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ORIGINAL ARTICLE

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Stereochemistry of matairesinol formation by *Daphne* secoisolariciresinol dehydrogenase

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Abstract Secoisolariciresinol dehydrogenase activity was detected for the first time from *Daphne odora* and *Daphne genkwa* (Thymelaeaceae), which are known to produce optically pure (+)-matairesinol. In sharp contrast, (−)-matairesinol was formed selectively over the (+)-antipode by the secoisolariciresinol dehydrogenase preparation from both *D. odora* callus and *D. genkwa* shoots.

Key words Matairesinols · Secoisolariciresinol dehydrogenase · *Daphne odora* · *Daphne genkwa* · Stereochemistry

Introduction

Stereochemical analyses of lignans in various plants have revealed that there is a great diversity in enantiomeric compositions of lignans.[1,2] First, in the cases of pinoresinol, lariciresinol, and secoisolariciresinol, which are the farthest upstream on the biosynthetic pathway, not only the sign of optical rotation (i.e., predominant enantiomers of the lignans) but also the enantiomeric composition, the percent enantiomeric excess (% e.e.) value, vary greatly with plant species. In addition, there have been no examples reported of optically pure pinoresinol and lariciresinol. Second, in marked contrast, dibenzylbutyrolactone lignans including matairesinol, which follow pinoresinol, lariciresinol, and secoisolariciresinol on the biosynthetic pathway, exhibit simpler enantiomeric compositions. Thus, all dibenzylbutyrolactone lignans of which enantiomeric compositions have so far been determined by chiral high-performance liquid chromatography (HPLC) have been found to be optically pure. Furthermore, it is noteworthy that the optically pure dibenzylbutyrolactone lignans isolated from various plant species are levorotatory and have the same absolute configuration in terms of C8 and C8′, whereas those isolated from Thymelaeaceae plants (e.g., *Wikstroemia* spp. and *Daphne* spp.) have the opposite configuration and are dextrorotatory.

These results indicate that the stereochemistry at C8 and C8′ are controlled by both the entrance step mediated by dirigent protein and subsequent metabolic steps, the last of which is the formation of matairesinol from secoisolariciresinol catalyzed by secoisolariciresinol dehydrogenase (SIRD).[3,4]

SIRD was first reported by Umezawa et al. using *Forsythia intermedia* as an enzyme source,[5,6] and its cDNA has been cloned.[7] SIRD from *F. intermedia* plants and the corresponding recombinant enzyme produced optically pure (−)-matairesinol following incubation with racemic (±)-secoisolariciresinols.[6,7] Suzuki et al. detected SIRD from *Arctium lappa*,[4] which shows the same stereochemical selectivity as the *Forsythia* SIRD.[6,7]

On the other hand, the SIRD-catalyzed oxidation of secoisolariciresinol to matairesinol in Thymelaeaceae plants has not yet been described. To understand the whole stereochemical mechanism of lignan biosynthesis, it is important to elucidate the stereochemical difference in the SIRD-catalyzed reactions between Thymelaeaceae and other plants. In the present study, we characterized for the first time the stereochemistry of secoisolariciresinol oxidation by *Daphne* SIRDs.

Experimental

Instruments and chromatography

^{1}H-Nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL).

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Gas chromatography-mass spectrometry (GC-MS) was conducted with a JMS-DX303HF mass spectrometer (JEOL) equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system [electron impact mode 70 eV; gas chromatographic column, Shimadzu Hicap CBP10-M25-025 (5 m × 0.22 mm); temperature 40°C at t = 0–2 min, then to 240°C at 30°C/min; carrier gas He; splitless injection][3,8–12] or with a GC-MS QP5050A mass spectrometer (Shimadzu) equipped with a Shimadzu GC-17A gas chromatograph [electron impact mode 70 eV; gas chromatographic column, Shimadzu Hicap CBP10-M25-025 (20 m × 0.22 mm); temperature 40°C at t = 0–2 min, then to 240°C at 20°C/min; carrier gas He; splitless injection]. Reversed-phase HPLC and chiral HPLC were conducted exactly as previously described.[3,9–12] Semimicro chiral LC-atmospheric pressure chemical ionization (APCI)-MS was conducted with a JMS-LCmate LCMS system (JEOL) with a Chiralcel OD-H (250 × 1.0 mm; Daicel Chemical). The solvent used was ethyl alcohol/1% acetic acid in *n*-hexane (15:85) at 0.05 ml/min. The eluate was made up to 0.35 ml/min by the addition of ethyl alcohol and was introduced to the mass spectrometer operated at negative ion mode. Silica gel thin-layer chromatography (TLC) employed Kieselgel 60 F_{254} (Merck; 20 × 20 cm, 0.50 and 0.25 mm). All the chemicals used were of reagent grade unless otherwise described.

Preparation of compounds and chemicals

(±)-Pinoresinols,[13] (±)-lariciresinols,[12] (±)-secoisolariciresinols,[13] (±)-matairesinols,[14] (±)-wikstromols,[10] (±)-[9,9,9′,9′-$^2H_4$]pinoresinols,[11] (±)-[9,9,9′,9′-$^2H_4$]lariciresinols,[11] (±)-[aromatic-^{2}H]secoisolariciresinols,[11] and (±)-[3-OC$^2H_3$]matairesinols[15] were prepared previously.

Plant material

The callus culture of *Daphne odora* Thumb. prepared previously[15] was maintained for 3 years on Wolter and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (1.0 μM) and 6-benzyladenine (1.0 μM). It was used for lignan isolation, a feeding experiment, and as the source for enzyme preparation. *Daphne genkwa* Sieb. et Zucc. was obtained from a local nursery, maintained in the experimental forest of Wood Research Institute, Kyoto University, and used for the source for enzyme preparation.

Isolation of lignans

Daphne odora callus (fresh weight 62.9 g) was freeze-dried and extracted with hot MeOH. The MeOH extracts thus obtained were incubated with β-glucosidase (Sigma; G-0395, 230 units) in 0.1 M NaOAc buffer (pH 5.0, 35 ml) at 37°C for 24 h and extracted with CH_2Cl_2. The CH_2Cl_2 extracts (216.4 mg) were submitted to purification by TLC and reversed-phase HPLC to afford pure lignans.

Determination of enantiomeric composition

Enantiomeric compositions of lignans were determined by chiral HPLC analysis. Lignans that gave only a single peak corresponding to one enantiomer on the chiral HPLC chromatogram were expressed as optically pure or >99% e.e. Enantiomeric compositions of lignans with peaks corresponding to (+)- and (−)-enantiomers in the chiral HPLC analysis were determined by GC-MS using deuterium-labeled racemic lignans as internal standards as described previously[3,9,11,16] or by semimicro chiral LC-APCI-MS.

Enzyme preparation

All procedures were conducted at about 4°C or on an ice bath. *D. odora* callus (53.0 g) was ground for a few minutes in the presence of polyclar AT (2.8 g), acid-washed sea sand, and a potassium phosphate buffer (0.1 M, pH 8.0) containing 10 mM dithiothreitol (20.0 ml). The slurry thus obtained was filtered through four layers of gauze, and the filtrate (45.0 ml) was centrifuged (10 000 g, 20 min). The supernatant (43.5 ml) was filtered through Whatman GF/A glass fiber filter. Solid ammonium sulfate was added to the filtrate up to 70% saturation. The precipitate obtained after centrifugation (14 000 g, 15 min) was redissolved in 50 mM Tris-HCl buffer (pH 8.0, 4.0 ml), and the solution so obtained was applied to a Sephadex G-25 column [Pharmacia, particle size 100–300 (coarse)] preequilibrated in 50 mM Tris-HCl buffer (pH 8.0). The protein fraction (6.0 ml) excluded from the gel was collected and used as the *D. odora* SIRD preparation. GC-MS analysis showed that the EtOAc extract of the cell-free preparation did not contain any detectable amounts of the lignans, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, or wikstromol.

Cell-free extracts from *D. genkwa* were prepared, partially purified exactly as previously reported,[3] and used as the *D. genkwa* SIRD preparation. The protein content of the enzyme preparation was measured by the method of Bradford[17] using bovine serum albumin as the standard.

Enzymatic conversion of (±)-secoisolariciresinols and (±)-[aromatic-^{2}H]secoisolariciresinols

The assay mixture (500 μl) consisted of 5 μl of the solution of (±)-secoisolariciresinols in MeOH (25 mM), 15 μl of NADP solution (50 mM in 50 mM Tris-HCl buffer, pH 8.0), and *D. odora* enzyme preparation (480 μl). After 1 h of incubation at 30°C, the reaction mixture was extracted with EtOAc containing (±)-[3-OC$^2H_3$]matairesinols as an internal standard. EtOAc solubles were evaporated to dryness, and the formed matairesinol was identified and quantified by GC-MS. In a separate experiment, (±)-secoisolariciresinols (0.75 μmol) were incubated as above except that all volumes were scaled up proportionately. The formed matairesinol was purified by TLC and HPLC and submitted to chiral HPLC and semimicro chiral LC-APCI-MS analyses. (±)-[Aromatic-^{2}H]secoisolariciresinols were incubated in the same manner as above with *D. genkwa* enzyme preparation,

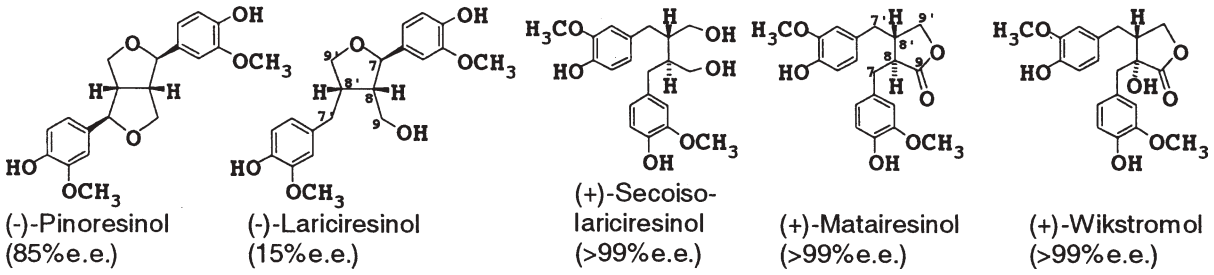

Fig. 1. Lignans isolated from *Daphne odora* callus culture and their enantiomeric compositions. Note that only the predominant enantiomers are shown

and the formed matairesinol was identified and analyzed by chiral HPLC as above.

Results and discussion

In a previous study, four lignans, pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol (Fig. 1) were isolated from *Daphne odora* shoots, and their enantiomeric compositions were determined: (−)-pinoresinol (95% e.e.), (−)-lariciresinol (89% e.e.), (+)-secoisolariciresinol (>99% e.e.), and (+)-matairesinol (>99% e.e.).[3] These lignans and wikstromol (Fig. 1) were also detected by GC-MS in MeOH extracts of *D. odora* callus and suspension cultures.[15] However, because different enantiomers of secoisolariciresinol occur predominantly in different organs of *Arctium lappa*,[4,8,9] the lignans in the *D. odora* callus culture were isolated and their enantiomeric compositions determined and compared with those from the *D. odora* shoots.

Thus, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, and wikstromol were isolated and identified by comparing their ^{1}H-NMR spectral data with those of synthesized authentic samples. Figure 2 contains chiral HPLC chromatograms of the lignans, showing that two dibenzylbutyrolactone lignans (matairesinol and wikstromol) and a dibenzylbutane lignan (secoisolariciresinol) isolated from *D. odora* callus were optically pure (>99% e.e.) and dextrorotatory (Figs. 1, 2); the enantiomeric compositions of pinoresinol and lariciresinol isolated from the callus were 85% and 15% e.e. in favor of (−)-enantiomers, respectively (Fig. 1).

The percent e.e. values indicate that there is no difference in the predominant enantiomers between the lignans from the callus and the shoots. In addition, the results accord well with the general features of the enantiomeric compositions of lignans.[1] First, pinoresinol and lariciresinol are not optically pure, whereas dibenzylbutyrolactone lignans are. Some plants produce optically pure secoisolariciresinol, whereas the others do not.[1] Second, the finding that matairesinol and wikstromol isolated were dextrorotatory is characteristic of dibenzylbutyrolactone lignans of Thymelaeaceae plants.[1,3,10,11]

Next, before the enzyme assay was carried out, [aromatic-$^{13}C_6$]phenylalanine was administered to *D. odora* callus, and the following transformation was observed by

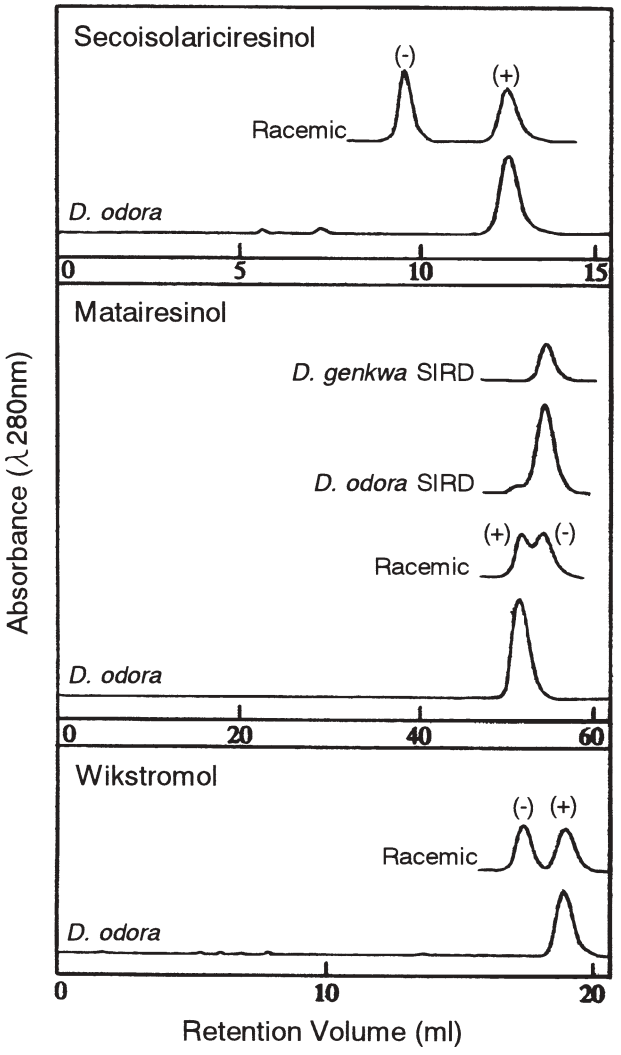

Fig. 2. Chiral high-performance liquid chromatograms of lignans isolated from *Daphne odora* callus and enzymatically formed matairesinol. *D. odora*, lignans isolated from *D. odora*. *Racemic*, racemic authentic sample. *D. odora SIRD* and *D. genkwa SIRD*, obtained following incubation of (±)-secoisolariciresinols with *D. odora* and *D. genkwa* SIRDs, respectively. (+) and (−), dextrorotatory and levorotatory enantiomers, respectively. The elution details are as follows: Chiralcel OD column (Daicel; 250 × 4.6 mm) with EtOH/1% AcOH in *n*-hexane (30:70) at 0.8 ml/min for secoisolariciresinol; Chiralcel OD column with EtOH/1% AcOH in *n*-hexane (15:85) at 1.0 ml/min for matairesinol; Chiralcel OC column (Daicel, 250 × 4.6 mm) with EtOH/*n*-hexane (50:50) at 0.5 ml/min for wikstromol

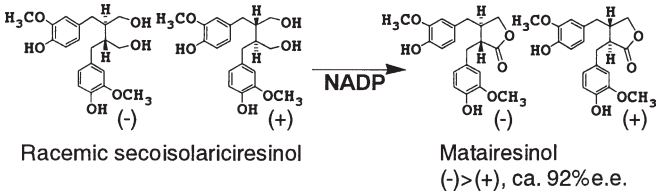

Fig. 3. Formation of (−)-matairesinol with *Daphne odora* secoisolariciresinol dehydrogenase

Table 1. Enzymatic formation of matairesinol from (±)-secoisolariciresinols

Assay	Cofactor	Matairesinol formation[a]
Complete	NADP	1.8
Controls[b]	None	0.1
	Boiled enzyme/NADP	0

[a] Expressed in nmol h^{-1} mg^{-1} protein
[b] Control experiments refer to the complete assay with the omission of NADP or with the denatured enzyme (boiled for 10 min). One other control experiment was done 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 amount of formed matairesinol was 0.2 nmol mg^{-1} protein

GC-MS analysis of methanol extracts obtained afterward (data not shown): [aromatic-$^{13}C_6$]phenylalanine → pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol. This result confirmed active lignan biosynthesis in the *D. odora* callus used in the experiment.

We then assayed SIRD activity. Matairesinol was formed following incubation of (±)-secoisolariciresinols in the presence of NADP with SIRD preparation from *D. odora* callus culture but not in the control systems without NADP or with an enzyme denatured by boiling, indicating that the matairesinol formation is enzymatic (Table 1). The enantiomeric composition of the formed matairesinol was estimated as about 92% e.e. in favor of (−)-enantiomer (Figs. 2, 3) by chiral HPLC analysis.[18] The result was further confirmed by semimicro chiral LC-APCI-MS: Both (−)- and (+)-matairesinols were detected by selected ion monitoring of the quasi-molecular ion (*m/z* 357), and the (−)-enantiomer predominated (data not shown).

The SIRDs have been detected in *Forsythia intermedia*[5–7] and *Arctium lappa*.[4] Both *Forsythia* and *Arctium* SIRDs catalyzed the formation of optically pure (−)-matairesinol from (±)-secoisolariciresinols; and optically pure (−)-matairesinol was also isolated from both plants. Thus, the enantiomer is consistent between the enzymatically formed and naturally occurring ones. Taking these facts into account, it seemed likely that (+)-matairesinol was formed from (±)-secoisolariciresinols by *D. odora* SIRD. However, the present result indicates that this is not the case: The (−)-enantiomer, which is opposite to that isolated from the plant, was formed preferentially during the enzymatic reaction.

Such an inconsistency was also observed with another Thymelaeaceae plant, *Daphne genkwa*. Lignans isolated from *D. genkwa* shoots had the following enantiomeric compositions: (−)-pinoresinol (88% e.e.), (−)-lariciresinol (88% e.e.), (+)-secoisolariciresinol (97% e.e.), and (+)-matairesinol (>99% e.e.).[3] On the other hand, optically pure (−)-matairesinol (Fig. 2) was formed following incubation of (±)-secoisolariciresinols as above with the NADP and *D. genkwa* SIRD preparation.

Thus, (−)-matairesinol was formed preferentially in the in vitro reactions with enzyme preparations of both *Daphne* species (Fig. 3), whereas the opposite (+)-enantiomer was isolated from *D. odora* callus (Fig. 1) and shoots[3] and *D. genkwa* shoots.[3] The results suggest that the present in vitro reaction system lacks factors that direct formation of the (+)-enantiomer or that the system employed a physiologically incorrect substrate. If the latter is the case, for example, secoisolariciresinol glycoside might be a true substrate, and a glycoside of (+)-secoisolariciresinol – but not of (−)-enantiomer – might be oxidized selectively to the corresponding matairesinol glycoside by *Daphne* SIRD. A preliminary experiment indicated that significant parts of the lignans of *D. odora* callus were present as glycosides.[15]

The possible mechanism that optically pure (+)-matairesinol accumulated in the *Daphne* plants following selective conversion of (−)-matairesinol to some other lignans seems unlikely. This is because a possible product from matairesinol, wikstromol (8′-hydroxymatairesinol), isolated from *D. odora* callus was found to be optically pure and dextrorotatory, which thus has the same absolute configuration as that of (+)-matairesinol.

In any event, these results present a new question about the lignan biosynthesis in Thymelaeaceae plants. Further experiments are required to elucidate the mechanisms for (+)-matairesinol formation in Thymelaeaceae plants: *Daphne* spp. and *Wikstroemia* spp.

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