Pergamon

PII: S0031-9422(97)00458-5

Phytochemistry, Vol. 47, No. 4, pp. 665–670, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

FORMATION OF (+)-EUDESMIN IN *MAGNOLIA KOBUS* DC. VAR. *BOREALIS* SARG.*

TERUHISA MIYAUCHI and SHUJI OZAWA†

Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060, Japan

(*Received* 15 *January* 1997; *in revised form* 22 *April* 1997)

Key Word Index—*Magnolia*; Magnoliaceae; *O*-methyltransferase; lignans; eudesmin; pinoresinol; momomethyl pinoresinol; biosynthesis; enantioselectivity; chiral separation.

Abstract—The formation of the non-phenolic furofuran lignan, (+)-eudesmin, in *Magnolia kobus* var. *borealis* was investigated. Chiral analyses by HPLC revealed that the pinoresinol isolated from the shoots was a mixture of (+)- and (−)-enantiomers with the former being predominant (77.1% enantiomeric excess). In contrast, eudesmin was present only as the (+)-antipode. *In vivo* labelling experiments of *M. kobus* var. *borealis* shoots with [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol have shown that pinoresinol was derived from the coupling of two intact coniferyl alcohol molecules. Studies in a cell-free system established that the conversion of pinoresinol to eudesmin was achieved by step-wise methylation in the presence of *S*-adenosyl-L-methionine. However, the *in vitro* transmethylation reactions observed were not enantioselective, since both (+)- and (−)-eudesmins were formed. However, the naturally occurring (+)-enantiomer was the predominant product. Consequently, it is suggested that the sequence of reactions *in vivo* probably begins with a highly stereoselective coupling step that yields the furofuran skeleton [i.e., (+)-pinoresinol]. Then the subsequent non-enantioselective methylation of this skeleton could result in formation of non-phenolic furofuran lignans such as (+)-eudesmin. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

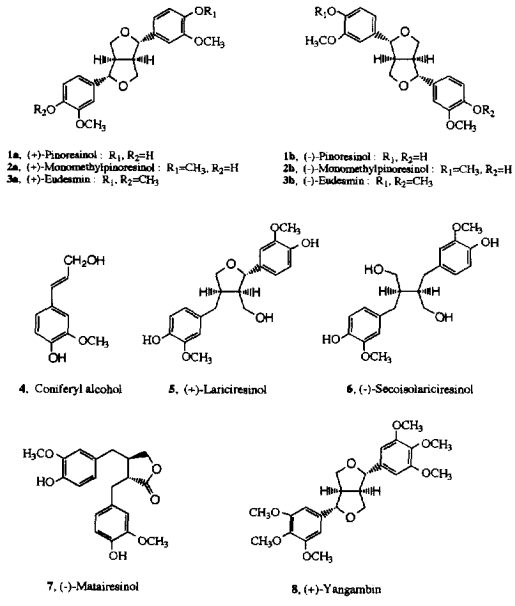
Lignans are widely distributed secondary metabolites that consist of two phenylpropane units, linked by 8,8′ bonds. Because most naturally occurring lignans are produced in an optically active form, it is likely that the relevant biosynthetic reactions are both stereoselective and enantioselective, in contrast to the random free-radical couplings that are involved in the biosynthesis of lignin. Therefore, a central problem in the biosynthesis of lignans is the stereochemical mechanism responsible for their formation.

In 1990, Umezawa *et al.* demonstrated the first example of a cell-free system from *Forsythia intermedia* for the enantioselective formation of the lignans (−)-secoisolariciresinol (**6**) and (−)-matairesinol (**7**) [1, 2, 3]. Lewis *et al.* then showed that, in *F. intermedia*, (+)-pinoresinol (**1a**) undergoes sequential enantioselective NAD(P)H-dependent benzylic ether reduction to yield first (+)-lariciresinol (**5**) [4, 5] and then (−)-secoisolariciresinol (**6**) [6]. Also, they reported the purification and characterization of (+)-pinoresinol/(+)-lariciresinol reductase from *F. intermedia* and the cloning of its cDNA, and the overexpression of catalytically active recombinant protein [7]. More recently, Davin *et al.* isolated a 78 kDa protein from *Forsythia* that, in the presence of an oxidase or one electron oxidant, confers stereoselective bimolecular phenoxy radical coupling to give (+)-pinoresinol [8].

Recent studies of lignan biosynthesis have focused on the enzymology and stereochemistry of the above-mentioned lignan transformations [7, 8]. However, the biosynthesis of non-phenolic furofuran lignans has received little attention to date. In a previous investigation of the constituents of *Magnolia kobus* var. *borealis*, we found that it contains various furofuran lignans, such as (+)-eudesmin (**3a**) and (+)-yangambin (**8**) [9]. The lignans in this plant have 4-hydroxy-3-methoxy-, 3,4-dimethoxy-, 3,4-methylenedioxy-, 4-hydroxy-3,5-dimethoxy- and 3,4,5-trimethoxy-substituted pendant aromatic rings [9]. In general, during the biosynthesis of lignin, three substitution patterns with 4-hydroxy, 4-hydroxy-3-methoxy and 4-hydroxy-3,5-dimethoxy phenyl groups, are gradually created at the cinnamic acid level by a sequence of hydroxylations and methylations [10]. A similar sequence might operate in the formation of lignans. However, formation of 3,4-

*This paper is dedicated to Clarence (Bud) Ryan on the occasion of his sixty-fifth birthday.

† Author to whom correspondence should be addressed.



dimethoxy, 3,4-methylenedioxy and 3,4,5-trimethoxy phenyl compounds involve reactions that are, as yet, only poorly understood.

Thus, as an example of the biosynthesis of non-phenolic furofuran lignans, we chose to investigate the formation of eudesmin in this plant at the cell-free level. Although direct experimental evidence was lacking at the time, it was suggested initially that eudesmin (**3**) is formed from pinoresinol (**1**) by stepwise methylation. This set of reactions is of interest in connection with the question of the sequence of reactions that results in substituted aryl rings, such as those in the 3,4-dimethoxy and 3,4,5-trimethoxy series.

RESULTS AND DISCUSSIONS

The biosynthetic sequence leading to eudesmin (**3**) has not yet been established. From considerations of structures and possible transformations, we prepared several furofuran lignans in a racemic (±)-form by chemical synthesis, namely, (±)-pinoresinols (**1a**, **1b**), (±)-monomethylpinoresinols (**2a**, **2b**), and (±)-eudesmins (**3a**, **3b**). Although a number of elegant methods have been reported for the synthesis of (±)-2,6-diaryl-3,7-dioxabicyclo[3.3.0]octane lignans [11, 12], we chose simple methods for the preparation of the above compounds. (±)-Pinoresinols (**1a**, **1b**) were synthesized by a general method, namely, the oxidative dimerization of coniferyl alcohol (**4**) by a horseradish peroxidase–hydrogen peroxide system [13]. The (±)-pinoresinols (**1a**, **1b**) obtained were methylated to yield (±)-monomethylpinoresinols (**2a**, **2b**) and (±)-eudesmins (**3a**, **3b**) by treatment with Me_2SO_4 in aqueous alkali. Each racemic lignan was separated into its distant enantiomeric forms on a Chiralcel OD column

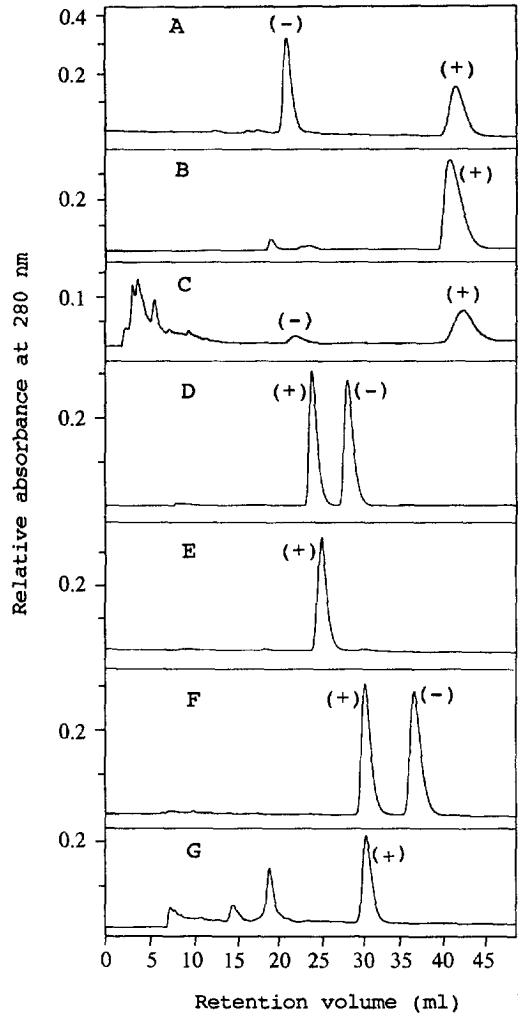


Fig. 1. Chiral HPLC separations of selected lignans. (A) Synthetic (±)-pinoresinols (**1a**, **1b**). (B) (+)-Pinoresinol (**1a**) isolated from *Abies sachalinensis*. (C) Pinoresinol (**1**) isolated from *M. kobus* var. *borealis*. (D) Synthetic (±)-monomethylpinoresinols (**2a**, **2b**). (E) Synthetic (+)-monomethylpinoresinol (**2a**). (F) Synthetic (±)-eudesmins (**3a**, **3b**). (G) (+)-Eudesmin (**3a**) isolated from *M. kobus* var. *borealis*. HPLC conditions: Chiralcel OD column (250 × 4.6 mm, Daicel) eluted with EtOH–*n*-Hexane, flow rate 0.5 ml min^{-1}.

(Fig. 1), with the optical form of each antipode determined as follows. The (+)- and (−)-pinoresinols (**1a** and **1b**) were distinguished by comparison with authentic (+)-pinoresinol (**1a**) that had been isolated from *Abies sachalinensis* [14] [Figs 1(A) and 1(B)]. Also, (+)- and (−)-monomethylpinoresinols (**2a** and **2b**) were distinguished by use of compound **2a** that had been obtained by methylation of compound **1a** [Figs 1(D) and 1(E)], whereas (+)- and (−)-eudesmins (**3a** and **3b**) were identified by comparison with compound **3a** that had been isolated from *M. kobus* var. *borealis* [Figs 1(F) and 1(G)].

As a preliminary experiment, we performed labelling experiments *in vivo*. Thus, we administered [9,9-

$^2H_2$,OC^2H_3] coniferyl alcohol (**4**) to shoots of *M. kobus* var. *borealis* and allowed metabolism to continue for 12 hr. Pinoresinol (**1**) and eudesmin (**3**) were isolated by reversed-phase HPLC and subjected to mass spectrometric examination. Also, the enantiomeric composition of the isolated pinoresinol (**1**) and eudesmin (**3**) were analysed by chiral HPLC. As shown in Fig. 2(B), the mass spectrum of synthetic unlabelled (±)-pinoresinols (**1a**, **1b**) included a large $[M]^+$ at *m/z* 358 and a base ion at *m/z* 151 $[ArCO]^+$. By contrast, the pinoresinol isolated from shoots of *M. kobus* var. *borealis*, to which deuterated coniferyl alcohol (**4**) had previously been administered, gave enhanced signals at *m/z* 368 $[M+10]^+$ and 154 $[ArCO+3]^+$ [Fig. 2(A)], an indication that it was derived from two intact deuterated coniferyl alcohol (**4**) units. The chromatogram obtained by chiral HPLC indicated that the pinoresinol isolated from shoots of *M. kobus* var. *borealis* was a mixture of (+)- and (−)-enantiomers (**1a** and **1b**) with the former predominating (77.1% enantiomeric excess) [Fig. 1(C)]. A possible explanation for this result is that formation of pinoresinol in *M. kobus* might involve two enzymes, a specific enzyme that yields (+)-pinoresinol (**1a**) and a non-specific peroxidase that synthesizes racemic (±)-pinoresinols (**1a**, **1b**), with the former activity predominating to some extent, in contrast, chiral HPLC analysis of the eudesmin isolated from shoots of *M. kobus* var. *borealis* revealed only the presence of the (+)-enantiomer (**1a**) [Fig. 1(G)]. Also, preliminary feeding experiments suggested that no incorporation of deuterated coniferyl alcohol (**4**) into eudesmin (**3**) was observed.

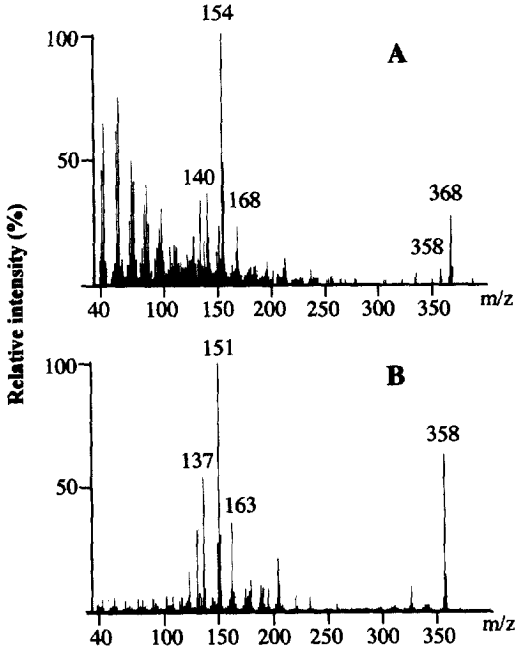


Fig. 2. Mass spectra of pinoresinol (**1**). (A) Deuterated pinoresinol obtained following administration of [9,9-$^2H_2$,OCH_3]coniferyl alcohol to *M. kobus* var. *borealis* shoots; (B) Synthetic unlabeled (±)-pinoresinols (**1a**, **1b**).

Next, we attempted to demonstrate the transformation of pinoresinol (**1**) to eudesmin (**3**). To examine this conversion and in order to determine whether a cell-free extract of *M. kobus* var. *borealis* could catalyse the methylation of furofuran lignans that contained a *p*-hydroxy moiety, we assayed *O*-methyltransferase (OMT) activity with (±)-pinoresinols (**1a**, **1b**) and (±)-monomethylpinoresinols (**2a**, **2b**) as substrates. Thus, we incubated (±)-pinoresinols (**1a**, **1b**) with a cell-free extract of *M. kobus* var. *borealis* in the presence of *S*-adenosyl-L-[methyl-^{14}C]methionine ([Me-^{14}C]SAM; 2.5 mM; 6.67 kBq). After incubation for various times, the enzymatic reaction was terminated by addition of HOAc, and unlabelled (±)-monomethylpinoresinols (**2a**, **2b**) and (±)-eudesmins (**3a**, **3b**) were added as carriers. The (±)-monomethylpinoresinols (**2a**, **2b**) and (±)-eudesmins (**3a**, **3b**) were separated by reversed-phase HPLC and fractions were subjected to determination of radioactivity in a liquid scintillation counter. As shown by the results in Table 1, we established that (±)-pinoresinols (**1a**, **1b**) had been converted to monomethylpinoresinol (**2**) by the action of an *O*-methyltransferase. No significant amounts of lignans were detected in control assays with heat-denatured protein, indicating that the formation of lignan was enzymatic. The rate of formation of monomethylpinoresinol was approximately constant for up to 120 min. Chiral HPLC and radiochemical analysis of the monomethylpinoresinol formed during a 120 min incubation revealed that (+)-pinoresinol (**1a**) was the preferred substrate, as compared to the (−)-antipode (**1b**), for the formation of monomethylpinoresinol (Table 2). Moreover, no evidence was found for the formation of eudesmin (**3**) when (±)-pinoresinols (**1a**, **1b**) were used as the substrates.

In an analogous manner, we incubated (±)-monomethylpinoresinols (**2a**, **2b**) with a cell-free extract of *M. kobus* var. *borealis* for 120 min in the presence of [Me-^{14}C]SAM. This reaction resulted in the formation of eudesmin (**3**) (101 pmol mg^{-1} protein). Chiral HPLC and radiochemical analysis of the eudesmin (**3**)

Table 1. Transmethylation of (±)-pinoresinols (**1a**, **1b**) by a cell-free extract of *M. kobus* var. *borealis*

Enzyme assay	Incubation period (min)	Formation of monomethyl-pinoresinol (**2**) (pmol mg^{-1} protein)
1	30	55
2	60	77
3	120	108
Control*	120	0

(±)-Pinoresinols (**1a**, **1b**) were incubated with a cell-free extract in the presence of [Me-^{14}C]SAM at 30°. The protein content of the cell-free extract was 4.0 mg ml^{-1}.

* Control experiments refer to the complete assay with heat-denatured proteins (boiled for 10 min).

Table 2. Enantioselectivity of the transmethylation of (±)-pinoresinols (**1a**, **1b**) and (±)-monomethylpinoresinols (**2a**, **2b**) by a cell-free extract of *M. kobus* var. *borealis*

Substrates	Products (pmol hr^{-1} mg^{-1} protein)	
(±)-Pinoresinols	Monomethylpinoresinol	
	(+) **2a**	40.1
	(−) **2b**	13.9
(±)-Monomethylpinoresinols	Eudesmin	
	(+) **3a**	33.7
	(−) **3b**	16.4

Substrates were incubated with a cell-free extract in the presence of [Me-^{14}C]SAM for 120 min at 30°.

The protein content of the cell-free extract was 4.0 mg ml^{-1}.

that had been formed enzymatically from monomethylpinoresinol (**2**) indicated that the cell-free extract had catalysed the preferential formation of (+)-eudesmin (**3a**), as compared to the unnatural (−)-antipode (**3b**) (Table 2). Thus, although the existence of monomethylpinoresinol (**2**) has not yet been established in *M. kobus* var. *borealis*, the formation of eudesmin appears to occur by the *O*-methylation of pinoresinol (**1**), via monomethylpinoresinol (**2**).

Taken together, our results show that a cell-free extract of *M. kobus* var. *borealis* can catalyse the *O*-methylation of pinoresinol (**1**) and monomethylpinoresinol (**2**), suggesting the conversion of pinoresinol (**1**) to eudesmin (**3**) by step-wise methylation in presence of *S*-adenosyl-L-methionine. Thus, methylation of 4-hydroxy-3-methoxy phenyl compounds to the corresponding compounds in the 3,4-dimethoxy series seems likely to occur in the biosynthetic pathway to furofuran lignans in *Magnolia*.

Judging from the results of the *in vitro* transmethylations, there is some level of enantioselectivity to (+)-pinoresinol (**1a**) and (+)-monomethylpinoresinol (**2a**) when racemic mixtures were used as substrate in the cell free reactions to give ultimately (+)-eudesmin (**3a**). However, in view of the lack of high enantioselectivity of the methylation reaction that is involved in the formation of eudesmin in *M. kobus* var. *borealis*, we suggest that a reasonable sequence of reactions begins with a highly stereoselective coupling step that yields the furofuran skeleton [i.e., (+)-pinoresinol (**1**)], and then the subsequent non-enantioselective methylation of the skeleton could result in formation of the non-phenolic furofuran [i.e., (+)-eudesmin (**3**)].

It is clear that cell-free extracts of *M. kobus* var. *borealis* contain a soluble *O*-methyltransferase system that can methylate phenolic furofuran lignans at the *para* position. This system must surely be different from the *O*-methyltransferase involved in the biosynthesis of lignin, which generally exhibit absolute specificity toward the *meta* hydroxy group of dihydroxycinnamate monomers, such as caffeic acid. Future studies will be directed towards the further characterization of these enzyme systems.

EXPERIMENTAL

Plant material. Magnolia kobus var. *borealis* plants for prepn of cell-free extracts were collected in August, 1996, on the campus of Hokkaido University. Plants for feeding experiments were maintained in the greenhouse facilities of Hokkaido University.

Chromatography materials and instrumentation. Prep. TLC was performed on plates on Kieselgel 60 F_{254} (Merck, Germany). All solvents and chemicals used were reagent or HPLC grade unless otherwise stated. HPLC was carried out with detection of 280 nm using either a column of Nova-Pak C_{18} (150 × 3.9 mm i.d.; Waters) for reversed-phase chromatography, or a column of Chiralcel OD (250 × 4.6 mm i.d.; Daicel) for chiral sepns. Sepn by reversed-phase HPLC of reaction products and substrates was performed with CH_3CN–3%HOAc in H_2O (3:7) at a flow rate of 1 ml min^{-1}. Chiral HPLC was performed with EtOH–*n*-Hexane (1:1) at a flow rate of 0.5 ml min^{-1} for (+)- and (−)-pinoresinols (**1a** and **1b**), (+)- and (−)-monomethylpinoresinols (**2a** and **2b**) and (+)- and (−)-eudesmins (**3a** and **3b**). ^{1}H NMR spectra were recorded on an AM-500 system (Bruker) with tetramethylsilane as the int. standard. Electron impact mass spectrometry was performed with a JMS-DX 300 system (JEOL). Radioactive samples were mixed with AQUASOL-2 liquid scintillation fluid (DuPont/NEN Research Products) and radioactivity was determined in a LSC-1000 liquid scintillation system (Aloka).

Chemical syntheses. [9,9-$^2H_2$,OC$^2H_3$]Coniferyl alcohol (**4**) [3] and (±)-pinoresinols (**1a**, **1b**) [13] were prepd as previously described.

(±)-Monomethylpinoresinols (**2a**, **2b**) and (±)-eudesmins (**3a**, **3b**) were synthesized as follows: (±)-Pinoresinols (**1a**, **1b**) (51.7 mg, 0.143 mmol) were dissolved in 0.25 M NaOH (1.50 ml) under N_2, and then Me_2SO_4 (60 μl) was added with stirring. The reaction mixt. was refluxed for 1 hr, after which 0.25 M NaOH (1.0 ml) and Me_2SO_4 (60 μl) were added. After the mixt. had been refluxed for a further 30 min, H_2O (400 ml) was added and the soln was extracted with EtOAc (500 ml × 2). The extract was washed with satd NaCl (100 ml × 2), dried with Na_2SO_4, filtered and evapd to give a gum. Purification of the crude product by prep. TLC ($CHCl_3$–MeOH, 100:1) yielded (±)-monomethylpinoresinols (**2a**, **2b**) (14.8 mg, 28%) and (±)-eudesmins (**3a**, **3b**) (11.1 mg, 20%). Identifications were made as follows.

(±)-*Monomethylpinoresinols* (**2a**, **2b**). IR ν cm^{-1}: 3414, 2955, 1514, 1462, 1265, 1027, 761. EI-MS (*m/z*): 372 $[M]^+$, 219, 205, 177, 165, 151, 137. ^{1}H NMR ($CDCl_3$) δ: 3.11 (2H, *m*, H-8, H-8′), 3.87 (3H, *s*, OMe), 3.89 (3H, *s*, OMe), 3.90 (3H, *s*, OMe), 3.92 (2H, over-

lapped with OMe signals, Ha-9, Ha-9′), 4.23 (2H, *dd*, $J_1 = 6.9$ Hz, $J_2 = 9.0$ Hz, Hb-9, Hb-9′), 4.74 (1H, *d*, $J = 4.5$ Hz, H-7 or H-7′), 4.76 (1H, *d*, $J = 4.4$ Hz, H-7′ or H-7), 5.57 (1H, *s*, OH), 6.81–6.91 (6H, *m*, arom. H).

(±)-*Eudesmins* (**3a**, **3b**). IR ν cm^{-1}: 2951, 1515, 1255, 1137, 1024, 666. EI-MS (*m/z*): 386 [M], 219, 189, 177, 165 (base ion), 151. ^1H NMR ($CDCl_3$) δ 3.12 (2H, m, H-8, H-8′), 3.87 (6H, *s*, OMe), 3.90 (6H, *s*, OMe), 3.88–3.91 (2H, overlapped with OMe signals, Ha-9, Ha-9′), 4.24 (2H, *dd*, $J_1 = 6.8$ Hz, $J_2 = 9.0$ Hz, Hb-9, Hb-9′), 4.75 (2H, *d*, $J = 4.3$ Hz, H-7, H-7′), 6.83–6.91 (6H, arom. H).

Feeding experiments. Shoots of *M. kobus* var. *borealis* (*ca* 10 cm long with two leaves) were excised with a razor. The cut end of each shoot was immersed in a soln of [9,9-$^2H_2$,OC^2H_3]coniferyl alcohol (2.5 mg) in 0.1 M K-Pi buffer (pH 7.0, 530 μl). After uptake and metabolism for 6 hr, additional 0.1 M K-Pi buffer (pH 7.0, 530 μl) was added. The shoots were then allowed to metabolize for an additional 6 hr. Then leaves were removed and stems were freeze-dried. The resulting dried stems were crushed with scissors and extracted with hot MeOH (20 ml × 1, 10 ml × 4, for 10 min each). The combined MeOH soluble frs were evapd to dryness *in vacuo* and the residue was dissolved in distilled H_2O (20 ml). The soln was then extracted with CH_2Cl_2 (20 × 3 ml). The combined extracts were dried and then subjected to reversed-phase HPLC on an Inertsil ODS column (20.0 × 250 mm; Gasukuro Kogyo Inc., Japan) with a linear gradient of acetonitrile and 3% HOAc in H_2O as follows: $t = 0$ min (3:7) to $t = 5$ min (7:13), to $t = 10$ min (2:3), to $t = 20$ min (1:1) and finally to $t = 40$ min (1:0) with a flow rate of 8.0 ml min^{-1}. Frs corresponding to pinoresinol (R_t 22.87 min) and eudesmin (R_t 33.68 min) were individually collected and subjected to chiral HPLC analysis.

Extraction of enzymes. All manipulations were carried out at 4° unless otherwise stated. Young shoots (5–10 cm) of *M. kobus* var. *borealis* were defoliated and the resulting stems (18.85 g) were washed with both tap and distilled H_2O. They were sectioned (*ca* 5 mm), frozen in liquid N_2 and pulverized with a mortar and pestle. The resulting powder was homogenized with polyvinylpolypyrrolidone (20%), acid-washed sea sand (*ca* 10 g) and K-Pi buffer (0.1 M, pH 7.5, 35.0 ml) that contained 10 mM dithiothreitol. The homogenate was filtered through four layers of cheesecloth. After centrifugation (13 000 *g*, 15 min), the supernatant was filtered (Whatman GFA glass fibre filter). The resulting filtrate was slowly brought to 20% satn with $(NH_4)_2SO_4$. After centrifugation (13 000 *g*, 15 min) the supernatant was adjusted to 80% satn. The pptd protein was collected by centrifugation and resuspended in K-Pi buffer (0.1 M, pH 7.5, 5.0 ml). An aliquot (2.5 ml) of this soln was applied to a PD-10 column (Pharmacia, Sephadex G-25) that had been equilibrated with K-Pi buffer (0.1 M, pH 7.5), and the excluded fr. (3.5 ml) was used as the prepn of enzymes. Protein content was determined by the method of Bradford [15] using the Bio-Rad's protein assay dye.

Assay of enzymatic activity. The standard assay mixt. consisted of 5.0 mM substrate [(±)-pinoresinols (**1a**, **1b**) or (±)-monomethylpinoresinols (**2a**, **2b**), 50 μl in MeOH–H_2O (1:1)], 2.5 mM *S*-adenosyl-L-[methyl-^{14}C]methionine (50 μl, 6.67 kBq; DuPont/NEN Research Products), and the soln of enzymes (400 μl). The reaction was started by the addition of enzyme. After incubation at 30°, the reaction was terminated by the addition of glacial HOAc (75 μl). The reaction mixt. was extracted with EtOAc (1.0 ml) that contained the racemic lignan [30 μg, (±)-monomethylpinoresinols (**2a**, **2b**) or (±)-eudesmins (**3a**, **3b**)] as carrier. The EtOAc soluble materials from two assays were combined and evapd to dryness *in vacuo*. The resulting residue was dissolved in MeOH (300 μl) and filtered, and an aliquot (30 μl) was subjected to reversed-phase HPLC. For chiral sepns, aliquots (60 μl × 4) were first subjected to reversed-phase HPLC, with frs that contained lignans being individually collected and evapd to dryness *in vacuo*. Each sample of lignan was dissolved in MeOH (150 μl), filtered, and subjected to chiral HPLC analysis and liquid scintillation counting.

REFERENCES

1. Umezawa, T., Davin, L. B. and Lewis, N. G., *Biochemistry and Biophysics Research Communications*, 1990, **171**, 1008.
2. Umezawa, T., Davin, L. B., Yamamoto, E., Kingston, D. G. I. and Lewis, N. G., *Journal of the Chemical Society, Chemical Communications*, 1990, 1405.
3. Umezawa, T., Davin, L. B. and Lewis, N. G., *Journal of Biological Chemistry*, 1991, **266**, 10 210.
4. Katayama, T., Davin, L. B. and Lewis, N. G., *Phytochemistry*, 1992, **31**, 3875.
5. Chu, A., Dinkova, A., Davin, L. B., Bedgar, D. L. and Lewis, N. G., *Journal of Biological Chemistry*, 1993, **268**, 27 026.
6. Katayama, T., Davin, L. B., Chu, A. and Lewis, N. G., *Phytochemistry*, 1993, **33**, 581.
7. Dinkova, A. T., Gang, D. R., Davin, L. B., Bedgar, D. L., Chu, A. and Lewis, N. G., *Journal of Biological Chemistry*, 1996, **271**, 29 473.
8. Davin, L. B., Wang, H.-B., Crowell, A. L., Bedgar, D. L., Martin, D. M., Sarkanen, S. and Lewis, N. G., *Science*, 1997, **275**, 362.
9. Kim, Y.-G., Ozawa, S., Sano, Y. and Sasaya, T., *Research Bulletin of Hokkaido University Forest*, 1996, **53**, 1.
10. Higuchi, T., *Biosynthesis and Biodegradation of Wood Components*. Academic Press, London, 1985, p. 141.
11. Ayres, D. C. and Loike, J. D., *Chemistry and*

Pharmacology and Natural Products. Lignans: Chemical, Biological and Clinical Properties. Cambridge University Press, Cambridge, 1990, p. 303.
12. Ward, R. S., *Chemical Society Review*, 1982, **11**, 75.
13. Katayama, Y. and Fukuzumi, T., *Mokuzai Gakkaishi*, 1978, **24**, 664.
14. Ozawa, S. and Sasaya, T., *Mokuzai Gakkaishi*, 1988, **34**, 851.
15. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.