiviral properties,[114−116] thus further contributing to seed protection.

Isorhapontin, Astringin, and Piceid. The hydroxystilbene glucosides, isorhapontin (isorhapontigenin-*O*-glucoside), astringin (piceatannol-*O*-glucoside), and piceid (resveratrol-*O*-glucoside), have been recently found incorporated into the lignin in Norway spruce (*Picea abies*) bark.[75] Interestingly, a previous paper had also reported the occurrence of hydroxystilbene glucosides in a "milled bark tannin-lignin" fraction isolated from Norway spruce bark, although the authors suggested that they were linked to the condensed tannin moiety instead of the lignin polymer.[117] The incorporation of hydroxystilbene glucosides into the lignin was evidenced by

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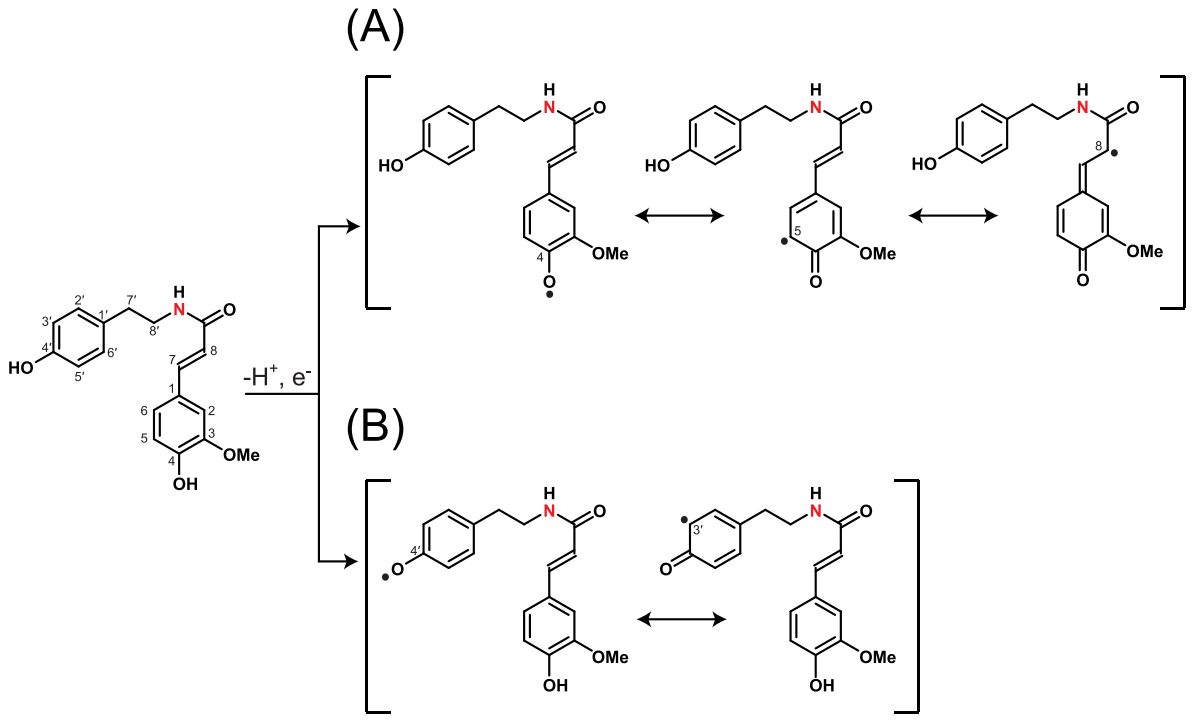

Figure 5. (A) Oxidative radicalization of feruloyltyramine, and resonance forms at 4-*O*-, 5-, and 8-positions of the ferulate moiety. (B) The tyramine moiety may also be oxidized in the same manner, producing a radical that is stabilized by resonance and can couple at its 4′-*O*- and 3′-positions.

the release of the corresponding aglycones (mostly isorhapontigenin and piceatannol, together with lower amounts of resveratrol), along with glucose, by DFRC degradation of a lignin preparation from Norway spruce bark, indicating that at least a fraction of the hydroxystilbene glucosides (particularly isorhapontin and astringin) is incorporated into the lignin through *β*-ether linkages. Additional NMR studies confirmed that hydroxystilbene glucosides were present in the lignin of Norway spruce bark and provided information on their modes of incorporation.[75] Several structures involving hydroxystilbene glucosides, similar to those formed for the incorporation of piceatannol into the lignins of palm fruit endocarps (Figure 4), were found in the lignin of Norway spruce bark, including benzodioxane ($\mathbf{P_b}$) and phenylcoumaran ($\mathbf{P_c}$) structures involving coupling of two hydroxystilbene glucosides (mostly isorhapontin and astringin), as well as a minor amounts of a benzodioxane structure (**V**) formed by cross-coupling of astringin and coniferyl alcohol. The occurrence of these structures involving hydroxystilbene glucosides indicates that these phenolic compounds, particularly isorhapontin and astringin, can also be considered as authentic lignin monomers in Norway spruce bark. The hydroxystilbene glucosides isorhapontin and astringin, as occurs with their respective aglycones isorhapontigenin and piceatannol, can also be oxidized by laccases or peroxidases to form radicals that are stabilized by resonance, similarly to those shown for piceatannol in Figure 4; these radicals can then undergo radical coupling reactions with other hydroxystilbene glucoside radicals, with monolignols, or with the growing lignin chain to be incorporated into the lignin polymer, forming the structures shown above. The hydroxystilbene glucoside isorhapontin contains a guaiacyl ring, and therefore it can also easily cross-couple with coniferyl alcohol or lignin oligomers in different ways, forming *β*-*O*-4′-alkyl aryl ethers, *β*-5′-phenylcoumarans, or 5-5′/4-*O*-*β*″-dibenzodioxocins.[75]

Lignin Monomers from Hydroxycinnamamides. Hydroxycinnamic amides (hydroxycinnamamides) are present in numerous plants in which they contribute to many developmental processes as well as plant responses against biotic and abiotic stress.[118,119] Among them, several ferulamides have been shown to be incorporated into the lignin structure in some plants, where they appear to behave as true lignin monomers.

Feruloyltyramine. Feruloyltyramine was the first hydroxycinnamamide described to behave as a true lignin monomer in several plants from the Solanaceae. Feruloyltyramine was found in the lignin of tobacco (*Nicotiana tabacum*) plants,[48,120,121] and was also shown to be covalently linked to the cell wall in potato (*Solanum tuberosum*) tubers.[122] Identification of feruloyltyramine was accomplished by detailed thioacidolysis and NMR analyses that indicated that it was mainly incorporated into the lignin through the ferulate moiety. The ferulate moiety of feruloyltyramine, as in other ferulate conjugates, can be oxidized by peroxidases and/or laccases, forming radicals that are stabilized by resonance (Figure 5A) and that can participate in radical coupling reactions (with other ferulates, monolignols, or the growing lignin polymer), to become incorporated into the polymer.[31] However, feruloyltyramine has two phenolic ends, and the phenolic group of the tyramine moiety may also be oxidized in parallel, forming another radical that is delocalized over the tyramine aromatic ring system (Figure 5B), providing additional sites for radical coupling. As with other non-methoxylated rings, the phenol might be expected to undergo radical transfer rather than radical coupling, but its coupling with other ferulates, monolignols, or the growing lignin polymer has already been shown with lignin model substrates.[123,124] Thus, the major part of feruloyltyramine is attached to the cell wall of potato tuber through 8-*O*-4′- ether and 8-5′-phenylcoumaran linkages involving the ferulate moiety; however, a significant part (~20%) of the feruloyltyramine is apparently attached to the cell

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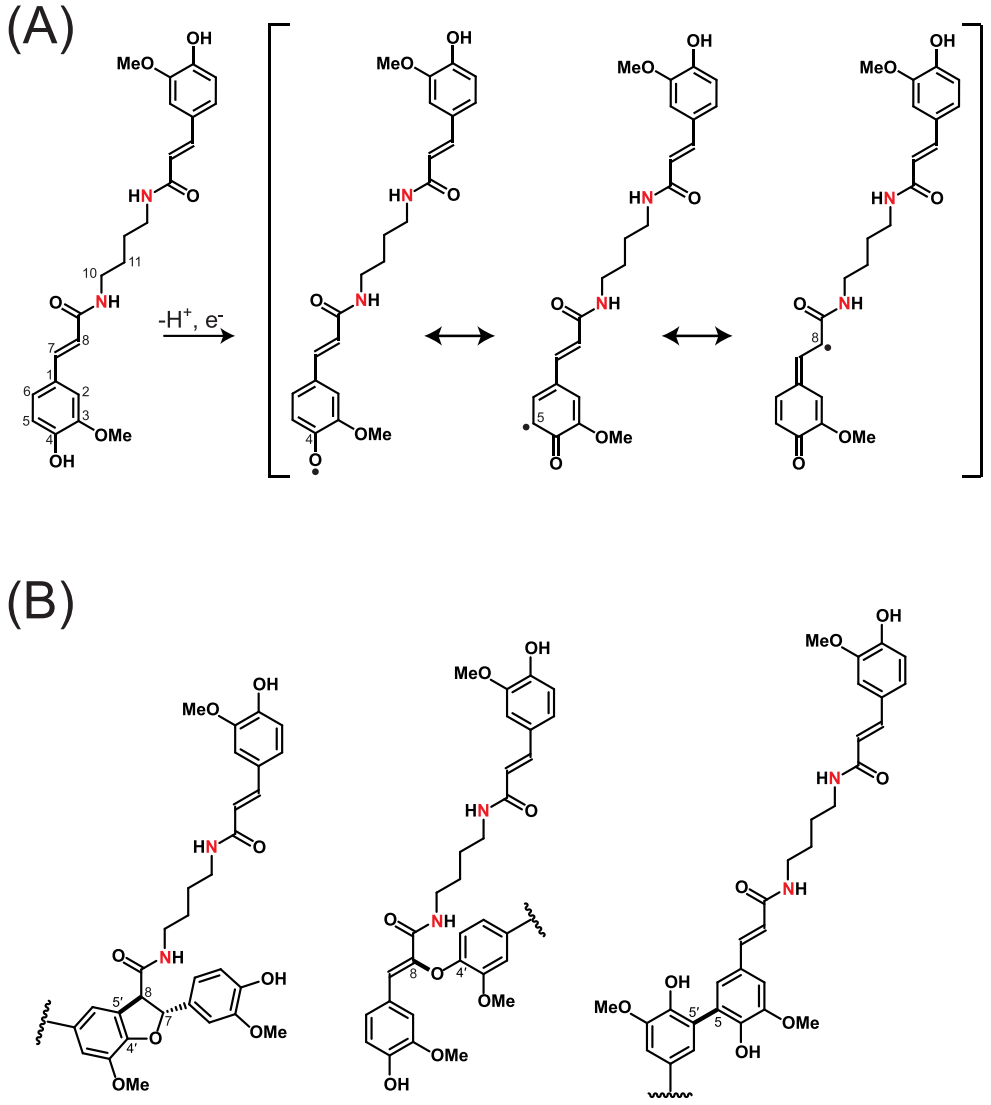

Figure 6. (A) Oxidative radicalization resulting from one-electron oxidation of diferuloylputrescine stabilized by delocalization; resonance forms are displayed in which the single-electron density is shown to localize at the 4-*O*-, 5-, and 8-positions of one ferulate moiety. The other ferulate moiety can also be oxidized in the same manner, allowing it also to undergo independent radical coupling. (B) Modes of incorporation of diferuloylputrescine into the lignin polymer through cross-coupling at the 4-*O*-, 5-, and 8-positions with other ferulates, monolignols, or the growing lignin chain.

wall through the phenolic group of the tyramine moiety.[122] The synthesis of feruloyltyramine is catalyzed by the hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase, which has been found in tobacco and potato.[125] It has been observed that the abundance of feruloyltyramine in these plants increases in response to plant wounding and pathogenic attack; it is therefore possible that under these circumstances the biosynthetic pathway leading to the production of this metabolite is up-regulated to produce a functional lignin polymer used for wound healing.

Diferuloylputrescine. More recently, the ferulamide diferuloylputrescine has also been found to be incorporated into the lignin polymer of maize kernels.[70] The identification was accomplished by detailed NMR analysis that established the structure of diferuloylputrescine and indicated that it was incorporated into the lignin through the ferulate moiety. As occurs with feruloyltyramine and other ferulate conjugates, each ferulate moiety in the diferuloylputrescine molecule can be oxidized by peroxidases and/or laccases forming radicals that are stabilized by resonance (Figure 6). These radicals can couple and cross-couple with another ferulate moiety, or with monolignols and the growing lignin polymer via radical coupling reactions, to become incorporated into the lignin polymer. The modes of incorporation of diferuloylputrescine into the lignin of maize kernels were elucidated by NMR analyses, and different types of linkages and structures involving diferuloylputrescine were found (Figure 6). Diferuloylputrescine is mostly incorporated into the lignin through 8-5′ linkages involving one of the ferulate moieties and producing a phenylcoumaran structure; the unambiguous identification of this structure in the lignin of maize kernel by NMR provided compelling evidence for the participation of diferuloylputrescine in radical coupling reactions, and its incorporation into the cell wall by covalent linkages.[70] Other coupled structures involving incorporation of diferuloylputrescine through 5-5′, 8-*O*-4′, and other linkages were also evidenced by the presence of characteristic signals for feruloyl amides in the HMBC spectrum of maize kernels.[70] The occurrence of all these linkages demonstrates that diferuloylputrescine behaves as a true lignin monomer, participating in

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radical coupling reactions during lignification and being incorporated into the lignin in maize kernels.

EXTENSION OF THE LIGNIN PARADIGM

Lignin has long been considered as derived from the oxidative polymerization of the three canonical monolignols, as well as of other related compounds also derived from the classical monolignol biosynthetic pathway. However, as shown above, different polyphenolic compounds from beyond the canonical monolignol biosynthetic pathway (flavonoids, hydroxystilbenes, hydroxystilbene glucosides, ferulamides) have been found to behave as authentic lignin monomers in several plants participating in radical coupling reactions during lignification and being incorporated into the lignin polymer, thus expanding and challenging the traditional definition of lignin. All these discoveries indicate that the plant is capable of using a wide variety of phenolic compounds, other than the canonical monolignols, for the formation of the lignin polymer. As was noted in a casual article some time ago,[126] the best alternative monomers are those capable of radical coupling in the (unsaturated) side chain to form β-ether analogs, not just on the aromatic ring whereby the monomer can only become a polymer starting unit, or being coupled with the growing polymer into 4-*O*-5 or 5-5 units that do not constitute true branching. The discovery of "non-conventional" phenolic precursors beyond the established phenylpropanoid biosynthetic pathway demonstrates that the mechanism of lignification is particularly flexible and demonstrates that any phenolic compound that is transported to the cell wall may be oxidized and incorporated into the lignin polymer via radical coupling reactions, subject exclusively to simple chemical compatibility, as is increasingly being recognized.[11,12,14,16,31,127] In short, the plant can accommodate all these phenolic compounds, each of which is simply *another brick in the wall*, for copolymerization with monolignols to produce a functional lignin polymer. Whether or not the properties of such copolymers, particularly those that are created by our engineering efforts, provide advantages to the plant/tissue can be debated. Those copolymers that evolved early on and remain as distinct features in various "natural" plants surely attest that they either do provide advantages or at least do not have significant disadvantages to the plant's viability.

SOURCES OF VALUABLE BIOACTIVE COMPOUNDS

The aromatic structure of lignin makes it an interesting renewable raw material for producing useful chemicals, fuels, and other commodities, in the context of a lignocellulosic biorefinery. Conversion of lignins to low-molecular-weight aromatic compounds offers a promising route for the valorization of lignins. The discovery of valuable phenolic compounds incorporated into the lignin of several plants provides new knowledge of the chemical structure and variability of the lignins from different lignocellulosic residues and will promote additional interest in deriving value from lignins from lignocellulosic materials, particularly from agricultural waste products. As shown above, the lignins from several agricultural and forest residues contain significant amounts of valuable compounds, such as the flavone tricin in cereal straws, the hydroxystilbenes piceatannol, resveratrol, and isorhapontigenin in palm fruit shell residues, hydroxystilbene glucosides in Norway spruce barks, or diferuloylputrescine in maize fibers, a byproduct of the wet-milling of maize grain, that will allow the development of optimized and sustainable uses of agroforestry residues, improving the competitiveness of agricultural and forest-based sectors. We have shown that these compounds can be (partially) released using analytical methods. Although it remains to be determined how efficiently and at what cost some of these potentially valuable compounds can be recovered industrially, many of the wastes generated in agricultural processes may be considered as renewable feedstocks for the production of value-added chemicals and other products from lignin. Among the non-classical phenolic compounds incorporated into the lignin structure, tricin has been the most studied. Tricin can be found in plants in extractable form, as free tricin, forming glycosides, or in low-molecular-weight flavonolignans (or flavonolignols). A quantitative study found that the content of tricin incorporated into the lignin polymer was much higher than the content of extractable tricin, reaching up to 4841, 1304, 5250, and 980 mg/kg in wheat, maize, oat, and rice straws (in comparison to only 1014, 274, 1377, and 195 mg/kg of extractable tricin),[73] which indicates that grass straw lignins, an abundant material that is usually regarded as waste, may be an attractive and potential source for the extraction of this valuable compound. On the other hand, the palm oil industry also generates high amounts of palm fruit shell residues, and similarly, large amounts of bark from Norway spruce are generated by the timber and pulp and paper industries. These wastes, which are mostly burned to co-generate power, or are basically left in the ground, can also be considered as an important source for obtaining high-value hydroxystilbenes. The particularly high levels of simple *p*-hydroxybenzoate esters in oil palm empty fruit bunches can provide a source of the acid for parabens and even pharmaceuticals production; a popularized example is the production of the commercial pain reliever and fever reducer Tylenol (paracetamol, acetaminophen) by a much shorter and more efficient pathway than it is produced from fossil-derived benzene today.[128]

LIGNIN ENGINEERING TO ENHANCE THE PRODUCTION OF HIGH-VALUE PRODUCTS

Metabolic engineering is a useful tool that can be used to increase the amounts of valuable compounds that can be extracted from the aforementioned agroforestry residues; theoretically, the content of flavonoids/hydroxystilbenes in the lignins that contain them could be enhanced by overexpression of CHS and other enzymes. As is now becoming appreciated, other valuable polyphenolic compounds could also be produced through metabolic engineering. This could be the case of the flavonoids naringenin and apigenin that, although not (yet) found naturally in the lignins of wild plants, can be readily incorporated into the lignin polymer in some mutant and transgenic plants. A recent study in rice showed that disruption of FNSII, the enzyme that catalyzes the direct conversion of the flavanone naringenin to the flavone apigenin (see Figure 3), resulted in an altered cell wall lignin incorporating the intermediate naringenin, instead of tricin, into the lignin polymer.[99] Interestingly, the *fnsII* mutant plants presented normal growth performances, similar to the wild-type plants, suggesting that incorporating naringenin instead of tricin into the lignin structure did not affect normal growth and development. More recently, the flavone apigenin was also incorporated into the lignin of culm tissues in a rice mutant lacking F3′H, the enzyme that hydroxylates the B-ring of apigenin to produce luteolin (see Figure 3), an intermediate in the biosynthesis of tricin.[129] These two examples illustrate how

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metabolic engineering of plant lignins could be used to introduce high-value products that may then be isolated from their lignins, thus enhancing their value. On the other hand, incorporation of non-conventional lignin monomers not usually present in plant lignins, as is the case of the phenolic compounds derived from the flavonoid, hydroxystilbene, or the ferulamide pathways described above, can open new ways to design and engineer the structure of the lignin to produce polymers with new or improved properties, as already considered with other phenolic compounds.[14,16,127,130,131] Metabolic engineering to introduce tricin (or other flavonoids) into the lignin of plants could provide lignins with special properties, such as a conferring higher UV protection as well as antioxidant, antifungal, and antimicrobial properties. Incorporation of piceatannol could confer hardness and rigidity to the polymer, besides antioxidant and antimicrobial properties; incorporation of hydroxystilbene glucosides could also confer hardness and rigidity as well as antioxidant, antifungal, and antimicrobial properties and, in addition, could provide hydrophilic properties to the lignin polymer, due to the glucose moiety, that have been shown to be beneficial for wall saccharification.[131] Introduction of diferuloylputrescine into the lignin of plants could provide a higher degree of plasticity and flexibility to the lignin polymer, as well as hydrophobicity due to the butane bridge; it could also provide antifungal and antimicrobial properties and may offer a way of adding a stabilized source of N to soils. In conclusion, all the phenolic compounds described above that have already been proven to participate in lignification, and presumably many more that may be considered or that Nature has yet to reveal, can be used to engineer and modify plants to produce tailor-made polymers with desired properties or lignins containing valuable compounds that could be produced at scale if the means to extract them from the polymer can be devised.

AUTHOR INFORMATION

Corresponding Author

José C. del Río − *Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, 41012 Seville, Spain;* orcid.org/0000-0002-3040-6787; Phone: (+34) 954624711; Email: delrio@irnase.csic.es

Authors

Jorge Rencoret − *Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, 41012 Seville, Spain;* orcid.org/0000-0003-2728-7331

Ana Gutiérrez − *Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, 41012 Seville, Spain;* orcid.org/0000-0002-8823-9029

Thomas Elder − *Forest Service, Southern Research Station, USDA, Auburn, Alabama 36849, United States*

Hoon Kim − *Department of Energy Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, University of Wisconsin, Madison, Wisconsin 53726, United States;* orcid.org/0000-0001-7425-7464

John Ralph − *Department of Energy Great Lakes Bioenergy Research Center, Wisconsin Energy Institute and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53726, United States;* orcid.org/0000-0002-6093-4521

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.0c01109

Notes

The authors declare no competing financial interest.

Biographies

José C. del Río is a Senior Research Professor at the Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC), Spain. After accomplishing his Ph.D. in Chemistry at the University of Seville in 1989, he completed several postdoctoral stays at the Oklahoma University (USA), the University of Bristol (UK), and the Pennsylvania State University (USA). His research activity is aimed at the chemistry of the plant cell-wall components and the study of the mechanisms of their chemical, microbial, and/or enzymatic transformations. He has high expertise in the structural characterization of lignins from woody and non-woody plants. His investigations led to the discovery of different phenolic compounds derived from outside the canonical monolignol biosynthetic pathway, namely the flavonoid and stilbene pathways, that behave as authentic lignin monomers, and that challenged the traditional definition of lignin. This experience has resulted in the publication of nearly 200 papers and the issuing of several patents of invention.

Jorge Rencoret is a Senior Researcher at the Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC, Spain), expert in structural elucidation of lignins by 2D NMR, DFRC, thioacydolysis, Py-GC/MS, and GPC. As a Ph.D. student, to learn the most advanced analytical techniques on plant cell-wall characterization, he completed stays at the Biological Research Center (CIB-CSIC, Spain) and the Royal Institute of Technology (KTH, Sweden). After finishing his Ph.D. (2008), he joined Prof. John Ralph's Lab at the University of Wisconsin−Madison (USA) and worked on the characterization of lignin in genetically modified plants (2009−2011). His research work has been recognized by the Royal Academy of Sciences of Seville (distinguished young researcher, 2013), the University of Seville (US-Bruker prizes, 2013 and 2015), and the Spanish National Research Council (recognition awards, 2013 and 2016). At present, his research is aimed at the characterization and valorization of lignins for their

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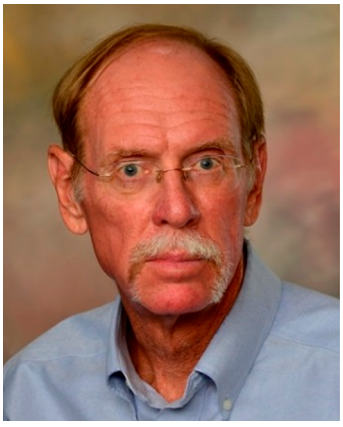
improved industrial use and also the development of biotechnological tools for plant cell-wall deconstruction.

Ana Gutiérrez obtained her B.S. and M.S. degrees in Pharmacy and Biotechnology from University Complutense of Madrid (Spain) in 1988 and 1990, respectively, and her Ph.D. in Pharmacy from the University of Seville (Spain) in 1995. She has a CSIC permanent position at the Institute of Natural Resources and Agrobiology of Seville (IRNAS) as a Research Professor, where she is Vice-Director. Her CV includes about 160 JCR articles and 10 patents. She is am Elected Member of the International Academy of Wood Science (IAWS) since January 2015, and of the Academy Board of IAWS since January 2016. Dr. Gutiérrez's research is focused on wood chemistry (mainly lipids and lignin) and development of biotechnological tools for removal/valorization of these wood components. She has performed outstanding contributions in the field of plant lipids, also including their biotechnological removal and enzymatic oxyfunctionalization for a better use of these renewable feedstocks. A second area of expertise includes the biotechnological removal of lignin from lignocellulosic materials by different enzymatic systems.



Thomas Elder is a Research Scientist with the United States Department of Agriculture—U.S. Forest Service. He holds a Ph.D. from Texas A&M University. He is an Emeritus Professor in the School of Forestry and Wildlife Sciences and Affiliate Professor in Biosystems Engineering at Auburn University. He is also an Adjunct Professor at the Center for Renewable Carbon at the University of Tennessee—Knoxville. He has been a visiting scientist at the University of Wisconsin, the University of Copenhagen, and the University of Natural Resources and Life Sciences, Vienna (BOKU). His research is concerned with the chemical characterization and utilization of wood. He is active in the application of computational chemistry to the reactions and structure of lignin.

Hoon Kim is a Distinguished Scientist at the DOE Great Lakes Bioenergy Research Center (GLBRC) and the University of Wisconsin—Madison, USA. He joined Dr. John Ralph's lab as a Ph.D. student in 1995 and received his Ph.D. in wood (forest product)/organic chemistry at UW-Madison in 2001. He has worked nearly 25 years with the Ralph group as a post-doc, Associate Scientist, and Senior Scientist, most recently being awarded UW's Distinguished Scientist title. He has established himself as a scientist in lignin and plant cell wall chemistry with both substantial and practical experience in NMR techniques. Lignin chemistry is his main focus, and his recent study is the analysis of whole plant cell wall components, including polysaccharides and metabolites; his most notable contribution to this research area has been the development of a gel-state 2D NMR method for whole plant cell walls that has significantly enhanced biomass research at large. His expertise also includes oxidative radical reactions of monolignols, synthesis of lignin model compounds, and structural analysis of transgenic and mutant lignins.

John Ralph is a Professor of Biochemistry at the University of Wisconsin—Madison and, since 2015, a Distinguished Professor of the Tokyo University of Agriculture and Technology. He obtained his B.Sc.-Hons in Chemistry at Canterbury University, New Zealand, in 1976, and his Ph.D. in Chemistry/Forestry at the University of Wisconsin—Madison in 1982. Ralph's group is recognized for its work on lignin biosynthesis, including delineation of the pathways of monolignol biosynthesis, lignin chemistry, and lignin reactions. The chemical/structural effects of perturbing lignin biosynthesis have been a focus, and extensions are aimed at redesigning lignins to be more valuable or more readily degraded. The group develops analytical methods and synthetic methods for biosynthetic products, precursors, intermediates, molecular markers, and cell wall model compounds. Ralph was elected as a Fellow of the American Association for the Advancement of Science (AAAS) in 2005, and has been named by the

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Institute for Scientific Information as one of the 10 most cited authors in the plant and animal sciences every year since 2007.

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