Stereochemical Diversity in Lignan Biosynthesis and Establishment of Norlignan Biosynthetic Pathway

Shiro Suzuki

2002

STEREOCHEMICAL DIVERSITY IN LIGNAN BIOSYNTHESIS

AND

ESTABLISHMENT OF NORLIGNAN BIOSYNTHETIC PATHWAY

Shiro Suzuki

2002

Contents

Introduction

Lignans and norlignans are two major classes of wood extractives. The word "lignan" was introduced by Haworth [1], and he defined lignans as 8,8'-linked phenylpropanoid dimers (Fig. 1). Later, the word "lignan" was redefined by some researchers, which resulted in confusion of the definition [2]. However, the original definition by Haworth is usually used to refer to such compounds. The word "norlignan" was first used as "nor-lignan" for diphenylpentane compounds, sugiresinol and hydroxysugiresinol (Fig. 4), by Kai [3], because the prefix "nor" is used to indicate a compound which is regarded as derived from a parent compound by a loss of one carbon atom. Later, Hatam and Whiting described that sequirins (Fig. 4) belong to the non-lignan group [4]. On the other hand, Erdtman and Harmatha proposed the word "conioid" [5]. However, the word "norlignan" is now usually used for expressing the diphenylpentane compounds. Typical carbon framework structure of norlignan is 7,8'-binding (Fig. 1).

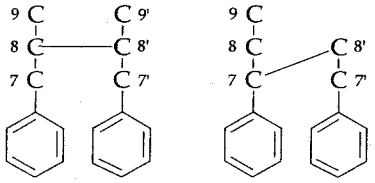

Fig. 1 Typical carbon frameworks of lignan and norlignan.

Lignans and norlignans have various important features. First, it has been well-known that both lignans and norlignans have various biological activities. For example, various lignans are antitumor, antiviral, and anti-oxidant or so forth. On the other hand, norlignans have biological activities such as antifungal [6,7], antiprotozoal [8] and estrogen-like activities [9]. Among them, an antitumor lignan, podophyllotoxin, is of special interest, because it is commercially important as a staring material of etoposide and teniposide, which are being used as anticancer

drugs in the hospital. The lignan has been supplied from a herbaceous perennial *Podophyllum hexandrum* growing in nature. However, the large-scaled exploitation is decreasing the amount of its natural resources [10]. 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.

Second feature of lignans and norlignans is their specific accumulation in heartwood of conifers. Heartwood, which is the colored tissue in inner trunks of trees, is composed of only dead cells and supports the trunk physically. Cambial initials are divided into phloem and xylem mother cells, and in turn xylem mother cells are differentiated into vessel elements, fibers, tracheids, and parenchyma cells within several weeks after cell division [11]. Lignification occurs strongly in vessel elements, fibers, and tracheids followed by their death, whereas parenchyma cells continue to live for a few years (*Robinia pseudoacacia* [12]) to 15-25 years (*Cryptomeria japonica* [13]). Prior to the death of parenchyma cells, secondary metabolites "heartwood substances" such as lignans, norlignans, and flavonoids are produced in these cells and spread out into other xylem elements, followed by the death of the cells resulting in the completion of heartwood formation. For example, Nobuchi et al. reported that the amounts of norlignans, hydroxysugiresinol and sequirin-C (Fig. 4), increased significantly in intermediate wood (the area between sapwood and heartwood) in *C. japonica* [13], and Takaku et al. demonstrated that a lignan, hinokinin, and a norlignan, (*E*)-hinokiresinol, distributed specifically in the heartwood [14]. These results strongly suggest that the biosynthesis of these heartwood substances is involved in the formation of heartwood. Importantly, the heartwood formation does not occur in herbaceous plants; the metabolic event is specific to woody plants but not to herbaceous plants. Heartwood formation probably plays an important role in preventing heartwood-rot by producing antifungal heartwood substances including lignans and norlignans. From an environmental point of view, such heartwood substances may contribute to retardation of CO_2 release from woody lignocellulosics

into the atmosphere.

Besides the antifungal activity, these heartwood substances like lignans and norlignans have marked effects on the physical and chemical properties of wood, such as acoustic properties, impregnation of preservatives, origin of the fragrance and color of wood [15]. Heartwood coloration of *C. japonica* (Japanese cedar) [16,17] and *Chamaecyparis obtusa* (hinoki cypress) [18], which account for 31 and 40 % of total artificial afforested area in Japan, respectively [19], is due to norlignans, while that of *Thuja plicata* (western red cedar) is attributed to the polymerization of lignans such as plicatic acid [20]. The normal heartwood coloration of *C. japonica* and *C. obtusa* is beautiful salmon pink, which is highly appreciated in Japan. However, black-discoloration often occurs in *C. japonica* heartwood, which lowers the value of the discolored wood.

Thus, heartwood and its formation are of special interest in both basic plant bioscience and the wood industry. However, little is known about the biochemical mechanisms of the formation. This is partly due to the recalcitrance of woody plants for biochemical studies. Fortunately, since heartwood formation is accompanied by the biosynthesis of lignans, norlignans, stilbenes, and/or flavonoids, elucidation of the molecular mechanisms involved in biosynthesis of these heartwood substances would be a clue to access heartwood formation mechanisms.

Third, stereochemistry of lignans is peculiar in two aspects. One is the stereochemical difference between lignins and lignans. They fundamentally differ in optical activity, although they are closely related in their chemical structures; lignans are optically active, whereas lignins are inactive. Therefore, lignan biosynthesis involves enantioselective process(es), which sharply contrasts with the non-enantioselective process of lignin polymerization. Hence, elucidation of difference in the stereochemical mechanisms is of great importance.

The other stereochemical feature of lignans is the stereochemical difference between lignans. In general, lignan molecules are chiral, and one enantiomer

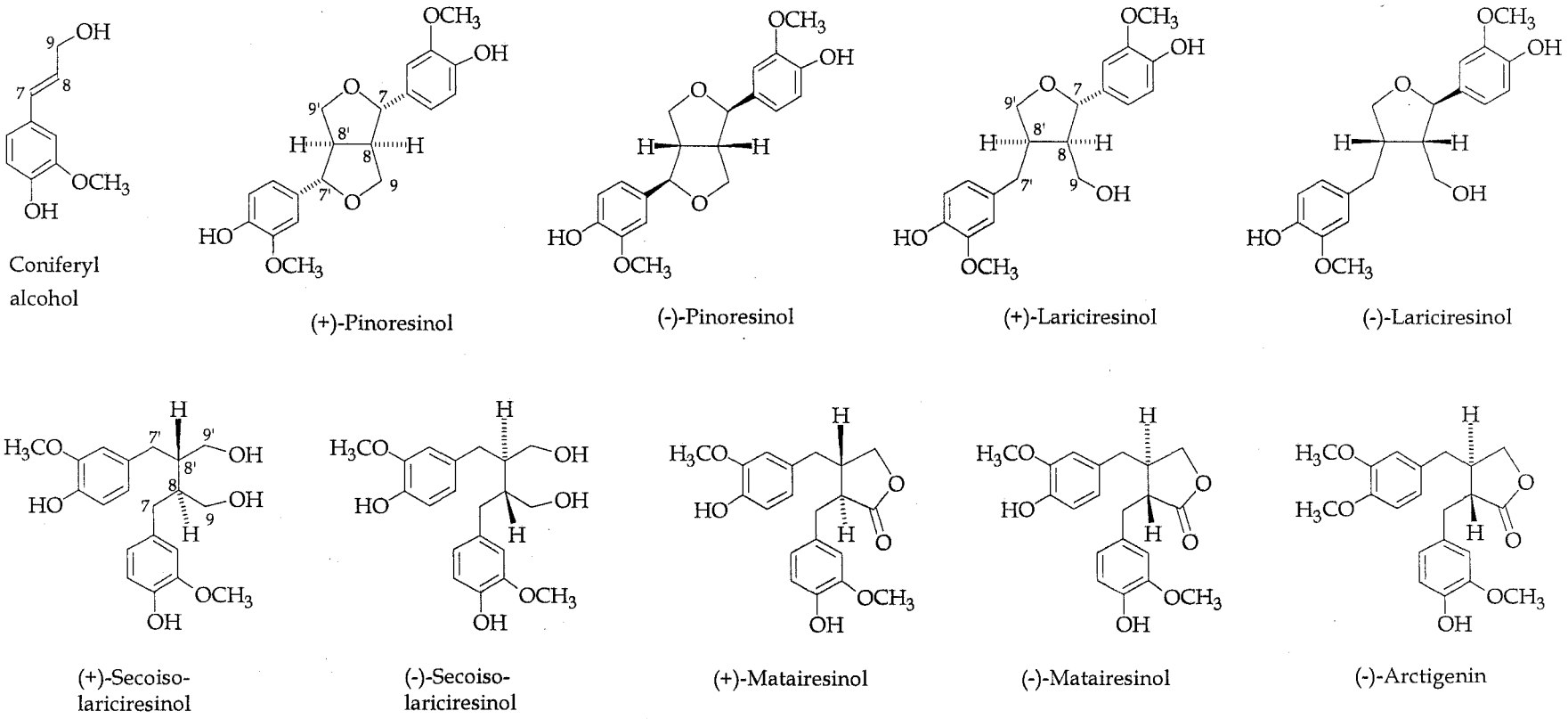

Fig. 2 Chemical structures of lignans and coniferyl alcohol.

predominates or only one enantiomer is present in each lignan sample isolated from plants. Interestingly, however, the predominant enantiomer can differ with the plant sources. For example, optically pure, levorotatory (-)-matairesinol (Fig. 2) was isolated from *Forsythia intermedia* [21], while the optically pure, dextrorotatory (+)-matairesinol (Fig. 2) was isolated from *Wikstroemia sikokiana* [22]. (-)-Secoisolariciresinol (Fig. 2) from *F. intermedia* [21] and *F. koreana* [63] is optically pure, whereas (-)-secoisolariciresinol isolated from *W. sikokiana* is not optically pure [45% enantiomer excess (e.e.)]. Furthermore, (+)-secoisolariciresinol (78% e.e., Fig. 2) was isolated from *Arctium lappa* petioles [23]. Thus, stereochemistry of lignan biosynthesis varies with the plant species, and elucidation of the stereochemical diversity in lignan biosynthesis is of special interest.

Because of these important features, biosynthesis of lignans and norlignans has been receiving widespread interest. In what follows, studies of lignan and norlignan biosynthesis which had been published before the author started the present work will be outlined.

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 [24]. They also demonstrated that the selective oxidation of (-)-secoisolariciresinol to optically pure (-)-matairesinol in the presence of NAD(P) [21].

Lewis and coworkers continued to investigate stereochemical mechanisms of secoisolariciresinol and matairesinol formation in *Forsythia*. First, Katayama et al. demonstrated that an extraordinary accumulation of (-)-pinoresinol in the assay mixture of secoisolariciresinol formation from coniferyl alcohol [25]. Later, they demonstrated that *F. intermedia* cell-free extracts catalyzed the selective reduction of (+)-pinoresinol to (-)-secoisolariciresinol via (+)-lariciresinol [26]. Then, Lewis and coworkers demonstrated that *pro*-R hydrogen of NADPH was transferred to the

pro-R position at C7 (and/or C7') of lariciresinol and secoisolariciresinol [27].

As for pinoresinol biosynthesis, Davin et al. reported the "insoluble residue" of *Forsythia suspensa* obtained after removal of soluble enzyme preparation could catalyze the enantioselective coupling of two molecules of coniferyl alcohol to afford the naturally occurring (+)-enantiomer of pinoresinol without addition of exogenously supplied cofactors [28]. Then, Paré et al. succeeded in solubilization of the (+)-pinoresinol synthase activity from the cell wall residue of *F. intermedia* and concluded that this was an enantioselective phenylpropanoid coupling oxidase in lignan formation with high enantioselectivity (>97% e.e.) in the (+)-pinoresinol formation from coniferyl alcohol [29]. This enzymatic conversion required O_2 as a cofactor. However, the purification of the peculiar oxidase was unsuccessful, and finally the enantioselective phenoxy radical coupling was found to be effected by a non-enantioselective laccase together with a 78-kDa protein without a catalytically active (oxidative) center [30]. They postulated that the role of the protein is to capture coniferyl alcohol-derived free-radical intermediates produced by a one-electron oxidant such as laccase, and to lead to the enantioselective coupling. They coined the word as "dirigent protein" (from a Latin verb, *dirigere*: to guide or align) for the protein [30].

Later, an enzyme (pinoresinol/lariciresinol reductase) catalyzing the reduction of (+)-pinoresinol to (-)-secoisolariciresinol was purified by Dinkova-Kostova et al [31]. They also reported its cDNA cloning and functional expression in *E. coli*. The enzyme had two isoforms and both (+)-pinoresinol and (+)-lariciresinol served as substrates [31]. The amino acid sequence of the enzyme exhibited a strong homology to isofravone reductase.

Very recently, cDNA cloning and functional expression of the enzyme catalyzing the formation of optically pure (-)-matairesinol from (-)-secoisolariciresinol named as (-)-secoisolarisiresinol dehydrogenase was reported [32].

O-Methylation of matairesinol (Fig. 3) was investigated with cell-free extracts of *F. intermedia* by Ozawa et al [33]. The conversion of matairesinol to arctigenin by the enzyme preparation was less selective; both (+)- and (-)-enantiomers of matairesinol served as substrates for methylation to give arctigenin, with the naturally occurring antipode slightly preferred. In addition, the enzyme preparation also catalyzed the formation of (+)- and (-)-isoarctigenins from the corresponding matairesinol enantiomers. However, because neither (+)- nor (-)-isoarctigenin was detected in *F. intermedia*, they proposed the mechanism of arctigenin formation in the plant as follows: the regioselective glycosylation of (-)-matairesinol affords matairesinoside, and the subsequent methylation followed by deglycosylation gave (-)-arctigenin.

As a whole, the conversion of coniferyl alcohol to the natural enantiomers of the *Forsythia* lignans, (+)-pinoresinol, (+)-lariciresinol, (-)-secoisolariciresinol, (-)-matairesinol, and (-)-arctigenin was, in large part, established (Fig. 3). Each step, except for the final methylation, is well controlled in terms of stereochemistry; (+)-pinoresinol is formed enantioselectively from achiral coniferyl alcohol with oxidase/oxidant in the presence of dirigent protein. The formed (+)-pinoresinol is transformed to (+)-lariciresinol and (-)-secoisolariciresinol with pinoresinol/lariciresinol reductase in the presence of NADPH, and (-)-secoisolariciresinol was in turn oxidized to (-)-matairesinol with secoisolariciresinol dehydorogenase in the presence of NADP.

However, little was known about the stereochemical diversity of lignan biosynthesis among different plant species, except for *Arctium lappa*. Thus, in contrast to cell-free extracts from *Forsythia* plants [24], Umezawa and Shimada isolated (+)-secoisolariciresinol (78% e.e.) from *Arctium 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 [23]. The result indicated that *A. lappa* has a different stereochemical control in lignan biosynthesis from that of *Forsythia* plants.

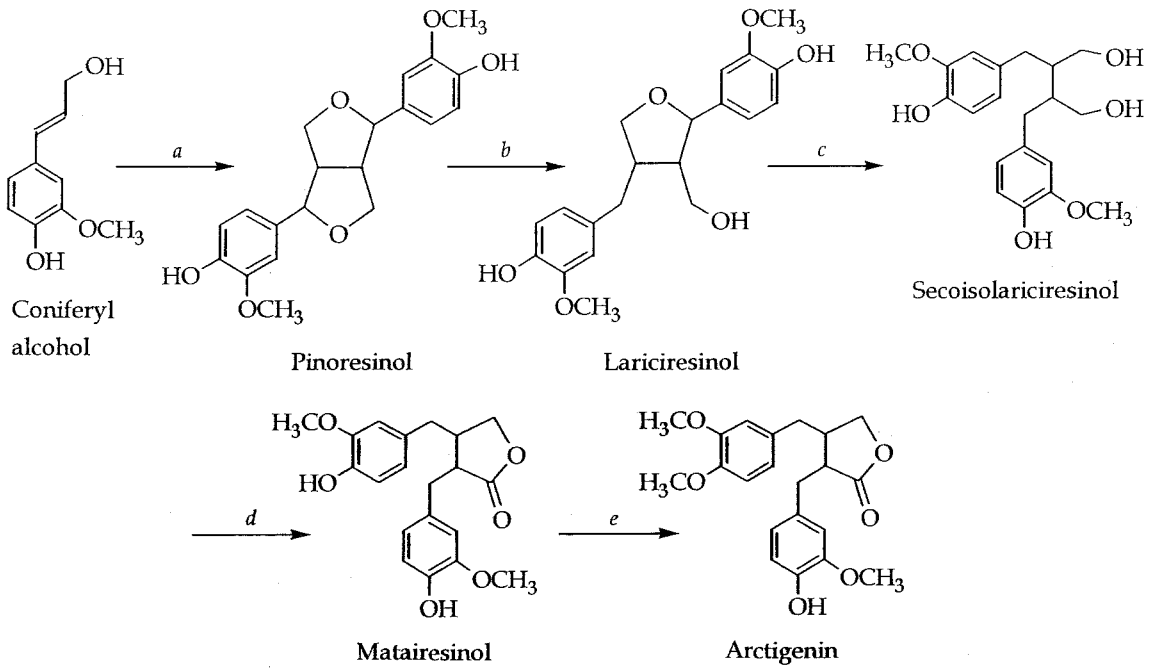

Fig. 3 Biosynthetic pathway of lignans from coniferyl alcohol.

As the continuation of the study [23], the present author tried to elucidate the mechanism of the stereochemical diversity in lignan biosynthesis using *A. lappa* as a plant material.

First, in Part A of Chapter 1, the author describes that (-)-secoisolariciresinol was isolated from *Arctium lappa* seeds and was formed enantioselectively from coniferyl alcohol with the enzyme preparation of the ripening seeds, in contrast to (+)-secoisolariciresinol formation with the petiole enzyme preparation [23]. Subsequently, the more detailed mechanism for this conversion was investigated with the seed and petiole enzyme preparations. Second, in Part B of Chapter 1, the lignan synthesis with enzyme preparations from another plant species, *Anthriscus sylvestris*, was studied. *A. sylvestris* is a good source of deoxypodophyllotoxin and yatein, both of which are precursors of podophyllotoxin in *Podophyllum* spp [85,86].

In addition, yatein is a typical heartwood lignan in conifers [5]. Hence, the knowledge of lignan biosynthesis in this species can be applied to both biotechnological production of podophyllotoxin and studies of heartwood lignan formation.

As for norlignan biosynthesis, several hypothetical biosynthetic pathways had been proposed based on the chemical structures of norlignans [5,35-41,108] before the author's study. First, Enzell and Thomas [39] 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 [40], and Beracierta and Whiting [41].

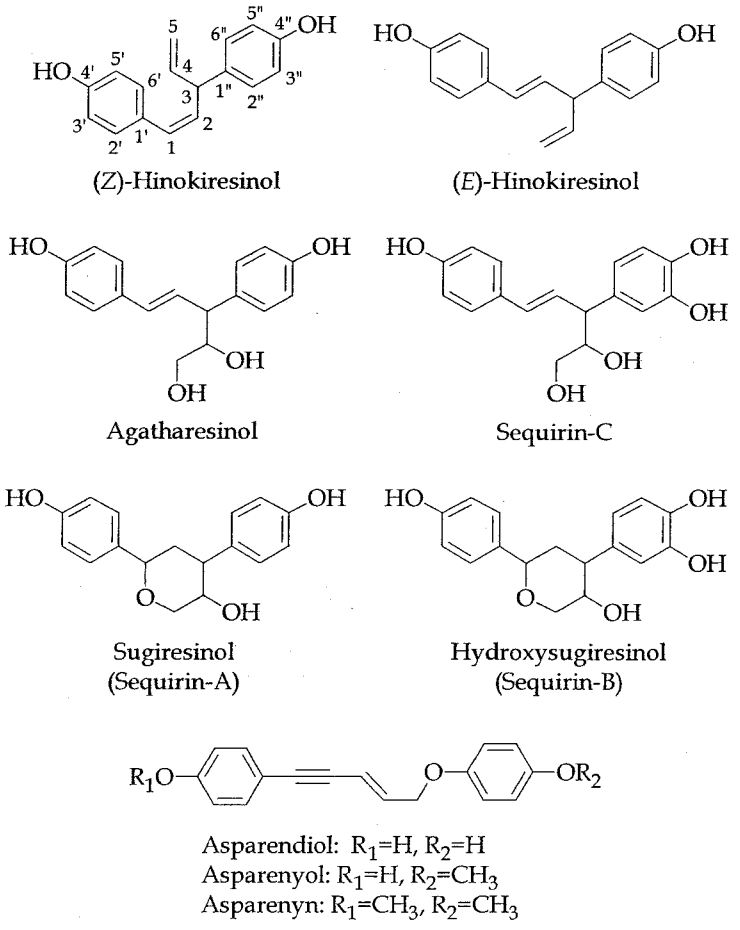

Fig. 4 Chemical structures of norlignans and related compounds.

Erdtman and Harmatha [5] subsequently assumed that C8-C8' linked lignans formed by the coupling of two phenylpropanoid monomers were converted to norlignans via 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. Within the last decade, Takasugi reported that a herbaceous plant, *Asparagus officinalis*, inoculated with a phytopathogen produced (Z)-hinokiresinol (Fig. 4) as a phytoalexin [42]. Terada et al. 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. 4), without any elicitor treatment [43]. 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 [44], and assumed hinokiresinol as a putative precursor of asparenyol, although without any experimental evidence [45].

These reports [42-46] stimulated the author to examine whether fungal treatment of *Asparagus* cell culture would elicit norlignan production, and to seek the possibility of using this system as a model to elucidate norlignan biosynthesis. The author established (Z)-hinokiresinol production system by fungal-elicited *A. officinalis* cells, and using this system, demonstrated that (Z)-hinokiresinol originates from two non-identical phenylpropanoid monomer: 4-coumaryl alcohol and a 4-coumaroyl compound in Part A of Chapter 2. In Part B of Chapter 2, the first *in vitro* norlignan formation with an enzyme preparation is described. 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 ester enolate Claisen rearrangement is proposed.

Chapter 1

Stereochemical diversity in lignan biosynthesis

Part A Stereochemical diversity in lignan biosynthesis of *Arctium lappa* L.

1.A.1 Introduction

During the last decade significant advances have been made in the field of lignan biosynthesis [2,47]. The following conversions by *Forsythia* enzymes have been demonstrated: coniferyl alcohol → pinoresinol (furofuran lignan) → lariciresinol (furan lignan) → secoisolariciresinol (dibenzylbutane lignan) → matairesinol (dibenzylbutyrolactone lignan) (Fig. 3) [21,24,27,28,30,31,34,48].

These studies with *Forsythia* plants revealed that the naturally occurring enantiomers of *Forsythia* lignans were formed by *Forsythia* enzymes and each enzymatic conversion was regulated in terms of stereochemistry. Thus, the entrance of lignan biosynthetic pathway following cinnamate pathway is already well-controlled stereochemically.

On the other hand, there are many examples of plants which produce the opposite enantiomers to those occurring in *Forsythia* spp [2,22,47,49-53]. In addition, phytochemical studies of lignans such as pinoresinol, lariciresinol, and secoisolariciresinol, which are the most upstream on the biosynthetic pathways, revealed that not only the sign of optical rotation, i.e. predominant enantiomers of the lignans, but also enantiomeric compositions, % enantiomer excess (e.e.) values, can vary drastically with plant species [47,50]. Furthermore, there have been no examples of optically pure pinoresinol and lariciresinol. In contrast, all dibenzylbutyrolactone lignans including matairesinol which follow pinoresinol and

lariciresinol in the biosynthetic pathway have been found to be optically pure [2,47,50]. These results suggest that not only the entrance step mediated by dirigent protein but also the subsequent metabolic steps are involved in the determination of the enantiomeric compositions.

In order to examine the stereochemical control in lignan biosynthesis in detail, *Arctium lappa* was chosen as a plant material, because the seeds of *Arctium* spp. contain significant amounts of dibenzylbutyrolactone lignans (and their glycosides) including arctiin [54-58] and matairesinol [56] as well as sesqui- and di-lignans [56,57,59-61] and because of its very good growth behavior. In addition, Umezawa et al. reported that the enzyme preparation from the petiole catalyzed the enantioselective formation of (+)-secoisolariciresinol from coniferyl alcohol and the isolation of (+)-secoisolariciresinol from the petiole [23]. The absolute configuration of (+)-secoisolariciresinol at C8 and C8' is the opposite to that of (-)-arctigenin [54,62] isolated from the seeds, which awaits further experimental studies. Herein, the author reports the first example showing that predominant enantiomers of a lignan, secoisolariciresinol, vary with organs of a plant species, *A. lappa*. In addition, the enantiomeric diversity of *A. lappa* lignans was demonstrated enzymatically.

1.A.2 Materials and Methods

Instrumentation

^{1}H NMR spectra were taken with a Varian XL-200 FT-NMR Spectrometer and a JNM-LA400MK FT-NMR System (JEOL Ltd.), using Me_4Si as an internal standard. Chemical shifts and coupling constants (*J*) are given in δ and Hz, respectively. GC-MS was conducted as previously described [34,52]. Reversed-phase HPLC separation [34] and chiral HPLC separation of pinoresinol [34], lariciresinol [52],

secoisolariciresinol [52], matairesinol [21], and arctigenin [63] were conducted as previously described. Silica gel TLC and silica gel column chromatography employed Kieselgel 60 F_{254} (Merck) and Kieselgel 60 (Merck), respectively.

Determination of enantiomeric compositions of lignans

Enantiomeric compositions of secoisolariciresinol isolated from *A. lappa* and lignans formed in enzymatic reactions were determined by chiral HPLC followed by GC-MS analysis. Briefly, each lignan sample was mixed with racemic unlabelled or deuterium-labelled lignans and the mixture was separated by chiral HPLC. Each enantiomer fraction was submitted to GC-MS measurement and the ratios of unlabelled/labelled lignan and the enantiomer excess were determined. Enantiomeric compositions of matairesinol and arctigenin were determined by chiral HPLC analysis as previously described [63].

Compounds

The following compounds had been prepared in the author's laboratory: [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol [34], [9,9-$^2H_2$]coniferyl alcohol [52], (±)-pinoresinols [34], (±)-[9,9,9',9'-$^2H_4$]pinoresinols [52], (±)-lariciresinols [64], (±)-[9,9,9',9'-$^2H_4$] lariciresinols [52], (±)-secoisolariciresinols [34], (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols [52], (±)-[*aromatic*-^{2}H]secoisolariciresinols [52], (±)-matairesinols [63], and (±)-arctigenins [63]. All solvents and reagents used were of reagent grade unless otherwise stated.

Plant materials

Arctium lappa L. cv. Kobarutogokuwase was used. Seeds of the plant from Atariya Noen Co. were sowed and the seedlings were maintained at a sunny laboratory supplemented with fluorescent light (*ca.* 10,000 lux, 40 cm apart from the

lumps), or in the experimental field in Wood Research Institute, Kyoto University, and used for lignan isolation and as enzyme sources. The seeds were also used for lignan isolation.

Isolation of (+)- and (-)-secoisolariciresinols from petioles

Petioles of *A. lappa* cv. Kobarutogokuwase (862.82 g in fresh weight) was freeze-dried. The dried material (62.31 g) was powdered with a Waring blender, extracted with hot MeOH (500 ml, 150 ml × 4, total 1.1 l), and the solvent was evaporated off to give MeOH extracts (19.69 g). The extracts were suspended in 400 ml of 0.1 M NaOAc buffer (pH 5.0), and incubated with β-glucosidase (SIGMA G-0395, 10,878.4 units) for 24 h at 37°C. Then, the reaction mixture was extracted with CH_2Cl_2 (150 ml × 4). The CH_2Cl_2 solution was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4, and the solvent was evaporated off. The CH_2Cl_2 extracts thus obtained (1.288 g) were submitted to successive purification by silica gel column chromatography, and a fraction corresponding to secoisolariciresinol was recovered. The fraction was further purified by reversed-phase HPLC to give (+)-secoisolariciresinol, which was identified by comparison of the ^1H NMR, mass spectrum, and t_R on GC with those of authentic (±)-secoisolariciresinols. Isolation of (+)-secoisolariciresinol was repeated with 36.8g (in fresh weight) of petioles of the plant. Enantiomeric compositions were determined as an average of those of the duplicate samples. In a separate experiment, yield of the lignan was determined using deuterium-labelled (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols as an internal standard. Yield, 0.0017% based on dried petioles; 81% e.e. [(+)>(-)].

(-)-Secoisolariciresinol was also isolated from MeOH extracts of the plants (736.89g in fresh weight) as above, but without the β-glucosidase treatment, and identified by comparison of the ^1H NMR, mass spectra, and t_R on GC with those of authentic (±)-secoisolariciresinol. Yield, 0.000020% based on dried petioles; 13% e.e. [(-)>(+)].

Isolation of (-)-secoisolariciresinol, (-)-matairesinol and (-)-arctigenin from seeds

Mature seeds of A. *lappa* cv. Kobarutogokuwase (4.00 g in fresh weight) were extracted with hot MeOH. From the MeOH extracts, (-)-matairesinol (0.065% based on dried seeds), (-)-arctigenin (2.6%), and (-)-secoisolariciresinol (0.0075%) were isolated as above after β-glucosidase treatment. The isolated lignans were identified by comparison of the ^1H NMR spectra with those of the authentic lignans. (-)-Matairesinol and (-)-arctigenin were optically pure (-)-enantiomers, respectively (Fig. 5). Enantiomeric composition of (-)-secoisolariciresinol was 65% e.e. [(-)>(+)].

In a separate experiment, the MeOH extracts (26.9 g) prepared as described above were suspended in 253 ml of 2.2 N H_2SO_4. After stirring at 95°C for 2 h, the reaction mixture was neutralized with 2.2 N NaOH and extracted with CH_2Cl_2. The extract was purified as above to afford (-)-secoisolariciresinol; 82% e.e. [(-)>(+)].

Enzyme preparation from *A. lappa* petioles

A. lappa petioles (102.68 g) were frozen (liquid N_2) and powdered with a Waring blender. The powder thus obtained was further ground with polyclar AT, acid-washed sea sand, and 0.1 M potassium phosphate buffer (pH 8.0) containing 10 mM dithiothreitol (DTT). The slurry thus obtained was filtered through 4 layers of gauze, and the filtrate (160 ml) was centrifuged (10,000×g, 20 min, 4°C). To the supernatant was added ammonium sulfate (0-70% saturation). The precipitate was collected by centrifugation (10,000×g, 15 min), redissolved into 15 ml of the same buffer containing DTT, and filtered through a Whatman GF/A glass fiber filter. The filtrate was applied to a Sephadex G-25 column that had been pre-equilibrated with the same buffer containing DTT. To the fraction (21 ml) excluded from the gel was added ammonium sulfate (0-70% saturation). The precipitate was collected by centrifugation (10,000×g, 15 min) and stored at 4°C until used. The same procedure was repeated twice with 207.02 g and 187.56 g of fresh petioles. To remove salts, the combined ammonium sulfate precipitates were redissolved in 0.1 M potassium

phosphate buffer (pH 7.0), and applied to a Sephadex G-25 column, which had been pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and eluted by the same buffer. The fraction excluded from the column was used as an enzyme preparation. The protein content of the enzyme preparation thus obtained was determined by Bradford method [65] using bovine serum albumin as a standard.

Enzyme preparation from *A. lappa* ripening seeds

A. lappa ripening seeds (4.04 g, ca. 6-20 days after the start of blooming), were frozen (liquid N_2) and powdered with a pestle and a mortar. The powder thus obtained was further ground as described in *Enzyme preparation from* A. lappa *petioles*. The slurry was filtered through 4 layers of gauze, and the filtrate thus obtained was centrifuged (10,000×g, 20 min, 4°C). The supernatant was filtered through a Whatman GF/A glass fiber filter, then to the supernatant was added ammonium sulfate (0-70% saturation). The precipitate was collected by centrifugation (10,000×g, 15 min), redissolved into the same buffer containing DTT. To remove salts, the ammonium sulfate precipitates were redissolved in 0.1 M potassium phosphate buffer (pH 7.0), and applied to a Sephadex G-25 column, which had been pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and eluted by the same buffer. The fraction excluded from the column was used as an enzyme preparation in the incubation with [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol, (±)-[9,9,9',9'-$^2H_4$]pinoresinols, and (±)-[9,9,9',9'-$^2H_4$]lariciresinols, individually, while the ammonium sulfate precipitates were desalted with 0.1 M Tris-HCl buffer (pH 8.8) and used for the incubation with (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols in the presence of NADP. The protein content of the enzyme preparation thus obtained (6.13-11.4 mg/ml) was determined by Bradford method [65] using bovine serum albumin as a standard.

Conversion of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol and [9,9-$^2H_2$]coniferyl alcohol with *A. lappa* enzyme preparations

The reaction mixture consisted of 50 μl of 25 mM [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol in 0.1 M potassium phosphate buffer (pH 7.0), 50 μl of 50 mM NADPH in the same buffer, 25 μl of 7.6 mM H_2O_2 in the same buffer, and 500 μl of the enzyme preparations prepared from petiole and ripening seeds, respectively. The reaction was initiated by adding H_2O_2. After incubating for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabelled racemic (±)-pinoresinols, (±)-lariciresinols, and (±)-secoisolariciresinols as internal standards. The EtOAc extract was dried under high vacuum and the lignans formed were identified and quantified by GC-MS analysis.

Next, [9,9-$^2H_2$]coniferyl alcohol was incubated with the petiole and seed enzyme preparations, individually, in the presence of H_2O_2 and NADPH, as described above except that each volume was proportionately scaled up. The products were separated with silica gel TLC and reversed-phase HPLC to give [$^2H_4$]pinoresinol, [$^2H_4$]lariciresinol, and [$^2H_4$]secoisolariciresinol, and their enantiomeric compositions were determined.

Conversion of (±)-[9,9,9',9'-$^2H_4$]pinoresinols and (±)-[9,9,9',9'-$^2H_4$]lariciresinols

The reaction mixture was composed of 5 μl of 50 mM of (±)-[9,9,9',9'-$^2H_4$] pinoresinols or (±)-[9,9,9',9'-$^2H_4$]lariciresinols in MeOH, 50 μl of 50 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.0), and 120 μl of the enzyme preparation. The reaction was initiated by adding (±)-[9,9,9',9'-$^2H_4$]pinoresinols or (±)-[9,9,9',9'-$^2H_4$]lariciresinols. After incubation for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabelled racemic lignans as internal standards. The EtOAc extract was dried under high vacuum and the formed lignans were identified and quantified by GC-MS analysis.

Next, (±)-[9,9,9',9'-$^2H_4$]pinoresinols were incubated as above, except that all the

volumes were proportionately scaled up. The products were purified with silica gel TLC and reversed-phase HPLC to give [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol, and their enantiomeric compositions were determined. Similarly, scaled-up incubation of (±)-[9,9,9',9'-$^2H_4$]lariciresinols with NADPH was carried out, and the enantiomeric composition of formed [$^2H_4$]secoisolariciresinol was determined.

Conversion of (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols

The reaction mixture consisted of 2.5 µl of 25 mM of (±)-[9,9,9',9'-$^2H_4$] secoisolariciresinols in MeOH, 2.5 µl of 50 mM NADP in 0.1 M Tris-HCl buffer (pH 8.8 at 30°C), and 167 µl of the enzyme preparation from *A. lappa* ripening seeds. The reaction was initiated by adding (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols. After incubating for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing (±)-1-(4-ethoxy-3-methoxyphenyl)-2-(2-mehoxyphenoxy)-1,3-propanediols [66] as an internal standard. The EtOAc extract was dried under high vacuum and analyzed by GC-MS.

Next, (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols were incubated as above with volumes scaled up proportionately and without the addition of the internal standard. The product was purified with silica gel TLC and reversed-phase HPLC [22] to give pure [$^2H_2$]matairesinol, which was subjected to chiral HPLC analysis.

1.A.3 Results and Discussion

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 these lignans [54-58]. Our survey of *A. lappa* lignans accorded well with the previous reports. Thus, preliminary GC-MS analysis of

β-glucosidase-treated MeOH extracts from petioles, root, and seeds of *A. lappa* indicated that the seeds contained significant amounts of matairesinol and arctigenin, and small amounts of secoisolariciresinol, while only small amounts of matairesinol and arctigenin were detected when the β-glucosidase treatment was omitted. The results suggest strongly that most of the lignans are present as glycosides. On the other hand, small amounts of secoisolariciresinol were detected from the petioles, probably as glycoside, and no lignans were detected in mature roots.

Next, lignans were isolated from *A. lappa* and their enantiomeric compositions were determined. (-)-Matairesinol and (-)-arctigenin isolated from the MeOH extracts of *A. lappa* seeds after β-glucosidase treatment were found to be optically pure (>99% e.e.) (Fig. 5). 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 [47].

In addition, small amounts of secoisolariciresinol were isolated from both seeds and petioles. Unexpectedly, the predominant enantiomers of the lignan isolated from the seeds and petioles were opposite each other. As summarized in Table 1, (+)-secoisolariciresinol (81% e.e.) was isolated from the MeOH extracts of *A. lappa* petioles after β-glucosidase treatment. In contrast, this lignan obtained after β-glucosidase treatment or acid hydrolysis from the MeOH extracts of mature seeds showed the enantiomeric compositions of 65% and 82% e.e. in favor of (-)-enantiomer, respectively (Table 1). To our knowledge, 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 mechanisms in *A. lappa*.

It is noteworthy that absolute configurations at C8 and C8' of (-)-secoisolariciresinol isolated from *A. lappa* seeds is the same as those of (-)-matairesinol and (-)-arctigenin isolated from the seeds (Fig. 2). In addition, the configurations at C8 and C8' of these enantiomers are the same as those of

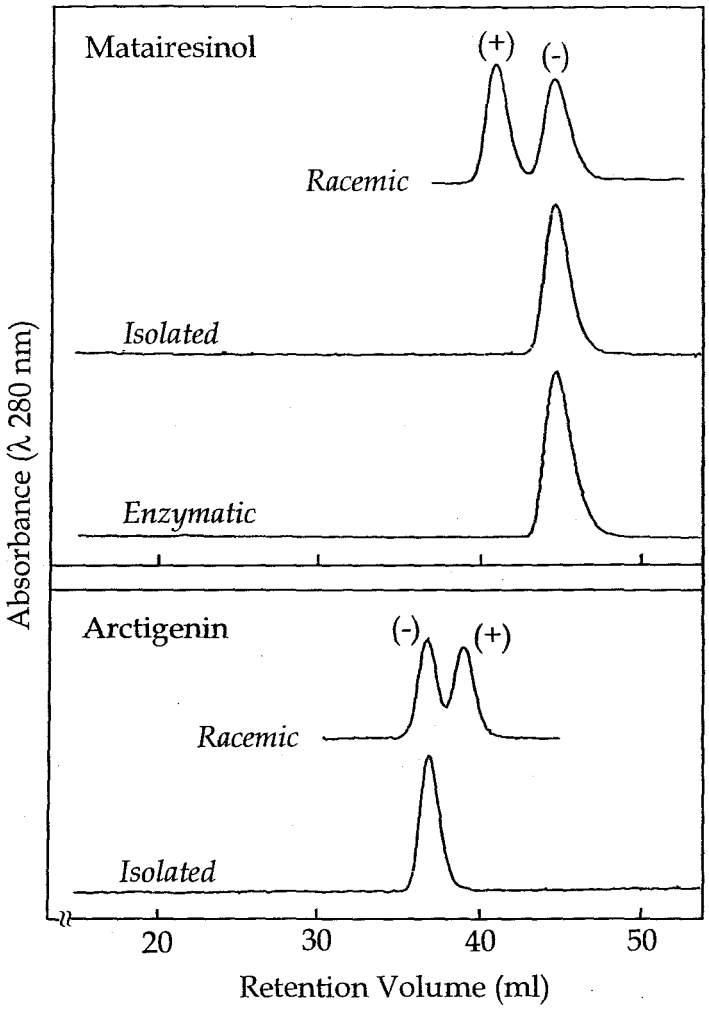

Fig. 5 Chiral HPLC chromatograms of matairesinol and arctigenin. *Racemic,* chemical synthesized (±)-matairesinol or (±)-arctigenin. *Isolated,* isolated matairesinol or arctigenin from *A. lappa* seeds. *Enzymatic,* enzymatically formed matairesinol with enzyme preparation obtained from *A. lappa* ripening seeds.

Table 1. The yield and enantiomeric composition (% e.e.) of secoisolariciresinol from *A. lappa* petioles and seeds.

Sources	Hydrolysis	Yield (%)[a]	% e.e.
Petioles	β-glucosidase	1.7×10^{-3}	81 [(+)>(-)]
Petioles	No	2.0×10^{-5}	13[(-)>(+)]
Seeds	β-glucosidase	7.5×10^{-3}	65 [(-)>(+)]
Seeds	2.2 N H_2SO_4	9.3×10^{-3}	82 [(-)>(+)]

[a]Based on dry weight.

(+)-antipodes of the upstream lignans, pinoresinol and lariciresinol [47].

The enantiomeric diversity of the lignans isolated from different organs of *A. lappa* is peculiar, and the author's next attention was directed to the enzymatic reconstitution of the enantiomeric compositions of secoisolariciresinol.

When the seed enzyme preparation was incubated with [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol, the (-)-enantiomer of [$^2H_{10}$]secoisolariciresinol was formed selectively (38% e.e., Tables 2 and 3, Fig. 2). In contrast, Umezawa and Shimada reported that the incubation of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol with the petiole enzyme preparation gave the opposite (+)-enantiomer of deuterated secoisolariciresinol (ca. 20% e.e.) [23]. They determined the enantiomeric composition by LC-MS. However, the chiral separation was incomplete. In the present study, the value was re-determined precisely under an improved separation condition (Table 3). The enzymatically formed predominant enantiomers coincide with those of secoisolariciresinol occurring naturally in the petioles and seeds, respectively (Table 3). The reaction system did not alter the enantiomeric composition of once formed secoisolariciresinol, because incubation of racemic (±)-[*aromatic*-^{2}H] secoisolariciresinols with each enzyme preparation under the same condition but with a half period of the incubation resulted in recovery of racemic (±)-[*aromatic*-^{2}H]secoisolariciresinols (petiole enzyme, 0.85% e.e. [(+)>(-)]; seed enzyme, 0.14% e.e [(+)>(-)]). Hence, the enzymatic formation of secoisolariciresinol, but not the enzymatic conversion of the once-formed secoisolariciresinol to some

Table 2. Enzymatic formation of [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol and [$^2H_{10}$]secoisolariciresinol from [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol with *A. lappa* ripening seed enzyme preparation.

Assay	Cofactor	[$^2H_{10}$]Pino-resinol formation[b]	Assay	Cofactor	[$^2H_{10}$]Larici-resinol formation[b]	[$^2H_{10}$]Secoiso-lariciresinol formation[b]
Complete	H_2O_2	94.5	Complete	H_2O_2/NADPH	11.7	26.7
Control[a]	H_2O_2/NADPH	15.1	Control[a]	H_2O_2	0	0
	NADPH	5.08		NADPH	8.00	16.3
	None	32.2		None	0	0
	Denatured enzyme/ H_2O_2/NADPH	1.94		Denatured enzyme/ H_2O_2/NADPH	0	0

[a]Control experiments refer to a complete assay with either the omission of cofactors or with the denatured enzyme (boiled for 5 min). One other experiment was carried out by using the complete assay, but the reaction was worked up by adding EtOAc as soon as possible (less than 10 sec) after the start of incubation. In this experiment, the amounts of pinoresinol, lariciresinol, and secoisolariciresinol formed were 26.9, 3.80 and 0 nmol mg^{-1} protein, respectively.

[b]Expressed in nmol h^{-1} mg^{-1} protein.

Table 3. Enantiomeric compositions (% e.e.) of deuterated pinoresinols, lariciresinols, secoisolariciresinols, and matairesinol formed following incubation of deuterated substrates with enzyme preparations from *A. lappa* petioles and ripening seeds.[a]

Enzyme sources	Substrate	Enantiomeric compositions of formed lignans (% e.e.)			
		[$^2H_4$]Pinoresinol or [$^2H_{10}$]pinoresinol	[$^2H_4$]Lariciresinol or [$^2H_{10}$]lariciresinol	[$^2H_4$]Secoisoariciresinol or [$^2H_{10}$]Secoisolariciresinol	[$^2H_2$]Matai-resinol
Petioles	[9,9-$^2H_2$]Coniferyl alcohol	33 [(+)>(-)]	30 [(+)>(-)]	20 [(+)>(-)][b]	—
	(±)-[9,9,9',9'-$^2H_4$] Pinoresinol	—	24 [(+)>(-)]	44 [(-)>(+)]	—
	(±)-[9,9,9',9'-$^2H_4$] Lariciresinol	—	—	37 [(-)>(+)]	—
Ripening seeds	[9,9-$^2H_2$, OC$^2H_3$] Coniferyl alcohol	22 [(-)>(+)]	>99 [(-)>(+)][c]	38 [(-)>(+)]	—
	(±)-[9,9,9',9'-$^2H_4$] Pinoresinol	—	85 [(+)>(-)]	99 [(-)>(+)]	—
	(±)-[9,9,9',9'-$^2H_4$] Lariciresinol	—	—	91 [(-)>(+)]	—
	(±)-[9,9,9',9'-$^2H_4$] Secoisolariciresinol	—	—	—	>99 [(-)>(+)]

[a]Incubation conditions are described in Materials and Methods.

[b]This value was reported previously [23] as about 20% e.e. based on chiral LC-MS analysis, where chiral separation was incomplete. In the present study, the value was re-determined precisely under an improved chiral separation condition.

[c](+)-Enantiomer was not detected under the conditions used.

Table 4. Enzymatic formation of [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol from [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol with *A. lappa* petiole ezyme preparation.

Assay	Cofactor	[$^2H_{10}$]Pinoiresinol formation[b]	Assay	Cofactor	[$^2H_{10}$]Lariciresinol formation[b]	[$^2H_{10}$]Secoiso-lariciresinol formation[c]
Complete	H_2O_2	4.36	Complete	H_2O_2/NADPH	0.20	0.73
Controls[a]	H_2O_2/NADPH	0.19	Controls	H_2O_2	0.059	0.075
	NADPH	0.12		NADPH	0	0
	None	0.40		None	0	0
	Denatured enzyme/ NADPH/H_2O_2	5.58		Denatured enzyme/ NADPH/H_2O_2	0.020	0

[a]Control experiments refer to the complete assay with either the omission of cofactors or with the denatured enzyme (boiled for 5 min). One other control experiment was conducted, using the complete assay but the reaction was worked up by adding EtOAc as soon as possible (less than 10 sec) after the start of incubation. In this experiment, the amounts of [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol formed were 0.32, 0.037, and 0 nmol mg^{-1}, respectively.

[b]Expressed n nmol h^{-1} mg^{-1} protein.

[c]From the data of Umezawa and Shimada [23].

other products, is responsible for its enantiomeric composition.

Besides (-)-[$^2H_{10}$]secoisolariciresinol, (-)-[$^2H_{10}$]pinoresinol (22% e.e.) and (-)-[$^2H_{10}$]lariciresinol (>99% e.e.) were also formed in the incubation of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol with the seed enzyme (Tables 2 and 3, Fig. 2). Again, in contrast, the opposite enantiomers, (+)-[$^2H_4$]pinoresinol (33% e.e.) and (+)-[$^2H_4$]lariciresinol (30% e.e.), were formed with the petiole enzyme (Tables 3 and 4, Fig. 2). Thus, 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*.

Pinoresinol/lariciresinol reductase (PLR) which can reduce pinoresinol to lariciresinol, and lariciresinol to secoisolariciresinol, was purified from *Forsythia intermedia* [31], and this enzyme was detected from *Zanthoxylum ailanthoides* [67] and *Daphne odora* [53]. Since the assay with only H_2O_2 as a cofactor exhibited significant activity of [$^2H_{10}$]pinoresinol formation from [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol (Tables 2 and 4), PLR-catalyzed reduction of the once-formed [$^2H_{10}$]pinoresinol probably resulted in [$^2H_{10}$]lariciresinol and [$^2H_{10}$]secoisolariciresinol in the incubation of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol with the *A. lappa* enzymes. This was confirmed by individual incubation of (±)-[9,9,9',9'-$^2H_4$]pinoresinols and (±)-[9,9,9',9'-$^2H_4$] lariciresinols with the seed enzyme preparation (Table 5, Fig. 2). Almost optically pure (-)-[$^2H_4$]secoisolariciresinol was formed from these racemic lignans [99% e.e. from (±)-[9,9,9',9'-$^2H_4$]pinoresinols, and 91% e.e. from (±)-[9,9,9',9'-$^2H_4$]lariciresinols (Table 3)]. The predominant formation of the (-)-enantiomer is in accordance with the results of incubation of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol with the seed enzyme. (+)-[$^2H_4$]lariciresinol (85% e.e.) was also obtained in the incubation of (±)-[9,9,9',9'-$^2H_4$]pinoresinols with the seed enzyme (Table 3).

The petiole enzyme also exhibited PLR activity giving rise to [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol from (±)-[9,9,9',9'-$^2H_4$]pinoresinols, and [$^2H_4$]secoisolariciresinol from (±)-[9,9,9',9'-$^2H_4$]lariciresinols (Table 5). Interestingly,

Table 5. Formation of deuterated lariciresinol, secoisolariciresinol, and matairesinol from deuterated lignans by enzyme preparations from ripening seeds and petioles of *A. lappa*.

Assay	Substrate	Cofactor	[$^2H_4$]Lariciresinol formation[b]		[$^2H_4$]Secoisolariciresinol formation[b]		[$^2H_2$]Matairesinol formation[b]
			Petiole enzyme	Seed enzyme	Petiole enzyme	Seed enzyme	Seed enzyme
Complete	(±)-[9,9,9',9'-$^2H_4$] Pinoresinols	NADPH	6.4	0.97	0.12	38.1	—
	(±)-[9,9,9',9'-$^2H_4$] Lariciresinols	NADPH	—	—	3.8	51.3	—
	(±)-[9,9,9',9'-$^2H_4$] Secoisolariciresinols	NADP	—	—	—	—	64.3
Control[a]	(±)-[9,9,9',9'-$^2H_4$] Pinoresinols	None	0	0.44	0.04	0.28	—
	(±)-[9,9,9',9'-$^2H_4$] Lariciresinols	None	—	—	0.14	0.81	—
	(±)-[9,9,9',9'-$^2H_4$] Secoisolariciresinols	None	—	—	—	—	5.98
	(±)-[9,9,9',9'-$^2H_4$] Pinoresinols	Denatured enzyme/ NADPH	0	0	0	0.54	—
	(±)-[9,9,9',9'-$^2H_4$] Lariciresinols	Denatured enzyme/ NADPH	—	—	0.02	0.94	—
	(±)-[9,9,9',9'-$^2H_4$] Secoisolariciresinols	Denatured enzyme/ NADP	—	—	—	—	0

[a]Control experiments refer to a complete assay with either the omission of a cofactor or with the denatured enzyme (boiled for 5 min).

[b]Expressed in nmol h^{-1} mg^{-1} protein.

however, the predominant enantiomers of the product lignans, (-)-[$^2H_4$]secoisolariciresinol and (+)-[$^2H_4$]lariciresinol, formed from (±)-[9,9,9',9'-$^2H_4$] pinoresinols and (±)-[9,9,9',9'-$^2H_4$]lariciresinols were the same as those obtained with the seed enzyme (Table 3), 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., Table 3) were much lower than those formed with the seed enzyme which are almost optically pure (99 and 91% e.e., Table 3).

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* [31]. 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 [31]. PLR of *Zanthoxylum ailanthoides* [67] 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 [53]. 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. [68], 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 [69] 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 (-)-[$^2H_2$]matairesinol following incubation of racemic (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols in the presence of NADP (Table 5 and Fig. 5). Thus, although formation of secoisolariciresinol is controlled stereochemically, the control is not enough strong to produce only one enantiomer of secoisolariciresinol. 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 lignan.

1.A.4 Summary

Stereochemistry of lignan biosynthesis in *Arctium lappa* L. was regulated organ-specifically. (+)-Secoisolariciresinol [81% enantiomer excess (e.e.)] was isolated from *A. lappa* petioles. In sharp contrast, lignans whose predominant enantiomers have the opposite absolute configurations to (+)-secoisolariciresinol [i.e.,

(-)-matairesinol (>99% e.e.), (-)-arctigenin (>99% e.e.), and (-)-secoisolariciresinol (65% e.e.)] were isolated from seeds of this species. The stereochemical diversity of secoisolariciresinol was also demonstrated with enzyme preparations from *A. lappa* petioles and seeds. Thus, a petiole enzyme preparation catalyzed the formation of (+)-pinoresinol (33% e.e), (+)-lariciresinol (30% e.e.), and (+)-secoisolariciresinol (20% e.e.) from achiral coniferyl alcohol in the presence of NADPH and H_2O_2, whereas that from ripening seeds catalyzed the formation of (-)-pinoresinol (22% e.e.), (-)-lariciresinol (>99% e.e.), and (-)-secoisolariciresinol (38% e.e.) under the same condition. In addition, the ripening seed enzyme preparation mediated selective formation of optically pure (>99% e.e.) (-)-enantiomer of matairesinol from racemic (±)-secoisolariciresinols in the presence of NADP.

These results indicated that stereochemical mechanisms of lignan biosynthesis of *A. lappa* vary with organs, suggesting that multiple PLR isozymes are involved in the stereochemical control of lignan formation in *A. lappa*. In addition, the optically pure lignan in *A. lappa* is formed in dehydrogenation of secoisolariciresinol giving rise to matairesinol.

Part B Stereochemistry of lignan biosynthesis in *Anthriscus sylvestris* (L.) Hoffm.

1.B.1 Introduction

An aryltetralin lignan, podophyllotoxin, isolated from *Podophyllum* plants [10,70,71] has been well-known to be antitumor. The lignan isolated from *Podophyllum hexandrum* is exploited commercially as a source of semisynthetic anticancer drugs, etoposide and teniposide [2,10,49,50,70-73]. However, due to the limited supply of the plant, much attention has been focused on the availability and biosynthesis of the lignan [10]. Several woody plants, *e.g. Juniperus savina, Thujopsis dolabrata, Callitris drummondii,* and *Hernandia ovigera* have been known to produce podophyllotoxin and/or its congeners [71]. Thus, biotechnological production of the antitumor lignan by the woody plants is a challenging subject in the field of wood chemistry and biochemistry. As the first step, it is critically important to understand the biosynthetic mechanisms. However, much remains unknown about the biosynthesis of podophyllotoxin.

Besides these woody plants and *Podophyllum* spp., some herbaceous plants such as *Linum* spp. (especially those belonging to the section *Syllinum* including *Linum flavum* and *Linum capitatum*) [49,74,75] and *Anthriscus* spp. [76-82] have been known to produce significant amounts of podophyllotoxin congeners. Studies of lignan biosynthesis in *Linum* spp. [83] which produce principally 5-methoxypodophyllotoxin besides podophyllotoxin [84] have been reported. On the other hand, although isolation of podophyllotoxin from *A. sylvestris* have not yet been reported, this species produces not only the precursor lignans of podophyllotoxin, deoxypodophyllotoxin (=desoxypodophyllotoxin or anthricin) [85] and yatein [86], but also the typical heartwood lignans, yatein and hinokinin, which are found specifically in heartwood region in conifers, *Libocedrus yateensis* [5] and

Chamaecyparis obtusa [14], respectively [76-78,80-82]. Thus, the knowledge of lignan biosynthetic mechanism in *A. sylvestris* can be applied to both biotechnological production of podophyllotoxin in woody and herbaceous plants and studies of heartwood lignan biosynthetic mechanisms. In addition, this species exhibits a good growth behavior and can be maintained easily in laboratories.

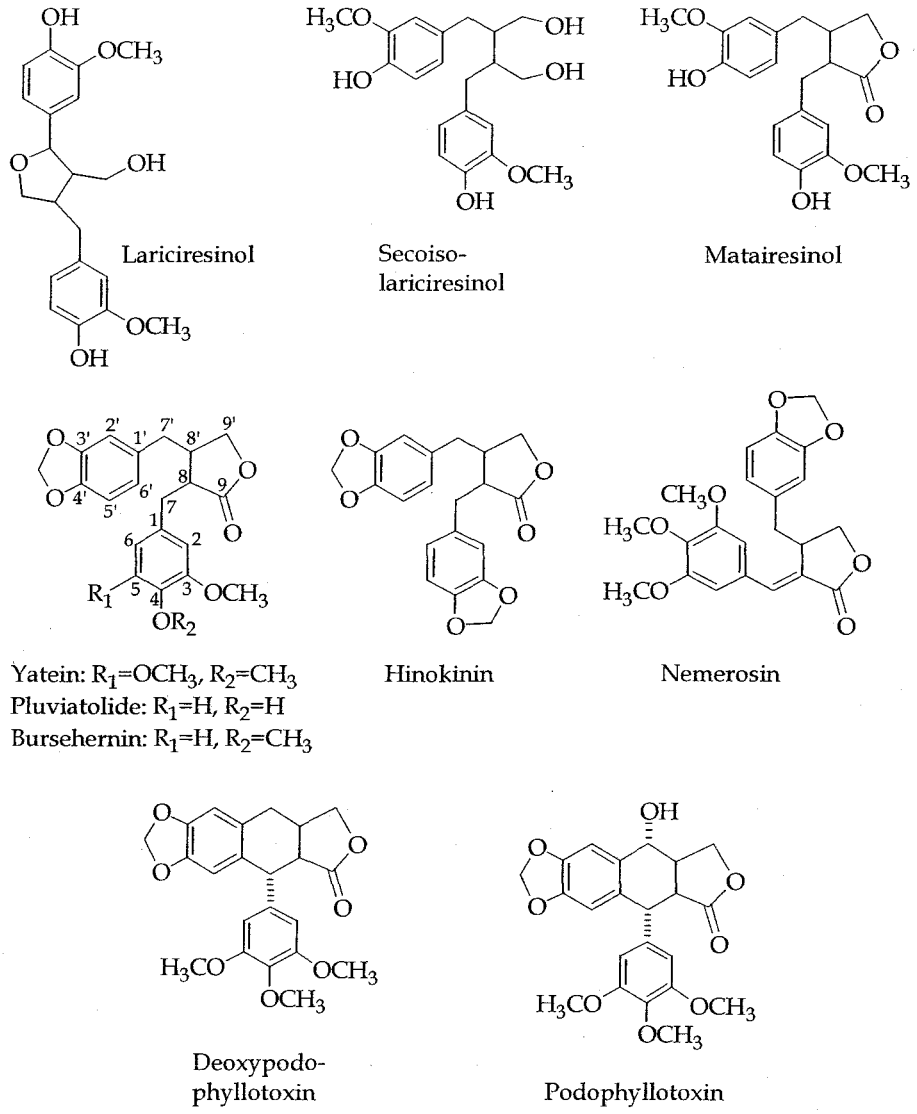

Fig. 6 Chemical structure of lignans.

Taken together, *A. sylvestris* is probably a good plant material for lignan biosynthetic studies to access mechanisms involved in antitumor and heartwood

lignan formation. In this study, as the first step for the elucidation of the lignan biosynthetic mechanism in this species, the author examined the stereochemical mechanism of the lignan biosynthesis in upstream steps (pinoresinol to secoisolariciresinol) in terms of stereochemistry, because the biological activities are often related to the stereochemical properties. As discussed in Part A of Chapter 1, it has been found that there is great stereochemical diversity in the upstream steps of lignan biosynthesis. Thus, the knowledge on the stereochemical property about biosynthetic mechanism of useful lignans having biological activities is of importance.

1.B.2 Materials and Methods

Instrumentation

^{1}H NMR spectra were taken with a JNM-LA400MK FT-NMR System (JEOL Ltd.), using Me_4Si as an internal standard. Chemical shifts and coupling constants (*J*) are given in δ and Hz, respectively. GC-MS was conducted as described in Part A of Chapter 1. Reversed-phase HPLC was conducted with the following elution conditions: column, a Waters Novapak C_{18} (150 mm × 3.9 mm), which was eluted with the following two solvent systems; (i) solvent system A was for gradient elution at 1 ml/min by a linear solvent gradient protocols of CH_3CN-H_2O at t=0 (23:77) to 15 min, and then to 50:50 at t=20 min, held at this ratio for an additional 5 min; and (ii) solvent system B was for gradient elution at 1 ml/min by a linear solvent gradient protocols of CH_3CN-H_2O at t=0 to 6 min from 15:85 to 17:83, at t=6 to 16 min from 17:83 to 20:80, 20:80 at t=16 to 20 min, then at t=20 to 26 from 20:80 to 50:50, held at this ratio for an additional 5 min. Chiral HPLC separation of lariciresinol and secoisolariciresinol were conducted as previously described [52]. Silica gel TLC and silica gel column chromatography employed Kieselgel 60 F_{254} (Merck) and Kieselgel

60 (Merck), respectively. All solvents and reagents used were of reagent or of HPLC grade, unless otherwise mentioned.

Compounds

The following compounds had been prepared in the author's laboratory, and used in the present study: [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol [34], (±)-[9,9,9',9'-$^2H_4$]pinoresinols [52], (±)-pinoresinols [34], (±)-[9,9,9',9'-$^2H_4$]lariciresinols [52], (±)-lariciresinols [64], (±)-[9,9,9',9'-$^2H_4$]secoisolariciresinols [52], (±)-secoisolariciresinols [34], (±)-matairesinols [63], (±)-hinokinins [14], (±)-pluviatolides [14], and (±)-bursehernins [52]. (±)-Yateins were prepared from (±)-4-demethylyatein benzyl ethers, which was a gift of Dr. Shingo Kawai, by debenzylation (10% Pd/C, H_2) followed by methylation (diazomethane). (±)-Yateins, δ_H ($CDCl_3$) 2.43-2.63 (3H, m, 7',8,8'-H), 2.88 (1H, dd, *J* 13.8 and 6.3, 7-H), 2.92 (1H, dd, *J* 13.8 and 5.4, 7-H), 3.817 (3H, s, OCH_3), 3.819 (6H, s, OCH_3×2), 3.87 (1H, dd, *J* 9.0 and 7.6, 9-H), 4.17 (1H, dd, *J* 9.1 and 7.2, 9-H), 5.92-5.93 (2H, m, -OCH_2O-), 6.35 (2H, s, 2,6-H), 6.45-6.47 (2H, m, 2',6'-H) and 6.69 (1H, dd, *J* 0.8 and 7.6, 5'-H).

Plant materials

Anthriscus sylvestris (L.) Hoffm. (Umbelliferae) plants were grown from seeds collected in a suburb of Brussels, Belgium in 1996, or at Kyoto University Forest in Ashiu, Kyoto in 1997. The plants were maintained in the experimental garden of Wood Research Institute, Kyoto University for about 6 months.

Lignans in *Anthriscus sylvestris*

Aerial parts (including leaves, petioles, and racemes) and roots of *A. sylvestris* were individually harvested, washed with tap and distilled water. Each sample (0.7-1.6 g) was frozen (liquid N_2), freeze-dried, disintegrated with scissors, and then

extracted with hot MeOH (11 ml). An aliquot (5 mg) of the MeOH extract was treated with β-glucosidase [from almonds, SIGMA G-0395, 10 units in 0.5 ml of 0.1 M NaOAc buffer (pH 5.0) for 24 h at 37°C]. The reaction mixture was extracted with CH_2Cl_2 (0.5 ml × 2), and the combined solution was evaporated off. The resulting residue was subjected to GC-MS analysis after derivatization with 7 μl of *N,O*-bis(trimethylsilyl) acetamide (BSA) for 45 min at 60°C.

In a separate experiment, aerial parts of *A. sylvestris* (from University Forest in Ashiu) were harvested and freeze-dried as above. The dried materials (48.7 g) were ground with a Waring blender, and extracted with hot MeOH (100 ml × 10), then the solvent was evaporated off. The MeOH extract (12.3 g) was dispersed in Et_2O (50 ml × 10), and Et_2O solubles were removed. The Et_2O insoluble residue (10.3 g) was suspended in 180 ml of 0.1 M NaOAc buffer (pH 5.0) containing in 2544 units of β-glucosidase, and the solution was incubated for 24 h at 37°C. Then, the solution was extracted with EtOAc (80 ml × 4), and the solvent was evaporated off. The EtOAc extract (721 mg) was fractionated into 6 factions by silica gel column (ϕ30 mm × 80 mm) chromatography. Each fraction was subjected to GC-MS analysis after TMS derivertization with BSA.

Enzyme preparation

Enzyme preparations from young leaves (including petioles) of *A. sylvestris* (grown from seeds collected in Belgium) exactly as described in *Enzyme preparation from* Arctium lappa *ripening seeds* of Part A of Chapter 1. The protein content of the enzyme preparation was 0.55-8.65 mg/ml, being measured by the method of Bradford [65] using bovine serum albumin as a standard.

Enzymatic conversion of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol

The reaction mixture was composed of 50 μl of 25 mM [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol in 0.1 M potassium phosphate buffer (pH 7.0), 50 μl of 50 mM NADPH in the

same buffer, 25 μl of 7.6 mM H_2O_2 in the same buffer, and 120 μl of the enzyme preparation from *A. sylvestris*. The reaction was initiated by adding H_2O_2. After incubation for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabelled (±)-pinoresinols, (±)-lariciresinols, and (±)-secoisolariciresinols as internal standards. The EtOAc extract was dried under high vacuum and dissolved in 7 μl of BSA. After standing at 60°C for 45 min, an aliquot (0.8 μl) of the BSA solution was subjected to GC-MS analysis, and the lignans formed were identified and quantified (Table 6).

Enzymatic conversion of (±)-[9,9,9',9'-$^2H_4$]pinoresinols and (±)-[9,9,9',9'-$^2H_4$] lariciresinols

The reaction mixture consisted of 150 μl of 50 mM of (±)-[9,9,9',9'-$^2H_4$]pinoresinols in MeOH, 1 ml of 50 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.0), and 16 ml of the cell-free extracts from *A. sylvestris* leaves. The reaction was initiated by adding (±)-[9,9,9',9'-$^2H_4$]pinoresinols. After incubating for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabelled (±)-lariciresinols and (±)-secoisolariciresinols as internal standards. An aliquot of the EtOAc extract was subjected to GC-MS analysis, and formed [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol were identified. The remainder was submitted to silica gel TLC [solvent, MeOH–CH_2Cl_2 (3:97)] followed by reversed-phase HPLC (solvent system A for the lariciresinol purification, and solvent system B for the secoisolariciresinol purification) to give [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol. Enantiomeric compositions of the deuterium-labelled lignans were determined as previously described using the corresponding unlabelled (±)-lariciresinols and (±)-secoisolariciresinols as internal standards, respectively (described in Part A of Chapter 1) [34,52].

In a separate experiment, the results shown in Table 7 were given under similar assay condition as above, but with the following scaled-down constituents: 5 μl of 25

mM of (±)-[9,9,9',9'-$^2H_4$]pinoresinols in MeOH, 50 μl of 50 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.0), and 200 μl of the cell-free extracts.

(±)-[9,9,9',9'-$^2H_4$]Lariciresinols were also incubated under the same assay condition as above, except that 5 μl of 25 mM (±)-[9,9,9',9'-$^2H_4$]lariciresinols were used as substrates.

1.B.3 Results and Discussion

GC-MS analysis of the β-glucosidase-treated MeOH extracts of both aerial parts and roots of *A. sylvestris* showed the presence of the lignans, yatein and secoisolariciresinol, which were identified by comparison of the mass spectra and t_R on GC with those of authentic samples. Yatein, *m/z* (EI) 400 (M^+, 100%), 368 (20), 239 (10), 223 (10), 181 (88) and 135 (31). Secoisolariciresinol (TMS ether), *m/z* (EI) 650 (M^+, 20%), 368 (43), 560 (10), 470 (22), 261 (52), 209 (100) and 179 (21). In addition, two GC peaks, A and B, were detected; A, *m/z* (EI) 398 (M^+, 23%), 263 (100), 235 (4), 207 (18), 176 (12), 161 (5), 135 (55), 209 (100) and 179 (21); and B, *m/z* (EI) 398 (M^+, 100%), 283 (5), 230 (13), 185 (18), 181 (30) and 173 (21). The mass spectra of peaks A and B essentially matched literature data of mass spectra of nemerosin [79] and deoxypodophyllotoxin [87], respectively, which were previously isolated from *Anthriscus* spp [76-82].

When partially fractionated MeOH extracts after glucosidase treatment from aerial parts of *A. sylvestris* by silica gel column chromatography were analyzed by GC-MS, the lignans, lariciresinol, matairesinol, hinokinin, and pluviatolide, were identified by comparison of the mass spectra and t_Rs with those of authentic samples. Lariciresinol (TMS ether), *m/z* (EI) 576 (M^+, 41%), 561 (10), 486 (26), 324 (31), 277 (31), 223 (46), 209 (45) and 179 (19). Matairesinol (TMS ether), *m/z* (EI) 502 (M^+, 94%), 209

(100), 235 (4), 179 (37), 176 (12), 161 (5), 135 (55), 209 (100) and 179 (21). Hinokinin, *m/z* (EI) 354 (M^+,58) and 135 (100). Pluviatolide (TMS ether), *m/z* (EI) 428 (M^+, 85), 179 (28) and 135 (26). In addition, the presence of small amounts of bursehernin was suggested by comparing mass chromatograms with those of the synthesized authentic sample. Secoisolariciresinol, lariciresinol, matairesinol, and pulviatolide were identified for the first time in *Anthriscus* spp.

When [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol was incubated with the *A. sylvestris* enzyme preparation in the presence of H_2O_2 and NADPH, the lignans, [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol, were formed (Table 6, Fig. 7). They were identified by comparing their mass spectra as TMS ethers and the t_Rs by GC with those of unlabelled authentic samples as previously reported [23,34]. Dehydrogenated dimers other than pinoresinol were not investigated. Next, the author incubated (±)-[9,9,9',9'-$^2H_4$]pinoresinols with the enzyme preparation in the presence of NADPH, because pinoresinol/lariciresinol reductase (PLR) have been known to be involved in production of lariciresinol and secoisolariciresinol from pinoresinol [27]. GC-MS analysis indicated that [$^2H_4$]lariciresinol was produced in the incubation (Table 7). [$^2H_4$]Lariciresinol was not formed in the omission of NADPH and with denatured enzyme, demonstrating that this reaction is enzymatic and requires NADPH as a cofactor. However, [$^2H_4$]secoisolariciresinol was not detected in this assay, probably due to the low enzyme activity. Therefore, (±)-[9,9,9',9'-$^2H_4$]lariciresinols were incubated with the preparation in the presence of NADPH, which afforded [$^2H_4$]secoisolariciresinol (Table 8). Control assays resulted in insignificant specific activity, demonstrating that this reaction is enzymatic and needs NADPH as a cofactor. Thus, these results show pinoresinol/lariciresinol reductase (PLR) activity in this species. In a separate experiment, when the author conducted the scaled-up incubation of (±)-[9,9,9',9'-$^2H_4$]pinoresinols with the enzyme preparation in the presence of NADPH, both [$^2H_4$]lariciresinol and [$^2H_4$]secoisolarici-

Table 6. Enzymatic formation of [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol and [$^2H_{10}$]secoisolariciresinol from [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol with *A. sylvestris* enzyme preparation.

Assay	Cofactor	[$^2H_{10}$]Pinoresinol formation[b]	Assay	Cofactor	[$^2H_{10}$]Lariciresinol formation[b]	[$^2H_{10}$]Secoisolariciresinol formation[b]
Complete	H_2O_2	144	Complete	H_2O_2/NADPH	13.6	36
Control[a]	H_2O_2/NADPH	20.7	Control[a]	H_2O_2	0	0
	NADPH	11.1		NADPH	10.7	16.9
	Denatured enzyme/ H_2O_2/NADPH	8.2		Denatured enzyme/ H_2O_2/NADPH	0	0

[a]Control experiments refer to a complete assay with either the omission of cofactors or with the denatured enzyme (boiled for 10 min).

[b]Expressed in nmol h^{-1} mg^{-1} proteins.

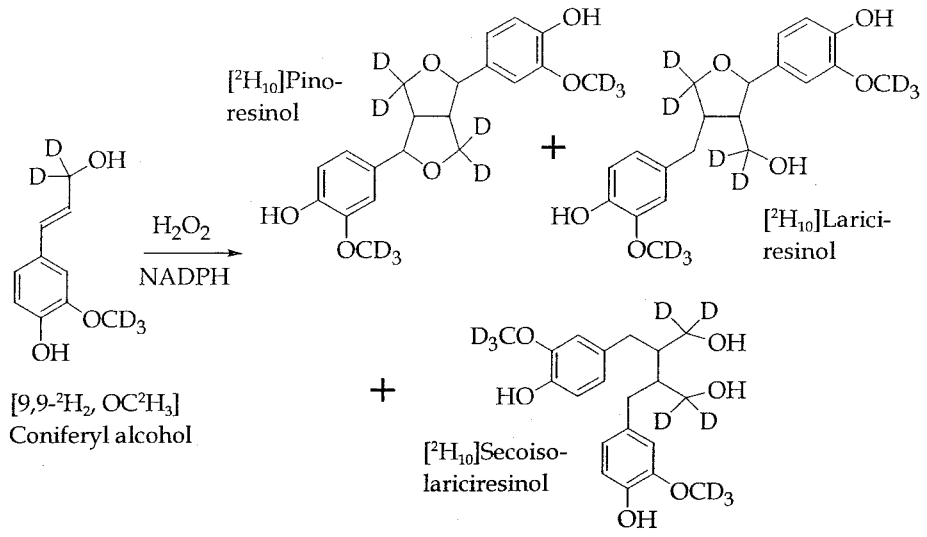

Fig. 7 Enzymatic conversion of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol to [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol.

Table 7. Enzymatic formation of [$^2H_4$]lariciresinol from (±)-[9,9,9',9'-$^2H_4$]pinoresinols.

Assay	Cofactor	[$^2H_4$]Lariciresinol formation[b]
Complete	NADPH	6.18
Control[a]	None	0
	Denatured enzyme/NADPH	0

[a]Control experiments refer to a complete assay with either the omission of a cofactor or with the denatured enzyme (boiled for 10 min).

[b]Expressed in nmol h^{-1} mg^{-1} protein.

Table 8. Enzymatic formation of [$^2H_4$]secoisolariciresinol from (±)-[9,9,9',9'-$^2H_4$] lariciresinols.

Assay	Cofactor	[$^2H_4$]Secoisolariciresinol formation[b]
Complete	NADPH	47.3
Control[a]	None	3.20
	Denatured enzyme/NADPH	11.8

[a]Control experiments refer to a complete assay with either the omission of a cofactor or with the denatured enzyme (boiled for 10 min).

[b]Expressed in nmol h^{-1} mg^{-1} protein.

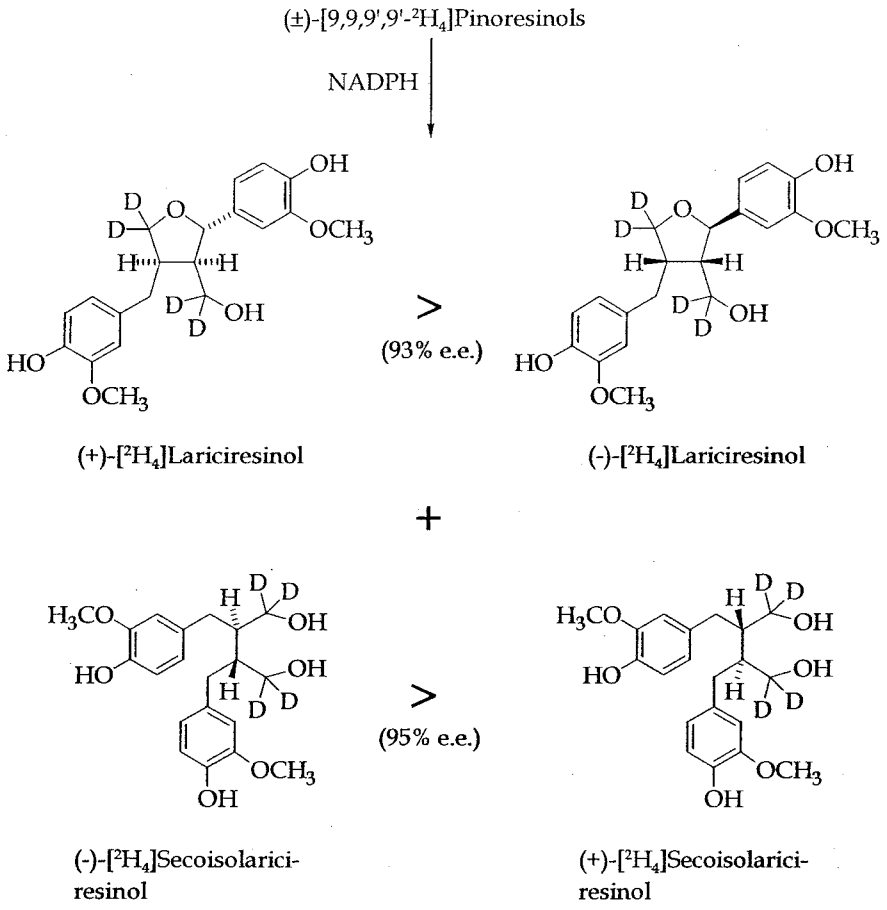

Fig. 8 Enantiomeric compositions of [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol formed enzymatically from (±)-[9,9,9',9'-$^2H_4$]pinoresinols.

resinol were formed, and their enantiomeric compositions were determined: [$^2H_4$]lariciresinol, 93% e.e. in favor of (+)-enantiomer; [$^2H_4$]secoisolariciresinol, 95% e.e. in favor of (-)-enantiomer (Fig. 8).

The PLR activity together with enzymatic formation of the lignans from coniferyl alcohol accorded well with those with *Arctium lappa* (in Part A of Chapter 1) and *Forsythia* spp [21,24-27,34]. 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 (Part A of Chapter 1).

The lignan formation by the *Anthriscus* enzyme preparation along with the detection of lariciresinol, secoisolariciresinol from the plant suggests strongly that the conversion, pinoresinol → lariciresinol → secoisolariciresinol, is operating in *A. sylvestris*, like *Forsythia* spp [2,88]. Although Dewick et al. reported the *in vivo* conversion of matairesinol to podophyllotoxin via yatein and deoxypodophyllotoxin [85,86,89], detailed pathway after secoisolariciresinol to yatein via matairesinol awaits enzymatic experiments in *A. sylvestris*, which are underway in the author's laboratory.

1.B.4 Summary

GC-MS analysis of the β-glucosidase-treated MeOH extracts of *Anthriscus sylvestris* showed, based on comparison of the mass spectra and retention times with those of authentic samples, the presence of lignans, yatein, secoisolariciresinol, lariciresinol, matairesinol, hinokinin, and pluviatolide. The existence of small amounts of bursehernin was suggested mass chromatographically. In addition, nemerosin and deoxypodophyllotoxin were tentatively identified by comparison of the mass spectra with those of literature data. Enzyme preparations from *A. sylvestris* catalyzed formation of secoisolariciresinol and lariciresinol from coniferyl alcohol. Furthermore, the enzyme preparation catalyzed the formation of lariciresinol from (±)-pinoresinols and the formation of secoisolariciresinol from (±)-lariciresinols, demonstrating pinoresinol/lariciresinol reductase (PLR) activity. Chiral HPLC analysis showed selective formation of (+)-lariciresinol and (-)-secoisolariciresinol from (±)-pinoresinols with the *A. sylvestris* PLR preparation, indicating that the stereochemical property of *A. sylvestris* PLR-catalyzed reduction was similar to those of *Forsythia* PLR and *Arctium lappa* ripening seed PLR.

Chapter 2

Establishment of norlignan biosynthetic pathway

Part A Norlignan biosynthesis in *Asparagus officinalis* L.: the norlignan originates from two non-identical phenylpropane units

2.A.1 Introduction

Typical norlignans having the 1,3-diphenylpentane [C_6-C_3(C_2)-C_6] structure {*e.g.* hinokiresinol [(*E*)-hinokiresinol], agatharesinol, and sequirin-C, Fig. 4} occur in coniferous trees (especially in heartwood) of Cupressaceae, Taxodiaceae, and Araucariaceae [15,35,90,91], while γ-lactonized 1,3-diphenylpentane norlignans (*e.g.* pueroside A and B) were isolated from two Leguminosae trees (*Pueraria lobata* and *Sophora japonica*) [92-94]. 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. 4) which is the geometrical isomer of a coniferous heartwood norlignan, (*E*)-hinokiresinol, was isolated from *Asparagus* and *Anemarrhena* [8,95,96].

During the last decade, metabolic engineering of trees has been developed significantly with the aid of the knowledge of herbaceous plant molecular biology. For example, a transgenic aspen (*Populus tremuloides*) with less lignin content and higher cellulose content has been produced by Chiang et al [97]. Now, exploiting knowledge of molecular mechanisms involved in metabolic events specific to woody plants is necessary to accelerate metabolic engineering of trees. However, nothing has been known about the molecular mechanisms specific to woody plants. Fortunately, the fact that norlignan biosynthesis is involved in heartwood formation

implies that the molecular mechanisms can be a clue to elucidate mechanisms of metabolisms specific to woody plants.

Using a fungal-elicited *A. officinalis* cell system, the author reports here the first evidence that (*Z*)-hinokiresinol is formed from the coupling of two units of phenylpropanoid monomers with the loss of one carbon atom at the 9-position of one of the monomers. In addition, the evidence indicating that two non-identical phenylpropane units, probably 4-coumaryl alcohol and a 4-coumaroyl compound, are the immediate C_6-C_3 precursors of (*Z*)-hinokiresinol was obtained based on simultaneous administration of two distinct phenylpropanoid monomers to the cultured cells.

2.A.2 Materials and Methods

Plant Material

Seeds of *Asparagus officinalis* cv. Akuseru (Takii Seed Co., Japan) were germinated aseptically on Linsmaier & Skoog agar medium (0.8% agar, 3% glucose, 4 × 10^{-4} g/l of 2,4-dichrolophenoxyacetic acid and 2 × 10^{-4} g/l of benzyladenine) [98]. Callus was formed directly from the seedlings. The cell suspension culture was obtained by transferring pieces of callus to the same medium but without agar followed by incubation under the same condition (96-98 rpm at 27°C in the dark) as reported by Terada et al [43]. The cells were subcultured every 4 weeks.

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a JNM-LA400MK FT-NMR System (JEOL Ltd.). Chemical shifts and coupling constants (*J*) are given in δ and Hz, respectively. GC-MS, DI-MS and HR-MS were performed on a JMS-DX303HF mass

spectrometer (JEOL Ltd.) equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system. GC-MS measurement conditions were as follows: electron impact mode, 70 eV; gas-chromatographic column, Shimadzu Hicap CBP-10M25-025 (5 m × 0.2 mm); temperature, 40 °C at t=0 to 2 min, then to 190 °C at 20°C/min; carrier gas, He; splitless injection. Samples dissolved in *N,O*-bis (trimethylsilyl)acetamide (BSA) were subjected to GC-MS measurement after heating at 60°C for 45 min.

Synthesis of labelled precursors

Synthesis of ^{13}C-labelled cinnamic acids. [7-^{13}C]Cinnamic acid was prepared by Knoevenagel condensation of unlabelled malonic acid and [*carbonyl*-^{13}C]benzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C). [7-^{13}C]Cinnamic acid (98 atom% excess): δ_H ($CDCl_3$; carbon numbers are shown in Fig. 10) 6.46 (1H, dd, *J* 16.0 and 1.1, 8-H), 7.40-7.42 (3H, m, Ar), 7.54-7.57 (2H, m, Ar) and 7.80 (1H, dd, *J* 156.6 and 15.9, 7-H); *m/z* (EI) 149.0549 (M^+, 85%. $C_8{}^{13}CH_8O_2$ requires *M*, 149.0558), 148 (100), 132 (23), 121 (6), 105 (11), 104 (53), 103 (21), 92 (18), 78 (24), 77 (21), 52 (13) and 51 (18). [8-^{13}C]Cinnamic acid was prepared from [2-^{13}C]malonic acid (Cambridge Isotope Laboratories, 99 atom% ^{13}C) and unlabelled benzaldehyde by a method similar to that for [7-^{13}C]cinnamic acid. [8-^{13}C]Cinnamic acid (98 atom% excess): δ_H ($CDCl_3$) 6.46 (1H, dd, *J* 162.8 and 16.1, 8-H), 7.38-7.42 (3H, m, Ar), 7.53-7.57 (2H, m, Ar) and 7.80 (1H, dd, *J* 15.9 and 2.9, 7-H); *m/z* (EI) 149.0546 (M^+, 79%), 148 (100), 132 (21), 121 (5), 105 (10), 104 (48), 103 (17), 91 (11), 78 (20), 77 (20), 52 (10) and 51 (16). [9-^{13}C]Cinnamic acid was prepared similarly from [1,3-$^{13}C_2$]malonic acid (Cambridge Isotope Laboratories, 99 atom% ^{13}C) and unlabelled benzaldehyde. [9-^{13}C]Cinnamic acid (98 atom% excess): δ_H ($CDCl_3$) 6.46 (1H, dd, *J* 15.9 and 2.7, 8-H), 7.40-7.41 (3H, m, Ar), 7.54-7.57 (2H, m, Ar) and 7.80 (1H, dd, *J* 15.9 and 6.8, 7-H); *m/z* (EI) 149.0546 (M^+, 85%), 148 (100), 132 (22), 120 (6), 104 (6), 103 (44), 102 (15), 91 (14), 77 (27) and 51 (20).

Synthesis of 4-[ring-$^{13}C_6$]coumaric acid. 4-[*ring*-$^{13}C_6$]Coumaric acid was prepared

from unlabelled malonic acid and 4-[*ring*-$^{13}C_6$]hydroxybenzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C) by a method similar to that for [7-^{13}C]cinnamic acid. 4-[*ring*-$^{13}C_6$]Coumaric acid (99 atom% excess): δ_H (acetone-d_6) 6.33 (1H, dd, *J* 16.0 and 5.2, 8-H), 6.66-7.11 (2H, m, Ar), 7.31-7.77 (2 H, m, Ar) and 7.60 (1H, ddt, *J* 16.0, 5.3 and 1.8, 7-H); *m/z* (EI) 170.0677 (M^+, 100%. $C_3{}^{13}C_6H_8O_3$ requires *M*, 170.0675), 169 (44), 168 (4), 167 (0.6), 166 (0.3), 165 (0.2), 164 (0.1), 153 (45), 152 (7), 125 (27), 124 (20), 113 (9), 96 (15) and 68 (6).

Synthesis of 4-[9,9-$^2H_2$, ring-$^{13}C_6$]coumaryl alcohol. 4-[*ring*-$^{13}C_6$]Hydroxybenzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C) was condensed with monoethyl malonate to give ethyl 4-[*ring*-$^{13}C_6$]coumarate, which was ethoxyethylated (ethyl vinyl ether, DL-10-camphorsulfonic acid, CH_2Cl_2, 0°C) to give ethyl 4-[*ring*-$^{13}C_6$]coumarate ethoxyethyl ether. This ether was reduced with $LiAl^2H_4$ (Aldrich, 98 atom% ^2H) to yield 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol ethoxyethyl ether, which was hydrolyzed with 0.1 N HCl-acetone (1:9, v/v) to afford 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol (99 atom% excess ^2H and 99 atom% excess ^{13}C): δ_H (acetone-d_6) 6.17 (1H, br d, *J* 16, 8-H), 6.49 (1H, m, 7-H), 6.55-6.99 (2H, m, Ar) and 7.02-7.48 (2H, m, Ar); *m/z* (EI) 158.1007 (M^+, 75%. $C_3{}^{13}C_6H_8{}^2H_2O_2$ requires *M*, 158.1008), 157 (10), 156 (3), 155(1), 154 (1), 153 (0.5), 152 (0.1), 151 (0.1), 150 (0.1), 139 (12), 138 (7), 114 (30), 113 (100), 112 (10), 101 (21), 100 (27), 82 (10), 70 (5) and 56 (6).

Synthesis of 4-[9-^2H, ring-$^{13}C_6$]coumaraldehyde. 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]Coumaryl alcohol ethoxyethyl ether was oxidized (activated MnO_2, CH_2Cl_2, room temperature) to afford 4-[9-^2H, *ring*-$^{13}C_6$]coumaraldehyde ethoxyethyl ether, which was hydrolyzed with HCl in acetone to give rise to 4-[9-^2H, *ring*-$^{13}C_6$]coumaraldehyde (>99 atom% excess ^2H and 99 atom% excess ^{13}C): δ_H (acetone-d_6) 6.59 (1H, dd, *J* 16.0 and 4.8, 8-H), 6.69-7.15 (2H, m, Ar), 7.37-7.83 (2H, m, Ar) and 7.58 (1H, ddt, *J* 15.9, 5.3 and 1.1, 7-H); *m/z* (EI) 155.0802 (M^+, 100%. $C_3{}^{13}C_6H_7{}^2HO_2$ requires *M*, 155.0788), 154 (85), 153 (21), 152 (2), 151 (0.4), 150 (0), 149 (2), 148 (0.8), 145 (0), 138 (29), 127 (27), 126 (24), 125 (20), 114 (7), 110 (6), 101 (17), 97 (25), 96 (17), 94 (6), 69 (11), 68 (9) and 54 (5).

Synthesis of 4-[7,9,9-$^2H_3$]coumaryl alcohol. Methyl 4-hydroxybenzoate was benzylated with benzyl bromide and K_2CO_3 to give methyl 4-hydroxybenzoate benzyl ether, which was reduced with $LiAl^2H_4$ in anhydrous THF at room temperature to yield 4-*O*-benzyl [α-$^2H_2$]benzyl alcohol. 4-*O*-Benzyl [α–$^2H_2$]benzyl alcohol was oxidized (activated MnO_2, benzene, 50°C) to afford 4-*O*-benzyl [α-^{2}H]benzaldehyde, which was hydrolyzed (conc. HCl in AcOH, refluxing temperature) to give 4-[α-^{2}H]hydroxybenzaldehyde. Then, 4-[7,9,9-$^2H_3$]coumaryl alcohol was prepared similarly to the preparation of 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol, but with 4-[α-^{2}H]hydroxybenzaldehyde as a starting material. 4-[7,9,9-$^2H_3$]Coumaryl alcohol (99 atom% excess): δ_H ($CDCl_3$) 6.17 (1H, s, 8-H), 6.76-6.80 (2H, m, Ar) and 7.24-7.27 (2H, m, Ar); *m/z* (EI) 153.0861 (M^+, 72%. $C_9H_7{}^2H_3O_2$ requires *M*, 153.0869), 152 (5), 151 (3), 150 (1), 136 (9), 109 (25), 108 (100), 95 (28), 94 (19), 78 (11), 66 (5) and 52 (5).

Preparation of fungal elicitors

An unidentified filamentous fungus was separated from the experimental field of Wood Research Institute, Kyoto University, by repeated streaking on a potato dextrose agar (Difco Laboratories). The isolated fungus was cultivated in a liquid medium (K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5g, KCl 0.5 g, Fe(III)-EDTA 20 mg, yeast extract 1 g, D-glucose 30 g, and L-asparagine 2 g per 1 l of distilled water [99]) at 27 °C for 2 weeks. *Fusarium oxysporum* f. sp. *asparagi* IFO31382 and *Fusarium solani* IFO5892 were obtained from Institute for Fermentation, Osaka. They were cultured in the same liquid media as described above. Mycelia of each fungus were collected by filtration, washed with distilled water, freeze-dried and powdered with a mortar and pestle. The powdered mycelia thus obtained were suspended in distilled water (5 g/l), autoclaved (121 °C, 20 min), and used as fungal elicitors, individually.

Isolation of (Z)-hinokiresinol

A. officinalis cells, harvested 19 days after subculture, were collected by filtration through a tea strainer. The cells (222 g, fresh weight) thus obtained were resuspended in the fungal elicitor suspension (40 ml). After incubation (120 rpm) with shaking for additional 42 h at 27°C in the dark, the cells were collected by filtration and freeze-dried. The resulting dried material (12.9 g) was powdered with a mortar and a pestle, and extracted with hot MeOH. The MeOH extract was suspended in 42 ml of 0.1 M AcONa buffer (pH 5.0) solution of β-glucosidase (SIGMA, 3.8 unit/mg, 0.175 g), and kept for 24 h at 37°C. Next, the reaction mixture was extracted with EtOAc, and the solvent was evaporated off. The resultant residue was purified with silica gel column chromatography and silica gel TLC giving rise to (Z)-hinokiresinol (1.5 mg, 0.01% based on dry cell weight): δ_H ($CDCl_3$) 4.48 (1H, dd, *J* 9.5 and 6.0, 3-H), 4.75 (1H, br s, -OH), 4.87 (1H, br s, -OH), 5.13-5.17 (2H, m, 5-H), 5.67 (1H, dd, *J* 11.3 and 10.1, 2-H), 5.96-6.04 (1H, m, 4-H), 6.51 (1H, d, *J* 11.4, 1-H), 6.76-6.79 (4H, m, Ar), 7.09 (2H, br d, *J* 8.6, Ar) and 7.16 (2H, br d, *J* 8.6, Ar); *m/z* (EI, (Z)-hinokiresinol TMS ether) 396 (M^+, 100), 382 (15), 381 (27), 230 (59), 217 (43) and 179 (38). δ_C ($CDCl_3$) is shown in Table 10.

Administration of labelled precursors

The following is the typical administration procedure for GC-MS analysis. After subculturing, *A. officinalis* cell suspension culture was incubated for 19-30 days. Then, the fungal elicitor suspension (1 ml) was aseptically added to the cells (3-5 g). The culture was incubated (120 rpm at 27°C in the dark) for additional 3 h. Next, the water solutions of labelled precursors (3 mg, dissolved in the minimal amount of 0.1 N KOH, then made up to 0.5 ml with distilled water) were added aseptically. After incubation under the same condition for additional 35 h, the cells were collected and freeze-dried. The resulting dried material (0.3-0.4 g) was powdered with a mortar and a pestle, and extracted with hot MeOH. The MeOH extract was treated with

β-glucosidase by a method similar to that for (Z)-hinokiresinol isolation, but scaled-down proportionately. An aliquot of EtOAc extracts thus obtained was submitted to GC-MS analysis after TMS derivertization with BSA.

In separate experiments, three batches of *A. officinalis* cell suspension culture were incubated for 24, 35, and 21 days, respectively. Then, the fungal elicitor suspensions (200, 200, and 80 ml) were aseptically added to the fresh cells (573, 595, and 615 g). The cultures were incubated (120 rpm at 27°C in the dark) for additional 3 h. Next, the water solutions of labelled precursors (247, 282, and 224 mg of [7-^{13}C]cinnmaic acid, [8-^{13}C]cinnmaic acid, and [9-^{13}C]cinnmaic acid, respectively, were dissolved in the minimal amount of 0.1 N KOH, then made up to 30, 26, and 18 ml with distilled water, respectively) were added aseptically. After incubation under the same condition for additional 35 h, the cells were collected and freeze-dried. The resulting dried materials (31.7, 22.1, and 45.7 g) were powdered with a mortar and a pestle, and extracted with hot MeOH. The MeOH extracts were treated with β-glucosidase, individually. Aliquots of the reaction products were subjected to GC-MS analysis, individually, as above, and the remainders were purified by a similar method to that for (Z)-hinokiresinol isolation. The purified (Z)-hinokiresinols were submitted to ^{13}C NMR measurements.

2.A.3 Results and Discussion

Asparagus officinalis cell suspension culture producing (Z)-hinokiresinol after elicitor treatment

First, *Asparagus* cell suspension culture was induced according to Terada et al. [43], and submitted to treatment with three fungal elicitors. Preliminary GC-MS analysis (data not shown) revealed that the amount of (Z)-hinokiresinol in the

cultured cells before the elicitor treatment was negligible, but increased significantly 24-45 h after any one of the elicitor treatments, indicating all the three fungal elicitors worked similarly.

Next, to confirm the production of (*Z*)-hinokiresinol unequivocally, (*Z*)-hinokiresinol was isolated chromatographically from MeOH extracts of the elicitor-treated cells followed by β-glucosidase treatment. The compound was identified by comparing the mass spectrum of its TMS ether, t_R of GC, and the ^1H NMR and ^{13}C NMR spectral data with those of (*E*)-hinokiresinol isolated from *Chamaecyparis obtusa* [14] and with the literature data of (*Z*)-hinokiresinol [9,95]. The yield was 0.01 % based on dry cell weight. GC-MS analysis of the β-glucosidase-treated MeOH extracts also showed the presence of trace amounts of (*E*)-hinokiresinol, which was identified by comparing the mass spectrum and retention time of GC with those of the authentic sample [14].

All carbon atoms of (*Z*)-hinokiresinol are derived from phenylpropanoid monomers

First, L-[*ring*-$^{13}C_6$]phenylalanine was administered (Icon, 98 atom% ^{13}C) 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 (Fig. 9*A*), 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 (Fig. 9*B*).

Similarly, cinnamic acids labelled with ^{13}C at the side chain were next administered to the *Asparagus* cells individually, and ^{13}C incorporation into (*Z*)-hinokiresinol was quantified. Table 9 shows relative intensities of molecular ion region of the mass spectra of unlabelled (*Z*)-hinokiresinol TMS ether and ^{13}C-enriched (*Z*)-hinokiresinol TMS ethers formed after the individual administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid. The high

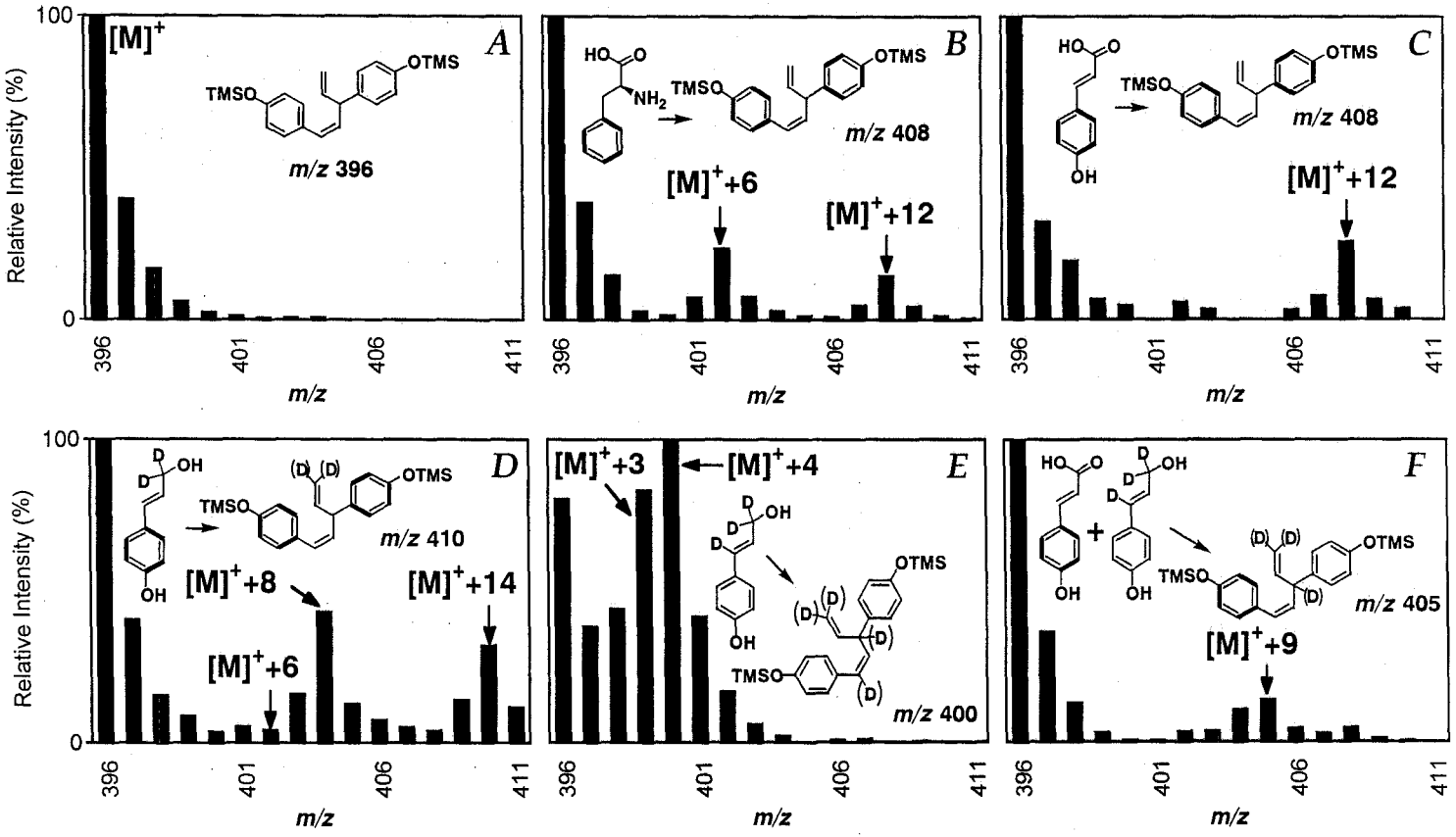

Fig. 9 Mass spectra of molecular ion region of (Z)-hinokiresinol TMS ethers. *A*, unlabelled. *B*, formed after L-[*ring*-$^{13}C_6$]phenylalanine administration. *C*, formed after 4-[*ring*-$^{13}C_6$]coumaric acid administration. *D*, formed after 4-[9,9-$^{2}H_2$, *ring*-$^{13}C_6$]coumaryl alcohol administration. *E*, formed after 4-[7,9,9-$^{2}H_3$]coumaryl alcohol administration. *F*, formed after simultaneous administration of 4-[*ring*-$^{13}C_6$]coumaric acid and 4-[7,9,9-$^{2}H_3$]coumaryl alcohol. ^{13}C atoms are shown as bold lines in aromatic rings, and D on the chemical structures represents ^{2}H.

intensities at *m/z* 398 were observed when [7-^{13}C]cinnamic acid and [8-^{13}C]cinnamic acid were administered, clearly indicating that two ^{13}C atoms were incorporated into (Z)-hinokiresinol from [7-^{13}C]cinnamic acid and [8-^{13}C]cinnamic acid, respectively. On the other hand, in the case of [9-^{13}C]cinnamic acid administration, the ion intensities of *m/z* 397 and 396 were almost equal, indicating the enrichment by one ^{13}C atom. At the same time, the intensity of *m/z* 398 was 39.7% of that of *m/z* 397, nearly identical to the ion intensity ratio between *m/z* 398 and 397 of the unlabelled one, and revealing that incorporation of two ^{13}C atoms into (Z)-hinokiresinol from [9-^{13}C]cinnamic acid did not occur.

Table 9. Mass spectral data of molecular ion region of unlabelled and labelled (Z)-hinokiresinols (TMS ether) formed by *A. officinalis*.

m/z	Relative intensity (%)[a]			
	Unlabelled	Administered cinnamic acids (CAs)		
		[7-^{13}C]CA	[8-^{13}C]CA	[9-^{13}C]CA
395	27.3			
396	100	100	100	100
397	39.9	67.1	59.6	100.4
398	15.8	114	55.7	39.9

[a]Relative intensity was calculated on the basis of peak intensity at *m/z* 396, which is the molecular ion of unlabelled (Z)-hinokiresinol. The values are the average of triplicated measurements.

In order to confirm the mass spectral analysis and to determine the ^{13}C-enriched position in the side chain of (Z)-hinokiresinol, ^{13}C-enriched (Z)-hinokiresinols formed after administration of the three [^{13}C]cinnamic acids were isolated in separate experiments, and then they were submitted to ^{13}C NMR measurements. As shown in Table 10, 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). Although the signal of C-5 of (Z)-hinokiresinol appears closely with C-3", C-5", C-3', and C-5', C-H correlation in HMQC spectra indicates that the enhanced signal in the [9-^{13}C]cinnamic acid administration was assigned to C-5 (data not shown). Thus, these ring (Fig. 9*B*) and side chain (Tables 9 and 10) ^{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. 10). Thus, intramolecular rearrangement of the side chain carbon atoms of the monomers did not occur in (Z)-hinokiresinol formation.

Table 10. ^{13}C enrichments of carbons in (Z)-hinokiresinol isolated following administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid in the elicited *A. officinalis* cells.

Carbon Number (*N*)	δ_C ($CDCl_3$)	Atom% ^{13}C excess[a]		
		Administered cinnamic acids (CAs)		
		[7-^{13}C]CA	[8-^{13}C]CA	[9-^{13}C]CA
4'	154.6	0	0	0
4"	154.1	0.04	-0.02	0.03
4	140.8	-0.10	31.2	-0.01
1"	135.7	-0.27	0.21	0.07
2	131.8	-0.19	32.3	0.20
2', 6'	130.1	-0.21	0.39	-0.01
1'	129.9	0.41	0.55	0.55
2", 6"	128.9	-0.02	0.29	0.15
1	128.7	11.7	-0.08	0.18
3", 5"	115.4	0.04	0.56	-0.04
3', 5'	115.2	0.07	0.36	0.00
5	115.1	0.17	-0.17	26.3
3	46.8	10.6	0.25	0.05

[a]Atom% ^{13}C excess = $\{R_N / R_{N(UL)}\} \times 1.1\text{-}1.1$, where R_N is the ratio of the peak intensity at *N*-position in labelled (Z)-hinokiresinol calculated on the basis of the peak intensity at 4'-position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at *N*-position in unlabelled (Z)-hinokiresinol. The value 1.1 is theoretical ^{13}C natural abundance (atom%).

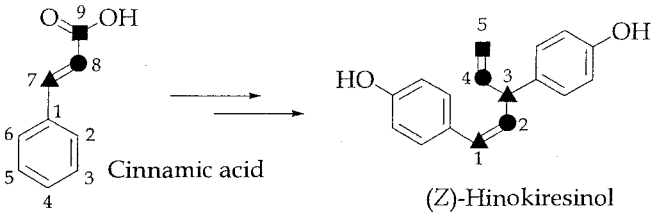

Fig. 10 ^{13}C-Labelling patterns of (*Z*)-hinokiresinol incorporating [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, or [9-^{13}C]cinnamic acid. ▲, ●, ■: ^{13}C.

The immediate C_6-C_3 precursors of (*Z*)-hinokiresinol

The author's attention was next focused on the immediate C_6-C_3 (phenylpropanoid monomer) precursor(s) of (*Z*)-hinokiresinol. The author 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 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) (Fig. 9*C*), 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 ($[M]^+$+14) was observed (Fig. 9*D*). This result indicated 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) (Fig. 9*D*) 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 (Fig. 9*B*). 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. Also, in the case of 4-[9-^{2}H, *ring*-$^{13}C_6$]coumaraldehyde feeding, the ion peak at *m/z* 403 ($[M]^+$+7) was increased in addition to the enhancement at *m/z* 409 ($[M]^+$+13) (data not shown). Interestingly, however, the ion peak at *m/z* 402 ($[M]^+$+6, (*Z*)-[$^{13}C_6$]hinokiresinol TMS ether) (Fig. 9*D*) 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 implies 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 [100-102]. 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-^{2}H, *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. 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. Again, as shown in Fig. 9*C*, 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}. Similarly, 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) (Fig. 9*E*) which corresponded to (*Z*)-[$^2H_2$, $^{13}C_{12}$]hinokiresinol TMS ether ($[M]^+$+14) and (*Z*)-[$^2H_2$, $^{13}C_6$]hinokiresinol ($[M]^+$+8), respectively, in the 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol administration (Fig. 9*D*). In sharp contrast, the simultaneous administration of the two precursors (Fig. 9*F*) 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 (Fig. 9*A*). The ion peak at *m/z* 405 ($[M]^+$+9) was prominent, and was derived by the coupling of one 4-[7,9,9-$^2H_3$]coumaryl alcohol unit and with 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. 11).

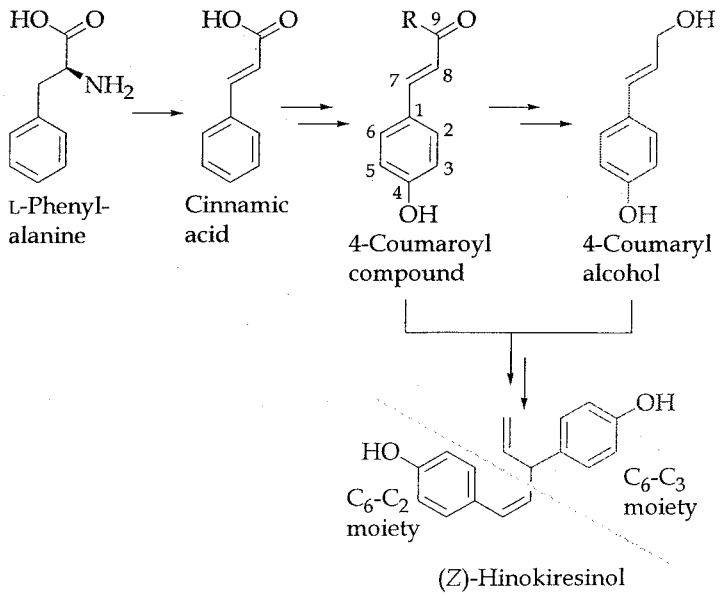

Fig. 11 Proposed biosynthetic pathway for (Z)-hinokiresinol.

Furthermore, the incorporation of four deuterium atoms into (Z)-hinokiresinol from 4-[7,9,9-$^2H_3$]coumaryl alcohol (Fig. 9*E*) indicates that the hydrogen atom at the 7-position of 4-coumaryl alcohol is retained in (Z)-hinokiresinol, which therefore eliminates the oxidation at the 7-position of the monomer to C-7 carbonyl group, and the intermediacy of 3-(4-hydroxyphenyl)-3-oxopropionic acid derivatives as precursors in the formation of (Z)-hinokiresinol (Fig. 12). Also, the results suggest that 1,3-bis(4-hydroxyphenyl)-4-pentene-1-one, which was isolated from *Anemarrhena asphodeloides* together with (Z)-hinokiresinol [103], is not a prerequisite intermediate of (Z)-hinokiresinol biosynthesis (Fig. 12), since this compound does not have a proton at the 1-position.

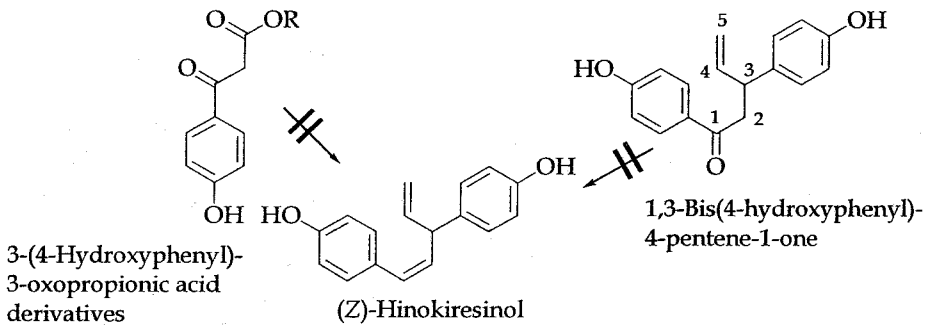

Fig. 12 The involvement of 3-(4-hydroxyphenyl)-3-oxopropionic acid derivatives and 1,3-bis(4-hydroxyphenyl)-4-pentene-1-one was not prerequisite in (Z)-hinokiresinol biosynthesis.

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

2.A.4 Summary

Little is known about the biosynthetic mechanism of norlignans with C_6-C_5-C_6 skeletons in spite of their important contributions to the heartwood formation in conifers. To clarify the mechanism, the author established cell suspension cultures of *Asparagus officinalis* that produce a norlignan, (Z)-hinokiresinol, after fungal elicitor treatment. Feeding experiments with ring or side chain ^{13}C-and/or ^{2}H-labelled phenylpropanoid monomers showed that two units of L-phenylalanine, cinnamic acid, 4-coumaric acid, or 4-coumaryl alcohol were efficiently incorporated into the norlignan. ^{13}C NMR of (Z)-hinokiresinols isolated after individual administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid 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. Thus, ring- and side chain-labelled tracer results indicated that all carbon atoms of (Z)-hinokiresinol were found to originate from C_6-C_3 (phenylpropanoid) monomers, and this compound was formed with a loss of one carbon atom at the 9-position of one of the coupling monomers. Furthermore, a competitive tracer experiment with simultaneous administration of 4-[*ring*-$^{13}C_6$]coumaric acid and 4-[7,9,9-$^{2}H_3$]coumaryl alcohol indicated that the C_6-C_3 moiety of (Z)-hinokiresinol was derived from 4-coumaryl alcohol, while the C_6-C_2 moiety originated from a 4-coumaroyl compound such as 4-coumaroyl CoA and not directly from 4-coumaryl alcohol.

Part B First *in vitro* norlignan formation with *Asparagus officinalis* enzyme preparation

2.B.1 Introduction

The author's next attention was focused on an enzyme which is responsible for the formation of norlignan carbon framework from phenylpropanoid monomers. In Part B of Chapter 2, the author reports for the first time the enzymatic formation of (*Z*)-hinokiresinol from two distinct phenylpropanoid monomers, 4-coumaryl alcohol and 4-coumaroyl CoA, and from a phenylpropanoid dimer, 4-coumaryl 4-coumarate.

2.B.2 Materials and Methods

Plant material

Cell suspension cultures of *Asparagus officinalis* L. cv. Akuseru were used as described in Part A of Chapter 2.

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a JNM-LA400MK FT-NMR System (JEOL Ltd.). Chemical shifts and coupling constants (*J*) are given in δ and Hz, respectively. GC-MS was performed exactly the same as described in Part A of Chapter 2. Samples dissolved in *N,O*-bis(trimethylsilyl)acetamide (BSA) were subjected to GC-MS measurement after heating at 60°C for 45 min.

Compounds

4-[7,9,9-$^2H_3$]Coumaryl alcohol was synthesized in Part A of Chapter 2. 4-Coumaroyl CoA was a gift of Mr. Tomoyuki Nakatsubo. 4-[7,9,9-$^2H_3$]Coumaryl 4-coumarate was synthesized by a similar method of Grabber et al. [104], using 4-[7,9,9-$^2H_3$]coumaryl alcohol and unlabelled 4-coumarate (purchased from Tokyo Kasei Kogyo Co.) as starting materials: δ_H (acetone-d_6, carbon numbers are shown in Fig. 14) 6.21 (1H, s, 8-H), 6.37 (1H, d, *J* 16.0, 8'-H), 6.81 (2H, d, *J* 8.8, Ar), 6.89 (2H, d, *J* 8.6, Ar), 7.33 (2H, d, *J* 8.6, Ar), 7.55 (2H, d, *J* 8.6, Ar) and 7.63 (1H, d, *J* 16.0, 7'-H).

Enzyme preparation

A fungal elicitor suspension derived from *Fusarium solani* IFO5892 was prepared as described in Part A of Chapter 1. The fungal elicitor suspension (5 ml) was added to *A. officinalis* cell suspension cultures. After incubation for 21-22 h (120 rpm, at 27°C in the dark), the cells were collected by filtration. The following procedure was carried out at 4°C. The cells (6.92 g) were frozen (liquid N_2) and powdered with a pestle and a mortar. The powder thus obtained was further ground with polyclar AT (1.38 g), acid-washed sea sand (3.59 g), and 9 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol (DTT). The slurry thus obtained was filtered through 4 layers of gauze, and the filtrate (5.5 ml) was centrifuged (10,000×g, 20 min). After centrifugation, the supernatant was filtered through a Whatman GF/A glass fiber filter. An aliquot of the supernatant (4 ml) was applied to a Sephadex G-25 column that had been pre-equilibrated with 0.1 M potassium phosphate buffer (pH 8.0) containing DTT. The fraction excluded from the column was collected, and to the fraction added ammonium sulfate (0-70% saturation). After kept for 1 h, the precipitates were collected by centrifugation (10,000×g, 15 min), and redissolved into 0.05 M potassium phosphate buffer (pH 7.0). Then, the solution was desalted by passing through a Sephadex G-25 column which had been pre-equilibrated and was eluted with 0.05 M potassium phosphate buffer

(pH 7.0). The excluded fraction from the column was used as an enzyme preparation.

Conversion of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA to (Z)-[$^2H_3$]hinokiresinol

Complete assay mixture consisted of 120 μl of the enzyme preparation (protein content was 2.6-6.4 mg/ml), 5 μl of 25 mM 4-[7,9,9-$^2H_3$]coumaryl alcohol in MeOH, and 15 μl of 5 mM 4-coumaroyl CoA in 0.05 M potassium phosphate buffer (pH 7.0). After incubating for 1 h at 27°C, the reaction mixture was extracted with EtOAc containing unlabelled (Z)-hinokiresinol isolated in Part A of Chapter 1 as an internal standard. The EtOAc extract was dried under high vacuum and analyzed by GC-MS after TMS derivertization with BSA.

Conversion of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate to (Z)-[$^2H_3$]hinokiresinol

Complete assay consisted of 120 μl of the enzyme preparation (protein content was 5.3 mg/ml), 120 μl of 0.05 M potassium phosphate buffer (pH 7.0), and 10 μl of 25 mM 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate in MeOH. The mixture was incubated for 1 h at 27 °C, then the reaction mixture was extracted with EtOAc containing unlabelled (Z)-hinokiresinol as described above. The EtOAc extract was dried under high vacuum and analyzed by GC-MS after TMS derivertization with BSA.

Administration of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate to (Z)-[$^2H_3$]hinokiresinol

After subculturing, *A. officinalis* cell suspension culture was incubated for 21 days. Then, the fungal elicitor suspension (5 ml) was aseptically added to the cells (5 g). The culture was incubated (120 rpm at 27°C in the dark) for additional 3 h. Next, solution of the labelled precursor (3 mg, dissolved in 250 μl of DMSO, then made up to 500 μl with distilled water) was added aseptically. After incubation under the same condition for additional 30 h, the cells were collected and freeze-dried. The

resulting dried material (0.3 g) was disintegrated with scissors and extracted with hot MeOH. The MeOH extract was treated with β-glucosidase as described in Part A of Chapter 2. An aliquot of EtOAc extracts thus obtained was submitted to GC-MS analysis after TMS derivertization with BSA.

2.B.3 Results and Discussion

First, *Asparagus officinalis* enzyme preparation was incubated with 4-[7,9,9,-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA but without exogenously supplied cofactors, and the reaction products were subjected to GC-MS analysis. The mass spectrum (Fig. 13*A*) of a GC peak of (*Z*)-hinokiresinol TMS ether shows ions at *m/z* 399, 233, and 219 besides ions (*m/z* 396, 230, and 217) derived from the internal standard unlabelled (*Z*)-hinokiresinol TMS ether, indicating the formation of (*Z*)-[$^2H_3$]hinokiresinol from 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA.

Proof that the formation of (*Z*)-[$^2H_3$]hinokiresinol was enzymatic was obtained by control experiments as summarized in Table 11A. Thus, 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 (boiled for 10 min) 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 (data not shown), 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.

The author next hypothesized 4-coumaryl 4-coumarate as an intermediate

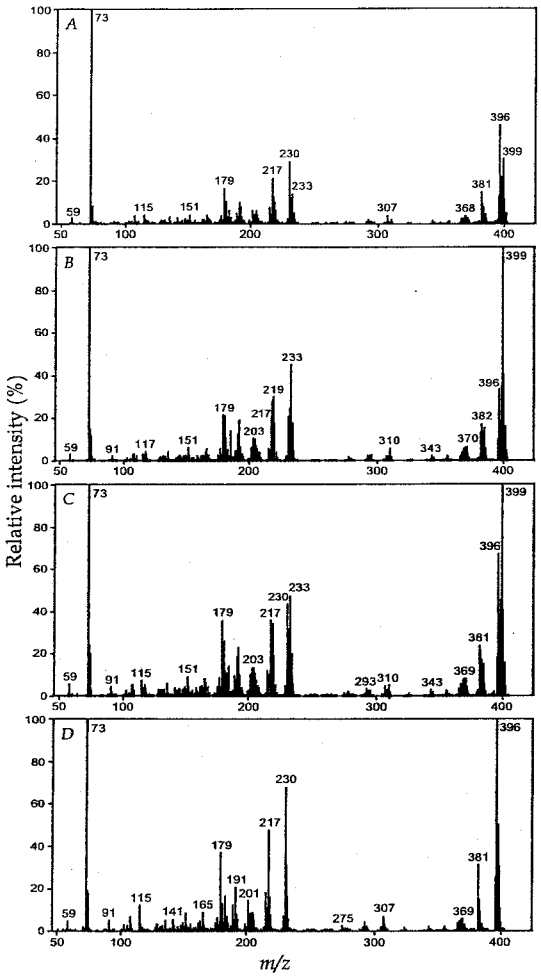

Fig. 13 Mass spectra of deuterated and unlabelled (Z)-hinokiresinol TMS ethers. *A*, formed after incubation of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA with the enzyme preparation. *B*, formed after administration of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate to *A. officinalis* cultured cells. *C*, formed after incubation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate with the enzyme preparation. *D*, unlabelled (=isolated from *A. officinalis*). Note that unlabelled (Z)-hinokiresinol (M^+=*m/z* 396) was added as an internal standard in the case of Figs. 13*A* and 13*C*.

compound in this enzymatic reaction. To test this hypothesis, the author synthesized 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate, then it was administered to the fungal-elicited *A. officinalis* cells. The mass spectrum (Fig. 13*B*) of (*Z*)-hinokiresinol (TMS ether) obtained following the administration shows the significant ions at *m/z* 399, 233, and 219 besides *m/z* 396, 230, and 217 which are ascribed to endogenous unlabelled (*Z*)-hinokiresinol, indicating the transformation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate into (*Z*)-[$^2H_3$]hinokiresinol.

Table 11. Enzymatic formation of (*Z*)-[$^2H_3$]hinokiresinol.

Substrate(s)		System	(*Z*)-[$^2H_3$]hinokiresinol formation (nmol h^{-1} mg^{-1} protein)
A	4-Coumaroyl CoA and 4-[7,9,9-$^2H_3$]coumaryl alcohol	Complete	3.4
		- Enzyme	0
		- 4-Coumaroyl CoA	0.2
		- 4-[7,9,9-$^2H_3$]Coumaryl alcohol	0.1
		- 4-Coumaroyl CoA and - 4-[7,9,9-$^2H_3$]coumaryl alcohol	0
		Denatured enzyme	0
B	4-[7,9,9-$^2H_3$]Coumaryl 4-coumarate	Complete	98.5
		- Enzyme	0.9
		- 4-[7,9,9-$^2H_3$]Coumaryl 4-coumarate	0
		Denatured enzyme	0

Furthermore, the conversion of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate to (*Z*)-[$^2H_3$]hinokiresinol was also demonstrated by an *in vitro* experiment; incubation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate with the enzyme preparation resulted in (*Z*)-[$^2H_3$]hinokiresinol formation (Fig. 13C and Table 11B).

These *in vivo* and *in vitro* experiments and the fact that esters are often biosynthesized by condensation between the corresponding CoA esters and alcohols [105] suggest strongly that 4-coumaryl 4-coumarate is the intermediate between the phenylpropanoid monomers (4-coumaryl alcohol and 4-coumaryl CoA) and

(Z)-hinokiresinol, although the author could detect neither *in vivo* nor *in vitro* formation of 4-coumaryl 4-coumarate from 4-coumaryl alcohol and 4-coumaroyl CoA. This may be due to 4-coumaryl 4-coumarate being unstable in the GC column under the condition used.

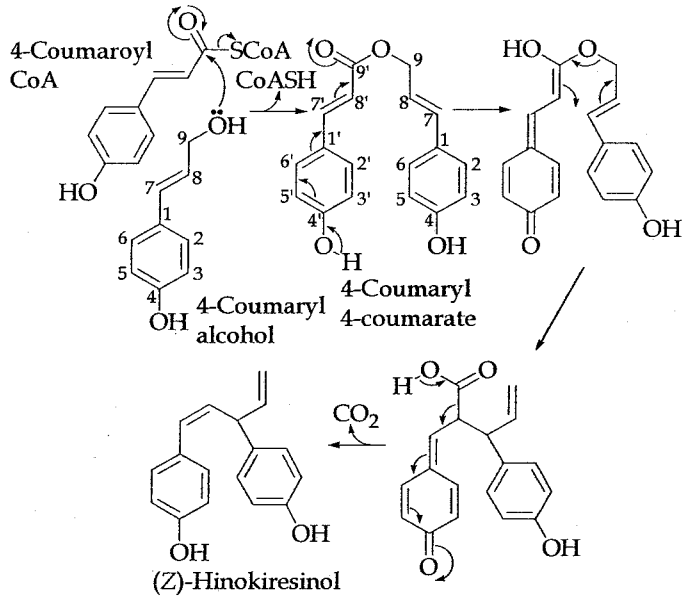

Fig. 14 Proposed mechanism of (Z)-hinokiresinol formation from phenylpropanoid monomers.

Since allyl esters can rearrange to γ,δ-unsaturated carboxylic acids in a reaction called the ester enolate Claisen rearrangement [106], the author proposes the mechanism illustrated in Fig. 14 for the formation of (Z)-hinokiresinol from the two phenylpropanoid monomers, 4-coumaryl alcohol and 4-coumaroyl CoA. This is initiated by esterification of the two monomers, followed by the ester enolate Claisen rearrangement (enolization of the ester 4-coumaryl 4-coumarate and [3,3]-sigmatropic rearrangement of the enol). Subsequent decarboxylation of the rearrangement product completes the formation of (Z)-hinokiresinol. This mechanism accords well with our previous finding that a loss of C-9 of a 4-coumaroyl type monomer such as 4-coumaroyl CoA occurred in the formation of (Z)-hinokiresinol as concluded in Part A of Chapter 2. An enzyme-catalyzed

[3,3]-sigmatropic rearrangement is rare [107], and, to the author's knowledge, there has been no report of enzymatic ester enolate Claisen rearrangement. Hence, it is of special interest to elucidate the detailed mechanism for the (Z)-hinokiresinol formation.

In conclusion, the present study has demonstrated for the first time the enzyme activity to form (Z)-hinokiresinol from 4-coumaryl alcohol and 4-coumaroyl CoA, and from 4-coumaryl 4-coumarate.

2.B.3 Summary

The author reported for the first time that an enzyme preparation from fungal-elicited *Asparagus officinalis* cultured cells catalyzes 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 exogenously supplied cofactors.

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 selectivities.

The biosynthetic pathway of a norlignan, (Z)-hinokiresinol, was proposed. Thus, the coupling of 4-coumaryl alcohol with 4-coumaroyl CoA afforded (Z)-hinokiresinol. In addition, the activity of enzyme catalyzing the formation of (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 biosynthetic mechanism of (Z)-hinokiresinol: (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 ester enolate Claisen rearrangement and subsequent decarboxylation.

Acknowledgements

The author wishes to thank Professor Dr. Mikio Shimada, Wood Research Institute, Kyoto University, for many helpful discussions and suggestions during this work. The author is also thankful to Professor Dr. Yasumasa Kuwahara, Graduate School of Agriculture, Kyoto University, and Professor Dr. Kanzo Sakata, Institute for Chemical Research, Kyoto University, for critical reading of this thesis. The author is greatly indebted to Associate Professor Dr. Toshiaki Umezawa, Wood Research Institute, Kyoto University, for many helpful discussions and critical reading of this thesis. The author is thankful to the following people: Dr. Guy Gusman, Faculty of Science, Université Libre de Bruxelles, for supplying *Anthriscus sylvestris* seeds; Associate Professor Dr. Shingo Kawai, Faculty of Agriculture, Gifu University, for supplying (±)-4-demethylyatein benzyl ethers; Mr. Tomoyuki Nakatsubo, Wood Research Institute, Kyoto University, for a kind gift of 4-coumaroyl CoA. Finally, the author would like to thank Instructor Dr. Takefumi Hattori and the other members in Laboratory of Biochemical Control, Wood Research Institute, Kyoto University, for their kind help and encouragements.

References

[1] Haworth, R.D. (1936) Annual Reports on the Progress of Chemistry 33, 266.

[2] Umezawa, T. (2001) Regulation of Plant Growth & Development 36, 57.

[3] Kai, Y. (1965) Mokuzai Gakkaishi 11, 23.

[4] Hatam, N.A.R. and Whiting, D.A. (1967) Tetrahedron Letters, 781.

[5] Erdtman, H. and Harmatha, J. (1979) Phytochemistry 18, 1495.

[6] Iida, Y., Oh, K.-B., Saito, M., Matsuoka, H., Kurata, H., Natsume, M. and Abe, H. (1999) Journal of Agricultural Food Chemistry 47, 584.

[7] Yamada, T. (1998) Bulletin of the Forestry and Forest Products Research Institute, 69.

[8] Oketch-Rabah, H.A., Dossaji, S.F., Christensen, S.B., Frydenvang, K., Lemmich, E., Cornett, C., Olsen, C.E., Chen, M., Kharazmi, A. and Theander, T. (1997) Journal of Natural Products 60, 1017.

[9] Minami, E., Taki, M., Takaishi, S., Iijima, Y., Tsutsumi, S. and Akiyama, T. (2000) Chemical and Pharmaceutical Bulletin 48, 389.

[10] Canel, C., Moraes, R.M., Dayan, F.E. and Ferreira, D. (2000) Phytochemistry 54, 115.

[11] Shimaji, K. (1985) in: Structure of Wood, pp. 119 (Harada, H. and Saeki, H., Eds.) Buneido Shuppan Co., Tokyo, Japan.

[12] Kawamura, I. and Higuchi, T. (1962) Mokuzai Gakkaishi 8, 148.

[13] Nobuchi, T., Takai, K. and Harada, H. (1987) Mokuzai Gakkaishi 33, 88.

[14] Takaku, N., Choi, D.-H., Mikame, K., Okunishi, T., Suzuki, S., Ohashi, H., Umezawa, T. and Shimada, M. (2001) Journal of Wood Science 47, 476.

[15] Umezawa, T. (2000) in: Wood and Cellulosic Chemistry, 2nd ed., pp. 213 (Hon, D.N.-S. and Shiraishi, N., Eds.) Marcel Dekker Inc., New York, USA.

[16] Kai, Y., Kuroda, H. and Teratani, F. (1972) Mokuzai Gakkaishi 18, 315.

[17] Takahashi, K. (1981) Mokuzai Gakkaishi 27, 654.

[18] Ohashi, H., Hayashi, H., Yamada, M. and Yasue, M. (1987) Research Bulletin of Faculty of Agriculture, Gifu University 52, 131.

[19] in: Japan Statistical Yearbook 2001, pp. 246 (Statistics Bureau, Management and Coordination Agency, Government of Japan, Ed.) Japan Statistical Association & The Mainichi Newspapers, Tokyo, Japan.

[20] Johansson, I., Saddler, J.N. and Beatson, R.P. (2000) Holzforschung 54, 246.

[21] Umezawa, T., Davin, L.B. and Lewis, N.G. (1991) The Journal of Biological Chemistry 266, 10210.

[22] Umezawa, T. and Shimada, M. (1996) Mokuzai Gakkaishi 42, 180.

[23] Umezawa, T. and Shimada, M. (1996) Bioscience, Biotechnology, and Biochemistry 60, 736.

[24] Umezawa, T., Davin, L.B. and Lewis, N.G. (1990) Biochemical and Biophysical Research Communications 171, 1008.

[25] Katayama, T., Davin, L.B. and Lewis, N.G. (1992) Phytochemistry 31, 3875.

[26] Katayama, T., Davin, L.B., Chu, A. and Lewis, N.G. (1993) Phytochemistry 33, 581.

[27] Chu, A., Dinkova, A., Davin, L.B., Bedgar, D.L. and Lewis, N.G. (1993) The Journal of Biological Chemistry 268, 27026.

[28] Davin, L.B., Bedgar, D.L., Katayama, T. and Lewis, N.G. (1992) Phytochemistry 31, 3869.

[29] Paré, P.W., Wang, H.-B., Davin, L.B. and Lewis, N.G. (1994) Tetrahedron Letters 35, 4731.

[30] Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S. and Lewis, N.G. (1997) Science 275, 362.

[31] Dinkova-Kostova, A.T., Gang, D.R., Davin, L.B., Bedgar, D.L., Chu, A. and Lewis, N.G. (1996) The Journal of Biological Chemistry 271, 29473.

[32] Xia, Z.-Q., Costa, M.A., Pelissier, H.C., Davin, L.B. and Lewis, N.G. (2001) The Journal of Biological Chemistry 276, 12614.

[33] Ozawa, S., Davin, L.B. and Lewis, N.G. (1993) Phytochemistry 32, 643.

[34] Umezawa, T., Kuroda, H., Isohata, T., Higuchi, T. and Shimada, M. (1994) Bioscience, Biotechnology, and Biochemistry 58, 230.

[35] Whiting, D.A. (1987) Natural Product Reports, 499.

[36] Nicoletti, M., Galeffi, C., Messana, I. and Marini-Bettolo, G.B. (1992) Journal of Ethnopharmacology 36, 95.

[37] Begley, M.J., Davies, R.V., Henley-Smith, P. and Whiting, D.A. (1973) Journal of the Chemical Society, Chemical Communications, 649.

[38] Enoki, A., Takahama, S. and Kitao, K. (1977) Mokuzai Gakkaishi 23, 579.

[39] Enzell, C.R. and Thomas, B.R. (1966) Tetrahedron Letters, 2395.

[40] Birch, A.J. and Liepa, A.J. (1978) in: Chemistry of Lignans, pp. 320 (Rao, C.B.S., Ed.) Andhra University Press, Andhra Pradesh, India.

[41] Beracierta, A.P. and Whiting, D.A. (1978) Journal of the Chemical Society Perkin Transactions 1, 1257.

[42] Takasugi, M. (1993) Kagaku To Seibutsu 31, 22.

[43] Terada, K., Honda, C., Suwa, K., Takeyama, S., Oku, H. and Kamisako, W. (1995) Chemical and Pharmaceutical Bulletin 43, 564.

[44] Terada, K., Suwa, K., Takeyama, S., Honda, C. and Kamisako, W. (1996) Biological and Pharmaceutical Bulletin 19, 748.

[45] Terada, K. and Kamisako, W. (1999) Biological and Pharmaceutical Bulletin 22, 561.

[46] Terada, K., Honda, C., Takeyama, S., Suwa, K. and Kamisako, W. (1995) Biological and Pharmaceutical Bulletin 18, 1472.

[47] Umezawa, T., Okunishi, T. and Shimada, M. (1997) Wood Research 84, 62.

[48] Umezawa, T., Davin, L.B., Yamamoto, E., Kingston, D.G.I. and Lewis, N.G.

(1990) Journal of the Chemical Society, Chemical Communications, 1405.

[49] Umezawa, T. (1996) Mokuzai Gakkaishi 42, 911.

[50] Umezawa, T. (1997) in: Biochemistry and Molecular Biology of Wood, pp. 181 (Higuchi, T., Ed.) Springer-Verlag, Berlin, Germany.

[51] Umezawa, T., Okunishi, T. and Shimada, M. (1998) in: ACS Symposium Series 697: Lignin and Lignan Biosynthesis, pp. 377 (Lewis, N.G. and Sarkanen, S., Eds.) The American Chemical Society, Washington, DC, USA.

[52] Okunishi, T., Umezawa, T. and Shimada, M. (2000) Journal of Wood Science 46, 234.

[53] Okunishi, T., Umezawa, T. and Shimada, M. (2001) Journal of Wood Science 47, 383.

[54] Shinoda, J. and Kawagoye, M. (1929) Yakugaku Zasshi 49, 565.

[55] Hänsel, R., Schultz, H. and Leuckert, C. (1964) Zeitschrift für Naturforschung 19, 727.

[56] Yamanouchi, S., Takido, M., Sankawa, U. and Shibata, S. (1976) Yakugaku Zasshi 96, 1492.

[57] Han, B.H., Kang, Y.H., Yang, H.O. and Park, M.K. (1994) Phytochemistry 37, 1161.

[58] Yakhontova, L.D. and Kibal'chich, P.N. (1971) Khimiya Prirodnykh Soedinenii 7, 299.

[59] Ichihara, A., Kanai, S., Nakamura, Y. and Sakamura, S. (1978) Tetrahedron Letters, 3035.

[60] Ichihara, A., Oda, K., Numata, Y. and Sakanuma, S. (1976) Tetrahedron Letters, 3961.

[61] Ichihara, A., Numata, Y., Kanai, S. and Sakanuma, S. (1977) Agricultural and Biological Chemistry 41, 1813.

[62] Omaki, T. (1935) Yakugaku Zasshi 55, 816.

[63] Umezawa, T., Isohata, T., Kuroda, H., Higuchi, T. and Shimada, M. (1992) in: Biotechnology in Pulp and Paper Industry, pp. 507 (Kuwahara, M. and Shimada, M., Eds.) Uni Publishing, Tokyo, Japan.

[64] Umezawa, T. and Shimada, M. (1994) Mokuzai Gakkaishi 40, 231.

[65] Bradford, M. (1976) Analytical Biochemistry 72, 248.

[66] Umezawa, T. and Higuchi, T. (1989) in: Modern Methods of Plant Analysis, Vol. 10, pp. 161 (Linskens, H.F. and Jackson, J.F., Eds.) Springer-Verlag, Berlin, Germany.

[67] Katayama, T., Masaoka, T. and Yamada, H. (1997) Mokuzai Gakkaishi 43, 580.

[68] Fujita, M., Gang, D.R., Davin, L.B. and Lewis, N.G. (1999) The Journal of the Biological Chemistry 274, 618.

[69] Gang, D.R., Costa, M.A., Fujita, M., Dinkova, K.A.T., Wang, H.B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B. and Lewis, N.G. (1999) Chemistry and Biology 6, 143.

[70] Ayres, D.C. and Loike, J.D. (1990) in: Lignans, Cambridge University Press, Cambridge, UK.

[71] Sackett, D. (1993) Pharmacology & Therapeutics 59, 163.

[72] MacRae, W.D. and Towers, G.H.N. (1984) Phytochemistry 23, 1207.

[73] Cragg, G., Boyd, M., Khanna, R., Newman, D. and Sausville, E. (1999) in: Recent Advances in Phytochemistry, Vol. 33, pp. 1 (Romeo, J., Ed.) Kluwer Academic/Plenum Publishers, New York, USA.

[74] Konuklugil, B. (1996) Fitoterapia 67, 379.

[75] Broomhead, A.J. and Dewick, P.M. (1990) Phytochemistry 29, 3839.

[76] Noguchi, T. and Kawanami, M. (1940) Yakugaku Zasshi 60, 629.

[77] Kozawa, M., Morita, N. and Hata, K. (1978) Yakugaku Zasshi 98, 1486.

[78] Kurihata, T., Kikuchi, M., Suzuki, S. and Hisamichi, S. (1978) Yakugaku Zasshi 98, 1586.

[79] Turabelidze, D.G., Mikaya, G.A., Kemertelidze, É.P. and Vul'son, N.S. (1982) Soviet Journal of Bioorganic Chemistry (English Translation) 8, 374.

[80] Ikeda, R., Nagao, T., Okabe, H., Nakano, Y., Matsunaga, H., Katano, M. and Mori, M. (1998) Chemical and Pharmaceutical Bulletin 46, 875.

[81] Ikeda, R., Nagao, T., Okabe, H., Nakano, Y., Matsunaga, H., Katano, M. and Mori, M. (1998) Chemical and Pharmaceutical Bulletin 46, 871.

[82] Koh, D. and Lim, Y. (1999) Agricultural Chemistry and Biotechnology 42, 208.

[83] Xia, Z.-Q., Costa, M.A., Proctor, J., Davin, L.B. and Lewis, N.G. (2000) Phytochemistry 55, 537.

[84] Weiss, S.G., Tin-Wa, M., Perdue, R.E., Jr. and Farnsworth, N.R. (1975) Journal of Pharmaceutical Sciences 64, 95.

[85] Jackson, D.E. and Dewick, P.M. (1984) Phytochemistry 23, 1037.

[86] Kamil, W.M. and Dewick, P.M. (1986) Phytochemistry 25, 2093.

[87] Pelter, A. (1968) Journal of the Chemical Society (c), 74.

[88] Lewis, N.G. and Davin, L.B. (1999) in: Comprehensive Natural Products Chemistry, Vol. 1, pp. 639 (Sankawa, U., Ed.) Elsevier Science Ltd., Oxford, UK.

[89] Broomhead, A.J., Rahman, M.M.A., Dewick, P.M., Jackson, D.E. and Lucas, J.A. (1991) Phytochemistry 30, 1489.

[90] Whiting, D.A. (1990) Natural Product Reports 7, 349.

[91] Ward, R.S. (1999) Natural Product Reports 16, 75.

[92] Kinjo, J., Furusawa, J. and Nohara, T. (1985) Tetrahedron Letters 26, 6101.

[93] Shirataki, Y., Tagaya, Y., Yokoe, I. and Komatsu, M. (1987) Chemical and Pharmaceutical Bulletin 35, 1637.

[94] Nohara, T., Kinjo, J., Furusawa, J., Sakai, Y., Inoue, M., Shirataki, Y., Ishibashi, Y., Yokoe, I. and Komatsu, M. (1993) Phytochemistry 33, 1207.

[95] Tsui, W.-Y. and Brown, G.D. (1996) Phytochemistry 43, 1413.

[96] Nikaido, T., Ohmoto, T., Noguchi, H., Kinoshita, T., Saitoh, H. and Sankawa, U. (1981) Planta Medica 43, 18.

[97] Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J. and Chiang, V.L. (1999) Nature Biotechnology 17, 808.

[98] Linsmaier, E.M. and Skoog, F. (1965) Physiologia Plantarum 18, 100.

[99] Komada, H. (1976) Bulletin of the Tokai-Kinki National Agricultural Experiment Station 29, 132.

[100] Gross, G.G., Stöckigt, J., Mansell, R.L. and Zenk, M.H. (1973) FEBS Letters 31, 283.

[101] Lüdewitz, T. and Grisebach, H. (1981) European Jounal of Biochemistry 119, 115.

[102] Sarni, F., Grand, C. and Boudet, A.M. (1984) European Jounal of Biochemistry 139, 259.

[103] Jeong, S.-J., Ahn, N.-H., Kim, Y.-C., Inagaki, M., Miyamoto, T. and Higuchi, R. (1999) Planta Medica 65, 367.

[104] Grabber, J.H., Quideau, S. and Ralph, J. (1996) Phytochemistry 43, 1189.

[105] Ulbrich, B. and Zenk, M.H. (1979) Phytochemistry 18, 929.

[106] Carey, F.A. and Sundberg, R.J. (1983) in: Advanced Organic Chemistry, 2nd ed., Part B: Reactions and Synthesis, pp. 341, Plenum Press, New York, USA.

[107] Rajagopalan, J.S., Taylor, K.M. and Jaffe, E.K. (1993) Biochemistry 32, 3965.

[108] Hatam, N.A.R. and Whiting, D.A. (1982) Journal of the Chemical Society, Perkin Transactions 1, 461.