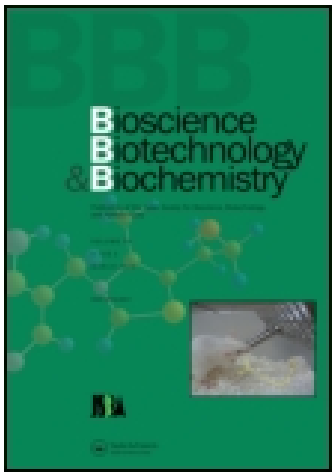

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Enantioselective Lignan Synthesis by Cell-free Extracts of Forsythia koreana

Toshiaki Umezawa[a], Hiroyuki Kuroda[a], Toshinari Isohata[a], Takayoshi Higuchi[ab] & Mikio Shimada[a]

[a] Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan

[b] Faculty of Agriculture and Veterinary Medicine, Nihon University, Shimouma, Setagaya-ku, Tokyo 154, Japan

Published online: 12 Jun 2014.

To cite this article: Toshiaki Umezawa, Hiroyuki Kuroda, Toshinari Isohata, Takayoshi Higuchi & Mikio Shimada (1994) Enantioselective Lignan Synthesis by Cell-free Extracts of Forsythia koreana, Bioscience, Biotechnology, and Biochemistry, 58:2, 230-234, DOI: 10.1271/bbb.58.230

To link to this article: http://dx.doi.org/10.1271/bbb.58.230

Biosci. Biotech. Biochem., **58** (2), 230–234, 1994

Enantioselective Lignan Synthesis by Cell-free Extracts of *Forsythia koreana*†

Toshiaki UMEZAWA, Hiroyuki KURODA, Toshinari ISOHATA,†† Takayoshi HIGUCHI,* and Mikio SHIMADA

Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan
**Faculty of Agriculture and Veterinary Medicine, Nihon University, Shimouma, Setagaya-ku, Tokyo 154, Japan*
Received April 5, 1993

The *Forsythia koreana* plant produces such lignans as (−)-secoisolariciresinol, and (+)-pinoresinol. Cell-free extracts from the plant catalyzed the enantioselective formation of (−)-[$^2H_{10}$]secoisolariciresinol from [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol in the presence of NADPH and H_2O_2. On the other hand, [$^2H_{10}$]lariciresinol isolated from the enzymatic reaction products was found to be predominantly composed of the unnatural (−)-enantiomer [88% enantiomer excess (*e.e.*)]. The stereoselectivity for the formation of these lignans can be explained, at least in part, by the finding that the enzyme system also catalyzed the stereoselective reduction of (+)-lariciresinol, but not its (−)-enantiomer, to (−)-secoisolariciresinol.

Lignans constitute an abundant class of phenylpropanoids,[1] and their biosynthetic mechanisms have been receiving widespread interest in a number of aspects. First, lignans and lignins fundamentally differ in optical activity, although they are closely related in their chemical structures; the former is optically active, whereas the latter is inactive. Therefore, lignan biosynthesis may involve enantioselective process(es), which sharply contrasts with the non-enantioselective process of lignin polymerization. Hence, elucidation of difference in the stereochemical mechanisms is very important. Second, a variety of lignans are known to have such biological activities as antitumor,[1,2] antimitotic,[1,2] and antiviral.[1-3] Antagonism toward the platelet activity factor[1] and inhibitory activities toward certain enzymes[1,2,4] have also been detected in some lignans. Thus, the elucidation of the enzyme systems for lignan biosynthesis provides an important basis for the biotechnological production of useful lignans. Third, understanding the mechanisms for gene expression in lignan synthesis may lead to an elucidation of the biochemical roles of lignans in higher plants.

Umezawa *et al.* have recently reported the first examples of enzyme systems for enantioselective lignan formation with cell-free extracts from *Forsythia intermedia*: one system catalyzed the coupling of two coniferyl alcohol units to afford an optically pure lignan, (−)-secoisolariciresinol ((−)-**2**), and the other catalyzed the stereoselective conversion of (−)-**2** to (−)-matairesinol.[5-7] However, little is known about the reaction mechanisms, particularly for stereochemical control of the processes. The present paper describes the involvement of (+)-lariciresinol ((+)-**3**) in the enantioselective formation of (−)-**2** from coniferyl alcohol (**1**) by cell-free extracts from *F. koreana*.

Materials and Methods

Instrumentation. ^{1}H-NMR spectra were taken with a Varian XL-200 NMR spectrometer, using tetramethylsilane as an internal standard. Chemical shifts and coupling constants are given in δ and Hz, respectively. Direct-inlet MS and GC-MS were conducted with a JMS-DX303HF mass spectrometer (JEOL Ltd.) equipped with a Hewlett-Packard 5890J gas chromatograph and with a JMA-DA5000 mass data system [electron impact mode, 70 eV; gas-chromatographic column, Shimadzu Hicap CBP10-M25-025 (5 m × 0.2 mm); temperature, 40°C at $t=0$ to 2 min, then to 240°C at 30°C/min; carrier gas, He; splitless injection]. Chiral LC-MS was performed with the same mass spectrometer, except that an HPLC system comprising of a Waters 6000A solvent delivery system and a Waters model 440 absorbance detector was fitted to the mass spectrometer instead of the gas chromatograph [Frit-fast atom bombardment (FAB)-mass spectrometery (negative ion mode) with xenon or argon gas; column, Chiralcel OD (Daicel Chemical Co., 250 × 4.6 mm); solvent, EtOH–*n*-hexane–glycerol (300 : 700 : 10) at 0.8 ml/min]. HPLC was conducted with the HPLC system or with a Shimadzu LC-6A liquid chromatograph, detection being at $\lambda=280$ nm. The reversed-phase column used was Waters Novapak C_{18} (150 × 3.9 mm), which was eluted with the following three solvent systems: (i) solvent system A was for gradient elution at 1 ml/min by two linear gradient protocols of CH_3CN–H_2O at $t=0$ to 6 min from 15 : 85 to 17 : 83, and then at $t=6$ to $t=16$ min from 17 : 83 to 40 : 60, the latter being held at this composition for an additional 4 min; (ii) solvent system B was for linear gradient elution at 1 ml/min by CH_3CN–H_2O (23 : 77) at $t=0$ to 10 min, and then to 50 : 50 at $t=15$ min, this latter composition being held for an additional 5 min; (iii) solvent system C was for linear gradient elution at 1 ml/min by CH_3CN–H_2O (23 : 77) at $t=0$ to 5 min, and then to 50 : 50 at $t=10$ min, this latter composition being held for an additional 5 min. The elution conditions for Chiral HPLC were as follows: secoisolariciresinol (**2**), Chiralcel OD column with EtOH–*n*-hexane (30 : 70) at 0.8 ml/min; lariciresinol (**3**), Chiralpak AD column (Daicel Chemical Co., Ltd., 250 × 4.6 mm) with iso-PrOH–*n*-hexane (35 : 65) at 0.4 ml/min; and pinoresinol (**4**), Chiralcel OD column with EtOH at 0.4 ml/min. Silica gel TLC and silica gel column chromatography employed Kieselgel 60 F_{254} (Merck, 20 × 20 cm, 0.25 mm) and Kieselgel 60 (Merck), respectively. All solvents and reagents used were of reagent grade, unless otherwise stated.

Chemical syntheses. [9,9-$^2H_2$, OC$^2H_3$]Coniferyl alcohol (**1**-d_5),[7]* (±)-secoisolariciresinols ((±)-**2**),[7] (±)-pinoresinols ((±)-**4**)[8] and 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol[9] were prepared as previously described. The preparation of (±)-lariciresinols ((±)-**3**), which will be reported in detail elsewhere,[10] is summarized as follows: (i) (±)-β-(4-benzyloxy-3-methoxybenzyl)-γ-butyrolactones/4-benzyloxy-3-methoxybenzaldehyde/lithium hexamethyldisilylamide/tetrahydrofuran, (ii) $LiAlH_4$/tetrahydrofuran, (iii) H_2/10% palladium on carbon/

† Part of this work was presented at the 37th Lignin Symposium, October, 19, 1992, Kyoto, Japan.

†† Present address: *Central Research Laboratory, New Oji Paper Co., Ltd., Koto-ku, Tokyo 135, Japan.*

Abbreviations: *e.e.*, enantiomer excess; TMS, trimethylsilyl; BSA, *N,O*-bis(trimethylsilyl)acetamide; FAB-MS, fast atom bombardment-mass spectrometry.

* d_5 and d_{10} in the compound numbers represent compounds labelled with five and ten deuterium atoms, respectively.

MeOH, and (iv) boron trifluoride etherate/CH_2Cl_2. (±)-**3**. ^{1}H-NMR δ ($CDCl_3$): 2.41 (1H, m), 2.54 (1H, dd, J=10.5, J=12.6), 2.72 (1H, m), 2.92 (1H, dd, J=4.5, J=12.6), 3.75 (1H, dd, J=8.5, J=6.0), 3.77 (1H, dd, J=11.0, J=6.1), 3.87 (3H, s), 3.89 (3H, s), 3.92 (1H, dd, J=11.0, J=7.2), 4.05 (1H, dd, J=8.5, J=6.4), 4.78 (1H, d, J=6.7), 6.7–6.9 (6H, m); EIMS m/z (%): 360 (M^+, 100), 236 (20.8), 221 (15.5), 219 (13.6), 206 (10.7), 194 (37.8), 191 (10.4), 190 (10.8), 180 (25.0), 175 (14.2), 164 (11.6), 153 (28.6), 151 (38.9), 137 (82.7), 124 (10.5), 122 (10.4). (+)-Lariciresinol ((+)-**3**) and (−)-lariciresinol ((−)-**3**) were obtained by chiral HPLC separation of (±)-**3** (Fig. 3). Repeated chiral chromatography (three times) afforded antipode-free (+)-**3** and (−)-**3**. Next, in order to remove impurities due to co-elution of the stationary phase of the chiral column, both the (+)- and (−)-lariciresinol fractions already obtained were individually purified by TLC (using a developing solvent of MeOH–CH_2Cl_2 (4:96)] and then through a silica gel column [3.5 × 0.5 cm (i.d.)] with MeOH–CH_2Cl_2 (3:97) to afford pure (+)-**3** and (−)-**3**. The sign for the optical rotation (+ or −) of each enantiomer was determined by comparing their t_R values from chiral HPLC with that of authentic (+)-**3**[11] supplied by Professor T. Sasaya of Hokkaido University, Japan.

Plant material. Forsythia koreana plants were grown from cuttings of the plant obtained from the campus of Wood Research Institute at Kyoto University, which were then maintained in the greenhouse facilities of Wood Research Institute and used as the source for enzyme preparation.

Enzyme preparation. Young shoots of *F. koreana* were excised with scissors, and the leaves were removed. The resulting stems (11.08 g in fresh weight) were successively washed with tap and distilled water, frozen (liquid N_2) and crushed with a pestle in a mortar. The powder obtained was further ground for 5 min with polyclar AT (2.2 g), acid-washed sea sand and a 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol (17.5 ml). The slurry was filtered through 4 layers of gauze, and the filtrate (13.0 ml) was centrifuged (15,000 × g, 20 min, 4°C). The supernatant was filtered (Whatmen GFA glass fibre filter), and the filtrate was applied to a Sephadex G-25 column that had been pre-equilibrated in a 0.1 M potassium phosphate buffer (pH 7.0). The fraction excluded from the gel was collected and used as the cell-free preparation, the protein content of the enzyme preparation being measured by the method of Bradford, using bovine serum albumin as a standard.[12]

Enzymatic conversion of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol (**1**-d_5). The reaction mixture contained 75 μl of 25 mM **1**-d_5 in a 0.1 M potassium phosphate buffer at pH 7.0 (1.88 μmol), 75 μl of 50 mM NADPH in the same buffer (3.75 μmol), 37.5 μl of 10 mM H_2O_2 in the same buffer, and the cell-free extracts from *F. koreana* (0.75 ml, with 0.84 mg/ml protein content). 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 internal standards [unlabelled (±)-**2** (5.0 μg) and unlabelled (±)-**4** (5.0 μg)]. The EtOAc extracts were dried under high vacuum and dissolved in *N,O*-bis(trimethylsilyl)acetamide (BSA, 5 μl). After standing at room temperature for more than 30 min, an aliquot of the BSA solution was subjected to the GC-MS analysis, and the lignans formed were identified and quantified.

Next, **1**-d_5 (6.9 mg, 37.3 μmol) was incubated as already described, except that all the volumes were proportionately scaled up. After incubating for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabelled (±)-**2** (50 μg). The EtOAc extracts were applied to silica gel TLC, using MeOH–CH_2Cl_2 (3:97) as the developing solvent, and a fraction corresponding to **2** was recovered. The other fractions were combined, again submitted to silica gel TLC separation as just described, and the fraction corresponding to **3** was recovered. These lignan fractions were further purified individually by reversed-phase HPLC using solvent system A for the secoisolariciresinol fraction, and solvent system C for the lariciresinol fraction. The fractions corresponding to **2** and **3** were collected, freeze-dried, and subjected to a chiral HPLC-mass spectrometric examination and chiral HPLC analysis, respectively.

In a separate experiment, after incubating **1**-d_5 with the cell-free extracts as already described, [$^2H_{10}$]pinoresinol (**4**-d_{10}) was isolated from the reaction products after adding unlabelled carriers [(±)-**4**] by reversed-phase HPLC with solvent system B, and subjected to chiral HPLC separation to obtain the (−)- and (+)-pinoresinol fractions. Both fractions were then analyzed individually by mass spectrometry.

Enzymatic conversion of (±)-, (+)-, *and* (−)-*lariciresinols* ((±)-**3**, (+)-**3**, *and* (−)-**3**). (±)-Lariciresinols ((±)-**3**; 42 μg, 0.12 μmol, in 7.5 μl acetone) were incubated at 30°C for 1 h with the enzyme preparation (150 μl, 0.82 mg/ml protein content) in the presence of 0.75 μmol of NADPH (50 mM, 15 μl) in a 0.1 M potassium phosphate buffer (pH 7.0) and 0.075 μmol of H_2O_2 (10 mM, 7.5 μl) in the same buffer. The reaction mixture was extracted with EtOAc containing the internal standard, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (2.5 μg). The EtOAc extracts were dried and then dissolved in BSA (3 μl). After standing at 60°C for 45 min, an aliquot of the solution was subjected to GC-MS analysis, and the products were identified and quantified.

Next, (+)-**3** (43 μg, 0.12 μmol) and (−)-**3** (38 μg, 0.11 μmol) were individually incubated as already described, but H_2O_2 was omitted from these reaction mixtures. The reaction products were then analyzed GC-MS as already described, compound **2** being isolated from the reaction products by reversed phase HPLC (solvent system C), and finally subjected to a chiral HPLC analysis.

Results and Discussion

The incubation of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol (**1**-d_5) with the cell-free extracts from *F. koreana* in the presence of NADPH and H_2O_2 yielded the reaction prod-

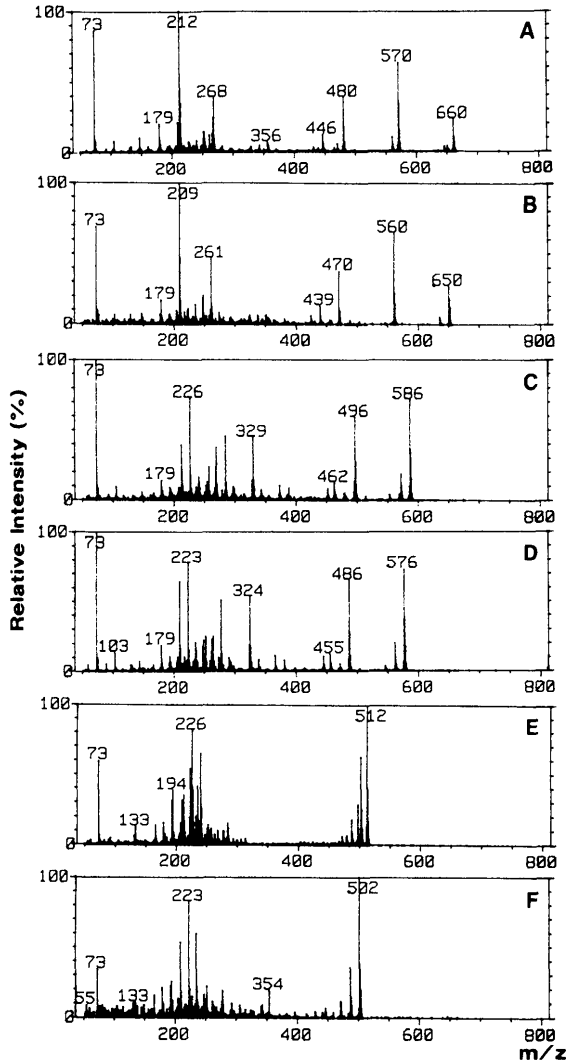

Fig. 1. Mass Spectra of the TMS Ethers of Secoisolariciresinols, Pinoresinols and Lariciresinols.

A, [$^2H_{10}$]secoisolariciresinol (**2**-d_{10}) formed from the incubation of [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol (**1**-d_5) with cell-free extracts of *F. koreana* in the presence of NADPH and H_2O_2. B, chemically synthesized (unlabelled) (±)-secoisolariciresinols ((±)-**2**). C, [$^2H_{10}$]lariciresinol (**3**-d_{10}) formed from the incubation of **1**-d_5 with the cell-free extracts in the presence of NADPH and H_2O_2. D, chemically synthesized (unlabelled) (±)-lariciresinols ((±)-**3**). E, [$^2H_{10}$]pinoresinol (**4**-d_{10}) formed from the incubation of **1**-d_5 with the cell-free extracts in the presence of NADPH and H_2O_2. F, chemically synthesized (unlabelled) (±)-pinoresinols ((±)-**4**). Note that unlabelled (±)-**2** and (±)-**4** were added as carriers in the cases of A and E, respectively.

Table I. Enzymatic Formation of [$^2H_{10}$]Secoisolariciresinol (**2**-d_{10}), [$^2H_{10}$]Lariciresinol (**3**-d_{10}), and [$^2H_{10}$]Pinoresinol (**4**-d_{10}) from [9,9-$^2H_2$, OC$^2H_3$]Coniferyl Alcohol (**1**-d_5)

Assay	Cofactor	[$^2H_{10}$]Secoisolariciresinol (**2**-d_{10}) formation[b]	[$^2H_{10}$]Lariciresinol (**3**-d_{10}) formation[b]	Assay	Cofactor	[$^2H_{10}$]Pinoresinol (**4**-d_{10}) formation[b]
Complete	$NADPH/H_2O_2$	75.5	4.3	Complete	$NADPH/H_2O_2$	16.0
Controls[a]	NADPH	9.8	0.9		H_2O_2	55.6
	H_2O_2	0	0	Controls[a]	NADPH	1.7
	None	0	0		None	13.8
	Denatured enzyme/ $NADPH/H_2O_2$	0	0		Denatured enzyme/ $NADPH/H_2O_2$	1.7

[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 using the complete assay, but the reaction was worked up by adding EtOAc as soon as possible (less than 10 s) after the start of incubation. In this experiment, the amounts of secoisolariciresinol, pinoresinol and lariciresinol formed were 0, 56.5 and 0 nmol mg^{-1} protein, respectively.

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

ucts, [$^2H_{10}$]secoisolariciresinol (**2**-d_{10}), [$^2H_{10}$]lariciresinol (**3**-d_{10}), and [$^2H_{10}$]pinoresinol (**4**-d_{10}). They were identified by comparing their mass spectra [trimethylsilyl (TMS) ethers] (Figs. 1A, 1C, and 1E) and retention times by GC with those of unlabelled authentic samples (Figs. 1B, 1D, and 1F). Figure 1A shows that the GC peak corresponding to **2** gave ion peaks at *m/z* 660, 570, 480, and 212, which are assigned to the molecular ion [M^+] of secoisolariciresinol (TMS ether) having ten deuterium atoms, [$M^+ - TMSOH$], [$M^+ - 2 \times TMSOH$], and to the fragment having three deuterium atoms derived from benzylic cleavage, respectively. The t_R value for the deuterated product (10.60 min) was practically identical to that of the accompanying unlabelled carrier ((±)-**2**, 10.62 min), but the former was always slightly smaller than that of the latter. Such a difference in t_R between a deuterated compound and its unlabelled analog is commonly known to be due to the isotope effect. Thus, the compound was identified as **2**-d_{10}. Similarly, the compounds having the mass spectra in Figs. 1C and 1E were identified as **3**-d_{10} and **4**-d_{10}, respectively.

Table I shows the amounts of these three lignans formed in the complete and control assay systems. No significant amounts of the lignans were determined from the control systems, indicating that the lignan formation was enzymatic. The smaller enzyme activity observed in the controls without H_2O_2 could have been due to contamination of the H_2O_2-producing activity in the crude enzyme preparation. In the presence of NADPH, the yield of **4**-d_{10} decreased significantly, suggesting that an NADPH-dependent reductive process hampered the formation of **4**-d_{10} and/or catalyzed further conversion of **4**-d_{10}. Under the assay condition employed, the specific activity forming **2**-d_{10} from **1**-d_5 was 75.5–140 nmol h^{-1} mg^{-1} protein, which is higher than that previously reported for cell-free extracts from *F. intermedia* [15.9 nmol h^{-1} mg^{-1} protein].[5] The pinoresinol formation is in accordance with the recent study on an *F. intermedia* enzyme preparation,[13] while the enzymatic lariciresinol formation from **1**-d_5 is a new finding.

The enantiomeric composition of the lignans formed enzymatically from **1**-d_5 was analyzed by chiral LC-MS or chiral HPLC. Figure 2 shows the chiral LC-MS chromatograms of the secoisolariciresinol fraction; unlabelled (±)-

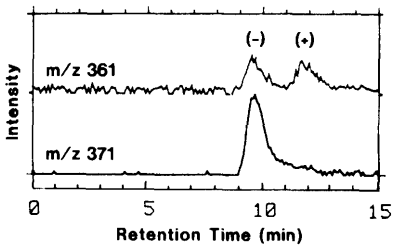

Fig. 2. Chiral LC Mass Chromatograms of the Deprotonated Molecular Ions of Secoisolariciresinols.

m/z 371, mass chromatogram of the deprotonated molecular ion of (−)-[$^2H_{10}$]secoisolariciresinol ((−)-**2**-d_{10}) formed from the incubation of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol (**1**-d_5) with cell-free extracts of *F. koreana* in the presence of NADPH and H_2O_2. *m/z* 361, mass chromatogram of the deprotonated molecular ion of the unlabelled carrier, (±)-secoisolariciresinols ((±)-**2**).

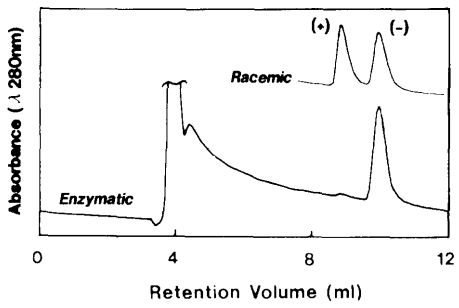

Fig. 3. Chiral HPLC Chromatograms of Lariciresinols.

Racemic, synthetic (unlabelled) (±)-lariciresinols ((±)-**3**). *Enzymatic*, [$^2H_{10}$]lariciresinol (**3**-d_{10}) formed from the incubation of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol (**1**-d_5) with cell-free extracts of *F. koreana* in the presence of NADPH and H_2O_2. Note that unlabelled (±)-lariciresinols ((±)-**3**) were not added as carriers.

2 and enzymatically formed **2**-d_{10} gave the deprotonated molecular ions at *m/z* 361 and at *m/z* 371, respectively. This result clearly indicates that (−)-**2**-d_{10} was formed, because the chromatographic peak of the ion at *m/z* 371 was coincident with that of (−)-**2**, which occurs as a natural enantiomer in the *F. koreana* plant.[14] This is in good agreement with the previous study using cell-free extracts from *F. intermedia* by Umezawa *et al.*[5]

Next, the lariciresinol fraction obtained from the enzymatic reaction products was analyzed by chiral HPLC (Fig. 3). It is noteworthy that unnatural (−)-enantiomer (−)-**3**-d_{10} predominated [88% enantiomer excess (*e.e.*)], because (+)-**3** has been isolated from *F. koreana* (manu-

script in preparation). The mass spectrometric examination confirmed that the small peak corresponding to the (+)-enantiomer on the chromatogram of enzymatically formed **3**-d_{10} was due to (+)-**3**-d_{10}, and not to impurities.

The fractions corresponding to (+)-pinoresinol ((+)-**4**) and (−)-antipode (−)-**4** were obtained individually from the enzymatic reaction products. By a mass spectrometric analysis of the fractions, both (+)-**4**-d_{10} and (−)-**4**-d_{10} were detected. However, the amount of unnatural (−)-enantiomer (−)-**4**-d_{10} predominated (91% *e.e.*), because (+)-**4** occurs naturally in the *F. koreana* plant.[14,15] Similar results have recently been reported by Katayama *et al.*, with cell-free extracts from *F. intermedia*.[13] On the other hand, racemic (±)-**4**-d_{10} ((+)-**4**-d_{10}:(−)-**4**-d_{10}, 49.5:50.5) were obtained when NADPH was omitted from the enzymatic reaction mixture. This result suggests that our enzyme system contained a peroxidase catalyzing non-enantioselective reaction, because this type of peroxidase (*e.g.*, horseradish peroxidase) is well known to oxidize **1** to form various racemic dimers including (±)-**4** *via* quinonemethides such as **5a** and **5b** as shown in Fig. 5.[16]

The reason for the higher observed yields of unnatural enantiomers (−)-**3**-d_{10} and (−)-**4**-d_{10} than those of natural antipodes (+)-**3**-d_{10} and (+)-**4**-d_{10} may be explained by postulating that (+)-**3**-d_{10} and (+)-**4**-d_{10} (or corresponding quinonemethides **6a** and **5a**) were selectively reduced

Table II. Enzymatic Formation of Secoisolariciresinol (**2**) from (±)-Lariciresinols ((±)-**3**)

Assay	Cofactor	Secoisolariciresinol (**2**) formation[b]
Complete	NADPH	96.7
	$NADPH/H_2O_2$	56.2
Controls[a]	None	0
	Denatured enzyme/NADPH	0

[a] Control experiments refer to the complete assay with the omission of cofactors or with the denatured enzyme (boiled for 5 min). One other control experiment was carried out, using the complete assay (cofactor of NADPH) but with a reaction period of less than 10 s. In this experiment, no secoisolariciresinol formation was observed.
[b] Expressed in nmol $h^{-1} mg^{-1}$ protein.

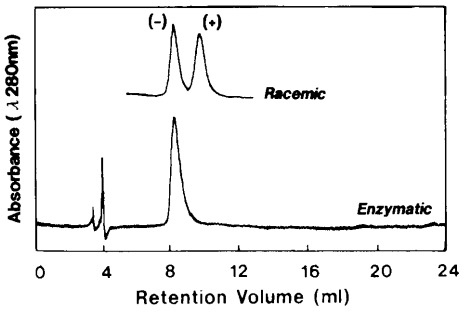

Fig. 4. Chiral HPLC Chromatograms of Secoisolariciresinols.
Racemic, synthetic (unlabelled) (±)-secoisolariciresinols ((±)-**2**). *Enzymatic*, (−)-secoisolariciresinol ((−)-**2**) formed from the incubation of (+)-lariciresinol ((+)-**3**) with cell-free extracts of *F. koreana* in the presence of NADPH.

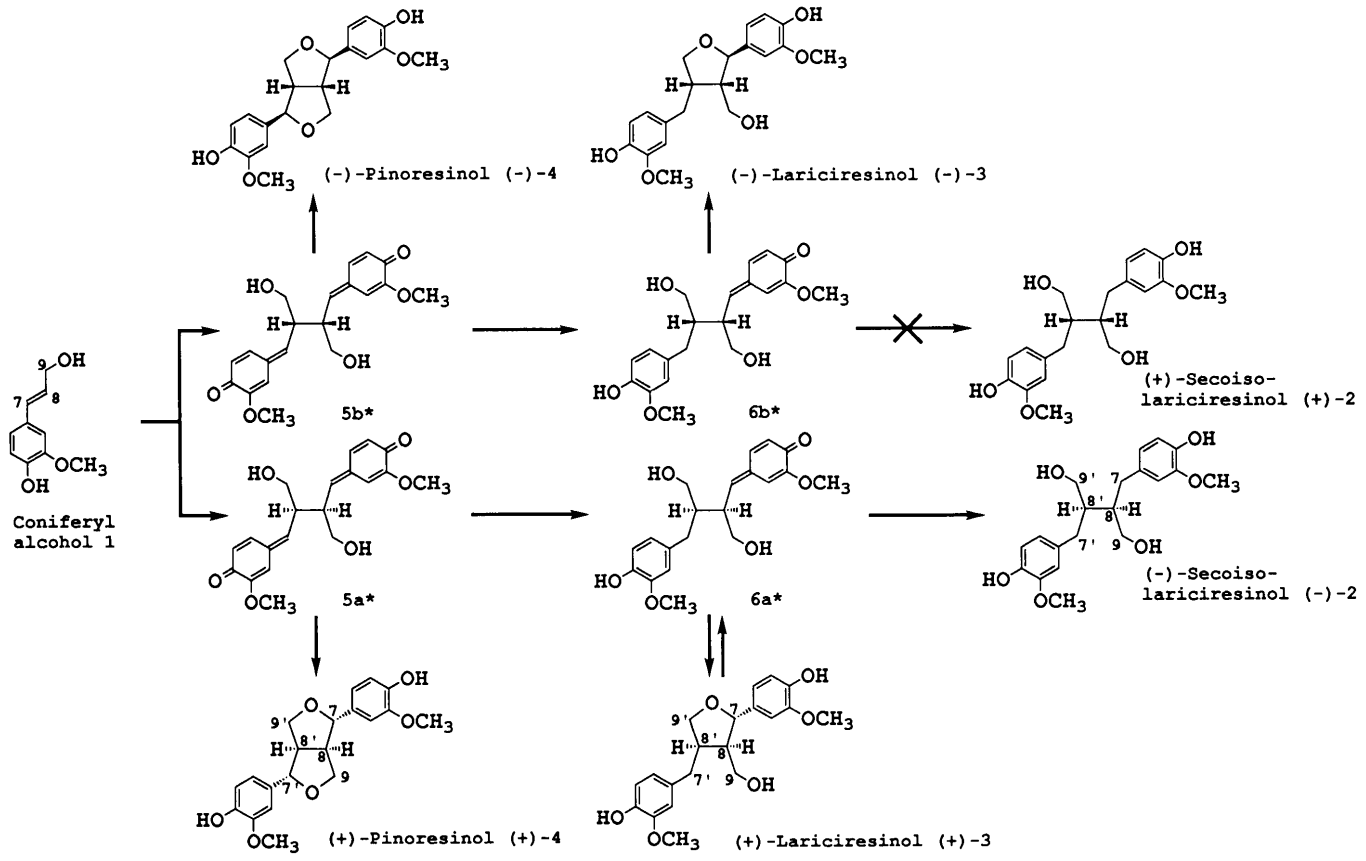

Fig. 5. (−)-Secoisolariciresinol ((−)-**2**) Formation from Coniferyl Alcohol (**1**) and (+)-Lariciresinol ((+)-**3**) with the Enzyme Preparation from *F. koreana*.

Note that (+)-secoisolariciresinol ((+)-**2**) was not detected as a product, and that the deuterium atoms derived from [9,9-$^2H_2$,OC^2H_3]coniferyl alcohol (**1**-d_5) were neglected. *Assumed compound.

to natural enantiomer (−)-**2**-d_{10}. Hence, the NADPH-dependent reduction of **3** to (−)-**2** was next examined.

GC-MS analysis of the products obtained after incubating (±)-**3** with the cell-free extracts showed the formation of **2**, which was identified by comparing the mass spectral data (TMS ether) [*m/z* (%), 650 (23.0), 560 (58.3), 470 (32.9), 439 (10.4), 261 (52.7), 209 (100), 179 (19.7), 73 (64.8)] and retention time (10.72 min) from GC with those of an authentic sample [mass spectrum, Fig. 1B; t_R, 10.70 min]. However, **4** was not formed from (±)-**3** by the incubation. The formation rate of **2** was measured by GC-MS [96.7 nmol h^{-1} mg^{-1} protein] (Table II). Secoisolariciresinol (**2**) was not formed either when NADPH was omitted or when a denatured enzyme was used (Table II).

In order to examine the stereoselectivity of the reduction process, (+)-**3** and (−)-**3** were individually incubated with the cell-free extracts. GC-MS analysis of the reaction products (TMS ether) showed that (+)-**3**, but not (−)-**3**, yielded **2**, which was found to consist only of (−)-enantiomer (−)-**2** by the chiral HPLC analysis (Fig. 4).

Thus, the selective reduction of (+)-**3**-d_{10} to (−)-**2**-d_{10} accounts for the accumulation of (−)-**3**-d_{10} in preference to (+)-antipode (+)-**3**-d_{10} during the incubation of **1**-d_{10}. In addition, the enantioselective formation of (−)-**2**-d_{10} from **1**-d_5 can be ascribed, at least in part, to the stereoselective reduction of (+)-**3**-d_{10} (Fig. 5). (±)-Pinoresinols ((±)-**4**) were also found to be reduced to **2** and **3** by the enzyme preparation in the presence of NADPH (data not shown). The preferential accumulation of (−)-**4**-d_{10} in the incubation of **1**-d_5 may be explained by stereoselective reduction of (+)-enantiomer (+)-**4**-d_{10} to (−)-**2**-d_{10}, which is consistent with the recent study on cell-free extracts from *F. intermedia*.[13]

In conclusion, this investigation has revealed that the stereoselective reduction step, (+)-lariciresinol ((+)-**3**) to (−)-secoisolariciresinol ((−)-**2**), plays an important role in the stereoselective formation of (−)-**2** from coniferyl alcohol **1** in the *in vitro* system.

Acknowledgments. This research was supported partly by a Grant-in-Aid for Scientific Research (No. 02404015) from the Ministry of Education, Science, and Culture of Japan, and by a Grant-in-Aid for Scientific Research from Kyoto University. We thank Professor T. Sasaya of Hokkaido University for supplying (+)-lariciresinol.

References

1) D. C. Ayres and J. D. Loike, "Lignans: Chemical, Biological and Clinical Properties," Cambridge University Press, Cambridge, 1990.
2) W. D. MacRae and G. H. N. Towers, *Phytochemistry*, **23**, 1207–1220 (1984).
3) W. D. MacRae, J. B. Hudson, and G. H. N. Towers, *Planta Medica*, **55**, 531–535 (1989).
4) S. Shimizu, H. Kawashima, K. Akimoto, Y. Shinmen, H. Sugano, and H. Yamada, *Phytochemistry*, **31**, 757–760 (1992).
5) T. Umezawa, L. B. Davin, and N. G. Lewis, *Biochem. Biophys. Res. Commun.*, **171**, 1008–1014 (1990).
6) T. Umezawa, L. B. Davin, E. Yamamoto, D. G. I. Kingston, and N. G. Lewis, *J. Chem. Soc., Chem. Commun.*, **1990**, 1405–1408.
7) T. Umezawa, L. B. Davin, and N. G. Lewis, *J. Biol. Chem.*, **266**, 10210–10217 (1991).
8) Y. Katayama and T. Fukuzumi, *Mokuzai Gakkaishi*, **24**, 664–667 (1978).
9) T. Umezawa and T. Higuchi, in "Modern Methods of Plant Analysis, New Series, Vol. 10, Plant Fibers," ed. by H. F. Linskens and J. F. Jackson, Springer-Verlag, Berlin, 1989, pp. 161–185.
10) T. Umezawa and M. Shimada, *Mokuzai Gakkaishi*, **40**, 231–235 (1994).
11) K. Miki, K. Ito, and T. Sasaya, *Mokuzai Gakkaishi*, **25**, 665–670 (1979).
12) M. M. Bradford, *Anal. Biochem.*, **72**, 248–254 (1976).
13) T. Katayama, L. B. Davin, and N. G. Lewis, *Phytochemistry*, **31**, 3875–3881 (1992).
14) T. Umezawa, T. Isohata, H. Kuroda, T. Higuchi, and M. Shimada, in "Biotechnology in Pulp and Paper Industry," ed. by M. Kuwahara and M. Shimada, Uni Publ., Tokyo, Japan, 1992, pp. 507–512.
15) S. Kitagawa, S. Nishibe, R. Benecke, and H. Thieme, *Chem. Pharm. Bull.*, **36**, 3667–3670 (1988).
16) K. Freudenberg, *Science*, **148**, 595–600 (1965).