J Wood Sci (1998) 44:244–246

RAPID COMMUNICATION

Takeshi Katayama · Yuki Kado

Formation of optically active neolignans from achiral coniferyl alcohol by cell-free extracts of *Eucommia ulmoides**

Received: July 23, 1997 / Accepted: December 17, 1997

Key words Biosynthesis · Neolignan · Lignan · Guaiacylglycerol-β-coniferyl ether · *Eucommia ulmoides*

We report the first enzymatic formation of optically active 8-*O*-4′ neolignans from achiral monolignols; that is, we determined the formation of (+)-*erythro*- and (−)-*threo*-guaiacylglycerol-β-coniferyl ethers [(+)-**2** and (−)-**3**, respectively] from coniferyl alcohol (**1**) by cell-free extracts of *Eucommia ulmoides* Oliv. (Fig. 1). Most lignans and neolignans isolated from higher plants are optically active, and they have been found to have diverse biological activities and important pharmacological actions. In contrast, lignins (whose substructures are similar to the structures of lignans and neolignans) are optically inactive and racemate-like polymers, the reason for which has been biosynthetically well defined. Biosynthesis of optically active lignans in *Forsythia* spp. has advanced over the past several years. In particular, Lewis and coworkers[1,2] reported that the enzymatic formation of (8*R*,8′*R*)-(+)-pinoresinol from **1** occurred enantioselectively, and they[3] clarified that the enantioselective radical coupling was catalyzed by the combination of a novel dirigent protein and an oxidase such as laccase. Little is known, however, about the biosynthesis of optically active neolignans.

Eucommia ulmoides is native to China, and its bark has been used as a crude drug (Eucommia Cortex). Deyama and colleagues isolated diverse optically active lignans and neolignans from the bark.[4,5] It is noteworthy that most of these substances were initial coupling products of monolignols [e.g., (+)-pinoresinol (**5**), (+)-syringaresinol, (−)-dehydrodiconiferyl alcohol (**4**), (+)-guaiacylglycerol-β-(sinapyl alcohol) ether] and compounds formed by one-step transformation from the initial coupling products [e.g., (+)-dihydrodehydrodiconiferyl alcohols, *erythro*- and *threo*-guaiacylglycerol-β-(coniferyl aldehyde) ethers]. To determine the enzymatic activity of stereoselective coupling of the monolignols, cell-free extracts from *E. ulmoides* were incubated with **1** in the presence of H_2O_2.

Eucommia ulmoides plants (obtained from Sanyo Nouen Inc.) were maintained at the Faculty of Agriculture, Kagawa University. Cell-free extracts were prepared from stems of the defoliated young shoots, as described previously.[6] Protein contents were determined by the method of Bradford.[7] The cell-free extracts were incubated with [8-^{14}C]-**1** (5.68 MBq/mmol)[6,8] in the presence of H_2O_2 at 30°C. Each assay mixture (230 μl) consisted of 2.6 mM of [8-^{14}C]-**1** (30 mM, 20 μl) and 0.43 mM of H_2O_2 (10 mM, 10 μl), previously dissolved in potassium phosphate (K-Pi) buffer (0.1 M, pH 7.0), and the cell-free extracts (200 μl). Assays were conducted in quintuplicate, and the reaction was initiated by adding H_2O_2. After incubation for 1 h, AcOH (20 μl) was added, and the mixture was then extracted with EtOAc (500 μl × 2). The EtOAc solubles from five assays were combined and evaporated in vacuo. The residue was reconstituted with MeOH containing a mixture of (±)-**2** and (±)-**3**, as cold carriers, and filtered. An aliquot (10 μl) of the filtrate was subjected to C_{18} high-performance liquid chromatography (HPLC) [ultraviolet (UV) detector at 280 nm] with a gradient elution of CH_3CN/3% AcOH in H_2O (v/v): at 0 min, 4:96; at 6 min, 10:90; at 16 min, held at 10:90; at 26 min, 40:60; at 30 min, held at 40:60; and at 45 min, 100:0. The eluate was collected at 30-s intervals from 5 to 35 min, with each fraction subjected to a liquid scintillation counter (LSC).[8] Larger aliquots were further applied to the C_{18} HPLC column, with fractions containing **2**, **3**, **4**, and **5** individually collected and evaporated in vacuo. MeOH solubles of the four fractions were then subjected to chiral column HPLC with UV (280 nm) and chiral detectors. Elution conditions employed were as follows (column, eluent, flow rate): **2**, Daicel Chiralcel OD, EtOH/*n*-hexane = 15:85,

T. Katayama (✉) · Y. Kado
Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan
Tel. +81-87-891-3083; Fax +81-87-891-3021
e-mail: katayama@ag.kagawa-u.ac.jp

*This paper was presented at the 41st Lignin Symposium at Nagoya, October 1996, and the 47th annual meeting of the Japan Wood Research Society, Kochi, April 1997

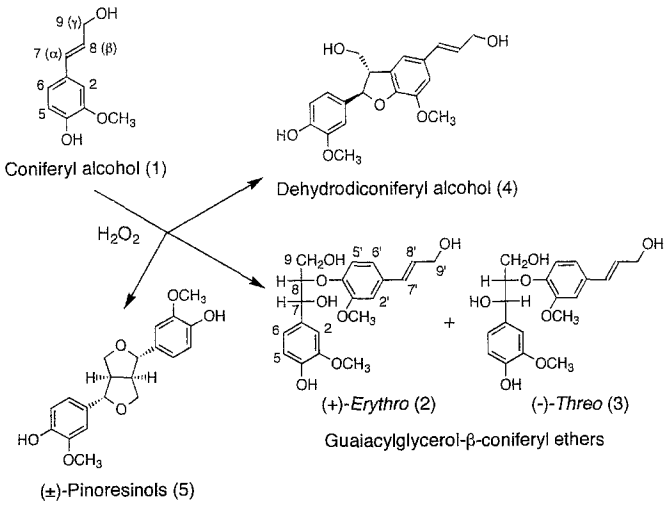

Fig. 1. Formation of (+)-*erythro*- and (−)-*threo*-guaiacylglycerol-β-coniferyl ethers [(+)-**2** and (−)-**3**, respectively], dehydrodiconiferyl alcohol (**4**), and (±)-pinoresinols [(±)-**5**] from coniferyl alcohol (**1**) catalyzed by *E. ulmoides* cell-free extracts in the presence of H_2O_2. Absolute configurations of (+)-**2** and (−)-**3** are unknown. The structural formulas of (−)-**4** and (+)-**5** are shown for convenience

Table 1. Formation of *erythro*- and *threo*-[^{14}C]guaiacylglycerol-β-coniferyl ethers, [^{14}C]dehydrodiconiferyl alcohol, and [^{14}C]pinoresinol, following incubation of [8-^{14}C]coniferyl alcohol (**1**) with cell-free extracts from *E. ulmoides* in the presence of H_2O_2

Enzyme assay	Neolignans and lignans present after 1 h of incubation (nmol/mg protein)			
	2	**3**	**4**	**5**
Complete	69.2	56.6	337	173
Minus H_2O_2	0.0	0.0	31.7	13.2
Complete, but denatured	0.0	0.0	44.2	26.2

2, **3**, *Erythro*- and *threo*-[^{14}C]guaiacyl glycerol-β-coniferyl ethers, respectively; **4**, [^{14}C]dehydrodiconiferyl alcohol; **5**, [^{14}C]pinoresinol.

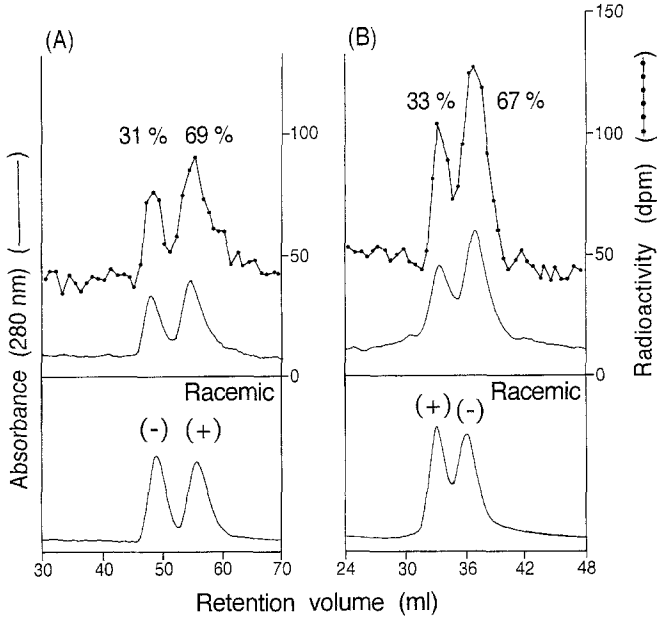

Fig. 2. Chiral high-performance liquid chromatography separations of (**A**) *erythro*-[^{14}C]guaiacylglycerol-β-coniferyl ether (**2**) and (**B**) *threo*-[^{14}C]guaiacylglycerol-β-coniferyl ether (**3**) formed following incubation of [8-^{14}C]coniferyl alcohol (**1**) with *E. ulmoides* cell-free extracts in the presence of H_2O_2. *Racemic*, racemic authentic samples

1.0 ml/min; **3**, Daicel Chiralcel OD (without a precolumn), EtOH/*n*-hexane = 17:83, 0.6 ml/min; **4**, Daicel Chiralcel OF, 2-PrOH/*n*-hexane (0.3% AcOH) = 50:50, 1.0 ml/min; **5**, see Davin et al.[1] Eluate was collected at 30- or 60-s intervals with each fraction subjected to LSC.

The products, [^{14}C]-**2**, [^{14}C]-**3**, [^{14}C]-**4**, and [^{14}C]-**5** (Fig. 1), which were tentatively identified by co-HPLC and co-thin layer chromatography (TLC) with the authentic samples (obtained by dehydrogenation of **1** with horseradish peroxidase and H_2O_2, or by organic synthesis) were quantified by LSC counting (Table 1). The formation of **4** was most abundant followed by that of **5** and then the sum of **2** and **3**. *Erythro* isomer **2** was more than *threo* isomer **3**. When H_2O_2 was omitted or the cell-free extracts were boiled for 10 min, no significant formation of the products was observed.

To confirm the structures of the radioactive products, unlabeled **1** was incubated with the cell-free extracts and H_2O_2 in a larger scale. After separation of unlabeled products (**4**, **5**, and a mixture of **2** and **3**) by silica gel TLC, they were subjected to ^{1}H nuclear magnetic resonance (NMR) and ^{1}H-^{1}H correlated spectroscopy (COSY); the spectra at 400 MHz were recorded. [Chemical shifts and coupling constants (J) were expressed in δ and Hz, respectively. Data of **4** and **5** are not shown.] **2** and **3** [as a mixture (3:2)] (CD_3OD); **2**, δ 3.74–3.79 (1H, H-9$_a$), 3.795 [3H, singlet (s), OCH_3], 3.801 (3H, s, OCH_3), 3.81–3.87 (1H, H-9$_b$), 4.17–4.20 (2H, H-9′), 4.353 [1H, double triplet (dt), J = 5.7, J = 3.8, H-8], 4.823 [1H, doublet (d), J = 5.6, H-7], 6.232 (1H, dt, J = 15.9, J = 5.7, H-8′), 6.508 (1H, d, J = 15.9, H-7′), 6.722 (1H, d, J = 8.3, H-5), 6.830 [1H, double doublet (dd), J = 8.2, J = 1.8, H-6], 6.86–6.87 (1H, H-2), and 6.98–7.03 (3H, H-2′, H-5′, H-6′); **3**, δ 3.464 (1H, dd, J = 12.1, J = 5.0, H-9$_a$), 3.742 (1H, dd, J = 12.2, J = 3.9, H-9$_b$), 3.818 (3H, s, OCH_3), 3.878 (3H, s, OCH_3), 4.19–4.21 (2H, H-9′), 4.287 (1H, dt, J = 4.1, J = 5.5, H-8), 4.85–4.90 (1H, H-7), 6.257 (1H, dt, J = 15.9, J = 5.7, H-8′), 6.532 (1H, d, J = 15.9, H-7′), 6.749 (1H, d, J = 8.1, H-5), 6.84–6.87 (1H, H-6), 6.86–6.87 (1H, H-2), 6.904 (1H, dd, J = 8.2, J = 1.8, H-6′), 6.98–7.01 (1H, H-5′), and 7.058 (1H, d, J = 2.0, H-2′). These spectra were identical to those of authentic samples. The control solution without the unlabeled substrate **1**, C_{18} HPLC peak areas (UV 280 nm) corresponding to **2**, **3**, **4**, and **5** were 0.049%, 0.060%, 0.046%, and 0.014% of those of the enzymatic products, respectively. Such unlabeled compounds derived from the cell-free extracts are negligible for the NMR and HPLC analyses of the enzymatic products.

Enantiomeric compositions of the products were then examined with chiral column HPLC. Figure 2 shows the preferential formations of (+)-*erythro*-[^{14}C]-**2** [69% (69%)] and (−)-*threo*-[^{14}C]-**3** [67% (72%)], respectively. The signs of the optical rotation were determined with a chiral detector. Absolute configurations of (+)-**2** and (−)-**3** are unknown at this time. The tiny difference of the retention volume between enzymatic and authentic (−)-**3** would be within experimental error. [Retention volumes of both (+) and (−) peaks of the product **3** after 30 min of incubation were identical to those of the authentic **3**.] Because the enantiomeric peaks of **2** and **3** partially overlap each other, the enantiomeric compositions were corrected (uncorrected values are in parentheses) by use of an observed enantiomeric composition of each racemate. On the other hand, [^{14}C]-**5**, formed by the cell-free extracts, was racemate, although the (+)-form was a natural enantiomer in this plant.[4] The unlabeled products **2**, **3**, and **5** had enantiomeric compositions similar to the ones noted above. It is under study whether the enzymatic reaction product **4** has low (−)-enantiomeric excess. (The antipode assignment had been done by Hirai et al.[9]) Compound **4** from the control solution was racemate.

This is the first example of enzymatic formation of optically active neolignans at large in woody plants. The facts show that enantioselective coupling of **1** by the enzyme occurred to give (+)-**2** and (−)-**3** [Note: the presence of the opposite enantiomers, (−)-**2** and (+)-**3**, would be due to formation of (±)-**2** and (±)-**3** by nonspecific peroxidases in the crude cell-free extracts.], or that formation of (±)-**2** and (±)-**3** by the nonspecific peroxidase was followed by the stereospecific turnover of (−)-**2** and (+)-**3** in the racemates to other compounds, thereby accumulating (+)-**2** and (−)-**3**. These results are in contrast to those of enantioselective (+)-pinoresinol (**5**) formation (due to a combination of a dirigent protein and an oxidase in *Forsythia* spp.) because (1) the enzyme system in *Forsythia* was originally obtained from the "insoluble cell-wall residue" of the stems, (2) it did not require H_2O_2, and (3) it catalyzed the formation of racemic **2**, **3**, and **4** but not that of optically active ones.[1–3] The isolation of **2** and **3** from *E. ulmoides* is under study, although the *erythro*- and *threo*-guaiacylglycerol-β-(coniferyl aldehyde) ethers obtained as an optically active mixture[4] would be derived from **2** and **3**, respectively. Orr and Lynn[10] reported that incubation of **1** with cell-free extracts of transformed *Vinca rosea* tumor cells in the presence of H_2O_2 gave **4**, whose enantiomeric ratio was determined to be 0.52:1.0.

Acknowledgment We thank Dr. N. Hirai, Faculty of Agriculture, Kyoto University, for supplying the (+)- and (−)-dehydrodiconiferyl alcohols.

References

1. Davin LB, Bedgar DL, Katayama T, Lewis NG (1992) On the stereo selective synthesis of (+)-pinoresinol in *Forsythia suspensa* from its achiral precursor, coniferyl alcohol. Phytochemistry 31:3869–3874
2. Paré PW, Wang H-B, Davin LB, Lewis NG (1994) (+)-Pinoresinol synthase: a stereoselective oxidase catalysing 8,8′-lignan formation in *Forsythia intermedia*. Tetrahedron Lett 35:4731–4734
3. Davin LB, Wang H-B, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275:362–366
4. Deyama T, Ikawa T, Kitagawa S, Nishibe S (1987) The constituents of *Eucommia ulmoides* Oliv. V. Isolation of dihydroxydehydrodiconiferyl alcohol isomers and phenolic compounds. Chem Pharm Bull (Tokyo) 35:1785–1789
5. Deyama T, Ikawa T, Kitagawa S, Nishibe S (1987) The constituents of *Eucommia ulmoides* Oliv. VI. Isolation of a new sesquilignan and neolignan glycosides. Chem Pharm Bull (Tokyo) 35:1803–1807
6. Katayama T, Davin LB, Lewis NG (1992) An extraordinary accumulation of (−)-pinoresinol in cell-free extracts of *Forsythia intermedia*: evidence for enantiospecific reduction of (+)-pinoresinol. Phytochemistry 31:3875–3881
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 72:248–252
8. Katayama T, Masaoka T, Yamada H (1997) Biosynthesis and stereochemistry of lignans in *Zanthoxylum ailanthoides*. I. (+)-Lariciresinol formation by enzymatic reduction of (±)-pinoresinols. Mokuzai Gakkaishi 43:580–588
9. Hirai N, Okamoto M, Udagawa H, Yamamuro M, Kato M, Koshimizu K (1994) Absolute configuration of dehydrodiconiferyl alcohol. Biosci Biotech Biochem 58:1679–1684
10. Orr JD, Lynn DG (1992) Biosynthesis of dehydrodiconiferyl alcohol glucosides: implications for the control of tobacco cell growth. Plant Physiol 98:343–352