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Formation of Lignans (−)-Secoisolariciresinol and (−)-Matairesinol with *Forsythia intermedia* Cell-free Extracts*

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In vivo **labeling experiments of *Forsythia intermedia* plant tissue with [8-^{14}C]- and [9,9-$^{2}H_2$,OC$^{2}H_3$]coniferyl alcohols revealed that the lignans, (−)-secoisolariciresinol and (−)-matairesinol, were derived from two coniferyl alcohol molecules; no evidence for the formation of the corresponding (+)-enantiomers was found. Administration of (±)-[Ar-^{3}H]secoisolariciresinols to excised shoots of *F. intermedia* resulted in a significant conversion into (−)-matairesinol; again, the (+)-antipode was not detected. Experiments using cell-free extracts of *F. intermedia* confirmed and extended these findings. In the presence of NAD(P)H and H_2O_2, the cell-free extracts catalyzed the formation of (−)-secoisolariciresinol, with either [8-^{14}C]- or [9,9-$^{2}H_2$,OC$^{2}H_3$]coniferyl alcohols as substrates. The (+)-enantiomer was not formed. Finally, when either (−)-[Ar-^{3}H] or (±)-[Ar-^{2}H]secoisolariciresinols were used as substrates, in the presence of NAD(P), only (−)- and not (+)-matairesinol formation occurred. The other antipode, (+)-secoisolariciresinol, did not serve as a substrate for the formation of either (+)- or (−)-matairesinol. Thus, in *F. intermedia*, the formation of the lignan, (−)-secoisolariciresinol, occurs under strict stereochemical control, in a reaction or reactions requiring NAD(P)H and H_2O_2 as cofactors. This stereoselectivity is retained in the subsequent conversion into (−)-matairesinol, since (+)-secoisolariciresinol is not a substrate. These are the first two enzymes to be discovered in lignan formation.**

Lignans are a structurally diverse class of aromatic phenylpropanoid compounds widely distributed in gymnosperms (*e.g.* softwoods) and angiosperms (*e.g.* hardwoods). By 1978, lignans had been found in 46 families, 87 genera, and 146 species (1, 2); many more have since been isolated, and the structures of several hundred are now known.

Lignans are most frequently encountered as "dimers" elaborated from two "phenylpropanoid" monomers (1, 2), although higher oligomers have been isolated (3–5). Typically, "dimeric" lignans (1, 2) are grouped according to structural type. Among the most common groups are diarylbutanes (*e.g.* secoisolariciresinol **1**), 1,2-dibenzylbutyrolactones (*e.g.* matairesinol **2**, arctigenin **3**, arctiin **4**), furofurans (*e.g.* pinoresinol **5**, epipinoresinol **6**), 1-phenylnaphthalenes and tetralins (*e.g.* podophyllotoxin **7**) (see Fig. 1).

Like the closely related polymeric lignins, lignans have been isolated from all parts of plant material (roots, leaves, stems, bark, etc.) but are mainly located in woody tissue, particularly heartwood (6–9). Currently, we have no knowledge regarding the actual site of lignan formation (biosynthesis) and the subcellular location where they are initially deposited or stored. It is often assumed that lignans are deposited first in the vacuole and are then ultimately secreted into the cell wall following vacuole collapse. This has never been rigorously proven.

In terms of their biosynthetic pathways and structures, lignans and lignins are products of the shikimate/chorismate and phenylpropanoid pathways, and both are structurally related. Many substructures in lignins contain the structural elements of isolated lignans.

Lignans and lignins, however, apparently differ in one fundamentally important aspect, namely optical activity. For the most part, dimeric lignans (*e.g.* secoisolariciresinol **1**, pinoresinol **5**, matairesinol **2**, and podophyllotoxin **7**) are optically active (1, 2), whereas isolated lignins are not. It is perhaps significant that higher oligomeric forms of lignans (trimers, tetramers, etc.) typically have only very small $[\alpha]_D$ values (5). Indeed, the exact point of demarcation between oligomeric lignans and lignins is not well defined.

The optical rotation of a particular lignan can vary with plant source; *e.g. Forsythia suspensa* (10, 11) contains (+)-pinoresinol **5a**, whereas *Xanthoxylum ailanthoides* (12) has the (−)-enantiomer **5b**. No satisfactory explanation has been proffered to account for this stereochemical control leading to optical activity, other than that the reaction is somehow enzymatically mediated. Such control is *not* possible via intercession of a *typical* peroxidase/H_2O_2-catalyzed reaction, a reaction often implicated in lignin synthesis (13).

Surprisingly, the biosynthesis of lignans has been a neglected area, even for medicinally important compounds such as podophyllotoxin **7**, a chemical precursor for the drugs etoposide and teniposide in cancer chemotherapy (14, 15). Indeed, not a single enzymatic step in the initial coupling of monomers, or any of the subsequent modifications (oxidations, ring closures, etc.), has ever been reported. This is all the more surprising because of the close chemical relationship between lignans and lignins.

In spite of substantive efforts (16, 17), unambiguous proof of the exact chemical nature of the phenylpropanoid monomers undergoing coupling to afford the lignan dimer skeleton had not been obtained. From our standpoint, two possibilities were under consideration: the lignans, matairesinol **2**, arctigenin **3**, arctiin **4**, and podophyllotoxin **7**, could be formed

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This paper is dedicated to the memory of Professor K. V. Sarkanen.

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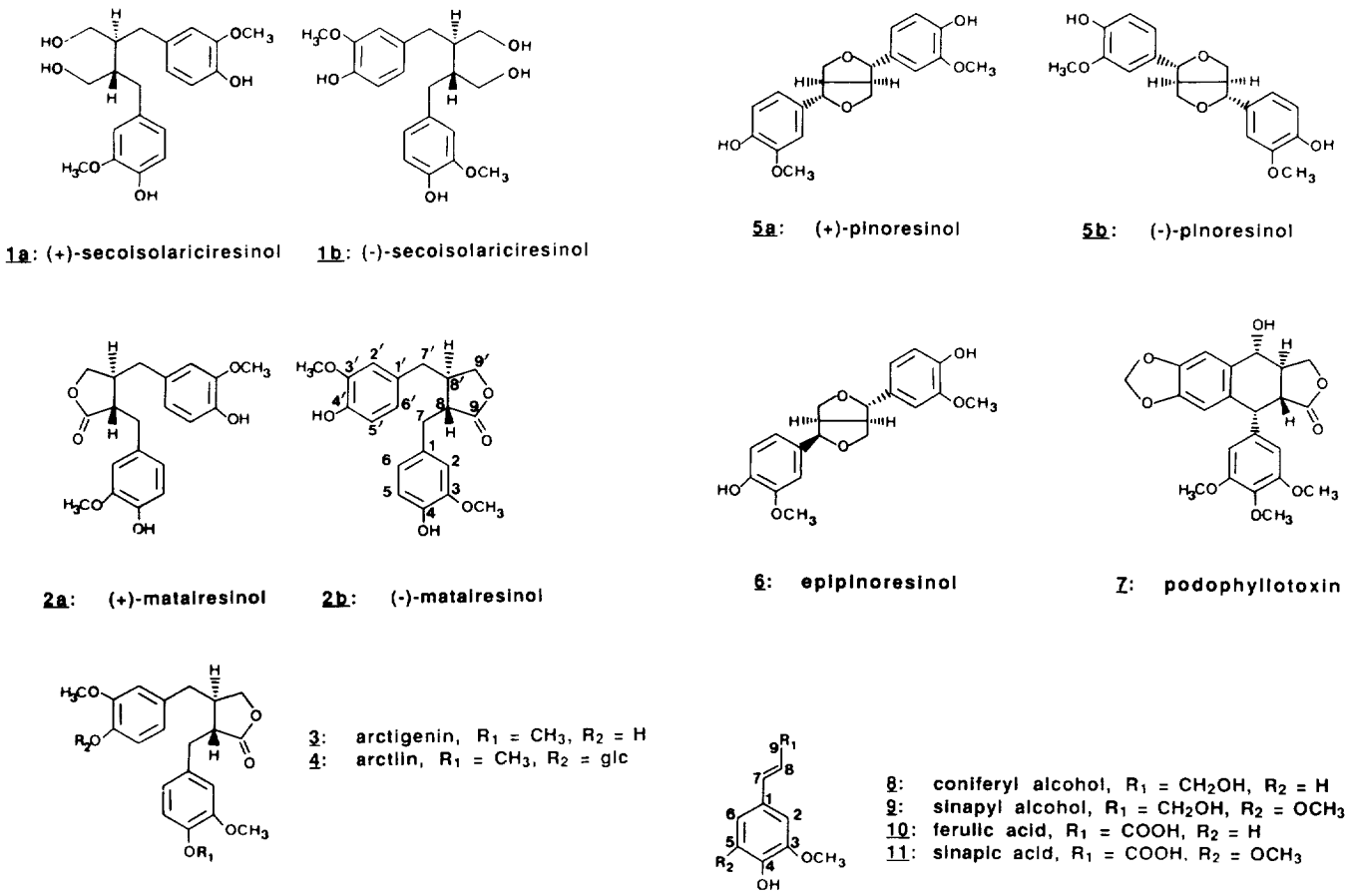

FIG. 1. **Representative lignans, monolignols, and hydroxycinnamic acids.**

via coupling of a monolignol (*e.g.* coniferyl **8** or sinapyl **9** alcohol) and a hydroxycinnamic acid (*e.g.* ferulic **10** or sinapic **11** acid). Alternatively, their formation could arise via direct coupling of either two monolignols or two hydroxycinnamic acids, with subsequent transformations occurring post-coupling. For example, secoisolariciresinol **1**, pinoresinol **5**, and epipinoresinol **6** could arise via direct coupling of the two monolignol molecules, coniferyl alcohol **8**.

It must be emphasized that this uncertainty, as regards identity of the phenylpropanoid monomer(s) undergoing coupling, was a key issue, since none of the possibilities described above could be ruled out. Herein, we describe the direct coupling of two coniferyl alcohol **8** moieties affording only (−)-secoisolariciresinol **1b**, which is then stereoselectively converted into (−)-matairesinol **2b**. These conversions have been demonstrated using cell-free preparations from *F. intermedia*.

EXPERIMENTAL PROCEDURES[1]

RESULTS AND DISCUSSION

The first goal of our research was to identify the key enzymatic reaction affording entry into the specialized biosynthetic pathway to the *Forsythia* lignans. This required identification of (i) the phenylpropanoid monomer(s) undergoing coupling (*i.e.* the substrate or substrates); (ii) the type of enzymatic coupling reaction (oxidative or reductive); and (iii) the immediate coupling product and its stereochemistry. For a molecule such as matairesinol **2**, its formation could occur either by coupling of one molecule of coniferyl alcohol **8** and one molecule of ferulic acid **10** followed by spontaneous lactone formation or via direct coupling of two coniferyl alcohol moieties to afford secoisolariciresinol **1** with subsequent dehydrogenation to give matairesinol **2**. Alternatively, ferulic acid **10** or coniferaldehyde could serve as immediate precursors.

Based on structural considerations, we rationalized that the initial coupling product was either secoisolariciresinol **1** or matairesinol **2**, and both lignans were obtained in racemic (±)-form by total synthesis. (±)-Matairesinols **2a/2b** were formed using the method of Brown and Daugan (21) with the following exception: reduction of methyl 2-carboxymethyl-3-(4-hydroxy-3-methoxyphenyl)propionate was carried out in 38.7% yield using a reducing agent, made *in situ* from *n*-butyllithium and diisobutyllithium aluminium hydride, rather than $Ca(BH_4)_2$, which, in our hands, consistently gave low yielding reactions. (±)-Secoisolariciresinols **1a/1b** were obtained by $LiAlH_4$ reduction of (±)-matairesinols **2a/2b**. Each racemic lignan was resolved into its separate enantiomeric forms following passage through a Chiralcel OD column (Figs. 2*A* and 3*A*). (−)-Secoisolariciresinol **1b** was synthesized from (−)-matairesinol **2b** as above (Fig. 2*B*).

With a method to rapidly determine chirality, we next examined *F. intermedia* plant extracts to establish the optical purity of the secoisolariciresinol **1** and matairesinol **2** present. Each lignan was isolated from methanol extracts of *F. intermedia* stems. Matairesinol **2** was relatively plentiful (1.05 mg g^{-1} dry plant tissue), whereas secoisolariciresinol **1** was less abundant (<0.036 mg g^{-1} dry plant tissue). Chiral HPLC[2]

[1] The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: HPLC, high performance liquid chromatography; m.pt, melting point; lit.m.pt., literature melting point; THF, tetrahydrofuran; TLC, thin layer chromatography.

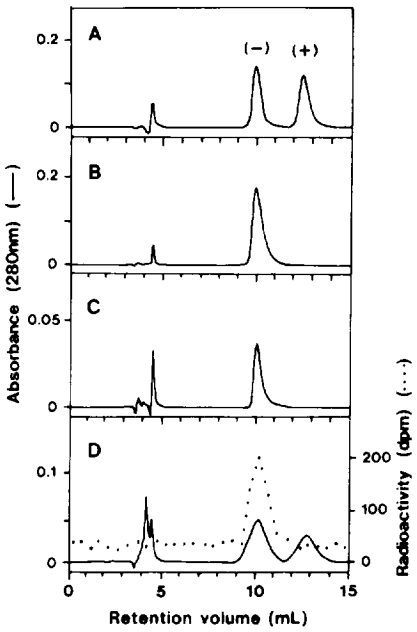

FIG. 2. **Chiral HPLC separations of secoisolariciresinols.** *A*, synthetic (±)-secoisolariciresinols **1a/1b**. *B*, synthetic (−)-secoisolariciresinol **1b**. *C*, unlabeled (−)-secoisolariciresinol **1b** isolated from *F. intermedia*. *D*, (−)-[^{14}C]secoisolariciresinol obtained following administration of [8-^{14}C]coniferyl alcohol to *F. intermedia*. Note that unlabeled (±)-secoisolariciresinols **1a/1b** were added as radiochemical carriers. Column, Chiralcel OD (Daicel) elution details: hexanes:EtOH (70:30); flow rate, 0.5 ml min^{-1}.

analysis of both lignans (before recrystallization) revealed only the presence of the (−)-, and not (+)-, antipodes (Figs. 2*C* and 3*B*). This suggested that only the (−)-form was being synthesized *in vivo*, although a rapid interconversion of (+)- into the (−)-forms, or into other metabolites, could not be ruled out.

Attention was next directed to establishing the chemical identity of the phenylpropanoid moiety undergoing coupling. In the first instance, [8-^{14}C]coniferyl alcohol (1.30 mg, 23 KBq mg^{-1}) was administered to *F. intermedia* shoots. Following its metabolism for 3 h, the plant material was homogenized, with unlabeled (±)-secoisolariciresinols **1a/1b** (100 μg) added as radiochemical carriers. The lignans were isolated as described in the Miniprint. First, secoisolariciresinol **1** and matairesinol **2** were separated by reversed phase HPLC, using both radiochemical and UV detection. In this way, it was established that [8-^{14}C]coniferyl alcohol had been incorporated into secoisolariciresinol **1** (0.3%) and matairesinol **2** (1.8%), respectively, based upon coincidence of radioactivity peaks with eluted lignans. Next, secoisolariciresinol **1** and matairesinol **2** were collected (by HPLC separation) and subjected to chiral HPLC analysis. As can be seen from the radiochemical elution profile (Fig. 2*D*), only radiolabeled (−)-secoisolariciresinol **1b** was detected *in vivo*. (Note that the UV elution profile shows the presence of both (+)- and (−)-forms since unlabeled (±)-secoisolariciresinols **1a/1b** were added as radiochemical carriers.) In a similar manner to secoisolariciresinol **1**, [8-^{14}C]coniferyl alcohol was only incorporated into (−)-matairesinol **2b**, as evidenced by the radiochemical elution profile (Fig. 3*C*). (Again, the UV profile of matairesinol **2** shows the presence of both (+)- and (−)-antipodes due to the addition of unlabeled carrier for chiral HPLC analysis; the large preponderance of the (−)-form reflects the amount of naturally occurring (−)-matairesinol **2b** already present in *F. intermedia* tissue.)

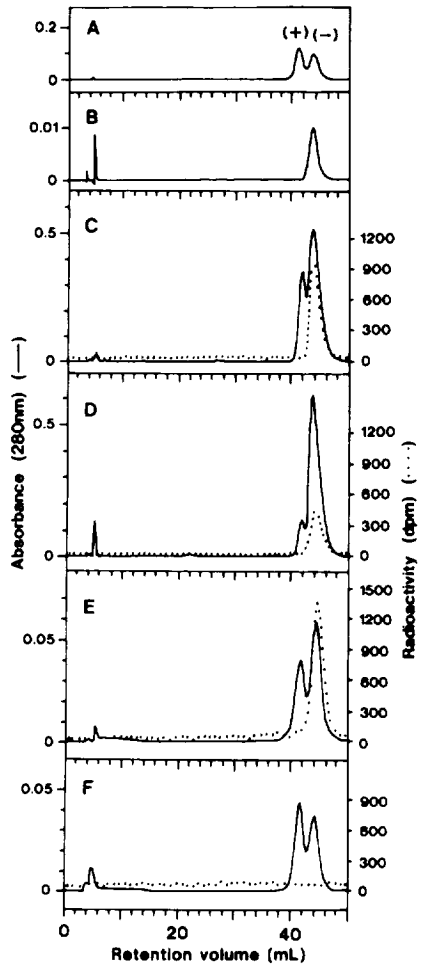

FIG. 3. **Chiral HPLC separations of matairesinols.** *A*, synthetic (unlabeled) (±)-matairesinols **2a/2b**. *B*, unlabeled (−)-matairesinol **2b** isolated from *F. intermedia*. *C*, (−)-[^{14}C]matairesinol obtained following administration of [8-^{14}C]coniferyl alcohol to *F. intermedia*. *D*, (−)-[^{3}H]matairesinol obtained following administration of (±)-[^{3}H]secoisolariciresinols to *F. intermedia*. *E* and *F*, Matairesinol **2** fractions isolated after incubation of (−)-[^{3}H] and (+)-[^{3}H] secoisolariciresinols, respectively, with cell-free extracts of *F. intermedia* in the presence of NADP. Note that unlabeled (±)-matairesinols **2a/2b** were added as radiochemical carriers in the cases of *C*, *D*, *E*, and *F*. Column, Chiralcel OD (Daicel) elution details: 1% AcOH in hexanes:EtOH (85:15); flow rate: 1 ml min^{-1}.

These experiments did not, however, prove that coniferyl alcohol **8** had been incorporated intactly into either lignan; enzymatic conversion of this alcohol to the acid or aldehyde could have occurred prior to coupling. Clearly, this uncertainty could be resolved by administration of [9,9-^{2}H$_2$,OC^{2}H$_3$] coniferyl alcohol to *F. intermedia* plant tissue actively synthesizing the lignans, (−)-secoisolariciresinol **1b** and (−)-matairesinol **2b**. If intact incorporation of coniferyl alcohol **8** occurred, then the (−)-secoisolariciresinol **1b** and (−)-matairesinol **2b** formed *de novo* would contain 10 and 8 deuterium atoms, respectively. This could be proven by mass spectrometry. If, however, oxidation to the aldehyde or acid occurred prior to coupling, then the C_9 position of the monomer would contain either one or no deuterium atom.

[9,9-^{2}H$_2$,OC^{2}H$_3$]Coniferyl alcohol obtained by total synthesis, as described in the Miniprint, was administered to excised *F. intermedia* shoots (0.59 mg/shoot) which were then allowed to metabolize for 3 h. Following this period, the lignans, (−)-secoisolariciresinol **1b** and (−)-matairesinol **2b** were isolated from *F. intermedia*, but without addition of unlabeled carrier,

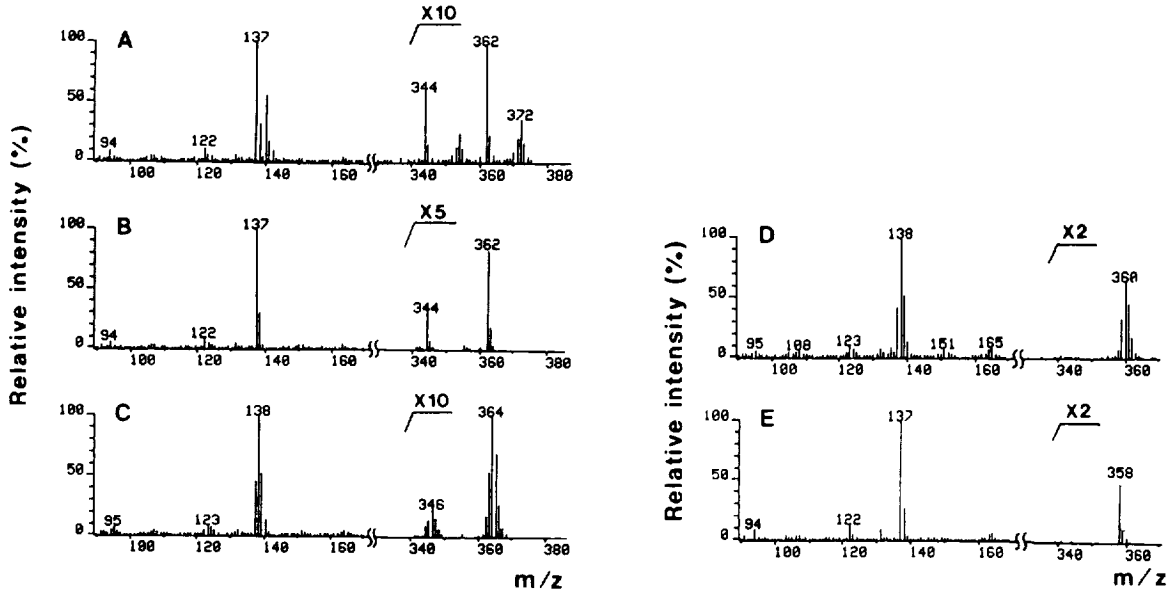

FIG. 4. **Mass spectra of secoisolariciresinols and matairesinols.** *A*, deuterated (−)-secoisolariciresinol, obtained following administration of [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol to *F. intermedia*. *B*, synthetic (unlabeled) (±)-secoisolariciresinols **1a/1b**. *C*, synthetic (±)-[Ar-^{2}H]secoisolariciresinols. *D*, deuterated (−)-matairesinol obtained following incubation of (±)-[Ar-^{2}H]secoisolariciresinols with cell-free extracts of *F. intermedia* in the presence of NADP. *E*, unlabeled (−)-matairesinol **2b** isolated from *F. intermedia*.

TABLE I

*Mass spectrometric fragmentation patterns of natural abundance and partially deuterated (−)-matairesinols **2b** from F. intermedia*

m/z	Relative intensities of molecular ion and base ion regions	
	Natural abundance (−)-matairesinol **2b** isolated from *F. intermedia*	(−)-Matairesinol **2b** isolated following administration of [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol to *F. intermedia*
137	100.0	100.0
138	25.6	18.8
139	2.3	1.8
140	0.3	3.4
141	0.2	1.0
142	0.0	0.3
358	21.1	26.1
359	4.6	6.0
360	0.7	1.0
361	0.0	0.1
362	0.0	0.1
363	0.0	0.1
364	0.0	0.0
365	0.0	0.3
366	0.0	0.6
367	0.0	0.2

TABLE II

*Enzymatic formation of (−)-matairesinol **2b** from (±)-secoisolariciresinols **1a/1b***

Enzyme assay[a]	Cofactor	Absolute incorporation of radioactivity into (−)-matairesinol **2b**	(−)-Matairesinol **2b** formation
		%	*nmol h^{-1} mg^{-1} protein*
1	NADP	2.0	14.1
2	NAD	2.9	19.9
Controls[b]			
3 no cofactors	None	0.08	0.6
4 denatured	NADP	0	0

[a] Standard assay conditions are described under "Experimental Procedures" and differ only in choice of NADP or NAD as shown. Protein content was 2.0 mg/ml.

[b] Control experiments refer to the complete assay with either omission of cofactors or with denatured enzyme (boiled for 5 min). One other control was carried out, using the complete assay (with NADP) but with a reaction period of 10 s. In this experiment, the incorporation of radioactivity into (−)-matairesinol **2b** was 0.03%.

and then subjected to mass spectrometry. For comparison purposes, spectra were also recorded for synthetic (±)-unlabeled lignans, secoisolariciresinols **1a/1b** and matairesinols **2a/2b**. Thus, Fig. 4*B* shows the mass spectrum of synthetic (±)-secoisolariciresinols **1a/1b**. As can be seen, there are three main signals at *m/z* 362 (M^+), 344 ($M^+ - 18$, loss of H_2O), and 137 (which corresponds to a fragment derived from benzylic cleavage). In contrast, the (−)-secoisolariciresinol **1b** obtained from *F. intermedia* plant tissue (Fig. 4*A*), which had previously been administered [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol, gave signals at *m/z* 372, 362, 354, 344, 140, and 137. The signals observed at *m/z* 362, 344, and 137 correspond to natural abundance (−)-secoisolariciresinol **1b** already present in the plant tissue. The signals at *m/z* 372, 354 and 140 reveal that (−)-secoisolariciresinol **1b** was formed from two [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol molecules without prior C_9 oxidation. This is because signals at *m/z* 372 ($M^+ + 10$) and 354 ($M^+ + 10$, less H_2O) prove that the newly formed (−)-secoisolariciresinol **1b** contains *ten deuterium atoms*. Additionally, the peak at *m/z* 140, corresponding to a fragment derived from benzylic cleavage, reveals that the methoxyl group was fully deuterated. Formation of (−)-secoisolariciresinol **1b** can, therefore, occur only via coupling of two *intact* coniferyl alcohol **8** moieties.

Comparison of the mass spectrum of synthetic matairesinol **2** to that obtained following [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol feeding to *F. intermedia* was also informative (see Table I). As shown in Fig. 4*E* and Table I, unlabeled (−)-matairesinol **2b** has two main signals at *m/z* 358 (M^+) and at 137 (derived from cleavage of the benzylic fragment). On the other hand, the (−)-matairesinol **2b** isolated from *F. intermedia* previously treated with [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol gave signals at *m/z* 366, 358, 140, and 137 (Table I). The peaks at *m/*

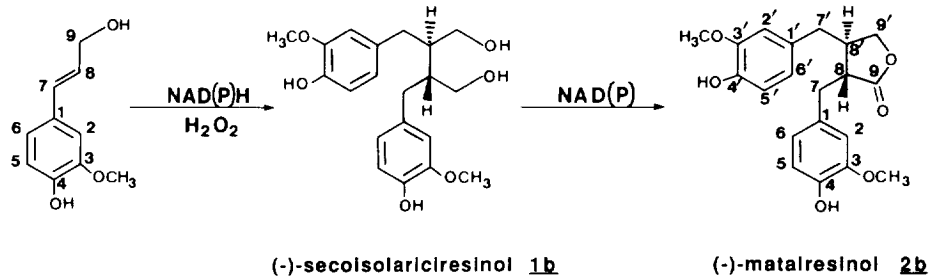

FIG. 5. **Biosynthetic pathway to (−)-secoisolariciresinol 1b and (−)-matairesinol 2b from coniferyl alcohol 8.**

z 358 and 137 again correspond to natural abundance (−)-matairesinol **2b**, whereas the small signals at *m/z* 366 and 140 suggest that eight deuterium atoms had been incorporated, six of which were associated with the two methoxyl groups. (The relatively low intensities of the deuterated peaks are a consequence of unlabeled (−)-matairesinol **2b** previously accumulated in *F. intermedia* tissue. This is in contrast to that observed for (−)-secoisolariciresinol **1b**.)

Having established that both halves of the (−)-secoisolariciresinol **1b** and (−)-matairesinol **2b** molecules were derived from coniferyl alcohol **8**, it was next of interest to determine whether (−)-matairesinol **2b** was formed *in vivo* by direct dehydrogenation of (−)-secoisolariciresinol **1b**. To answer this question, (±)-[Ar-^{3}H]secoisolariciresinols (17 KBq mg^{-1}) were synthesized (from unlabeled synthetic material by exchange with $CF_3CO_2{}^3H$) and administered to *F. intermedia* plant tissue. After a 3-h metabolism, matairesinol **2** was isolated and subjected to reversed phase HPLC. Analysis of the resulting radiochemical elution profile revealed that the incorporation of (±)-[Ar-^{3}H]secoisolariciresinols into matairesinol **2** was 0.94%. The isolated [Ar-^{3}H]matairesinol was subsequently subjected to chiral HPLC analysis, which demonstrated that only the (−)-antipode **2b** was radiolabeled (Fig. 3*D*). No radioactivity was detected in (+)-matairesinol **2a**. (Note that the UV profile shows the presence of both enantiomers due to the addition of unlabeled (±)-matairesinols **2a/2b** for chiral HPLC analysis.) These sets of experiments, therefore, suggest the following sequence of events *in vivo*: coupling of two coniferyl alcohol **8** molecules to afford (−)-secoisolariciresinol **1b** and subsequent dehydrogenation to give (−)-matairesinol **2b**.

Our next objective was to determine whether such transformations (*i.e.* coupling and dehydrogenation) could be demonstrated *in vitro* using cell-free extracts from *F. intermedia*. Thus, incubation of [8-^{14}C]coniferyl alcohol with *F. intermedia* cell-free extracts for 1 h at 30 °C was carried out next (24). Following a series of experiments with appropriate cofactors (*i.e.* H_2O_2 and NAD(P)H), it was found that secoisolariciresinol **1** formation only occurred in the presence of H_2O_2 (0.4 mM) and NAD(P)H (4 mM). Subsequent chiral HPLC analysis of the isolated lignan revealed only formation of (−)-secoisolariciresinol **1b**, and not its (+)-antipode **1a**. The rate of formation of (−)-secoisolariciresinol **1b** was 15.9 nmol h^{-1} mg^{-1} protein. Significantly, no formation of (−)-secoisolariciresinol **1b** was observed when either cofactor was omitted (NAD(P)H or H_2O_2) or when the enzyme was denatured (boiled 5 min). To further confirm that the enzymatic product was indeed (−)-secoisolariciresinol **1b**, [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol (6.97 mg) was incubated with the cell-free extract, in the presence of NADPH and H_2O_2. The enzymatic product was confirmed to be (−)-[$^2H_{10}$]secoisolariciresinol by comparison of its mass spectrum with that of natural abundance (±)-secoisolariciresinols **1a/1b** (24). It can, thus, be concluded that in this species, coupling of coniferyl alcohol **8** *in vivo* and *in vitro* permits only the formation of (−)-secoisolariciresinol **1b**. The precise nature of the enzymatic process in this key coupling reaction is under active investigation.

In an analogous manner, we wished to demonstrate the *in vitro* conversion of secoisolariciresinol **1** into matairesinol **2**. Thus, cell-free extracts of *F. intermedia* were incubated individually with both (+)- and (−)-[Ar-^{3}H]secoisolariciresinols (17 KBq mg^{-1}) for 1 h in the presence of NADP; both enantiomers of (±)-[Ar-^{3}H]secoisolariciresinols had been separated previously by chiral HPLC . Following incubation with each enantiomer, unlabeled (±)-matairesinols **2a/2b** (35.8 μg) were added (as radiochemical carriers) to each assay mixture. As can be seen from the radiochemical elution profiles, (−)-secoisolariciresinol **1b** was converted into (−)-matairesinol **2b**, but not its (+)-enantiomer **2a** (Fig. 3*E*). We were unable to demonstrate the conversion of (+)-secoisolariciresinol **2a** into either (+)- or (−)-matairesinol **2a** or **2b** (Fig. 3*F*). Furthermore, no conversion into (−)-matairesinol **2b** occurred when the enzyme was denatured (boiled 5 min) or when NAD(P) was omitted (Table II). This again demonstrates the strict stereochemical control (or preference) of this enzymatic dehydrogenation. When (±)-[Ar-^{3}H]secoisolariciresinols (1.94 KBq) were incubated with the enzyme preparation in the presence of NADP and NAD (Table II), it was established that the rate of formation of (−)-matairesinol **2b** was 14.1 and 19.9 nmol h^{-1} mg^{-1} protein, respectively.

To confirm and extend these radiochemical observations, we next undertook to demonstrate the conversion of [Ar-^{2}H]secoisolariciresinol into [Ar-^{2}H]matairesinol. Thus, (±)-[Ar-^{2}H]secoisolariciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignan with $CF_3CO_2{}^2H$. The (±)-[Ar-^{2}H]secoisolariciresinols, so obtained, were subjected to mass spectroscopic analysis. As can be seen (Fig. 4*C*), the parent molecular ion (M^+) for unlabeled secoisolariciresinol **1**, previously noted at *m/z* 362 (Fig. 4*B*), was now shifted to an ion cluster centered at *m/z* 364, *i.e.* a partial aromatic substitution of H by D had occurred. This corresponds to the replacement of two to three aromatic hydrogens by deuterium. This observation was also confirmed by ^{1}H NMR analysis. Following incubation of the (±)-[Ar-^{2}H]secoisolariciresinols with the *F. intermedia* cell-free extract in the presence of NADP, the matairesinol **2b** so obtained gave a cluster of ions now centered at *m/z 360 (Fig. 4D)*. This cluster is centered two to three mass units higher than that of natural abundance (−)-matairesinol **2b** (M^+, 358; Fig. 4*E*) indicating the presence of two to three deuterium atoms in the enzymatically formed (−)-matairesinol. Thus, the stereoselective conversion of (−)-secoisolariciresinol **1b** into (−)-matairesinol **2b** had now been unequivocally demonstrated at the cell-free level.

In summary, we have detected enzymatic activities for lignan formation (Fig. 5), one of which is involved in the stereochemically controlled formation of (−)-secoisolaricire-

sinol **1b** from coniferyl alcohol **8** and the other in the conversion of lignan **1b** to (−)-matairesinol **2b**. More needs to be known about the coupling of the two phenylpropanoid units, in terms of how this enzyme (or enzymes) differ from typical peroxidase reactions. This is currently under investigation. Research directed to the elucidation of the biosynthetic pathways (intermediates and enzymes) involved in the formation of the more highly functionalized lignans, such as arctiin **4** and podophyllotoxin **7**, is currently under way in these laboratories. The current findings, as regards lignan biosynthesis, raise obvious questions with respect to the lignification process. At what point (if any) does the pathway leading to the optically active lignans and (purportedly) optically inactive lignins differ? This important point awaits clarification.

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Supplementary Material to
Formation of Lignans,
(-)-Secoisolariciresinol and (-)-Matairesinol
with *Forsythia intermedia* Cell-free Extracts

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Chromatography Materials and Instrumentation - Silica gel thin-layer chromatography (TLC) and silica gel column chromatography employed Kieselgel 60 F_{254} (Merck, 20 x 20 cm, 0.25 or 0.5 mm) and silica gel 60 (EM Science) respectively. All solvents and chemicals used were reagent grade, unless otherwise stated. High-performance liquid chromatography (HPLC) was performed on two Waters Model 510 solvent delivery systems fitted with a Model 721 system controller, a WISP Model 710B automatic injection module and a Model 990 photodiode array detector equipped with a NEC Power Mate 2, a Waters 990 Plotter and a NEC pinprinter CP6. The lignans, (-)-secoisolariciresinol 1b and (-)-matairesinol 2b were separated using a reversed phase column [Waters Novapak C_{18} (150 x 3.9 mm,) stainless steel] with detection at λ=280 nm. Elution details were as follows: a gradient solvent system (1 ml min^{-1}) consisting of CH_3CN: 3% AcOH in H_2O (20:80) at t = 0 min to 32:68 at t = 10 min, and which was held at that composition for an additional 10 min. Chiral column HPLC separations of lignans were performed using a Daicel Chiralcel OD column (250 x 4.6 mm). The mixture of (+)- and (-)-matairesinols 2a and 2b was separated following elution with EtOH:1% AcOH in hexanes (15:85) at a flow rate of 1 ml min^{-1}. The mixture of (+)- and (-)-secoisolariciresinols 1a and 1b was separated following elution with hexanes:EtOH (70:30) at a flow rate of 0.5 ml min^{-1}. The EtOH used for chiral HPLC separations was denatured with either MeOH (5%) and 2-propanol (4.5%) (Pierce, HPLC Spectrograde) or with MeOH (4.7%) and 2-propanol (4.7%) (Fisher, HPLC grade). ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Brüker WP-270SY spectrometer and a Varian Unity 400 spectrometer, respectively, using tetramethylsilane as an internal standard. Electron impact mass spectrometry was performed on a VG analytical 7070E-HF mass spectrometer (ionizing voltage, 70eV). Optical rotations were measured with a Perkin-Elmer 241 Polarimeter. UV spectra were obtained using a Perkin-Elmer Lambda 6 UV/VIS Spectrophotometer. Radioactive samples were counted in Ecolume (ICN Biomedicals) liquid scintillation fluid using a Beckman LS-250 Liquid Scintillation System. Counting efficiencies were determined using *n*-[^{14}C]hexadecane and [^{3}H]toluene respectively for calibration. All melting points are uncorrected.

Plant Material - *Forsythia intermedia* (var. Lynwood Gold) plants, grown on the campus of Virginia Polytechnic Institute and State University (VPI&SU), were used as a source of (-)-matairesinol 2b. *Forsythia intermedia* plants (var. Lynwood Gold, obtained from Cox's Nursery, Blacksburg, Virginia and Greensboro Nursery, Greensboro, North Carolina) were maintained in VPI & SU greenhouse facilities and used as a source of (-)-secoisolariciresinol 1b, and of enzyme preparations.

Chemical Syntheses - [8-^{14}C]Coniferyl alcohol (23 KBq mg^{-1}) was prepared as previously described via reduction of the methyl ester of [8-^{14}C]ferulic acid, obtained from vanillin and [2-^{14}C]malonic acid [18]. [9,9-$^2H_2$, OC^{2}H$_3$]Coniferyl alcohol was synthesized in four steps as follows:

(1) 4-Benzyloxy-3-[OC^{2}H$_3$]methoxybenzaldehyde: To a stirred solution of 3,4-dihydroxybenzaldehyde (2.076 g) in *N,N*-dimethylformamide (30 ml), under N_2, were added benzylbromide (3.421 g) and K_2CO_3 (2.076 g). The reaction mixture was stirred at room temperature for 12.5 h, poured into distilled water (100 ml), with the whole then extracted with Et_2O (3 x 100 ml). The combined Et_2O extracts were washed with a saturated NaCl solution, dried (anhydrous Na_2SO_4), and the solvent evaporated *in vacuo*. The product was purified on a silica gel column (30 x 2 cm) eluted with EtOAc:hexanes (1:4). Fractions containing 4-benzyloxy-3-hydroxybenzaldehyde were evaporated to dryness *in vacuo*. Recrystallization from EtOAc-hexanes afforded the desired aldehyde (1.925 g, 56.3% yield). To a stirred solution of 4-benzyloxy-3-hydroxybenzaldehyde (1.573 g) and C^{2}H$_3$I (Aldrich, 99 atom % ^{2}H, 1 g) in *N,N*-dimethylformamide (10 ml), was added K_2CO_3 (1.38 g) under N_2. Following stirring at room temperature for 16 h, the resulting reaction mixture was poured into distilled water (75 ml), then extracted with Et_2O (3 x 75 ml). The combined Et_2O solubles were washed with a saturated NaCl solution, dried (anhydrous Na_2SO_4), and the solvent removed *in vacuo* to yield 4-benzyloxy-3-[OC^{2}H$_3$]methoxybenzaldehyde (1.67 g, 98.8%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$): δ 5.26 (2H, s, CH_2), 6.99 (1H, d,J = 8.1Hz, Ar-H), 7.3 - 7.5 (7H, m, Ar-H), 9.84 (1H, s, CHO); MS m/z (%): 245 (M^+, 5.3), 91 (100), 65 (9.8).

(2) *O-trideuteromethyl*[OC^{2}H$_3$]vanillin: To a stirred solution of 4-benzyloxy-3-[OC^{2}H$_3$]methoxybenzaldehyde (1.66 g) in AcOH (15 ml) under N_2 was added 30 wt% HBr in AcOH (0.5 ml). The reaction mixture was stirred at 90°C for 30 min, then at 130°C for 20 min. After cooling in an ice bath, the reaction mixture was diluted with EtOAc (200 ml), then washed successively with a saturated $NaHCO_3$ solution (until the washings became neutral) and a saturated NaCl solution (ca. 10 ml). The organic solubles were dried (anhydrous Na_2SO_4) and evaporated *in vacuo* to yield an oil which was applied to a silica gel column (7 x 7 cm). Following elution with EtOAc:hexanes (1:4), fractions containing [OC^{2}H$_3$]vanillin were combined and evaporated to dryness *in vacuo* to give the desired aldehyde (825 mg, 78.5%); ^{1}H-NMR ($CDCl_3$): δ 7.05 (1H, d, J = 8.6Hz, Ar-H) 7.43 (2H, m, Ar-H), 9.84 (1H, s, CHO); MS m/z (%): 156(8.9), 155 (100), 154 (100), 153 (1.7), 152 (0.5), 151 (0.5), 137 (7.5) 126 (14.0), 109 (17.2), 108 (9.1), 81 (22.6).

(3) Ethyl *O-trideuteromethyl*[OC^2H_3]ferulate. To a stirred solution of [OC^2H_3]vanillin (820 mg) in pyridine (2 ml) under N_2, were added monoethyl malonate (0.9 ml) [19], aniline (one drop) and piperidine (one drop). The reaction mixture was stirred at 52°C for 21 h, then cooled (to ice-bath temperature) and acidified to pH 2 with 2N HCl. To this solution was added distilled water (ca. 10 ml), and the whole was extracted with EtOAc (2 x 100 ml). The combined EtOAc solubles were washed successively with a saturated $NaHCO_3$ solution until the washings became neutral, then with a saturated NaCl solution (ca. 10ml, twice), and dried (anhydrous Na_2SO_4). The solvent was removed *in vacuo*, and the product applied to a silica gel column (8 x 7 cm). Following elution with EtOAc:hexanes (1:3), fractions containing the ethyl ester were combined, and the solvent removed *in vacuo* to give ethyl [OC^2H_3]ferulate (977 mg, 82.1%). ^{1}H-NMR ($CDCl_3$): δ 1.34 (3H, t, J = 7.1Hz, $CH_2C\underline{H}_3$), 4.26 (2H, q, J = 7.1Hz, $C\underline{H}_2CH_3$), 6.29 (1H, d, J = 15.9Hz, C8H), 6.92 (1H, d, J = 8.1Hz, Ar-H), 7.03 (1H, d, J = 1.9Hz, Ar-H), 7.07 (1H, dd, J_1 = 8.2Hz, J_2 = 1.9Hz, Ar-H), 7.62 (1H, d, J = 15.9Hz, C7H); MS m/z (%):225 (M^+, 100), 197 (16.5), 180 (83.5), 153 (58.4), 145 (43.8), 135 (11.5), 134 (14.7), 133 (14.3), 117 (23.1), 105 (14.4), 89 (25.8).

(4) Pentadeutero[9,9-$^2H_2$,OC^2H_3] coniferyl alcohol. To a stirred suspension of $LiAl^2H_4$ (800 mg, Aldrich, 98 atom % ^2H) in anhydrous Et_2O (15 ml, dried over Na metal), was added dropwise a solution of ethyl [OC^2H_3]ferulate (782 mg) in dry Et_2O (35 ml) over a period of 35 min at room temperature. Following stirring for 40 min at the same temperature, the resulting suspension was cooled (0°C) and to this was added dropwise EtOAc:Et_2O (6 ml, 1:5) to decompose excess $LiAl^2H_4$. The resulting slurry was poured onto dry ice (ca. 10 g) and EtOAc (ca. 100 ml), to which distilled water (ca. 50 ml) was added. The whole was extracted with EtOAc (2 x 150 ml), with the combined EtOAc solutions next washed with a saturated NaCl solution (ca. 4 x 10 ml), and dried (anhydrous Na_2SO_4). The solvent was removed *in vacuo* to afford pale yellow crystals of coniferyl alcohol (700 mg). These were redissolved and applied to a silica gel column (8 x 7 cm) which was eluted with EtOAc:hexanes (1:1). Fractions containing coniferyl alcohol were combined and dried *in vacuo*. Recrystallization from CH_2Cl_2:hexanes afforded colorless crystals of [9,9-$^2H_2$,OC^2H_3]coniferyl alcohol (380 mg, 59.0%), m.pt.: 75.5-76.0°C, lit. m.pt.: 74-76°C (for unlabelled coniferyl alcohol) [20]. ^{1}H-NMR ($CDCl_3$): δ 6.21 (1H, d, J = 15.8 Hz, C8H), 6.53 (1H, d, J = 15.8Hz, C7H), 6.8-7.0 (3H,m,Ar-H); ^{13}C-NMR ($CDCl_3$): δ 55.0 (septet, OC^2H_3), 63.1 (quintet, $C9^2H_2$), 108.4 (C_2), 114.5 (C_5), 120.2 (C_6), 125.9 (C_8), 129.2 (C_1), 131.5 (C_7), 145.6 (C_4), 146.7 (C_3). MS m/z (%): 186 (11.0), 185 (81.4), 184 (3.6), 183 (1.1), 182 (1.9), 181 (0.6), 180 (0.3), 167 (53.8), 149 (26.6), 141 (24.5), 140 (100), 134 (16.3), 133 (18.5), 128 (37.5), 127 (15.2), 121 (33.7), 120 (17.9), 105 (20.1), 93 (49.5), 92 (21.7).

(±)-Matairesinols 2a/2b. These were synthesized by the method of Brown and Daugan [21] with the following exception. Methyl 2-carboxymethyl-3-(4-hydroxy-3-methoxyphenyl)propionate was reduced with the complex prepared from *n*-butyllithium and diisobutyl aluminum hydride [22] instead of $Ca(BH_4)_2$ used in the original method. Thus, to a stirred solution of the above methyl ester (4.717 g) in tetrahydrofuran (THF, 25 ml, freshly distilled over benzophenone and potassium metal) was added dropwise the reducing agent, prepared at 0°C from *n*-butyllithium (88 mmol) and diisobutyl aluminum hydride (88 mmol) in dry THF (25 ml), over a period of 30 min at -78°C. The reaction mixture was maintained at this temperature for 50 min more, then warmed to room temperature and stirred for an additional 2 h. EtOH (2 ml) was next added at 0°C, with the whole reaction mixture poured onto dry ice (~10 g) and EtOAc (100 ml). The resulting mixture was acidified to pH 2 with 1N HCl, extracted with EtOAc (5 x 200 ml), with the combined EtOAc solubles washed with saturated NaCl solution, and dried (anhydrous Na_2SO_4). The solvent was evaporated *in vacuo* to yield 3-hydroxymethyl-4-(4-hydroxy-3-methoxyphenyl)butyric acid, which was then redissolved in EtOAc (10 ml). Next 6 N HCl (4 ml) was added and stirred for 1.7 h at room temperature. The reaction mixture was washed with saturated NaCl solution until the washings became neutral, dried (anhydrous Na_2SO_4), and the solvent evaporated *in vacuo* to yield crude β-vanillyl-γ-butyrolactone. This was purified by silica gel column (41 x 2.5 cm) chromatography, eluted with MeOH:CH_2Cl_2 (gradient 0:100 → 2:98) to give, after combination of the appropriate fractions and evaporation to dryness, the pure lactone (1.513 g, 38.7%). The lactone was converted to (±)-matairesinols 2a/2b exactly as described [21]. ^{1}H-NMR ($CDCl_3$): δ 2.4-2.7 (4H, m, C7-H_2, C8H, C8-H), 2.87 (1H,dd,J_1 = 14.1Hz, J_2 = 6.9Hz, C7H), 2.95 (1H,dd,J_1 = 14.1Hz,J_2 = 5.1Hz, C7H), 3.81 (3H,s,OCH_3), 3.82 (3H,s,OCH_3), 3.89 (1H,dd,J_1 = 9.2Hz, J_2 = 6.8Hz, C9-H), 4.16 (1H, dd, J_1 = 9.2Hz, J_2 = 7.0Hz, C9-H), 6.41 (1H, d, J = 1.7Hz, Ar-H), 6.51 (1H, dd, J_1 = 8.0Hz, J_2 = 1.8Hz, Ar-H), 6.6 (2H, m, Ar-H) 6.78-6.85 (2H, m, Ar-H); ^{13}C-NMR ($CDCl_3$): δ 34.6, 38.2, 41.0, 46.5, 55.7, 55.8, 71.4, 111.1, 111.6, 114.3, 114.6, 121.3, 122.0, 129.4, 129.7, 144.4, 144.6, 146.8, 146.9, 179.1. MS m/z (%):358(M^+, 43.1), 137 (100), 122 (17.4), 94 (11.1).

(±)-Secoisolariciresinols 1a/1b. (±)-Matairesinols 2a/2b (100.4 mg) were dissolved in THF (9 ml, freshly distilled over benzophenone and potassium metal) under N_2. The resulting solution was added dropwise at room temperature over a period of 15 min to a stirred suspension of $LiAlH_4$ (88.6 mg) in dry THF (2 ml). Following stirring for an additional 1 h at the same temperature, the reaction mixture was cooled to 0°C. Next, EtOAc (3 ml) was added dropwise, and the whole was then poured onto dry ice. Distilled water (5 ml) was added to the resulting suspension, with the organic solvent then removed *in vacuo*. The sample was reconstituted in distilled water (10 ml) with the whole extracted with EtOAc (4 x 20 ml). The combined EtOAc solubles were washed with a saturated NaCl solution, dried (anhydrous Na_2SO_4) and evaporated *in vacuo* to yield crude (±)-secoisolariciresinols 1a/1b. The crude (±)-secoisolariciresinols 1a/1b were purified by silica gel column (15 x 2.7 cm) chromatography. Following elution with EtOAc:hexanes (gradient 1:1 → 2:1), fractions containing (±)-secoisolariciresinols 1a/1b were combined and evaporated to dryness *in vacuo*. Recrystallization from EtOAc:hexanes gave (±)-secoisolariciresinols 1a/1b as colorless crystals (54.5 mg, 53.7%), m.pt.: 127.5°C; ^{1}H-NMR ($CDCl_3$): δ 1.86 (1H x 2, m, C8H, C8-H), 2.66 (1H x 2, dd, J_1 = 13.9Hz J_2 = 6.6Hz, C7 and 7'-H), 2.75 (1H x 2, dd, J_1 = 13.9Hz, J_2 = 8.2Hz, C7 and 7'-H), 3.57 (1H x 2, dd, J_1 = 11.4Hz, J_2 = 4.3Hz, C9 and 9'-H), 3.82 (3H x 2, s, OCH_3), 3.83 (1H x 2, dd, J_1 = 11.2Hz, J_2 = 2.0Hz, C9 and 9'-H), 6.59 (1H x 2, d, J = 1.8Hz, Ar-H), 6.64 (1H x 2, dd, J_1 = 8.0Hz, J_2 = 1.9Hz, Ar-H), 6.82 (1H x 2, d, J = 7.9Hz, Ar-H). MS m/z (%): 362 (M^+, 20.6), 344 (7.3), 137 (100), 122 (7.1).

(-)-Secoisolariciresinol 1b. (-)-Matairesinol 2b (40 mg), obtained and purified from *F. intermedia*, was reduced as above to afford (-)-secoisolariciresinol 1b (27 mg, 66.6%); m.pt.:113.0-113.6°C; lit.m.pt.: 112-113.5°C [21]; $[\alpha]_D$ = -33° (c 0.74, acetone); lit. $[\alpha]_D$ = -32° (c 0.8, acetone) [21]. ^{1}H-NMR ($CDCl_3$): δ 1.86 (1H x 2, m, C8 and 8'-H), 2.65 (1H x 2, dd, J_1 = 13.8Hz, J_2 = 6.5Hz, C7 and 7'-H), 2.75 (1H x 2, dd, J_1 = 13.8Hz, J_2 = 8.1Hz, C7 and 7'-H), 3.56 (1H x 2, dd, J_1 = 11.3Hz, J_2 = 4.6Hz, C9 and 9'-H), 3.82 (3H x 2, s, OCH_3), 3.83 (1H x 2, dd J_1 = 11.2Hz, J_2 = 2.1Hz, C9 and 9'-H), 6.59 (1H x 2, d, J = 1.8Hz, Ar-H), 6.63 (1H x 2, dd, J_1 = 8.0Hz, J_2 = 1.9Hz, Ar-H), 6.81 (1H x 2, d, J = 8.0Hz Ar-H); ^{13}C-NMR (acetone-d_6): δ 36.1, 44.7, 56.1, 61.2, 113.3, 115.4, 122.4, 133.6, 145.4, 148.1; Chiral column (Daicel) HPLC retention volume : 10.0 ml (Fig. 2B).

(±)Aromatic-deuterated-[Ar-^{2}H]Secoisolariciresinols: (±)-Secoisolariciresinols 1a/1b (23.8 mg) were dissolved in $CF_3CO_2{}^2H$, generated from trifluoroacetic anhydride (1.41 ml) and ^2H_2O (MSD isotopes, minimum isotopic purity, 99.8 atom % ^2H, 199 µl) under N_2. The resulting solution was allowed to stand at room temperature for 46 h, then evaporated to dryness under reduced pressure. The residue was redissolved in MeOH (0.6 ml), and to this was added saturated $NaHCO_3$ solution (0.44 ml) under N_2. After stirring for 45 min, a small amount of dry ice (ca. 5 g) and distilled water (1 ml) were added, and the resulting solution was extracted with EtOAc (3 x 5 ml). The combined EtOAc solubles were washed with distilled water (1 ml), then with a saturated NaCl solution (2 x 1 ml), and dried (anhydrous Na_2SO_4). The solvent was removed *in vacuo* to afford (±)-[Ar-^{2}H]secoisolariciresinols, which were purified successively by silica gel TLC [MeOH:CH_2Cl_2 (6:94), developed once] and silica gel column chromatography [7 x 1 cm, solvent MeOH:CH_2Cl_2 (6:94)]. Recrystallization from EtOAc:hexanes gave (±)-[Ar-^{2}H]secoisolariciresinols as white crystals (12 mg, 49.9%), m.pt.:124.5°C. MS m/z (%): 367 (0.6), 366 (2.6), 365 (6.8), 364 (9.9), 363 (5.2), 362 (1.7), 348 (0.5), 347 (1.5), 346 (2.2), 345 (1.2), 344 (0.7), 140 (13.0), 139 (51.3), 138 (100), 137 (44.9), 123 (7.7).

(±)-, (+)- and (-)-Aromatic-tritiated[Ar-^{3}H]secoisolariciresinols. The ^{3}H-labelled compounds were prepared from (±)-secoisolariciresinols 1a/1b (29.7 mg) and purified as above [see (±)-[Ar-^{2}H]secoisolariciresinols synthesis] except that ^3H_2O (Dupont, 3.7 GBq ml^{-1}, 0.248 ml, 9.18 MBq) was used instead of ^2H_2O to give (±)-[Ar-^{3}H]secoisolariciresinols (15 mg, 50.5% yield, 17 KBq mg^{-1}); m.pt.128.0°C. The (±)-[Ar-^{3}H]secoisolariciresinols thus obtained (2.84 mg) were dissolved in MeOH (320 µl), aliquots of which (15-25 µl, 15 times) were applied onto a Chiralcel OD column and eluted as described in chromatography section. Combination of appropriate eluted fractions afforded pure (-)-[Ar-^{3}H]secoisolariciresinol (1035 µg). Fractions containing (+)-[Ar-^{3}H]secoisolariciresinol were contaminated with ≤ 1% of the (-)-enantiomer, and rechromatography afforded pure (+)-[Ar-^{3}H]secoisolariciresinol (959 µg).

Isolation of (-)-secoisolariciresinol 1b

F. intermedia (var. Lynwood gold) stems (63.77 g fresh weight) were freeze-dried with the resulting dried material (28.04 g) ground in a Waring blender and extracted sequentially by steeping with hot MeOH (200 ml, 5 x 100 ml). The methanol solubles were combined, concentrated to ~10 ml under reduced pressure, and to this was added distilled water (50 ml). The whole was next centrifuged (850 x *g*, 20 min), the supernatant decanted and extracted with Et_2O (4 x 50 ml). The combined Et_2O solubles were dried (1.054 g), then applied to a silica gel column (6 x 6 cm) and eluted with a gradient of EtOAc:hexanes (2:1) → EtOAc. Fractions containing (-)-secoisolariciresinol 1b were combined, dried (16 mg) and purified further as follows: (i) silica gel TLC, eluted with EtOAc:hexanes (2:1), developed three times; (ii) silica gel TLC, with eluting solvents MeOH:CH_2Cl_2 (5:95), developed twice; and (iii) reversed phase and chiral HPLC, as described in chromatography section, to give (-)-secoisolariciresinol 1b (~1 mg); ^{1}H-NMR ($CDCl_3$): δ 1.87 (1H x 2, m, C8 and 8'-H), 2.66 (1H x 2, dd, J_1 = 13.9Hz, J_2 = 6.5Hz, C7 and 7'-H), 2.76 (1H x 2, dd J_1 = 13.9Hz, J_2 = 8.1Hz, C7 and 7'-H), 3.57 (1H x 2, dd, J_1 = 11.3Hz, J_2 = 4.5Hz, C9 and 9'-H), 3.82 (3H x 2, s OCH_3), 3.84 (1H x 2, dd J_1 = 11.2Hz, J_2 = 2.1Hz, C9 and 9'-H), 6.59 (1H x 2, d, J = 1.8Hz, Ar-H), 6.64 (1H x 2, dd, J_1 = 8.0Hz, J_2 = 1.8Hz, Ar-H), 6.82 (1H x 2, d, J = 8.1Hz, Ar-H); MS m/z (%) 362 (M^+, 6.4), 344 (6.4), 137 (100), 122 (10.7); high resolution MS, found: M^+ 362.1704; calculated for $C_{20}H_{26}O_6$ 362.1729.; UV λmax(CH_3OH): 227, 281 nm; chiral HPLC retention volume: 10.0 ml (Fig. 2C).

Isolation of (-)-matairesinol 2b

F. intermedia stems (142 g fresh weight) were freeze-dried, and the resulting dried material (67.6 g) extracted as for (-)-secoisolariciresinol 1b. The Et_2O solubles were applied to a silica gel column (8 x 7 cm). Following elution with an EtOAc:hexanes gradient 1:2 → 1:1, the fractions containing (-)-matairesinol 2b were combined (202 mg). These were next subjected to preparative silica gel TLC [CH_2Cl_2:MeOH (99:1), developed five times] and silica gel column chromatography (12 x 2.5 cm), eluted with [EtOAc:$CHCl_3$, (1:4)], to afford (-)-matairesinol 2b (71 mg) which was recrystallized from CH_2Cl_2:hexanes, m.pt.:118.0°C; lit. m.pt.:117-119°C [23]; $[\alpha]_D$ = -44° (c 0.62, acetone); lit $[\alpha]_D$ = -43° (c 0.84, acetone) [21]. ^{1}H-NMR ($CDCl_3$): δ 2.4-2.7 (4H, m, C7-H_2, C8H, C8-H), 2.88 (1H, dd, J_1 = 14.1Hz, J_2 = 6.9Hz, C7-H), 2.95 (1H, dd, J_1 = 14.1Hz, J_2 = 5.2Hz, C7-H), 3.81 (3H, s, OCH_3), 3.82 (3H s OCH_3), 3.89 (1H, dd, J_1 = 9.1Hz, J_2 = 6.9Hz, C9-H), 4.15 (1H, dd, J_1 = 9.2Hz, J_2 = 7.0Hz, C9-H), 6.41 (1H, d, J = 1.9Hz, Ar-H), 6.51 (1H, dd, J_1 = 8.0Hz, J_2 = 2.0Hz, Ar-H), 6.6 (2H, m, Ar-H), 6.78-6.85 (2H, m, Ar-H); MS m/z (%) 358 (M^+, 21.1), 137 (100), 122 (11.4), 94 (7.1). High resolution MS, found : M^+ 358.1435, calculated for $C_{20}H_{22}O_6$ 358.1416; UV λmax (CH_3OH):229, 281 nm; Chiral HPLC retention volume: 43.8 ml (Fig 3B).

Administration of [8-^{14}C]coniferyl alcohol to F. intermedia shoots

F. intermedia shoots (10 cm long with 8-10 leaves), were excised by means of a razor, the cut end of which was placed directly into a solution of [8-^{14}C]coniferyl alcohol (1.30 mg, 23 KBq mg^{-1}) in 0.1 M potassium phosphate buffer (289 µl, pH 7.0). Following uptake and metabolism for 3 h, the leaves were removed and the stems freeze-dried. The resulting dried material (263 mg) was hand disintegrated (using scissors), and solvent extracted in the same way as previously described for (-)-secoisolariciresinol 1b isolation, except that unlabelled (±)-secoisolariciresinols

(100 μg) were added as radiochemical carriers. The resulting dried Et_2O solubles were dissolved in MeOH (250 μl), 10 μl of which were applied to a Waters Novapak C_{18} column and eluted with a gradient solvent system described in Chromatography Materials and Instrumentation section. The resulting eluate was collected at 30 s intervals from 0 to 20 min, with each fraction subjected to liquid scintillation counting. This was then repeated three times (with 10 μl, 2 x 25 μl injection aliquots), and fractions corresponding to secoisolariciresinol 1 and matairesinol 2 were separately collected. Each lignan was further analyzed by chiral HPLC.

Administration of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol to F. intermedia

[9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol (2.37 mg) in 0.1 M potassium phosphate buffer (pH 7.0, 526 μl) was administered to four excised *F. intermedia* shoots which were allowed to metabolize for 3 h as before. (-)-Matairesinol 2b was isolated as above without addition of unlabelled carrier, and analyzed for mass spectroscopic enrichment. In a separate experiment, [9,9-$^2H_2$,OC$^2H_3$]coniferyl alcohol (23.7 mg) was administered to forty *F. intermedia* shoots, which were then allowed to metabolize for an additional 3 h; (-)-secoisolariciresinol 1b was isolated by successive purification as before but with no addition of unlabelled carrier, and subjected to mass spectroscopic analysis.

Administration of (±)-[Ar-^3H]secoisolariciresinols 1a/1b to F. intermedia (±)-[Ar-^{3}H]Secoisolariciresinols 1a/1b (927 μg, 17 KBq mg^{-1}) were dissolved in MeOH (23μl) and 0.1 M potassium phosphate buffer (232 μl, pH 7.0) and administered to two shoots of *F. intermedia*. Following metabolism for 3 h, the leaves were removed and the stems freeze-dried. The resulting dried material was extracted in the same way as previously described for (-)-secoisolariciresinol 1b isolation. The Et_2O solubles containing both secoisolariciresinol 1 and matairesinol 2 were dissolved in MeOH (250 μl). An aliquot of this solution (20 μl) was applied to the Waters-Novapak C_{18} column, and eluted with the gradient solvent system described in the Chromatography Materials and Instrumentation section. Eluted fractions were collected every 30 s from t = 0 to 20 min and subjected to analysis by liquid scintillation counting. Next, aliquots (total 50 μl) were applied to the aforesaid column, and fractions corresponding to matairesinol 2 were collected, and subjected to chiral HPLC analysis.

Cell-free extract from F. intermedia

Young shoots (5-10 cm long) of *F. intermedia* were excised by means of a razor, then washed with both tap and distilled water, and the leaves removed. The resulting stems (2.7 g fresh weight) were cut into small pieces by hand (scissors), frozen (liq. N_2) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polyclar AT (0.54 g), acid-washed sea sand and 0.1 M potassium phosphate buffer (pH 7.0, 4 ml) containing 10 mM dithiothreitol. The slurry was filtered through 4 layers of cheese-cloth, and the filtrate (3 ml) centrifuged (15,000 x *g*, 20 min). The resulting supernatant (2.7 ml) was again filtered (Whatman GFA glass fibre filter), and an aliquot (1.5 ml) of the filtrate was applied to a Sephadex G-25 column [18.7 x 1 cm, Pharmacia, particle size 50-150 μm (medium)], pre-equilibrated in 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol. The fraction excluded from the gel (1.5 ml) was collected and used as the cell-free preparation. Protein content of the preparation was 2.0 mg ml^{-1} on the basis of a Bio-Rad Protein Assay using bovine serum albumin as standard [25].

Enzymatic oxidation of (±)-,(+)- and (-)-[Ar-^3H]secoisolariciresinols

The assay mixture contained 770 μl 0.1 M Tris-HCl buffer (pH 8.8, 30°C), 230 μl crude enzyme preparation and 10 μl 50 mM NAD or NADP solution in 0.1 M potassium phosphate buffer (pH 7.0). To initiate the enzymatic reaction, (±)-[Ar-^{3}H]secoisolariciresinols 1a/1b (114.9 μg, 17 KBq mg^{-1}) dissolved in MeOH (5μl) and 0.1 M potassium phosphate buffer (pH 7.0, 5μl) were added. Following enzymatic incubation for 1 h at 30°C, EtOAc (2 ml) containing unlabelled (±)-matairesinols 2a/2b (35.8 μg) as radiochemical carriers was added, and the EtOAc solubles were removed. Following evaporation to dryness, the EtOAc extract was redissolved in MeOH (80 μl), with an aliquot (40 μl) then applied to the Waters Novapak C_{18} column. Following elution with the solvent system described in the Chromatography Materials and Instrumentation section, fractions were collected every 30 s for t = 0 to 20 min, and subjected to liquid scintillation counting (Table 2). Next, optically pure samples of (-)-[Ar-^{3}H]secoisolariciresinol 1b (451 μg, 7.67 KBq) and (+)-[Ar-^{3}H]secoisolariciresinol 1a (385 μg, 6.55 KBq) were individually incubated exactly as above, but with NADP as cofactor. Unlabelled (±)-matairesinols 2a/2b (35.8 μg) were added as radiochemical carriers to each assay mixture before extraction, and the matairesinol 2 was then isolated by the use of reversed phase HPLC, and subjected to chiral HPLC and liquid scintillation counting analyses.

Enzymatic oxidation of (±)-[Ar-^2H]secoisolariciresinols

(±)-[Ar-^{2}H]secoisolariciresinols (6.87 mg) were incubated with the crude enzyme preparation (11.5 ml) from *F. intermedia*, in the presence of NADP or NAD with volumes scaled up proportionately. The (-)-matairesinol 2b was isolated with no addition of unlabelled carrier, by (i) silica gel TLC, eluted with EtOAc:hexanes (1:1), developed three times (ii) reversed phase HPLC and (iii) chiral HPLC, and then subjected to mass spectroscopic examination.