Stereochemical Diversity in Lignan Biosynthesis and Establishment of Norlignan Biosynthetic Pathway*[1]

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(Received May 31, 2002)

Contents

Introduction

Lignans and norlignans are two major classes of wood extractives, accumulating specifically in heartwood composed of only dead cells and occupying the most of the trunk. These secondary metabolites are called "heartwood substances", which are synthesized in parenchyma cells and spread out from the cells to other xylem elements, followed by the death of the cells. This sequence of metabolic events, heartwood formation, was specific to woody plants but not to herbaceous plants. The reason woody plants are long-lived is partly because they accumulate heartwood substances, some of which prevent wood-degrading fungi from rotting.

In addition to the antimicrobial activity, lignans and norlignans have various biological activities[1-4]. Among them, the antitumor lignan, podophyllotoxin, is of special interest, because it is commercially important as a staring material of etoposide and teniposide, which have been used as anticancer drugs in the hospital. However, the large-scaled exploitation of source plants is decreasing the amount of its natural resources[5]. Therefore, it is necessary to establish the efficient production system of podophyllotoxin by which we do not need to depend on the small natural resources. The studies on biosynthesis of lignans and norlignans would afford the essential knowledge for biotechnological production of these

(+)-Matairesinol   (-)-Matairesinol

(+)-Secoiso-   (-)-Secoiso-
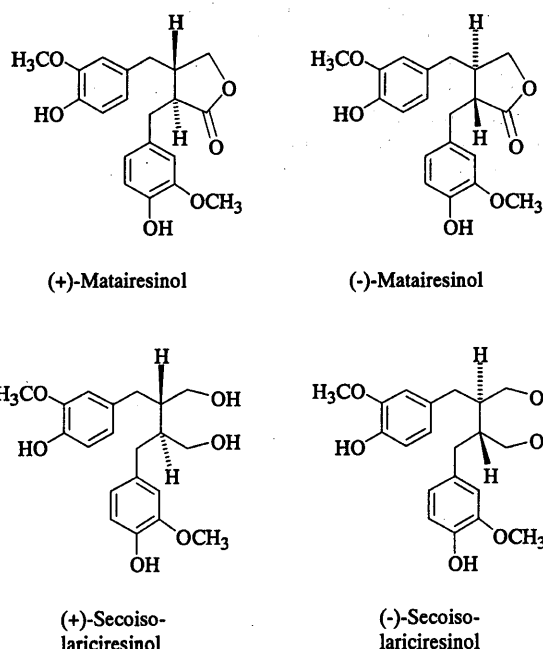lariciresinol   lariciresinol

Fig. 1. Chemical structures of (+)- and (−)-enantiomers of matairesinol and secoisolariciresinol.

compounds.

Biological activities are often related to stereochemistry of compounds. Lignans are stereochemically peculiar in natural products. In general, lignan molecules are chiral, and one enantiomer predominates or only one enantiomer is present in each lignan sample isolated from plants. Interestingly, however, the predominant enantiomer varies with the plant sources. For example, optically pure, levorotatory (−)-matairesinol (Fig. 1) was isolated from *Forsythia intermedia*[6], while the optically pure, dextrorotatory (+)-matairesinol (Fig. 1) was isolated from *Wikstroemia sikokiana*[7]. (−)-Secoisolariciresinol (Fig. 1) from *F. intermedia*[6] and *F. koreana*[8] is optically pure, whereas (−)-secoisolariciresinol isolated from *W. sikokiana* is not optically pure [45% enantiomer excess (e.e.)][9]. Furthermore, (+)-secoisolariciresinol (78% e.e., Fig. 1) was isolated from *Arctium lappa* petioles[10]. These findings strongly suggest that stereochemical control in lignan formation differs among plant species.

Stereochemical difference between lignan and lignin is also of interest. Both are synthesized by one-electron

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Coniferyl alcohol

Pinoresinol

Lariciresinol

Secoisolariciresinol

Matairesinol

Arctigenin

a dirigent protein/laccase/H_2O_2
b pinoresinol/lariciresinol reductase/NADPH
c secoisolariciresinol dehydrogenase/NADP
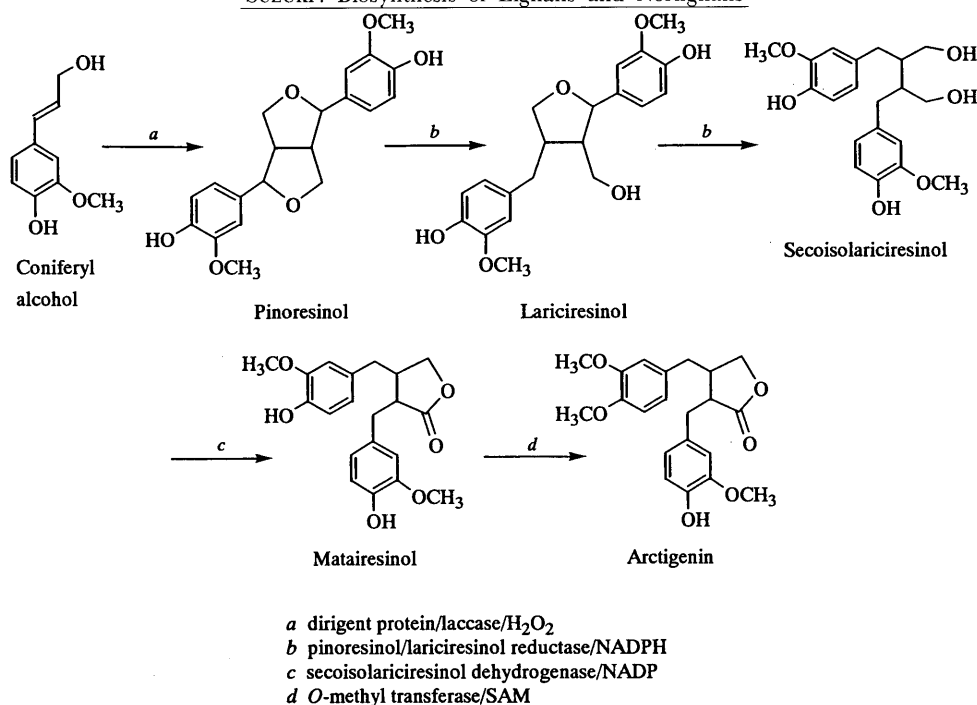d O-methyl transferase/SAM

Fig. 2.   Lignan biosynthetic pathway from coniferyl alcohol to arctigenin.

oxidative coupling of hydroxycinnamyl alcohols, but fundamentally differ in optical activity; lignans are optically active, whereas lignins are inactive. These results suggest that lignan biosynthesis involves stereochemically different process from that of lignin biosynthesis.

Because of these important features, biosynthesis of lignans and norlignans has been receiving widespread interest. In this review, the author describes the recent findings on stereochemical diversity in lignan biosynthesis and the establishment of norlignan biosynthetic pathway.

Chapter I Stereochemical diversity in lignan biosynthesis

The first enzymatic and enantioselective formation of an optically pure lignan, (−)-secoisolariciresinol, from achiral coniferyl alcohol with cell-free extracts from *Forsythia intermedia* in the presence of H_2O_2 and NAD(P)H was reported by Umezawa et al.[11] They also demonstrated the selective oxidation of (−)-secoisolariciresinol to optically pure (−)-matairesinol in the presence of NAD(P)[6].

Lewis and co-workers continued to investigate the processes of secoisolariciresinol and matairesinol formation in *Forsythia* and established the lignan biosynthetic pathway (Fig. 2). Each step, except for the final conversion from matairesinol to arctigenin[12], is well controlled in terms of stereochemistry; (+)-pinoresinol is formed enantioselectively from achiral coniferyl alcohol with oxidase/oxidant in the presence of dirigent protein[13]. The formed (+)-pinoresinol is transformed to (+)-lariciresinol and (−)-secoisolariciresinol with pinoresinol/lariciresinol reductase in the presence of NADPH[14], and (−)-secoisolariciresinol was in turn oxidized to (−)-matairesinol with secoisolariciresinol dehydrogenase in

the presence of NADP[15].

On the other hand, the recent studies in the author's laboratory have revealed that the stereochemistry of lignan biosynthesis varies with plant species. In contrast to cell-free extracts from *Forsythia* plants[11], Umezawa and Shimada[10] isolated (+)-secoisolariciresinol (78% e.e.) from *A. lappa* petioles and the cell-free extracts catalyzed the enantioselective formation of (+)-secoisolariciresinol from coniferyl alcohol in the presence of H_2O_2 and NADPH. This result indicates that *A. lappa* has a different stereochemical control in lignan biosynthesis from that of *Forsythia* plants.

In this chapter, the stereochemistry of lignan formation in two plant species other than *Forsythia*, e.g. *Arctium lappa* and *Anthriscus sylvestris*, is discussed. First, the author describes that the stereochemistry of lignan biosynthesis in *A. lappa* is regulated organ-specifically[16,17]. In contrast to the case of *A. lappa* petioles, (−)-secoisolariciresinol (65% e.e.) was isolated from seeds, and the enzyme preparations from ripening seeds catalyzed the formation of (−)-secoisolariciresinol (38% e.e.) from coniferyl alcohol in the presence of NADPH and H_2O_2. In addition, ripening seed enzyme preparation mediated the selective formation of the optically pure (>99% e.e.) (−)-enantiomer of matairesinol from (±)-secoisolariciresinols in the presence of NADP. Second, the author describes the stereochemistry of lignan formation in *A. sylvestris*, a herbaceous plant known to produce podophyllotoxin congeners and the same lignans as often found in conifer heartwood (= heartwood lignans, e.g. yatein, hinokinin)[18]. The author shows the formation of (+)-lariciresinol (93% e.e.) and (−)-secoisolariciresinol (95% e.e.) from (±)-pinoresinols in the presence of NADPH. This result indicates that the stereochemical property of reduction catalyzed by *A. sylvestris* pinoresinol/lariciresinol reductase (PLR) is similar to those of *Forsythia* PLR and *A. lappa* seed

PLR.

I-1 Stereochemical diversity in lignan biosynthesis of *Arctium lappa* L.

Since Shinoda and Kawagoye isolated arctiin, a glycoside of arctigenin, from seeds of *Arctium lappa* in 1929, seeds of *Arctium* spp. have been well-known to contain significant amounts of lignans[19-23]. Arctigenin isolated from the seed was levorotatory, but the optical purity has not been determined. Suzuki *et al.*[17] determined the optical purity of lignans (−)-matairesinol and (−)-arctigenin by chiral high-performance liquid chromatography (HPLC) after treating MeOH extracts from the seeds with β-glucosidase. Both lignans were optically pure (>99% e.e.). This is in good accordance with previous reports; all the dibenzylbutyrolactone lignans of which enantiomeric compositions have so far been determined precisely by chiral HPLC are optically pure[24].

In addition, small amounts of secoisolariciresinol were isolated from seeds. In contrast to (+)-seoisolariciresinol (78% e.e.) isolated from the petioles[10], (−)-secoisolariciresinol (65% e.e.) was isolated from MeOH extract of the seeds after glucosidase treatment[16]. Acid hydrolysis (H$_2$SO$_4$) also yielded (−)-secoisolariciresinol (82% e.e.) from the MeOH extracts[17]. This is the first example that different enantiomers of a particular lignan occur predominantly in different organs of a single plant species, indicating the stereochemical diversity of lignan biosynthetic mechanism in *A. lappa*.

Umezawa and Shimada[10] reported that the incubation of coniferyl alcohol with the petiole enzyme preparation

gave the (+)-enantiomer of secoisolariciresinol (ca. 20% e.e.) by liquid-chromatography mass spectrometry. However, because of the incomplete separation in the liquid-chromatography, the value was not accurate. Suzuki *et al.*[17] determined the enantiomeric compositions of lignans, pinoresinol, lariciresinol and secoisolariciresinol, by gas-chromatography mass spectrometry after chiral HPLC separation; (+)-pinoresinol (33% e.e.), (+)-lariciresinol (30% e.e.) and (+)-secoisolariciresinol (20% e.e.) were formed with the petiole enzyme from coniferyl alcohol. On the other hand, seed enzyme prepared from *A. lappa* ripening seeds catalyzed the formation of (−)-secoisolariciresinol (38% e.e.), (−)-pinoresinol (22% e.e.), and (−)-lariciresinol (>99% e.e.) from coniferyl alcohol in the presence of NADPH and H$_2$O$_2$[16]. The enzymatic experiments with coniferyl alcohol exhibited the stereochemical diversity, which is in line with the discordance of the predominant enantiomers of secoisolariciresinol isolated from different organs of *A. lappa* (Fig. 3).

Pinoresinol/lariciresinol reductase (PLR), responsible for reduction of pinoresinol to lariciresinol, and lariciresinol to secoisolariciresinol, was purified from *Forsythia intermedia*[14], and this enzyme was detected from *Zanthoxylum ailanthoides*[25] and *Daphne odora*[26]. Incubation of (±)-pinoresinols with the seed enzyme preparation yielded almost optically pure (−)-secoisolariciresinol (99% e.e.) and (+)-lariciresinol (85% e.e.), and (−)-secoisolariciresinol (91% e.e.) was formed from (±)-lariciresinols with the enzyme preparation[17]. Thus, the seed enzyme preparation had PLR activity. The predominant formation of (−)-secoisolariciresinol from
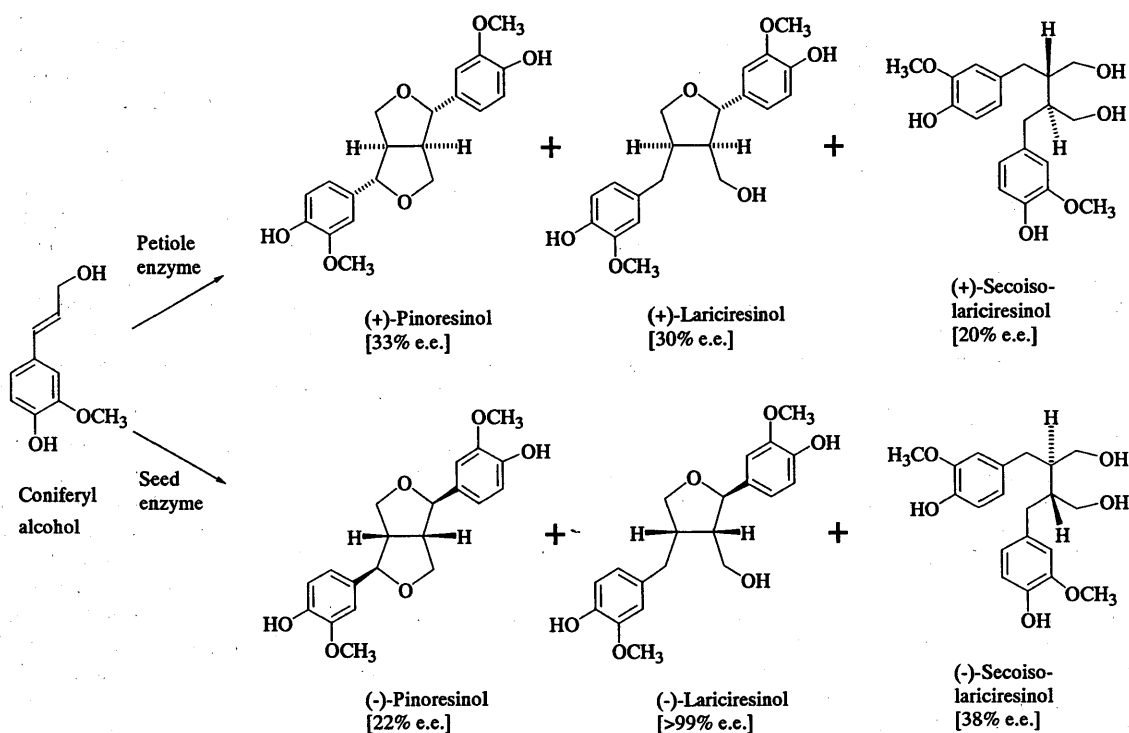

Fig. 3. Formation of pinoresinol, lariciresinol and secoisolariciresinol by *Arctium lappa* enzyme preparations. The petiole enzyme preparation catalyzes the lignan formation in favor of (−)-enantiomer, while the seed enzyme preparation does in favor of (+)-enantiomer.

these racemic lignans are in line with the results of incubation of coniferyl alcohol with the seed enzyme[16].

The petiole enzyme also exhibited PLR activity giving rise to lariciresinol and secoisolariciresinol from (±)-pinoresinols, and secoisolariciresinol from (±)-lariciresinols[17]. Interestingly, however, the predominant enantiomers of the product lignans, (−)-secoisolariciresinol and (+)-lariciresinol, formed from (±)-pinoresinols and (±)-lariciresinols were the same as those obtained with the seed enzyme, and (−)-secoisolariciresinol is opposite to the predominant enantiomer, (+)-secoisolariciresinol, isolated from the petiole. The enantiomer excess values of the formed (−)-secoisolariciresinol (44 and 37% e.e.) were much lower than those formed with the seed enzyme which are almost optically pure (99 and 91% e.e.).

These results can be accounted for by postulating that A. lappa has PLR isoforms showing different selectivity in terms of the substrates, (+)-pinoresinol and (−)-pinoresinol. Although final conclusions await further experiments, this view is in good accordance with recent findings on PLR of different plant species as follows : PLR was partially purified from Forsythia intermedia cv. Lynwood gold[27], and its cDNAs were cloned and expressed in E. coli[14]. Both plant and recombinant proteins exhibited the same stereochemical property ; each protein catalyzed selective formation of (+)-lariciresinol and (−)-secoisolariciresinol from (±)-pinoresinols, and (−)-secoisolariciresinol from (±)-lariciresinols in the presence of NADPH[14]. PLR of Zanthoxylum ailanthoides[25] also showed similar stereochemical selectivity to Forsythia PLR. In contrast, PLR activity from Daphne genkwa which exhibited the opposite stereochemical property to the Forsythia and Zanthoxylem PLRs ; the Daphne crude enzyme preparation catalyzed selective formation of (−)-lariciresinol (23% e.e.) from (±)-pinoresinols[26]. These results indicated that different PLRs which have opposite stereochemical properties with respect to lariciresinol and secoisolariciresinol formation distribute in different plant species. Furthermore, the presence of cDNAs corresponding to the two stereochemically distinct PLRs in a single plant species was demonstrated by Fujita et al.[28], although they did not mention the physiological roles of the two isoforms. On the other hand, the author's present results strongly suggest that the two PLR isoforms are expressed differentially in A. lappa.

As for the stereochemistry of pinoresinol formation, dirigent protein has not yet been isolated from A. lappa. However, a recent detection of a dirigent-protein-like gene from A. lappa using a PCR-guided strategy[29] suggests that stereochemistry of formation of pinoresinol from coniferyl alochol in A. lappa is also under control of dirigent protein.

In accordance with the presence of large amounts of two optically pure dibenzylbutyrolactone lignans, (−)-matairesinol and (−)-arctigenin, in A. lappa seeds, secoisolariciresinol dehydrogenase activity was detected in the seed enzyme preparation which gave rise to optically pure (−)-matairesinol following incubation of racemic (±)-secoisolariciresinols in the presence of NADP[17]. Thus, although formation of secoisolariciresinol is controlled stereochemically, the control is not enough strong to produce only one enantiomer of secoisolari-

ciresinol. Formation of optically pure lignan is finally achieved in the conversion of secoisolariciresinol to matairesinol in this plant species.

Taken together, there is a great stereochemical diversity in lignan biosynthesis, and not only the enantioselective coupling of coniferyl alcohol assisted by dirigent protein but also the subsequent several steps must play substantial roles in production of optically pure lignans.

I-2 Stereochemistry of lignan biosynthesis in Anthriscus sylvestris (L.) Hoffm.

Due to the limited supply of podophyllotoxin (Fig. 4) from natural resources[5], the alternative sources have been searched. Anthriscus sylvestris (L.) Hoffm. might be one of the candidates, because it produces angeloyl podophyllotoxin[30] as well as significant amounts of a precursor of podophyllotoxin, deoxypodophyllotoxin[31–36] (Fig. 4). In addition, it produces the typical heartwood lignans yatein and hinokinin[34] (Fig. 4), which are found specifically in the heartwood region in the conifers Libocedrus yateensis[37] and Chamaecyparis obtusa[38], respectively. Thus, A. sylvestris is probably a good plant material for lignan biosynthesis studies to access mechanisms involved in antitumor and heartwood lignan formation. As the first step, it is necessary to characterize the precursor lignans of yatein and deoxypodophyllotoxin in A. sylvestris.

Suzuki et al.[18] preliminarily surveyed lignans in the β-glucosidase-treated MeOH extracts of both aerial parts and roots of A. sylvestris. GC-MS analysis revealed that the presence of the lignans, yatein and secoisolariciresinol. In addition, nemerosin and deoxypodophyllotoxin, which were previously isolated from Anthriscus spp.[31–36,39], were identified by comparing their spectrometoric data[39,40].

After fractionating the β-glucosidase-treated MeOH extracts of A. sylvestris by silica gel column chromatography, lariciresinol, matairesinol, hinokinin, pluviatolide, and bursehernin (Fig. 4) were identified by GC-MS analysis[18]. Secoisolariciresinol, lariciresinol, matairesinol, pulviatolide and brusehernin were identified for the first time in Anthriscus spp.

When coniferyl alcohol was incubated with the A. sylvestris enzyme preparation in the presence of H_2O_2 and NADPH, the lignans, pinoresinol, lariciresinol, and secoisolariciresinol, were formed. Furthermore, the enzymatic conversion of (±)-pinoresinol to (+)-lariciresinol (93% e.e.) and (−)-secoisolariciresinol (95% e.e.) by PLR was demonstrated in the presence of NADPH[18].

The PLR activity together with enzymatic formation of the lignans from coniferyl alcohol accorded well with those with A. lappa and Forsythia spp[6,8,11,27,41,42]. In addition, the PLR-catalyzed selective formation of (+)-lariciresinol and (−)-secoisolariciresinol from (±)-pinoresinols with the A. sylvestris enzyme preparation suggested that the stereochemical property of A. sylvestris PLR-catalyzed reduction was similar to those of Forsythia PLR[27] and A. lappa ripening seed PLR[17].

The lignan formation by the Anthriscus enzyme preparation along with the detection of lariciresinol and secoisolariciresinol from the plant suggests strongly that the conversion, pinoresinol→lariciresinol→secoisolaricire-
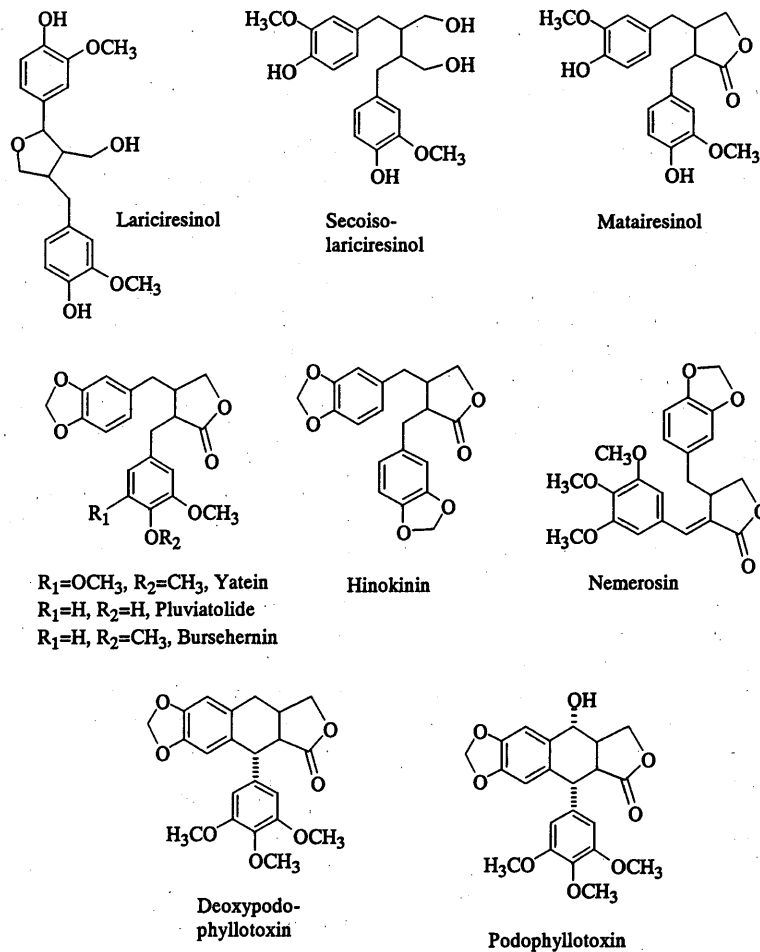
Fig. 4. Chemical structures of podophyllotoxin and *Anthriscus sylvestris* lignans.

sinol.

Chapter II Establishment of norlignan biosynthetic pathway

Typical norlignans having the 1, 3-diphenylpentane [C_6-C_3(C_2)-C_6] structure (*e.g.* hinokiresinol [(*E*)-hino-kiresinol], agatharesinol, and sequirin-C, Fig. 5) occur in coniferous trees (especially in heartwood) of Cupressaceae, Taxodiaceae, and Araucariaceae[43–46], while γ-lactonized 1, 3-diphenylpentane norlignans (*e.g.* pueroside A and B) were isolated from two Leguminosae trees (*Pueraria lobata* and *Sophora japonica*)[47–49]. Some monocotyledonous Liliaceae and Hypoxidaceae plants are also good sources of 1, 3-diphenylpentane and 1, 5-diphenylpentane norlignans. For instance, (*Z*)-hinokiresinol (=nyasol) (Fig. 5) which is the geometrical isomer of a coniferous heartwood norlignan, (*E*)-hinokiresinol, was isolated from *Asparagus* and *Anemarrhena*[3,50,51].

It is well known that norlignans accumulate specifically in conifer heartwood. Heartwood coloration of *C. japonica* (Japanese cedar)[52,53] and *Chamaecyparis obtusa* (hinoki cypress)[54] is due to norlignans. The normal heartwood coloration of *C. japonica* and *C. obtusa* is pale salmon pink, which has been appreciated in Japan. However, black-discoloration often occurs in *C. japonica* heartwood, which lowers the value of the wood. To solve the problem, much
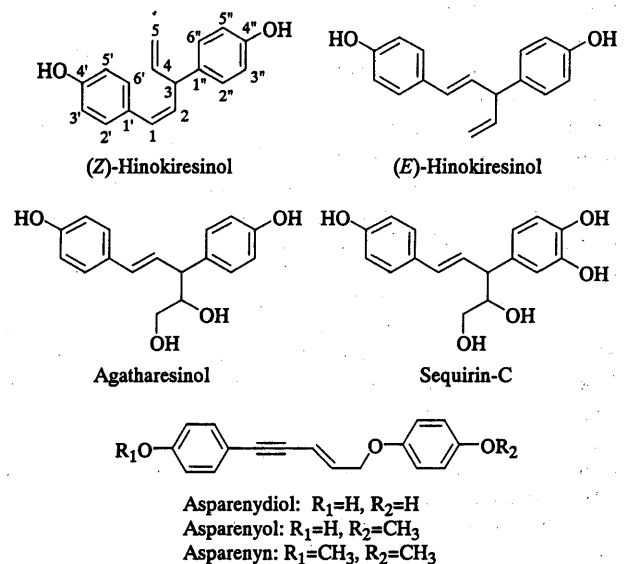

Fig. 5. Chemical structures of norlignans and related compounds.

attention has been paid to regulating the norlignan biosynthesis.

Several hypothetical pathways for norlignan biosyn-

thesis had been proposed based on the chemical structures of norlignans[37,43,55–60]. First, Enzell and Thomas[58] suggested the coupling of two phenylpropane units followed by a loss of one carbon atom giving rise to agatharesinol. Later, a coupling of 4-coumaric acid with 4-coumaryl alcohol that involved the loss of the carbon atom at the 9-position of 4-coumaric acid was proposed independently by Birch and Liepa[59], and Beracierta and Whiting[60]. Erdtman and Harmatha[37] subsequently assumed that C8-C8′ linked lignans formed by the coupling of two phenylpropanoid monomers were converted to norlignans via an intramolecular rearrangement of the side chain of the carbon skeleton. Despite these proposals of coupling modes of two phenylpropane units, none of them were supported by any concrete experimental evidence.

In this chapter, the establishment of norlignan biosynthetic pathway is described. Using a fungal-elicited *Asparagus officinalis* cell system, it has been demonstrated that (Z)-hinokiresinol originates from two non-identical phenylpropanoid monomer : 4-coumaryl alcohol and a 4-coumaroyl compound[61]. Furthermore, the first *in vitro* norlignan formation with an enzyme preparation has been demonstrated[62]. The enzyme preparation from fungal-elicited *A. officinalis* cells catalyzed the formation of (Z)-hinokiresinol from two non-identical phenylpropanoid monomers, 4-coumaryl alcohol and 4-coumaroyl CoA, and from a dimer, 4-coumaryl 4-coumarate, without any additional cofactors. Based on the results of the enzymatic reaction, the novel biosynthetic mechanism for (Z)-hinokiresinol via the ester enolate Claisen rearrangement is proposed.

II-1 Pathway of norlignan biosynthesis

Within the last decade, Takasugi[63] reported that a herbaceous plant, *Asparagus officinalis*, inoculated with a phytopathogen produced (Z)-hinokiresinol (Fig. 5) as a phytoalexin. Terada *et al.*[64] reported that cell cultures of the plant produced norlignan-related C_6-C_5-O-C_6 compounds, asparenydiol and its methylated compounds (asparenyol and asparenyn) (Fig. 5), without any elicitor treatment. Later, they demonstrated that asparenyol was derived from two units of phenylalanine with a loss of one carbon atom at the 9-position of phenylalanine based on[13]C tracer experiments[65], and assumed hinokiresinol as a putative precursor of asparenyol, although without any experimental evidence[66].

Suzuki *et al.*[61] established a *A. officinalis* cell system producing a norlignan, (Z)-hinokiresinol (yield : 0.02% based on dried cell weight). They isolated (Z)-hinokiresinol from MeOH extract of elicitor-treated *A. officinalis* cells and identified it by NMR analysis.

Next, L-[ring-$^{13}C_6$]phenylalanine was administered to the elicitor-treated *A. officinalis* cells, and the β-glucosidase-treated MeOH extract was submitted to GC-MS analysis to examine the incorporation of ^{13}C. Compared with the mass spectrum of unlabelled (Z)-hinokiresinol TMS ether ([M]$^+$ = m/z 396), the enhanced ion peak at m/z 408 ([M]$^+$ + 12) was observed, indicating unequivocally that two aromatic rings of (Z)-hinokiresinol were derived from L-phenylalanine.

Similarly, cinnamic acids labelled with ^{13}C at the side chain were next administered to the *Asparagus* cells
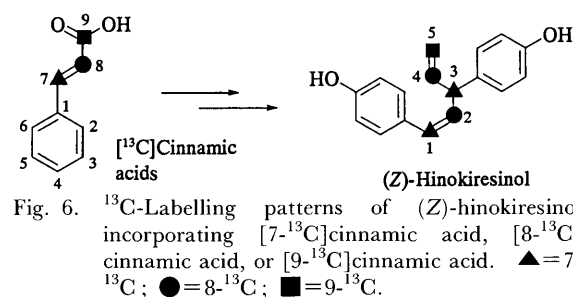

Fig. 6. ^{13}C-Labelling patterns of (Z)-hinokiresinol incorporating [7-^{13}C]cinnamic acid, [8-^{13}C] cinnamic acid, or [9-^{13}C]cinnamic acid. ▲=7-^{13}C ; ●=8-^{13}C ; ■=9-^{13}C.

individually, and the ^{13}C-enriched position in the side chain of (Z)-hinokiresinol was determined by GC-MS and ^{13}C NMR. When [7-^{13}C]cinnamic acid was administered, specific ^{13}C enrichments at C-1 (11.7 atom% excess) and C-3 (10.6 atom% excess) of (Z)-hinokiresinol were observed. Similarly,^{13}C enrichments at C-2 (32.3 atom% excess) and C-4 (31.2 atom% excess) occurred when [8-^{13}C]cinnamic acid was fed. As for the feeding of [9-^{13}C] cinnamic acid, significant ^{13}C enrichment at only C-5 (26.3 atom% excess) was observed. ^{13}C enrichments at other positions were negligible (−0.27–0.56 atom% excess).

These ^{13}C-tracer experiments unequivocally established that all 17 carbon atoms of (Z)-hinokiresinol are derived from phenylpropanoid monomers. Also, it was conclusively demonstrated that the side chain, 7-C, 8-C, and 9-C atoms of cinnamic acid were incorporated into C-1 and C-3, C-2 and C-4, and C-5 of (Z)-hinokiresinol, respectively (Fig. 6). Thus, intramolecular rearrangement of the side chain carbon atoms of the monomers did not occur in (Z)-hinokiresinol formation.

Suzuki *et al.*[61] next demonstrated the immediate precursor(s) in (Z)-hinokiresinol formation. First, they synthesized the following ^{13}C and/or ^{2}H labelled compounds, 4-[ring-$^{13}C_6$]coumaric acid, 4-[9, 9-^{2}H$_2$, ring-$^{13}C_6$]coumaryl alcohol, 4-[7, 9, 9-^{2}H$_3$]coumaryl alcohol, and 4-[9-^{2}H, ring-$^{13}C_6$]coumaraldehyde, and then administered the compounds individually to the elicited *Asparagus* cells.

When 4-[ring-$^{13}C_6$]coumaric acid was fed, GC-MS analysis of the formed (Z)-hinokiresinol showed the significant enhancement of ion peak at m/z 408 ([M]$^+$ + 12), indicating that 4-coumaric acid was on the metabolic pathway leading to (Z)-hinokiresinol. When 4-[9, 9-^{2}H$_2$, ring-$^{13}C_6$]coumaryl alcohol was fed to the cells, great enhancement of ion peak at m/z 410 was observed. This result demonstrated that two units of 4-coumaryl alcohol were converted ultimately to (Z)-hinokiresinol with the loss of the two 9-positioned deuterium atoms from one of the monomers, but did not imply that two units of the alcohol were directly involved in dimerization giving rise to (Z)-hinokiresinol.

Importantly, when 4-[9, 9-^{2}H$_2$, ring-$^{13}C_6$]coumaryl alcohol was administered, enhancement at m/z 404 ([M]$^+$ + 8) was also observed, which was assigned to (Z)-[^{2}H$_2$, $^{13}C_6$]hinokiresinol TMS ether, i.e. the product of coupling of one unit of exogenous 4-[9, 9-^{2}H$_2$, ring-$^{13}C_6$] coumaryl alcohol with an endogenous unlabelled phenylpropane unit. This endogenous precursor-induced dilution effect is rather common in feeding experiments, and, in fact, also occurred in the case of L-[ring-$^{13}C_6$] phenylalanine administration. In addition to the

significant enhancement of the ion peak at m/z 408 ($[M]^{+}+12$), due to the incorporation of two $[^{13}C_6]$ phenylalanine units into (Z)-hinokiresinol, great enhancement was also observed at m/z 402 ($[M]^{+}+6$), and may be ascribed to coupling of one $[^{13}C_6]$phenylalanine unit and one endogenous unlabelled phenylpropane unit. Interestingly, however, the ion peak at m/z 402 ($[M]^{+}+6$, (Z)-$[^{13}C_6]$hinokiresinol TMS ether) after 4-[9, 9-$^2H_2$, ring-$^{13}C_6$]coumaryl alcohol administration was not significant. If one such labelled 4-coumaryl alcohol unit and one endogenous unlabelled 4-coumaryl alcohol unit are directly involved in the dimerization, both $[M]^{+}+8$ and $[M]^{+}+6$ ions must appear with equal intensity. This suggests that two 4-coumaryl alcohol units were not involved directly in coupling, and that the coupling of one 4-coumaryl alcohol unit and another phenylpropane unit which can be formed from 4-coumaryl alcohol.

It is established that the reduction of cinnamaldehyde and cinnamoyl CoA by cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl CoA reductase (CCR), respectively, is reversible[67–69]. Hence, it was hypothesized that some of the exogenously administered 4-[9, 9-$^2H_2$, ring-$^{13}C_6$] coumaryl alcohol were converted to 4-[9-^2H, ring-$^{13}C_6$] coumaraldehyde and 4-[ring-$^{13}C_6$]coumaroyl CoA, which in turn coupled with 4-[9, 9-$^2H_2$, ring-$^{13}C_6$]coumaryl alcohol to afford (Z)-$[^2H_2, ^{13}C_{12}]$hinokiresinol.

To test this hypothesis, the simultaneous administration of two distinct, possible precursors was carried out[61]. Thus, equal molar amounts of 4-[ring-$^{13}C_6$]coumaric acid and 4-[7, 9, 9-$^2H_3$]coumaryl alcohol were administered to elicited cells in a single flask, and the results were compared with those obtained after individual administration of the two precursors as positive controls.

Administration of 4-[7, 9, 9-$^2H_3$]coumaryl alcohol alone resulted in formation of (Z)-$[^2H_4]$hinokiresinol TMS ether ($[M]^{+}+4$) and (Z)-$[^2H_3]$hinokiresinol TMS ether ($[M]^{+}+3$) which corresponded to (Z)-$[^2H_2, ^{13}C_{12}]$ hinokiresinol TMS ether ($[M]^{+}+14$) and (Z)-$[^2H_2, ^{13}C_6]$ hinokiresinol TMS ether ($[M]^{+}+8$), respectively, in the 4-[9, 9-$^2H_2$, ring-$^{13}C_6$]coumaryl alcohol administration. Similarly, administration of only 4-[ring-$^{13}C_6$]coumaric acid resulted in the enhanced ion peaks of $[M]^{+}+12$ {(Z)-$[^{13}C_{12}]$hinokiresinol TMS ether} as already described. In sharp contrast, the simultaneous administration of the two precursors provided no significant evidence in coupling products of two units of 4-[7, 9, 9-$^2H_3$]coumaryl alcohol ($[M]^{+}+4$, (Z)-$[^2H_4]$hinokiresinol TMS ether). In addition, the ion peak at m/z 408 ($[M]^{+}+12$, (Z)- $[^{13}C_{12}]$hinokiresinol TMS ether) showed only a small increase, compared with the unlabelled one. The ion peak at m/z 405 ($[M]^{+}+9$) was prominent, and was derived by coupling one 4-[7, 9, 9-$^2H_3$]coumaryl alcohol unit with one 4-[ring-$^{13}C_6$]coumaric acid unit, confirming our hypothesis that (Z)-hinokiresinol is not formed by the direct dimerization of two units of 4-coumaryl alcohol. Instead, the C_6-C_3 moiety of (Z)-hinokiresinol is derived from 4-coumaryl alcohol unit, while the C_6-C_2 moiety is from a 4-coumaroyl compound (HO–C_6H_5–CH=CH– CO–R) such as 4-coumaric acid, 4-coumaroyl CoA, or 4-coumaraldehyde (Fig. 7).

In conclusion, it has been shown for the first time that all carbon atoms of a norlignan, (Z)-hinokiresinol, are derived
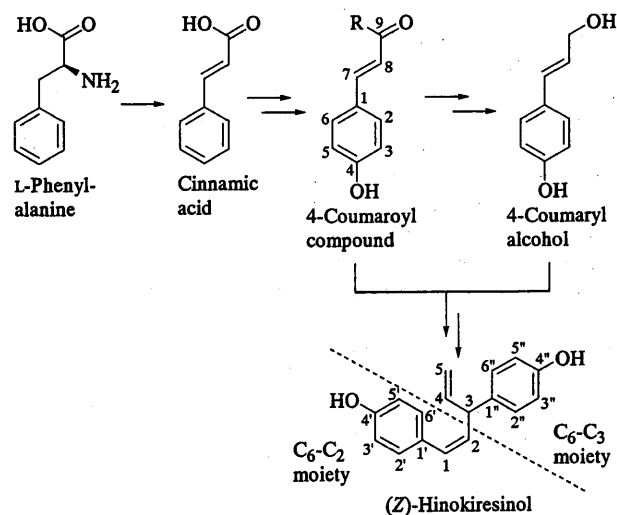

Fig. 7. Proposed biosynthetic pathway for (Z)-hinokiresinol. The C_6-C_3 moiety of (Z)-hinokiresinol was derived from 4-coumaryl alcohol, while the C_6-C_2 moiety was from a 4-coumaroyl compound (4-coumaric acid, R=OH; 4-coumaroyl CoA, R=SCoA; 4-coumaral-dehyde, R=H).

from phenylpropanoid monomers with the loss of one carbon atom at the 9-position of one of the monomers. The C_6-C_3 moiety of (Z)-hinokiresinol is originated from 4-coumaryl alcohol, while the C_6-C_2 moiety is from a 4-coumaroyl compound.

II-2 First enzymatic formation of the norlignan

Suzuki et al.[62] demonstrated in vitro norlignan formation for the first time. Thus, an enzyme preparation from fungal-elicited *Asparagus officinalis* cultured cells catalyzed the formation of a norlignan, (Z)-hinokiresinol, from two non-identical phenylpropanoid monomers, 4-coumaryl alcohol and 4-coumaroyl CoA, and from a dimer, 4-coumaryl 4-coumarate, without any additional cofactors.

Proof that the formation of (Z)-hinokiresinol was enzymatic was obtained by control experiments; the formation of (Z)-$[^2H_3]$hinokiresinol from 4-[7, 9, 9-$^2H_3$] coumaryl alcohol and 4-coumaroyl CoA did not occur when the denatured enzyme preparation was used, and barely occurred when the enzyme preparation or the substrate(s) were omitted from the complete assay. On the other hand, incubation of 4-[7, 9, 9-$^2H_3$]coumaryl alcohol and unlabelled 4-coumarate with the enzyme preparation did not afford (Z)-$[^2H_3]$hinokiresinol, eliminating the mechanism that 4-coumaroyl CoA was first hydrolyzed to 4-coumarate, which coupled with 4-coumaryl alcohol to afford (Z)-hinokiresinol. These results demonstrate for the first time a norlignan synthase activity.

Since esters are often biosynthesised by acyltransferase-catalyzed condensation between the corresponding CoA esters and alcohol[70], Suzuki et al.[62] next hypothesized that (Z)-hinokiresinol was formed via the coupling of 4-coumaryl alcohol and 4-coumaroyl CoA to afford 4-coumaroyl 4-coumarate followed by C7-C8' bond formation and C9' decarboxylation (Fig. 8). To test this hypothesis, 4-[7, 9, 9-$^2H_3$]coumaryl 4-coumarate was
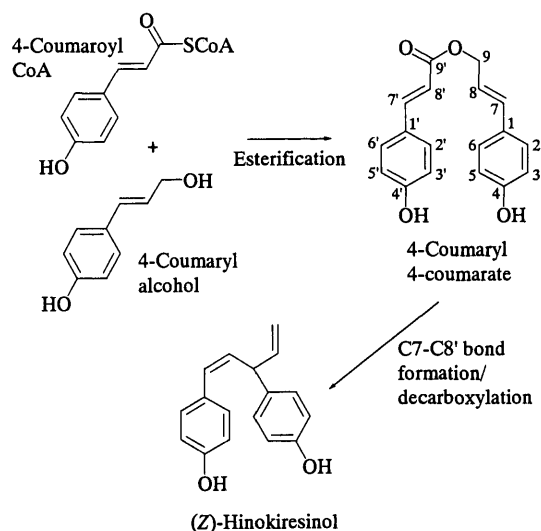
Fig. 8. A putative mechanism for the formation of (Z)-hinokiresinol with *A. officinalis* enzyme preparation.

administered to the fungal-elicited *A. officinalis* cells. 4-Coumaryl 4-coumarate is found to be efficiently converted to (Z)-hinokiresinol.

Furthermore, the conversion of 4-coumaryl 4-coumarate to (Z)-hinokiresinol was also demonstrated by an *in vitro* experiment[62]; incubation of 4-[7, 9, 9-^{2}H$_3$]coumaryl 4-coumarate with the enzyme preparation resulted in enzymatic (Z)-[^{2}H$_3$]hinokiresinol formation in a high yield. These *in vivo* and *in vitro* experiments strongly suggest that 4-coumaryl 4-coumarate is the intermediate between the phenylpropanoid monomers (4-coumaryl alcohol and 4-coumaroyl CoA) and (Z)-hinokiresinol (Fig. 8).

Incubation of 4-coumaryl 4-coumarate with horseradish peroxidase in the presence of H_2O_2 did not afford (Z)-hinokiresinol, indicating the peroxidase does not initiate the C7-C8′ bond formation and ultimate decarboxylation. This is in sharp contrast to C8-C8′ bond formation between hydroxycinnamyl alcohol units in lignan and lignin biosynthesis which is mediated by peroxidase or laccase[71]. Instead, 4-coumaryl 4-coumarate might be converted to (Z)-hinokiresinol via the ester enolate Claisen rearrangement (the [3, 3]-sigmatropic rearrangement of allyl esters to γ, δ-unsaturated carboxylic acids)[72] followed by decarboxylation. However, the true mechanism awaits further experiments with purified enzymes.

In conclusion, the present study has demonstrated for the first time the enzymatic formation of (Z)-hinokiresinol from 4-coumaryl alcohol and 4-coumaroyl CoA, and from 4-coumaryl 4-coumarate.

Conclusions

The enzyme preparations derived from *Arctium lappa* petioles and seeds respectively showed pinoresinol/lariciresinol reductase (PLR) activity, but their selectivity were different in terms of substrate enantiomers. On the other hand, *Anthriscus sylvestris* PLR enzyme preparation revealed the similar selectivity to that of *A. lappa* seed

enzyme. These results suggest that the great diversity in enantiomeric compositions of lignans among plant species are at least partly due to the differential expression of PLR isozymes which have distinct stereochemical selectivity.

The biosynthetic pathway for a norlignan, (Z)-hinokiresinol, was proposed. Thus, the coupling of 4-coumaryl alcohol with 4-coumaroyl CoA afforded (Z)-hinokiresinol. In addition, the enzyme activity forming (Z)-hinokiresinol from 4-coumaryl alcohol and 4-coumaroyl CoA was detected. In this reaction process, 4-coumaryl 4-coumarate is probably an intermediate compound, and in fact, the compound was transformed to (Z)-hinokiresinol enzymatically. These results strongly suggest the following mechanism for (Z)-hinokiresinol biosynthesis: (i) 4-coumaryl alcohol couples with 4-coumaroyl CoA to afford 4-coumaryl 4-coumarate. (ii) 4-Coumaryl 4-coumarate are transformed to (Z)-hinokiresinol via the ester enolate Claisen rearrangement and subsequent decarboxylation.

References

1) Y. IIDA, K.-B. OH, M. SAITO, H. MATSUOKA and H. KURATA : *Planta Med.*, **66**, 435–438 (2000).
2) T. YAMADA : *Bull. For. & For. Prod. Res. Inst.*, 69–162 (1998).
3) H.A. OKETCH-RABAH, S.F. DOSSAJI, S.B. CHRISTENSEN, K. FRYDENVANG, E. LEMMICH, C. CORNETT, C.E. OLSEN, M. CHEN, A. KHARAZMI and T. THEANDER : *J. Nat. Prod.*, **60**, 1017–1022 (1997).
4) E. MINAMI, M. TAKI, S. TAKAISHI, Y. IIJIMA, S. TSUTSUMI and T. AKIYAMA : *Chem. Pharm. Bull.*, **48**, 389–392 (2000).
5) C. CANEL, R.M. MORAES, F.E. DAYAN and D. FERREIRA : *Phytochemistry*, **54**, 115–120 (2000).
6) T. UMEZAWA, L.B. DAVIN and N.G. LEWIS : *J. Biol. Chem.*, **266**, 10210–10217 (1991).
7) T. UMEZAWA and M. SHIMADA : *Mokuzai Gakkaishi*, **42**, 180–185 (1996).
8) T. UMEZAWA, H. KURODA, T. ISOHATA, T. HIGUCHI and M. SHIMADA : *Biosci. Biotechnol. Biochem.*, **58**, 230–234 (1994).
9) T. OKUNISHI, T. UMEZAWA and M. SHIMADA : *J. Wood Sci.*, **46**, 234–242 (2000).
10) T. UMEZAWA and M. SHIMADA : *Biosci. Biotechnol. Biochem.*, **60**, 736–737 (1996).
11) T. UMEZAWA, L.B. DAVIN and N.G. LEWIS : *Biochem. Biophys. Res. Commun.*, **171**, 1008–1014 (1990).
12) S. OZAWA, L.B. DAVIN and N.G. LEWIS : *Phytochemistry*, **32**, 643–652 (1993).
13) L.B. DAVIN, H.B. WANG, A.L. CROWELL, D.L. BEDGAR, D.M. MARTIN, S. SARKANEN and N.G. LEWIS : *Science*, **275**, 362–366 (1997).
14) A.T. DINKOVA-KOSTOVA, D.R. GANG, L.B. DAVIN, D.L. BEDGAR, A. CHU and N.G. LEWIS : *J. Biol. Chem.*, **271**, 29473–29482 (1996).
15) Z.-Q. XIA, M.A. COSTA, H.C. PELISSIER, L.B. DAVIN and N.G. LEWIS : *J. Biol. Chem.*, **276**, 12614-12623 (2001).
16) S. SUZUKI, T. UMEZAWA and M. SHIMADA : *Biosci. Biotechnol. Biochem.*, **62**, 1468–1470 (1998).
17) S. SUZUKI, T. UMEZAWA and M. SHIMADA : *Biosci. Biotechnol.*

Biochem., **66**, 1262–1269 (2002).

18) S. SUZUKI, N. SAKAKIBARA, T. UMEZAWA and M. SHIMADA : *J. Wood Sci.* (2002), in press.

19) J. SHINODA and M. KAWAGOYE : *Yakugaku Zasshi*, **49**, 565–575 (1929).

20) R. HÄNSEL, H. SHULTZ and C. LEUCKERT : *Z. Naturforsch.*, **19**, 727–734 (1964).

21) S. YAMANOUCHI, M. TAKIDO, U. SANKAWA and S. SHIBATA : *Yakugaku Zasshi*, **96**, 1492–1493 (1976).

22) B.H. HAN, Y.H. KANG, H.O. YANG and M.K. PARK : *Phytochemistry*, **37**, 1161–1163 (1994).

23) L.D. YAKHONTOVA and P.N. KIBALCHICH : *Khim. Prir. Soedin.*, **7**, 299–301 (1971).

24) T. UMEZAWA, T. OKUNISHI and M. SHIMADA : *Wood Res.*, **84**, 62–75 (1997).

25) T. KATAYAMA, T. MASAOKA and H. YAMADA : *Mokuzai Gakkaishi*, **43**, 580–588 (1997).

26) T. OKUNISHI, T. UMEZAWA and M. SHIMADA : *J. Wood Sci.*, **47**, 383–388 (2001).

27) A. CHU, A. DINKOVA, L.B. DAVIN, D.L. BEDGAR and N.G. LEWIS : *J. Biol. Chem.*, **268**, 27026–27033 (1993).

28) M. FUJITA, D.R. GANG, L.B. DAVIN and N.G. LEWIS : *J. Biol. Chem.*, **274**, 618–627 (1999).

29) D.R. GANG, M.A. COSTA, M. FUJITA, K.A.T. DINKOVA, H.B. WANG, V. BURLAT, W. MARTIN, S. SARKANEN, L.B. DAVIN and N.G. LEWIS : *Chem. Biol.*, **6**, 143–151 (1999).

30) Y.-H. LIM, M.-J. LEEM, D.-H. SHIN, H.-B. CHANG, S.-W. HONG, E.-Y. MOON, D.-K. LEE, S.-J. YOON and W.-S. WOO : *Arc. Pharm. Res.*, **22**, 208–212 (1999).

31) M. KOZAWA, N. MORITA and K. HATA : *Yakugaku Zasshi*, **98**, 1486–1490 (1978).

32) T. NOGUCHI and M. KAWANAMI : *Yakugaku Zasshi*, **60**, 629–636 (1940).

33) T. KURIHATA, M. KIKUCHI, S. SUZUKI and S. HISAMICHI : *Yakugaku Zasshi*, **98**, 1586–1591 (1978).

34) R. IKEDA, T. NAGAO, H. OKABE, Y. NAKANO, H. MATSUNAGA, M. KATANO and M. MORI : *Chem. Pharm. Bull.*, **46**, 875–878 (1998).

35) R. IKEDA, T. NAGAO, H. OKABE, Y. NAKANO, H. MATSUNAGA, M. KATANO and M. MORI : *Chem. Pharm. Bull.*, **46**, 871–874 (1998).

36) D. KOH and Y. LIM : *Agric. Chem. Biotechnol.*, **42**, 208–209 (1999).

37) H. ERDTMAN and J. HARMATHA : *Phytochemistry*, **18**, 1495–1500 (1979).

38) N. TAKAKU, D.-H. CHOI, K. MIKAME, T. OKUNISHI, S. SUZUKI, H. OHASHI, T. UMEZAWA and M. SHIMADA : *J. Wood Sci.*, **47**, 476–482 (2001).

39) D.G. TURABELIDZE, G.A. MIKAYA, P. KEMERTELIDZE and N.S. VUL'SON : *Sov. J. Bioorg. Chem.*, **8**, 374–379 (1982).

40) A. PELTER : *J. Chem. Soc. (c)*, 74–79 (1968).

41) T. KATAYAMA, L.B. DAVIN, A. CHU and N.G. LEWIS : *Phytochemistry*, **33**, 581–591 (1993).

42) T. KATAYAMA, L.B. DAVIN and N.G. LEWIS : *Phytochemistry*, **31**, 3875–3881 (1992).

43) D.A. WHITING : *Nat. Prod. Rep.*, 499–525 (1987).

44) T. UMEZAWA : "Wood and cellulosic chemistry, 2nd ed."
(Hon, D.N.-S. and Shiraishi, N., eds.), Marcel Dekker Inc., New York, 213–241 (2000).

45) D.A. WHITING : *Nat. Prod. Rep.*, **7**, 349–364 (1990).

46) R.S. WARD : *Nat. Prod. Rep.*, **16**, 75–96 (1999).

47) J. KINJO, J. FURUSAWA and T. NOHARA : *Tetrahedron Lett.*, **26**, 6101–6102 (1985).

48) Y. SHIRATAKI, Y. TAGAYA, I. YOKOE and M. KOMATSU : *Chem. Pharm. Bull.*, **35**, 1637–1640 (1987).

49) T. NOHARA, J. KINJO, J. FURUSAWA, Y. SAKAI, M. INOUE, Y. SHIRATAKI, Y. ISHIBASHI, I. YOKOE and M. KOMATSU : *Phytochemistry*, **33**, 1207–1210 (1993).

50) W.-Y. TSUI and G.D. BROWN : *Phytochemistry*, **43**, 1413–1415 (1996).

51) T. NIKAIDO, T. OHMOTO, H. NOGUCHI, T. KINOSHITA, H. SAITOH and U. SANKAWA : *Planta Med.*, **43**, 18–23 (1981).

52) Y. KAI, H. KURODA and F. TERATANI : *Mokuzai Gakkaishi*, **18**, 315 (1972).

53) K. TAKAHASHI : *Mokuzai Gakkaishi*, **27**, 654–657 (1981).

54) H. OHASHI, H. HAYASHI, M. YAMADA and M. YASUE : *Res. Bull. Fac. Agr. Gifu Univ.*, **52**, 131–139 (1987).

55) M. NICOLETTI, C. GALEFfi, I. MESSANA and G.B. MARINI-BETTOLO : *J. Ethnopharmacology*, **36**, 95–101 (1992).

56) M.J. BEGLEY, R.V. DAVIES, P. HENLEY-SMITH and D.A. WHITING : *J. Chem. Soc., Chem. Commun.*, 649–650 (1973).

57) A. ENOKI, S. TAKAHAMA and K. KITAO : *Mokuzai Gakkaishi*, **23**, 579–586 (1977).

58) C.R. ENZELL and B.R. THOMAS : *Tetrahedron Lett.*, 2395–2402 (1966).

59) A.J. BIRCH and A.J. LIEPA : "Chemistry of lignans" (Rao, C.B.S., ed.), Andhra University Press, Andhra Pradesh, 307–327 (1978).

60) A.P. BERACIERTA and D.A. WHITING : *J. Chem. Soc., Perkin Trans. 1*, 1257–1263 (1978).

61) S. SUZUKI, T. UMEZAWA and M. SHIMADA : *J. Chem. Soc., Perkin Trans. 1*, 3252–3257 (2001).

62) S. SUZUKI, T. NAKATSUBO, T. UMEZAWA and M. SHIMADA : *J. Chem. Soc., Chem. Commun.*, 1088–1089 (2002).

63) M. TAKASUGI : *Kagaku To Seibutsu*, **31**, 22–29 (1993).

64) K. TERADA, C. HONDA, K. SUWA, S. TAKEYAMA, H. OKU and W. KAMISAKO : *Chem. Pharm. Bull.*, **43**, 564–566 (1995).

65) K. TERADA, K. SUWA, S. TAKEYAMA, C. HONDA and W. KAMISAKO : *Biol. Pharm. Bull.*, **19**, 748–751 (1996).

66) K. TERADA and W. KAMISAKO : *Biol. Pharm. Bull.*, **22**, 561–566 (1999).

67) G.G. GROSS, J. STÖCKIGT, R.L. MANSELL and M.H. ZENK : *FEBS Lett.*, **31**, 283–286 (1973).

68) T. LÜDEWITZ and H. GRISEBACH : *Eur. J. Biochem.*, **119**, 115–124 (1981).

69) F. SARNI, C. GRAND and A.M. BOUDET : *Eur. J. Biochem.*, **139**, 259–265 (1984).

70) M.J.C. RHODES and L.S.C. WOOLTORTON : *Phytochemistry*, **15**, 947–951 (1976).

71) T. UMEZAWA : *Regul. Plant Growth Dev.*, **36**, 57–67 (2001).

72) F.A. CAREY and R.J. SUNDBERG : "Advanced organic chemistry, 2nd edition", Plenum Press, New York, 341 (1983).