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REVIEW ARTICLE

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Biosynthesis of lignans and norlignans

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Abstract Lignans and norlignans constitute abundant classes of phenylpropanoids. Biosynthesis of these compounds has received widespread interest, mainly because they have various clinically important biological activities. In addition, lignans and norlignans are often biosynthesized and deposited in significant amounts in the heartwood region of trees as a metabolic event of heartwood formation, probably preventing heart rot by heart-rot fungi. Furthermore, biosynthetic reactions of lignans and norlignans involve unique stereochemical properties that are of great interest in terms of bioorganic chemistry and are expected to provide a model for biomimetic chemistry and its application. We outline the recent advances in the study of lignan and norlignan biosynthesis.

Key words Lignan · Norlignan · Phenylpropanoid · Heartwood formation · Biosynthesis

Introduction

Lignans and norlignans constitute abundant classes of phenylpropanoids. Lignans are phenylpropanoid dimers in which the monomers are linked by the central carbon (C8) atoms,[1,2] and are distributed widely in the plant kingdom.[1,3] Norlignans are a class of natural phenolic compounds with diphenylpentane carbon skeletons (C_6-C_5-C_6) that are found mainly in conifers and monocotyledons.[4] Biosynthesis of these compounds has received widespread interest, mainly because they have various clinically important biological activities. Some lignans are used in medicine and nutritional supplements, such as podophyllotoxin-derived semisynthetic lignans in cancer therapies and sesamin in health and nutrition. In addition, lignans and norlignans are often biosynthesized and deposited in significant amounts in the heartwood region of trees as a metabolic event of heartwood formation, probably preventing heart rot by heart-rot fungi. Because heartwood formation is specific to trees and does not occur in herbaceous plants, biosynthesis of lignans and norlignans can be a clue to elucidating heartwood formation mechanisms.

Furthermore, biosynthetic reactions of lignans and norlignans involve unique stereochemical properties that are of great interest in terms of bioorganic chemistry and are expected to provide a model for biomimetic chemistry and its application. Herein, we outline the recent advances in the study of lignan and norlignan biosynthesis.

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Lignans

Lignans are classified into the following eight subgroups based upon the way in which oxygen is incorporated into the skeleton and the cyclization pattern: furofuran, furan, dibenzylbutane, dibenzylbutyrolactone, aryltetralin, arylnaphthalene, dibenzocyclooctadiene, and dibenzylbutyrolactol (Fig. 1).[1] Lignans of each subgroup vary substantially in oxidation levels of both the aromatic rings and the propyl side chains. In addition, these lignans can be further classified into three categories depending on the oxidation state of the C9(C9′) positions, which are at the terminal of the propyl side chain: lignans with 9(9′)-oxygen, lignans without 9(9′)-oxygen, and dicarboxylic acid lignans, as shown in Fig. 2.

Most lignans have oxygen at C9 (C9′), while some lignans of furan, dibenzylbutane, and dibenzocyclooctadiene do not (Figs. 1 and 2). Lignans with and without C9 (C9′) are formed from the corresponding phenylpropanoid monomers with and without C9 oxygen, respectively. Thus, their biosynthesis diverges in the biosynthetic pathways of the phenylpropanoid monomers, while the diversity of the aromatic substitutions is probably introduced after phenylpro-

panoid dimerization, that is, in the conversion of lignans (Fig. 2).

Biosynthetic pathway of lignans with 9(9′)-oxygen

Of the three lignan categories, lignans with 9(9′)-oxygen, lignans without 9(9′)-oxygen, and dicarboxylic acid lignans (Fig. 2), the study of the biosynthesis of lignans with 9(9′)-oxygen is the most advanced.[1] This type of lignan is formed by enantioselective dimerization of two coniferyl alcohol units with the aid of dirigent protein to give rise to pinoresinol (furofuran). Pinoresinol is then reduced by pinoresinol/lariciresinol reductase, via lariciresinol (furan), to secoisolariciresinol (dibenzylbutane), which is in turn oxidized to afford matairesinol (dibenzylbutyrolactone) by secoisolariciresinol dehydrogenase (Fig. 2). The conversion from coniferyl alcohol to matairesinol has been demonstrated in various plant species, which strongly suggests that this is the general lignan biosynthetic pathway.[1] Many other subclasses of lignans are formed from the lignans on the general pathway, while aromatic substituent modification also starts from these lignans.[1]

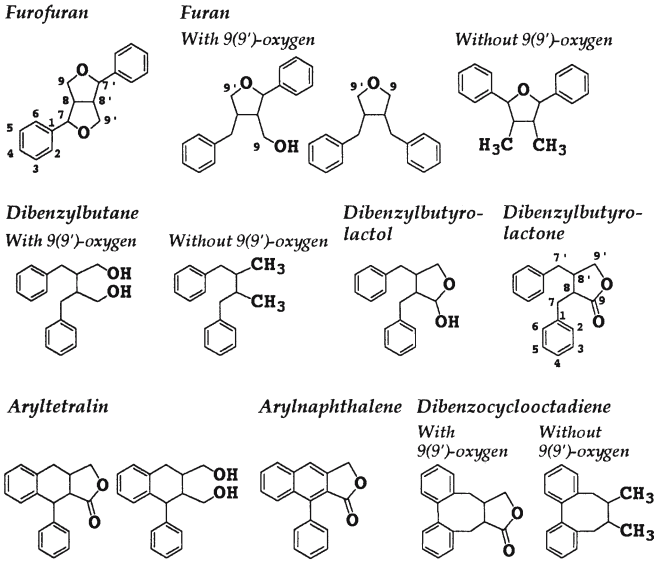


Fig. 1. Basic skeletons of lignans

Coniferyl alcohol formed via the cinnamate/monolignol pathway[5] is a precursor of not only lignans but also lignins, and actually its biosynthetic studies have mostly been conducted in relation to lignin biosynthesis.[5] Recently, Sakakibara et al.[6] carried out the comprehensive metabolic analysis of the cinnamate/monolignol pathway of *Carthamus tinctorius* seeds producing both lignins and lignans, and strongly suggested the intermediacy of ferulic acid in lignan biosynthesis in this plant. This is in sharp contrast to the current view that ferulic acid is not involved as a precursor in lignin biosynthesis.[7]

Dirigent protein

Although both lignans and lignins are biosynthesized from the same immediate monomeric precursor, coniferyl alco-

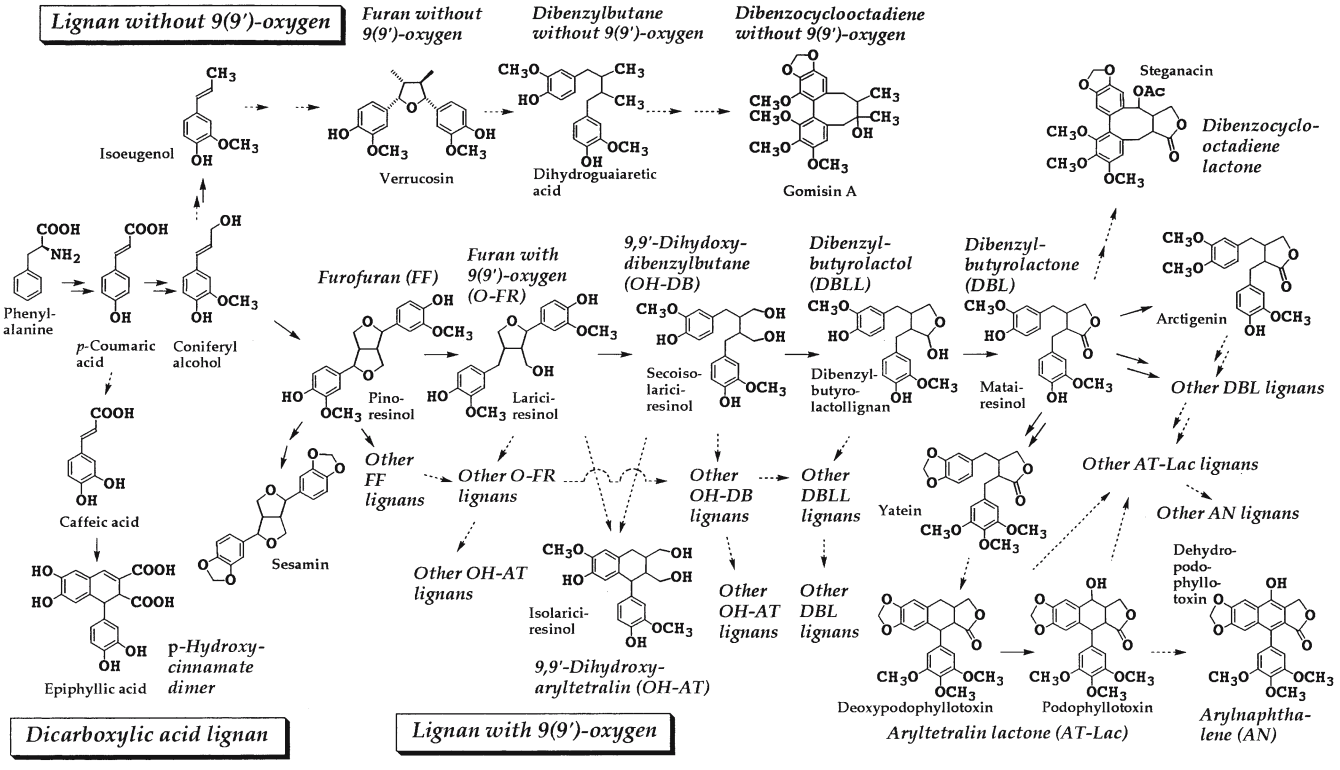


Fig. 2. Possible biosynthetic pathways for various types of lignans. *Solid arrows* represent pathways substantiated by experiments, *broken arrows* represent pathways assumed based on comparison of chemical structures

hol, they differ fundamentally in optical activity. Lignans are composed of only one enantiomer, or of both enantiomers but with one of them being in excess, and are therefore optically active.[1,3,5,8] In contrast, lignins consist of a number of substructures (dimeric or intermonomeric structures, e. g., *β-O*-4 and syringaresinol), each of which are in turn composed of equal numbers of both enantiomers [e.g., the (+)-*erythro*/*threo*-*β*-*O*-4 and (−)-*erythro*/*threo*-*β*-*O*-4 substructures, and the (+)-syringaresinol and (−)-syringaresinol substructures],[8,9] while polymeric lignin molecules are not equimolar mixtures of pairs of enantiomers, and are therefore not racemic.[8] Thus, lignins are racemate-like in terms of their substructures.[8] This implies that lignan biosynthesis is under strict enantioselective control of monomer coupling, whereas lignin biosynthesis is not. In this regard, the mechanisms for enantioselective dimerization in lignan biosynthesis have attracted much interest.

The first example of dirigent protein activity was (+)-pinoresinol synthase activity, which was obtained by dissolving *Forsythia intermedia* cell wall residues,[10] and this was then purified and named dirigent protein (DIR).[11] The protein with a molecular weight of 78 kDa does not have oxidase activity, and thereby in the presence of an oxidase or a one-electron oxidant it effects enantioselective bimolecular coupling of the coniferyl alcohol radical, giving rise to (+)-pinoresinol (Fig. 3).

Halls and Lewis[12] showed that the primary molecular form of the native *F. intermedia* DIR was a dimer of ca. 50 kDa composed of monomers of 23–25 kDa, while no cross-linking of DIR-oxidase complexes was observed following treatment with a cross-linking reagent. Next, they suggested that the coniferyl alcohol radical, rather than coniferyl alcohol, was the substrate for DIR, and that two coniferyl alcohol radical substrates were bound per protein dimer (Fig. 3).[13]

Cloning of cDNAs encoding DIRs was first reported by Gang et al.:[14] two clones (*psd-Fi1* and *psd-Fi2*) (psd: pinoresinol synthesizing dirigent protein) from *F. intermedia*, eight from *Thuja plicata* (western red cedar), two from *Tsuga heterophylla* (western hemlock), two from *Fraxinus mandschurica* (Manchurian ash), and two from *Populus tremuloides* (quaking aspen). Some more were reported later. Thus, DIR homologs have so far been detected in the following species: *F. intermedia*, *Pinus taeda* (loblolly pine), *T. plicata*, *T. heterophylla*, *Eucommia ulmoides* (eucommia), *F. mandshurica*, *P. tremuloides*, *Sesamum indicum* (sesame), *Oryza sativa* (rice), *Arabidopsis thaliana*, *Schisandra chinensis*, *Larrea tridentata* (creosote bush), *Piper futokadsura*, *Linum usitatissimum* (flax), *Nicotiana tabacum* (tobacco), *Podophyllum peltatum* (May apple), *Pisum sativum* (pea), and *Liquidambar styraciflua* (sweet gum).[14–16]

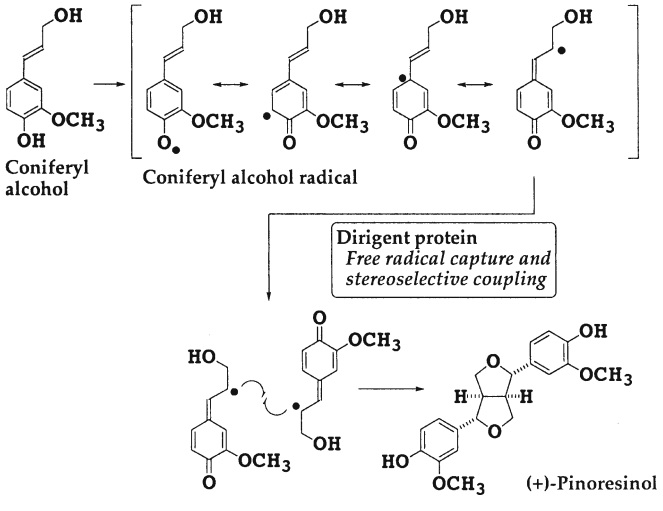


Fig. 3. Enantioselective formation of (+)-pinoresinol with the aid of dirigent protein

Recently, Ralph et al.[17] isolated 17 full length and 2 partial cDNAs encoding DIR and DIR-like genes from Sitka spruce (*Picea sitchensis*), white spruce (*Picea glauca*), and interior spruce (*P. glauca* × *engelmannii*). It was predicted that all 17 full-length spruce DIRs are targeted to the secretory pathway. They performed maximum likelihood analysis using 72 DIR and DIR-like sequences obtained by a GenBank search, which suggested that there may be five distinct DIR subfamilies, tentatively named DIR-a, DIR-b, DIR-c, DIR-d, and DIR-e. Only FiDIR1 (*psd-Fi1*: AF210061),[14] TpDIR5 (*TpDIR5*: AF210067), and TpDIR8 (*TpDIR8*: AF210070)[16] have been demonstrated to have the ability to direct enantioselective (+)-pinoresinol formation by in vitro assays, and all these clones were found to be included in the DIR-a subfamily. Nine out of 19 spruce dirigent homologs belong to DIR-a, while the other 10 clones are grouped into DIR-b.

When the *Forsythia* DIR cDNA sequence was subjected to a database search, there was significant identity (77%) to the *P. sativum* (pea) gene 206-d (U11716),[14] which shows disease resistance response.[18] Six spruce DIR-a genes were found to accumulate rapidly and strongly in wound-induced and weevil-induced stem bark and xylem, while constitutive expression of these genes was insignificant in xylem and cambium, but higher in lateral shoot tips, roots, and cortex.[17] The expression profile is in accordance with *T. plicata* dirigent homologs, which are not expressed in xylem or cambium.[16] Thus, the relative abundance of mRNA transcripts of *T. plicata* DIR homologs was examined by quantitative real-time polymerase chain reaction (PCR), showing that none of them expressed in xylem or cambium, while some are expressed significantly in female flowers, roots of 2-year-old trees, and cotyledons and hypocotyls of seedlings.[16] Taken together, in addition to the role of some DIRs as the asymmetric inducer in pinoresinol biosynthesis, it is clear that some DIR homologs are involved in plant stress responses.

In contrast, the main pattern of subcellular localization of immunolabeling detected by use of polyclonal antibodies raised against recombinant PSD-Fi1 was found to be associated with the S1 sublayer and compound middle lamella of lignified secondary xylem cells, and to a lesser extent inside the corresponding S3 layer of *F. intermedia.*[14,19] The immunolabeling localization is in accordance with initial sites of lignin deposition.[20–22] Based on the coincidence, Lewis and coworkers proposed that the initiation of lignin macromo-

lecular assembly processes could be under the control of arrays of monolignol or oligolignol radical binding sites, which were referred to as arrays of DIRs (or arrays of DIR sites).[14,15,19,23]

On the other hand, there have been strong objections to the concept of arrays of DIR sites.[24–27] Isolation of each gene and its expression product composing the arrays involved in initiation of lignification and their in vitro biochemical characterization will allow us to come to a final conclusion as to the existence and roles of DIR arrays in lignification.

Pinoresinol/lariciresinol reductase

In 1990, it was reported that cell-free extracts of *F. intermedia* mediated the formation of optically pure (−)-secoisolariciresinol from achiral coniferyl alcohol in the presence of NAD(P)H and hydrogen peroxide.[28] The enantioselective production of (−)-secoisolariciresinol observed under the conditions employed was accounted for by nonenantioselective formation of both (+)- and (−)-pinoresinols by peroxidase or some other single-electron oxidant, followed by selective reduction of (+)-pinoresinol, via (+)-lariciresinol, to give rise to (−)-secoisolariciresinol. Thus, in retrospect, pinoresinol/lariciresinol reductase (PLR) activity had been practically detected for the first time when exploiting the substrate formed in situ, although PLR activity itself was not described at that time. Next, using exogeneously supplied substrates, pinoresinol and lariciresinol, PLR activity with *F. intermedia* was described.[29,30] Later, (−)-secoisolariciresinol or (+)-lariciresinol-forming PLR activity was detected in *Forsythia koreana,*[31] *Arctium lappa,*[32–34] *Zanthoxylum ailanthoides,*[35] *Anthriscus sylvestris*,[36] and *Linum flavum.*[37,38] Interestingly, in contrast, PLRs that catalyze selective formation of (+)-secoisolariciresinol from lariciresinol and/or (−)-lariciresinol from pinoresinol were detected in *Daphne genkwa*[39] and *L. usitatissimum.*[38]

In the *F. intermedia*-PLR-catalyzed reaction, the 4-*pro-R* hydrogen of NADPH was found to be employed, and accommodated in the 7′-*pro-R* of formed lariciresinol and the 7-*pro-R* and 7′-*pro-R* of secoisolariciresinol.[40]

cDNA cloning of PLR was reported for the first time with *F. intermedia*,[41] and now many PLR homolog sequences have been deposited in the database. Some of them were functionally expressed and characterized biochemically, especially in terms of the stereochemistry; PLR-Fi1,[41] PLR-Tp2,[42] and PLR-La1[38] catalyze the stereoselective reduction of (+)-pinoresinol to (+)-lariciresinol and (−)-secoisolariciresinol, while PLR-Tp1[42] and PLR-Lu1[38] reduce (−)-pinoresinol to (−)-lariciresinol and (+)-secoisolariciresinol (Fig. 4). It is noteworthy that the selectivity in terms of substrate enantiomers does not correlate to the sequence homology; phylogenetic analysis indicates that angiosperm PLR genes, *PLR-Fi1*, *PLR-La1*, and *PLR-Lu1*, fall into a cluster, while gymnosperm *PLR-Tp1* and *PLR-Tp2* group into another cluster.[38]

Considering the degree of sequence similarity between PLR-Tp1 and PLR-Tp2, the overall secondary and tertiary structures of PLR-Tp1 and PLR-Tp2 can be expected to be

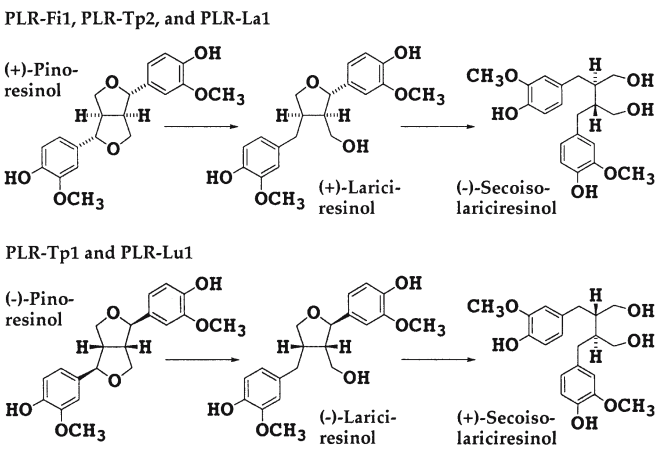


Fig. 4. Reactions catalyzed by pinoresinol/lariciresinol reductases

similar, and the only significant differences are expected to be in the substrate-binding and cofactor-binding sites, which accommodate different enantiomers of pinoresinol. In this regard, X-ray crystallographic analysis of PLR-Tp1 was performed by Min et al.[43] First, PLR-Tp1 was found to exist as a dimer. It was also suggested that Lys138 was involved in general base catalysis to produce a hypothetical enzyme-bound enone intermediate, which was attacked by the 4-*pro-R* hydrogen of NADPH.[43] Although PLR-Tp1 and PLR-Fi1 utilize opposite enantiomers of pinoresinol, it is of interest that the 4-*pro-R* hydrogen of NADPH is commonly used. The structure of PLR-Tp2 was deduced from the crystal structure of PLR-Tp1. By comparing these structures, the authors concluded that (−)-pinoresinol fits among the hydrophobic side chains of Phe164, Leu272, and Val268 in PLR-Tp1, but in PLR-Tp2 there is symmetric substitution between 164 and 272 residues, i.e., Leu164 and Phe272, as well as the residue 268 of Gly, thereby favoring binding of (+)-pinoresinol. Later, however, von Heimendahl et al.[38] reported that a Gly282Val and Tyr286Leu mutant from (+)-pinoresinol-specific PLR-La1 where 282 and 286 correspond to 268 and 272 in PLR-Tp1, respectively, still showed the same stereoselectivity in terms of substrate enantiomers as the wild-type PLR-La1. These results indicate the presence of other factors controlling the enantioselectivity of PLRs.

Secoisolariciresinol dehydrogenase

Secoisolariciresinol dehydrogenase was first detected in *F. intermedia.*[44,45] Cell-free extracts from *F. intermedia* catalyzed selective oxidation of (−)-secoisolariciresinol to (−)-matairesinol in the presence of NAD(P)H, while (+)-secoisolariciresinol did not serve as a substrate. The enzyme preparation exhibited slightly higher activity when NADH was used rather than NADPH (Fig. 5).[45] Later, the enzyme secoisolariciresinol dehydrogenase (SIRD) activities were detected in *L. flavum,*[37] *A. lappa* cv. Kobarutogokuwase,[34] *D. genkwa*, and *Daphne odora.*[46]

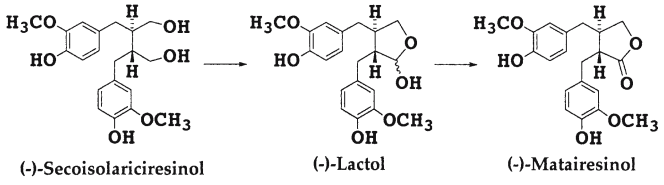


Fig. 5. Reactions catalyzed by secoisolariciresinol dehydrogenase

The stereoselectivity of SIRD in terms of substrate enantiomers is of special interest, because in lignan biosynthesis, it is in the conversion of secoisolariciresinol to matairesinol that the optically pure lignan is formed.[1] Stereochemical analyses of lignans in various plants have revealed that there is a great diversity in the enantiomeric compositions of lignans.[1,8] First, in the cases of pinoresinol, lariciresinol, and secoisolariciresinol, which are the farthest upstream on the biosynthetic pathway, not only the predominant enantiomers of the lignans but also the enantiomeric compositions vary greatly between plant species. In addition, there have been no reported examples of optically pure pinoresinol or lariciresinol. Second, in marked contrast, all dibenzylbutyrolactone lignans, including matairesinol, of which the enantiomeric compositions have so far been determined by chiral high-performance liquid chromatography (HPLC), have been found to be optically pure. Furthermore, it is noteworthy that the optically pure dibenzylbutyrolactone lignans isolated from various plant species are levorotatory and have the same absolute configuration in terms of C8 and C8′, whereas those isolated from Thymelaeaceae plants (e.g., *Wikstroemia* spp. and *Daphne* spp.) have the opposite configuration and are dextrorotatory.

Thus, there is no doubt that the stereochemistry at C8 and C8′ are controlled not only by the entrance step mediated by DIR but also by the subsequent metabolic steps catalyzed by PLR and SIRD. In addition, it was suggested that the variation in enantiomeric compositions of the upstream lignans (lariciresinol, secoisolariciresinol, and matairesinol) between different plant species can be ascribed, at least in part, to the characteristics of the reactions catalyzed by PLR and SIRD as well as their spatiotemporal expression patterns.[1,47] Furthermore, of particular importance is the SIRD-catalyzed reaction, because it is the last of the enantioselective steps giving rise to optically pure lignans.

Considering the consistency between the enantiomers of the naturally occurring and in vitro formed matairesinols in the cases of *Forsythia* and *Arctium*,[34,44,45] it seemed highly likely that (+)-matairesinol-forming SIRD would be detected in (+)-matairesinol-producing Thymelaeaceae plants. Interestingly, however, it was found that this is not the case; *D. genkwa* and *D. odora* SIRDs catalyzed selective formation of (−)-matairesinol from (±)-secoisolariciresinols. The mechanisms for the (+)-matairesinol formation remain unclear, but could be accounted for by the lack of factors that direct formation of the (+)-enantiomer in the assay system, although the assay system might employ a physiologically incorrect substrate.[46]

Forsythia intermedia SIRD was purified to apparent homogeneity, and by use of the amino acid sequence information, the cDNAs encoding *F. intermedia* and *P. peltatum* SIRDs were isolated.[48] Like plant FiSIRD, their functional recombinant proteins catalyzed the formation of (−)-matairesinol when incubated with (±)-secoisolariciresinol in the presence of NAD.[48] In addition, (−)-lactol was identified as the intermediate (Fig. 5).[48]

X-ray crystallographic analysis of recombinant PpSIRD showed that it was a homotetramer.[49] SIRD contains a highly conserved catalytic triad (Ser153, Tyr167, and Lys171) whose activity was abolished by site-directed mutagenesis of Tyr167Ala and Lys171Ala, whereas mutagenesis of Ser153Ala only resulted in a much-reduced catalytic activity giving rise to only lactol but not matairesinol.[50] The hydrogen atoms abstracted from the substrates are accommodated in the *pro-S* position of the formed NADH.[50]

Biosynthesis of podophyllotoxin and sesamin

Biosynthetic pathways for many lignans other than the upstream lignans, pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol, can be regarded as starting from these four lignans. Among them, the pathways toward an important antitumor lignan, podophyllotoxin, and a nutritional supplement lignan, sesamin, have been studied the most intensively.

In the 1980s, Dewick and coworkers conducted a series of feeding experiments and revealed the pathways from yatein to podophyllotoxin via deoxypodophyllotoxin (= desoxypodophyllotoxin, anthricin).[1,51] They also showed that matairesinol was metabolized to podophyllotoxin and proposed yatein as a possible intermediate between matairesinol and podophyllotoxin.[52]

The transformation of matairesinol to yatein involves four steps: 5-hydroxylation, dual methylation at C4OH and at C5OH, and methylenedioxy bridge formation between C3′ and C4′. Many possible sequences of these four steps can be envisaged. Based on metabolic profiling and a series of stable isotope tracer experiments using *A. sylvestris*, Sakakibara et al.[53] demonstrated a direct pathway and not a metabolic grid from matairesinol to yatein via thujaplicatin (Fig. 6).

The enzyme that converts yatein to deoxypodophyllotoxin is still unknown, while a number of enzymes involved in the further transformation of deoxypodophyllotoxin have been reported: the hydroxylase activity converting deoxypodophyllotoxin to podophyllotoxin,[54,55] deoxypodophyllotoxin 6-hydroxylase to give β-peltatin from deoxypodophyllotoxin,[56] and β-peltatin 6-*O*-methyltransferase.[57] On the other hand, there has been no report on the genes encoding enzymes involved in the yatein transformation (Fig. 6). Recently, cDNA encoding matairesinol *O*-methyltransferase (OMT), which catalyzes the regioselective methylation of matairesinol to give rise to arctigenin (Fig. 2), was isolated from *Carthamus tinctorius* (safflower) seeds, which is the first example of molecular cloning of a cDNA

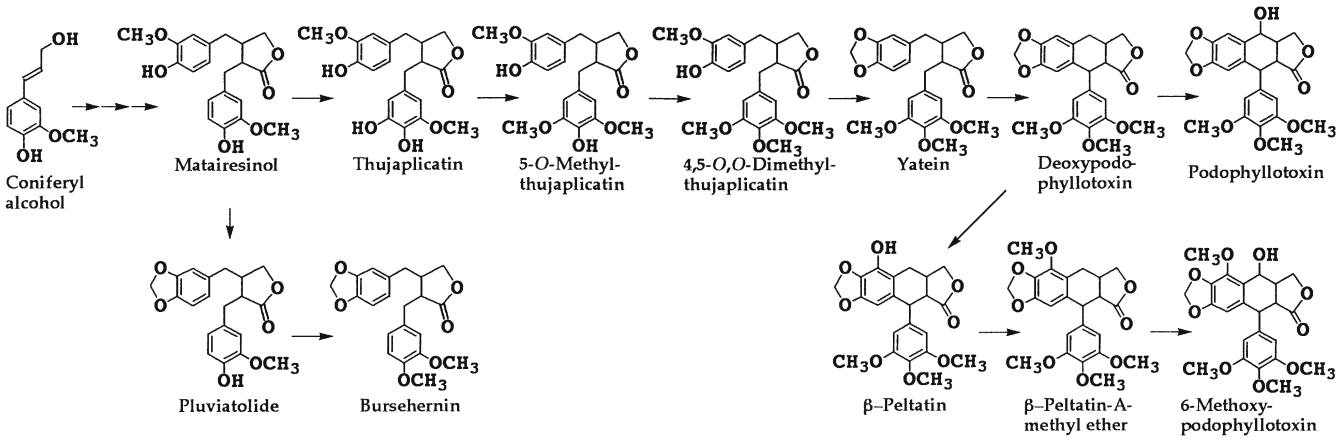


Fig. 6. Proposed biosynthetic pathways for podophyllotoxin, 6-methoxypodophyllotoxin, and bursehernin

encoding a lignan OMT. This gene can be exploited to isolate genes encoding OMTs involved in the biosynthesis of antitumor podophyllotoxin and heartwood lignans.[58]

Recently, de novo biosynthesis of podophyllotoxin in microorganisms has been reported.[59,60] Two endophytic fungal isolates, *Trametes hirsuta*[59] and *Phialocephala fortinii*,[60] isolated from *Podophyllum hexandrum* and *P. peltatum*, respectively, were found to produce podophyllotoxin. This is of special interest, because lignan biosynthesis has been regarded as a plant-specific metabolic event, although the production of another plant secondary metabolite, taxol, by an endophyte of *Taxus* spp., *Taxomyces andreanae*, is known.[61] These results may suggest the possibility that horizontal gene transfer from plants to endophytic organisms occurred, for which the mechanisms are of special interest. In addition, the production of podophyllotoxin by endophytes has significant commercial implications, because of the limited supply of the natural source of podophyllotoxin, *Podophyllum* plants.[62] In this respect, many reports on the production of podophyllotoxin and other lignans in plant cell and organ cultures have been published, and these have been summarized by Petersen and Alfermann,[54] Kuhlmann et al.,[55] Arroo et al.,[62] and Fuss.[63]

Another commercially important lignan is sesamin. The pathway from coniferyl alcohol to sesamin via pinoresinol and piperitol (Fig. 7) was proposed based on feeding experiments,[64] and a part of the conversion was demonstrated by an in vitro experiment; a cytochorome P-450 monooxygenase obtained from a microsomal fraction of *S. indicum* seeds catalyzed the conversion of pinoresinol to piperitol.[65] However, it failed to transform piperitol further to sesamin; therefore, the enzyme was referred to as piperitol synthase, and the presence of sesamin synthase, which converts piperitol to sesamin, was suggested.[65]

By contrast, Ono et al.[66] isolated a cDNA encoding a cytochrome P-450, CYP81Q1 from *S. indicum*, and its functionally expressed recombinant protein catalyzed the sequential methylenedioxy bridge formation of pinoresinol

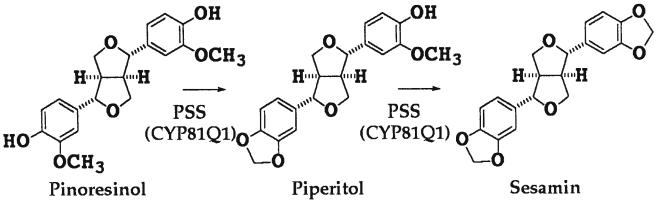


Fig. 7. Sesamin formation by piperitol/sesamin synthase

giving rise to sesamin, as in plant proteins. Thus, it is clear that CYP81Q1 encodes piperitol/sesamin synthase (PSS) (Fig. 7).

Biosynthesis of lignans without 9(9′)-oxygen

Based on the similarity between the phylogenetic distribution of plants producing lignans without 9(9′)-oxygen and that of plants producing allyl- and propenylphenols such as eugenol, safrole, and anethole, which have no oxygen at C9, it was suggested that lignans without 9(9′)-oxygen may be formed by coupling of propenylphenols such as isoeugenol,[1] as proposed by Gottlieb.[67] In addition, Moinuddin et al.[68] proposed coupling of *p*-anol (*p*-propenylphenol) to give several furan lignans without 9(9′)-oxygen in *Larrea tridentata*, while, based on a feeding experiment, Lopes et al.[69] showed that isoeugenol is converted to verrucosin, a furan lignan without 9(9′)-oxygen in *Virola surinamensis* (Fig. 8).

Recently, two articles that deal with enzymes responsible for allyl- and propenylphenol formation have been published.[70,71] One reported the NAD(P)H-dependant reductase in *Ocimum basilicum* (sweet basil), catalyzing the conversion of *p*-coumaryl *p*-coumarate and *p*-coumaryl acetate to chavicol (*p*-allylphenol),[71] while Koeduka et al.[70] isolated genes encoding NADPH-dependent reductases from *Petunia hybrida* cv. Mitchell (petunia) and *O. basilicum*; catalyzing conversion of coniferyl acetate to isoeuge-

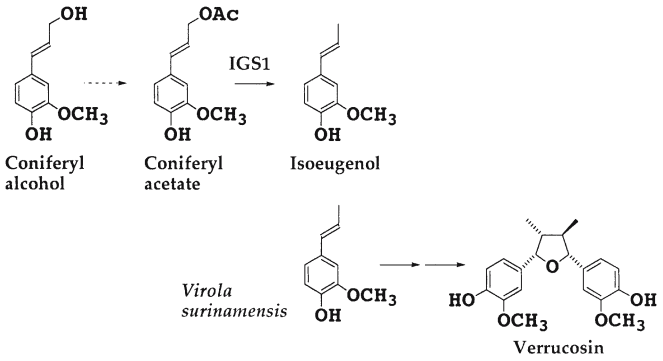


Fig. 8. Isoeugenol formation from coniferyl acetate by isoeugenol synthase and its conversion to lignan in *Virola surinamensis*

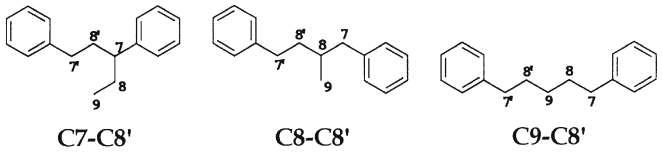


Fig. 9. Basic skeletons of norlignans

nol and eugenol, respectively, and named them PhIGS1 (isoeugenol synthase 1) and ObEGS1 (eugenol synthase 1), respectively. In addition, acetylation of coniferyl alcohol giving rise to coniferyl acetate was suggested. Thus, the biosynthetic pathway from coniferyl alcohol to eugenol or isoeugenol via coniferyl acetate in the plants was proposed, although it remains to be determined whether coniferyl acetate or a similar acylated form of coniferyl alcohol is the actual substrate in planta[70] (Fig. 8).

Norlignan

The chemical structures of norlignan are apparently composed of phenylpropane (C_6-C_3) and phenylethane (C_6-C_2) units. Based on the linkage position between the two units, the chemical structures are classified into three groups: (1) C7-C8′ linkage type, (2) C8-C8′ linkage type, and (3) C9-C8′ linkage type (Fig. 9).

Norlignans from conifers

Many norlignans have been found in the heartwood of coniferous trees.[72] The typical conifer norlignan has a C7-C8′ linkage type (Fig. 10). Taxodiaceae, Cupressaceae, and Araucariaceae are good sources of typical conifer norlignans. Several conifer species contain norlignans with a C8-C8′ linkage (yateresinol)[73] and a C9-C8′ linkage [sequirin-D and (*Z*,*Z*)-1,5-bis(4′-hydroxyphenyl)-1,4-pentadiene].[74–76] Other complicated lignans from Taxodiaceae species are also known.[77–79]

Norlignans from monocotyledons

Some monocotyledonous species classified as Liliaceae[4,80–82] and Hypoxidaceae[83] produce norlignans with a C7-C8′ linkage. It has been reported that Hypoxidaceae also produces C9-C8′ type norlignans.[83] On the other hand, *Asparagus officinalis* is known to produce Ph-O-C5-Ph compounds (asparenyol, asparenyn, and asparendiol) that are biogenetically related to norlignans with a C7-C8′ linkage[82] (Fig. 10).

Norlignans from other plant sources

Several norlignans have been derived from sources other than conifers and monocots (Fig. 10). Three glycosylated norlignans, pueroside-A, pueroside-B, and sophoraside-A, have been isolated from *Pueraria lobata* and *Sophora japonica*.[84–86] The hydroxylation at the 6-position and the C9-lactonization are unique and not found in norlignans from conifers and monocotyledons. *Daphne odora*[87] is known to produce the C9-C8′ norlignan (daphneolone) (Fig. 10).

Norlignan biosynthesis

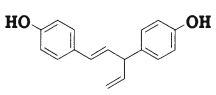
Because norlignans have two aromatic rings and a side chain with five carbons, it had been hypothesized that norlignan biosynthesis was partly related to lignan biosynthesis.[88] However, the following points must be discriminated from lignan formation. First, lignans have a C8-C8′ linkage but typical norlignans have a C8-C7′ linkage. Second, one carbon atom must be lost in norlignan biosynthesis if norlignans are derived from two phenylpropane units. Several hypothetical biosynthetic pathways were proposed in line with the above restrictions and norlignan chemical structures.[73,88,89] However, none of them were supported by any concrete experimental evidence.

Recently, Suzuki et al.[4,90,91] revealed for the first time that norlignans [(*Z*)- and (*E*)-hinokiresinol] are formed from phenylpropanoid monomers via *p*-coumaryl *p*-coumarate as a dimeric intermediate. A reaction mechanism via the ester enolate Claisen rearrangement (the [3,3]-sigmatropic rearrangement of allyl esters to γ,δ-unsaturated carboxylic acids) followed by decarboxylation to form hinokiresinols (Fig. 11, route a) has been hypothesized.[90] Another mechanism (Fig. 11, routes b and c) that cannot be ruled out is based on a quinonemethide intermediate from *p*-coumaryl alcohol reacting with *p*-coumarate.[88] This quinonemethide intermediate and *p*-coumarate might also be used for the formation of C9-C8′ norlignans (Fig. 11, route d) such as sequirin-D[89] (Fig. 11, route f) and hypoxoside[83] (Fig. 11, route e). More experimental evidence regarding the molecular mechanisms that produce norlignan main skeletons is needed.

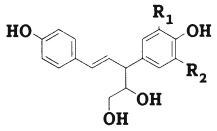
Sequirin-D has a structure that is distinct from those of other conifer heartwood norlignans. Interestingly, Zhang et al.[92] have recently discovered sequosemperivirin-A, a new spirocyclic compound from *Sequoia sempervirens*, which

Fig. 10. Chemical structures of norlignans and related compounds

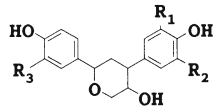
Norlignans from conifers



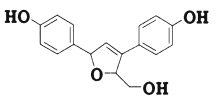
(*E*)-Hinokiresinol



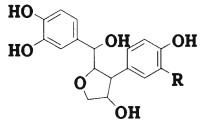
R_1=R_2=H: Agatharesinol
R_1=OH, R_2=H: Sequirin-C
R_1=OCH_3, R_2=H: Sequosempervirin-B
R_1=OCH_3, R_2=OCH_3: Sequosempervirin-C



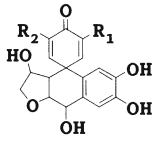
R_1=R_2=R_3=H: Sugiresinol
R_1=OH, R_2=R_3=H: Hydroxysugiresinol
R_1=R_2=H, R_3=OH: Sequirin-E
R_1=R_3=OH, R_2=H: Sequirin-F
R_1=R_2=R_3=OH: Sequirin-G
R_1=OCH_3, R_2=R_3=H: Sequosempervirin-F
R_1=R_2=OCH_3, R_3=H: Sequosempervirin-G



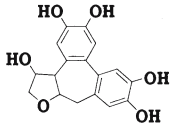
Cryptoresinol



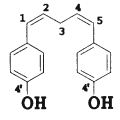
R=H: Metasequirin-A
R=OH: Hydroxymethasequirin-A



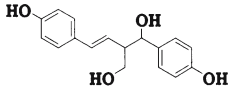
R_1=R_2=H: Athrotaxin
R_1 or R_2=OH: Hydroxyathrotaxin



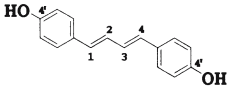
Metasequirin-B



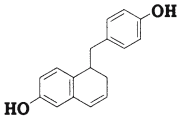
1,5-Bis(4'-hydroxyphenyl)-(*Z*,*Z*)-1,4-pentadiene



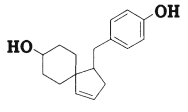
Yateresinol



1,4-Bis(4'-hydroxyphenyl)-(*E*,*E*)-1,3-butadiene

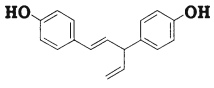


Sequirin-D

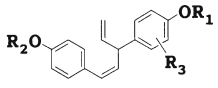


Sequosempervirin-A

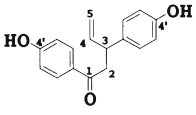
Norlignans from monocotyledons



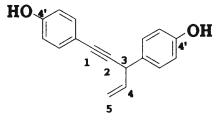
(*E*)-Hinokiresinol



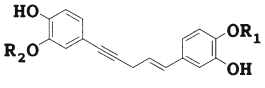
R_1=R_2=R_3=H: (*Z*)-Hinokiresinol (nyasol)
R_1=R_2=H, R_3=OH: (*Z*)-Hydroxyhinokiresinol
R_1=R_3=H, R_2=CH_3: (*Z*)-Monomethylhinokiresinol
R_1=R_2=Glc, R_3=H: Nyasoside
R_1=Apiose-Glc, R_2=Glc, R_3=H: Nyaside
R_1=Glc, R_2=R_3=H: Mononyasine-A
R_2=Glc, R_1=R_3=H: Mononyasine-B



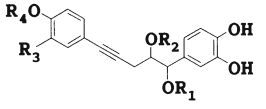
1,3-Bis(4'-hydroxyphenyl)-4-pentene-1-one



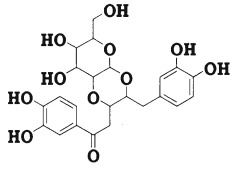
1,3-Bis(4'-hydroxyphenyl)-4-pentene-1-yne



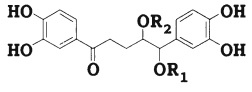
R_1=R_2=Glc: Hypoxoside
R_1=Glc, R_2=H: Obtuside-A
R_1=H, R_2=Glc: Obtuside-B



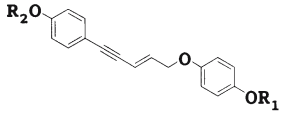
R_1=R_4=H, R_2=Glc, R_3=OH: Nyasicoside
R_1=R_3=R_4=H, R_2=Glc: 3"-Dehydroxynyasicoside
R_1=CH_3, R_2=Glc, R_3=OH, R_4=H: 1-*O*-Methylnyasicoside
R_1=H, R_2=*p*-coumaroyl-Glc, R_3=OH, R_4=Glc: Interjectin



Curcapicycloside

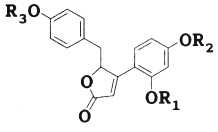


R_1=OH, R_2=Glc: Curculigine
R_1=CH_3, R_2=Glc: 1-*O*-Methylcurculigine
R_1=H, R_2=Glc, R_3=CH_3: 1-*O*-Methylnyasicoside

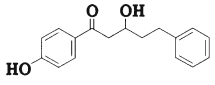


R_1=R_2=H: Asparenydiol
R_1=CH_3, R_2=H: Asparenyol
R_1=R_2=CH_3: Asparenyn

Norlignans from sources other than conifers and monocotyledons



R_1=Glc, R_2=CH_3, R_3=H: Sophoraside-A
R_1=Glc-Rha, R_2=R_3=H: Pueroside-A
R_1=R_3=Glc, R_2=CH_3: Pueroside-B



Daphneolone

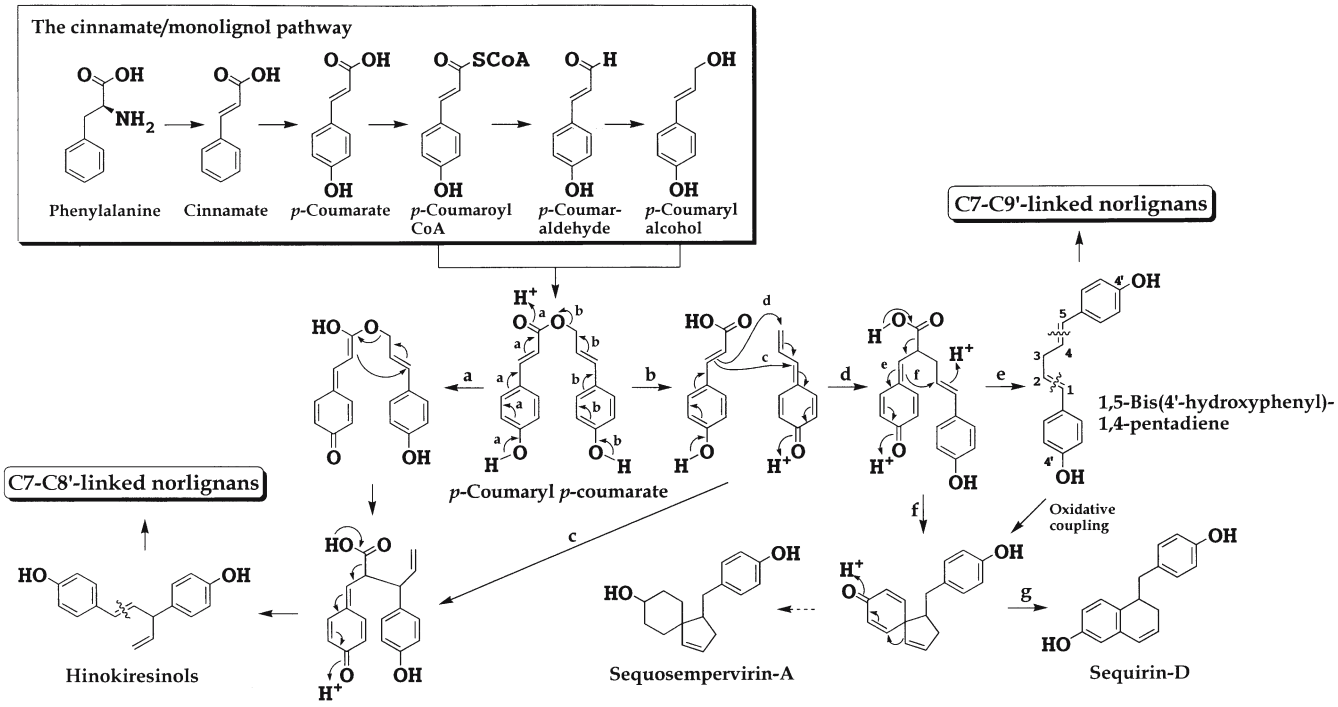


Fig. 11. Proposed biosynthetic pathway of C7-C8′-linked and C9-C8′-linked norlignans

appears to be derived from a biosynthetic intermediate toward sequirin-D (Fig. 11). The detection of this compound supports the hypothesis that sequirin-D may be formed via the dienone-phenol rearrangement (Fig. 11, route g) previously suggested.[89]

At least for C8-C7′ type norlignan biosynthesis, the mechanism appears to be distinct from that for lignan biosynthesis mediated by phenoxyradical coupling.[93] In this conversion scheme (Fig. 11, route a or c), *p*-coumaryl *p*-coumarate is a key intermediate bridging phenylpropanoid monomers with norlignans, and hinokiresinols appear to be entry compounds toward C8-C7′ norlignan biosynthesis. Imai et al.[94] recently proposed that agatharesinol originates from the phenylpropanoid pathway, but (*E*)-hinokiresinol is not a precursor of agatharesinol. By using *Cryptomeria japonica* drying sapwood sticks to generate agatharesinol, they demonstrated the incorporation of labeled phenylalanine and cinnamate into agatharesinol.[94] Subsequently, they administered *p*-[$^2H_2$]coumaryl alcohol and [^{2}H]hinokiresinol into the sticks. The incorporation of these compounds into agatharesinol was so poor that they concluded that these two compounds are not precursors of agatharesinol.[95] However, the following points must be discussed: (1) the compartmentalization of the administered precursors is uncertain; (2) due to the natural (^{29}Si+^{30}Si)/^{28}Si ratio being relatively high, small incorporation of a deuterium label into agatharesinol trimethylsilyl ether may not have been detected; and (3) the daughter ion of the deuterated agatharesinol trimethylsilyl ether that had been used for incorporation detection may not have retained the deuterium because of rearrangement during fragmentation. Thus, the conclusion that (*E*)-hinokiresinol is not a precursor of agatharesinol remains an open question and awaits further experiments.

Aromatic rings in conifer norlignans are often substituted with multiple hydroxyl groups (Fig. 10). The hydroxylation pattern of the phenyl groups is similar to that of phenylpropanoid monomers and flavonoids. Such hydroxylation is often mediated by cytochrome P450 monooxygenases. Imai et al.[96] recently reported the conversion of agatharesinol to sequirin-C by microsomal membranes prepared from *C. japonica* transition wood. This conversion required NADPH and O_2, which was consistent with the cofactors/cosubstrates required by cytochrome P450-monooxygenases together with their localization in microsomal membranes.

Yoshida et al.[97] have published suppression subtractive expressed sequence tag (EST) sequences from *C. japonica* drying sapwood producing norlignans. The EST sequences include several oxygenase/oxidoreductase homologs, which may possibly be involved in norlignan biosynthesis. The EST sequence, along with functional validation of the native/recombinant proteins, would help the biochemical and molecular biological understanding of norlignan biosynthesis.

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