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

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Toshiaki Umezawa, Laurence B. Davin§, and Norman G. Lewis§

From the Commonwealth Center for Wood Science and Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0323

In vivo labeling experiments of Forsythia intermedia plant tissue with [8-14C]- and [9,9-2H2,OC2H2]-coniferyl alcohol 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−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 H2O2, the cell-free extracts catalyzed the formation of (−)-secoisolariciresinol, with either [8-14C]- or [9,9-2H2,OC2H2]-coniferyl alcohols as substrates. The (+)-enantiomer was not formed. Finally, when either (−)-[Ar−H] or [±]-[Ar−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 H2O2 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), furufuran (e.g. pinoresinol 5, epipinoresinol 6), 1-phenylcycloheptanone 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, pinioresinol 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 [α]θ 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 (+)-pinioresinol 5a, whereas Xanthoxylum aizoides (12) has the (−)-enantiomer 5b. No satisfactory explanation has been proffered to account for this stereochemocontrol leading to optical activity; other than that the reaction is somehow enzymatically mediated. Such control is not possible via interference of a typical peroxidase/H2O2-catalyzed reaction, a reaction often implicated in lignin synthesis (13).

Surprisingly, the biosynthesis of lignans has been 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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1a: (+)-secoisolariciresinol
1b: (−)-secoisolariciresinol

2a: (+)-matairesinol
2b: (−)-matairesinol

3: arctigenin, R1 = CH3, R2 = H
4: arctin, R1 = CH3, R2 = glc

5: (+)-plnoresinol
5a: (+)-plnoresinol
5b: (−)-plnoresinol

6: epipinoresinol
7: podophyllotoxin

8: coniferyl alcohol, R1 = CH3OH, R2 = H
9: sinapyl alcohol, R1 = COOH, R2 = OCH3
10: ferulic acid, R1 = COOH, R2 = H
11: sinapic acid, R1 = COOH, R2 = OCH3

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 sinac 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, plnoresinol 5, and epipinoresinol 6 could arise via direct coupling of the two monolignol molecules, coniferol 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 stereo-selectively converted into (−)-matairesinol 2b. These conversions have been demonstrated using cell-free preparations from *F. intermedia*.

**EXPERIMENTAL PROCEDURES**

**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 coniferylaldehyde 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-methoxyphenetyl)propanoate was carried out in 38.7% yield using a reducing agent, made in situ from n-butyllithium and diisobutylaluminium hydride, rather than Ca(BH4)2, which, in our hands, consistently gave low yielding reactions. (±)-Secoisolariciresinols 1a/1b were obtained by LiAlH4 reaction of (±)-matairesinols 2a/2b. Each racemic ligan was resolved into its separate enantiomeric forms following passage through a Chiralcel OD column (Figs. 2A and 3A). (−)-Secoisolariciresinol 1b was synthesized from (−)-matairesinol 2b as above (Fig. 2B).

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 ligan 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 HPLC²

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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.p., melting point; lit.m.p., literature melting point; THF, tetrahydrofuran; TLC, thin layer chromatography.
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Fig. 2. Chiral HPLC separations of secoisolariciresinols. A, synthetic (±)-secoisolariciresinol 1a/1b. B, synthetic (-)-secoisolariciresinol 1b. C, unlabeled (-)-secoisolariciresinol 1b isolated from F. intermedia. D, (-)-[14C]secoisolariciresinol obtained following administration of [8,9-14C]coniferyl alcohol to F. intermedia. Note that unlabeled (±)-secoisolariciresinols 1a/1b were added as radiochemical carriers. Column, Chiralcel OD (Daicel) elution details: hexanes:EtOH (95:5); flow rate, 0.5 ml min⁻¹.

Analysis of both lignans (before recrystallization) revealed only the presence of the (-), and not (+), antipodes (Figs. 2C and 3B). 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, [8,9-14C]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 [8,9-14C]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. 2D), 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, [8,9-14C]coniferyl alcohol was only incorporated into (−)-matairesinol 2b, as evidenced by the radiochemical elution profile (Fig. 3C). (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, (−)-[14C]matairesinol obtained following administration of [8-14C]coniferyl alcohol to F. intermedia. D, (−)-[1H]matairesinol obtained following administration of (±)-[1H]secoisolariciresinol to F. intermedia. E, F, Matairesinol 2 fractions isolated after incubation of (−)-[1H] and (+)-[1H] 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 intact 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 [9,9-3H₂,OC']H₃] 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 C₆ position of the monomer would contain either one or no deuterium atom.

[9,9-3H₂,OC']H₃] 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,
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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. 4B 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 H2O), and 137 (which corresponds to a fragment derived from benzylic cleavage). In contrast, the (-)-secoisolariciresinol 1b obtained from F. intermedia plant tissue (Fig. 4A), which had previously been administered [9,9-2H3,OC3H]coniferyl alcohol, gave signals at m/z 372, 362, 354, 344, 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 [9,9-2H3,OC3H]coniferyl alcohol molecules without prior C6 oxidation. This is because signals at m/z 372 (M+ + 10) and 354 (M+ + 10, less H2O) 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 2b to that obtained following [9,9-2H3,OC3H]coniferyl alcohol feeding to F. intermedia was also informative (see Table I). As shown in Fig. 4E and Table I, unlabeled (-)-matairesinol 2b isolated from F. intermedia contained no radioactivity at m/z 362, 344, and 137 (derived from cleavage of the benzylic fragment). On the other hand, the (-)-matairesinol 2b isolated from F. intermedia previously treated with [9,9-2H3,OC3H]coniferyl alcohol gave signals at m/z 366, 358, 140, and 137 (Table I). The peaks at m/z

\[
\begin{array}{c|c|c|c}
\text{m/z} & \text{Relative intensity} & \text{Relative intensity} & \text{Relative intensity} \\
\hline
137 & 100.0 & 100.0 & 100.0 \\
138 & 25.6 & 18.8 & 18.8 \\
139 & 2.3 & 1.8 & 1.8 \\
140 & 0.3 & 3.4 & 3.4 \\
141 & 0.2 & 1.0 & 1.0 \\
142 & 0.0 & 0.3 & 0.3 \\
358 & 21.1 & 26.1 & 26.1 \\
359 & 4.6 & 6.0 & 6.0 \\
360 & 0.7 & 1.0 & 1.0 \\
361 & 0.0 & 0.1 & 0.1 \\
362 & 0.0 & 0.1 & 0.1 \\
363 & 0.0 & 0.1 & 0.1 \\
364 & 0.0 & 0.0 & 0.0 \\
365 & 0.0 & 0.3 & 0.3 \\
366 & 0.0 & 0.6 & 0.6 \\
367 & 0.0 & 0.2 & 0.2 \\
\end{array}
\]

Fig. 4. Mass spectra of secoisolariciresinols and matairesinols. A, deuterated (-)-secoisolariciresinol, obtained following administration of [9,9-2H3,OC3H]coniferyl alcohol to F. intermedia. B, synthetic (unlabeled) (+)-secoisolariciresinols 1a/1b. C, synthetic (+)-[Ar-2H]secoisolariciresinols. D, unlabeled (-)-matairesinol obtained following incubation of (+)-[Ar-2H]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

<table>
<thead>
<tr>
<th>m/z</th>
<th>Natural abundance (-)-matairesinol 2b isolated from F. intermedia</th>
<th>(-)-Matairesinol 2b isolated following administration of [9,9-2H3,OC3H]coniferyl alcohol to F. intermedia</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>138</td>
<td>25.6</td>
<td>18.8</td>
</tr>
<tr>
<td>139</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>140</td>
<td>0.3</td>
<td>3.4</td>
</tr>
<tr>
<td>141</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>142</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>358</td>
<td>21.1</td>
<td>26.1</td>
</tr>
<tr>
<td>359</td>
<td>4.6</td>
<td>6.0</td>
</tr>
<tr>
<td>360</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>361</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>362</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>363</td>
<td>0.0</td>
<td>0.1</td>
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<tr>
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<td>366</td>
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<td>0.6</td>
</tr>
<tr>
<td>367</td>
<td>0.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

TABLE II

Enzymatic formation of (-)-matairesinol 2b from (+)-secoisolariciresinols 1a/1b

<table>
<thead>
<tr>
<th>Enzyme assay</th>
<th>Cofactor</th>
<th>Absolute incorporation of radioactivity into (-)-matairesinol 2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADP</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>NAD</td>
<td>2.9</td>
</tr>
<tr>
<td>Controls</td>
<td>None</td>
<td>0.08</td>
</tr>
<tr>
<td>4 denatured</td>
<td>NADP</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*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.

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

(+)-secoisolariciresinols 2a/2b for chiral HPLC analysis. This is in contrast to the observed formation of (-)-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-H]secoisolariciresinols (17 KBq mg⁻¹) were synthesized (from unlabeled synthetic material by exchange with CF₃CO₂H) and administered to F. intermedia plant tissue. After a 3-h metabolism, matairesinol 2b was isolated and subjected to reversed phase HPLC. Analysis of the resulting radiochemical elution profile revealed that the incorporation of (+)-[Ar-H]secoisolariciresinols into matairesinol 2b was 0.94%. The isolated [Ar-H]matairesinol was subsequently subjected to chiral HPLC analysis, which demonstrated that only the (-)-antipode 2b was radiolabeled (Fig. 3D). 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 [8-¹³C]coniferyl alcohol with F. intermedia cell-free extracts for 1 h at 30 ºC was carried out next (24). Following a series of experiments with appropriate cofactors (i.e., H₂O₂ and NADP⁺H), it was found that secoisolariciresinol 1 formation only occurred in the presence of H₂O₂ (0.4 mM) and NADP⁺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 (-)-matairesinol 2b was observed when either cofactor was omitted (NADP⁺H or H₂O₂) or when the enzyme was denatured (boiled 5 min). To further confirm that the enzymatic product was indeed (-)-secoisolariciresinol 1b, [9,9-³H₂O,OC₂H₅]coniferyl alcohol (6.97 mg) was incubated with the cell-free extract, in the presence of NADPH and H₂O₂. The enzymatic product was confirmed to be (+)-[³H₁₇]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 vivo conversion of secoisolariciresinol 1 into matairesinol 2. Thus, cell-free extracts of F. intermedia were incubated individually with both (+)- and (-)-[Ar-H]secoisolariciresinols (17 KBq mg⁻¹) for 1 h in the presence of NADP⁺: both enantiomers of (+)-[Ar-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. 3E). We were unable to demonstrate the conversion of (+)-secoisolariciresinol 2a into either (+)- or (-)-matairesinol 2a or 2b (Fig. 3F). Furthermore, no conversion into (-)-matairesinol 2b occurred when the enzyme was denatured (boiled 5 min) or when NADP⁺ was omitted (Table II). This again demonstrates the strict stereochromic control (or preference) of this enzymatic dehydrogenation. When (+)-[Ar-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-H]secoisolariciresinols into [Ar-H]matairesinols. Thus, (+)-[Ar-H]secoisolariciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignan with CF₃CO₂H. The (+)-[Ar-H]secoisolariciresinols, so obtained, were subjected to mass spectroscopic analysis. As can be seen (Fig. 4C), the parent molecular ion (M⁺) for unlabeled secoisolariciresinol 1, previously noted at m/z 362 (Fig. 4B), 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 H NMR analysis. Following incubation of the (+)-[Ar-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. 4E) 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 stereochromically controlled formation of (-)-secoisolariciresinol 1b and is centered two to three mass units higher than that of natural abundance (-)-matairesinol 2b (M⁺; 358; Fig. 4E) 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.

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 methoxy 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.)

Thus, cell-free extracts of F. intermedia were incubated individually with both (+)- and (-)-[Ar-H]secoisolariciresinols (17 KBq mg⁻¹) for 1 h in the presence of NADP⁺: both enantiomers of (+)-[Ar-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. 3E). We were unable to demonstrate the conversion of (+)-secoisolariciresinol 2a into either (+)- or (-)-matairesinol 2a or 2b (Fig. 3F).

Furthermore, no conversion into (-)-matairesinol 2b occurred when the enzyme was denatured (boiled 5 min) or when NADP⁺ was omitted (Table II). This again demonstrates the strict stereochromic control (or preference) of this enzymatic dehydrogenation. When (+)-[Ar-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-H]secoisolariciresinols into [Ar-H]matairesinols. Thus, (+)-[Ar-H]secoisolariciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignan with CF₃CO₂H. The (+)-[Ar-H]secoisolariciresinols, so obtained, were subjected to mass spectroscopic analysis. As can be seen (Fig. 4C), the parent molecular ion (M⁺) for unlabeled secoisolariciresinol 1, previously noted at m/z 362 (Fig. 4B), 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 H NMR analysis. Following incubation of the (+)-[Ar-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. 4E) 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.
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 ligation 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

Yoshinori Umezawa, Laurence B. Davin and Norman G. Lewis

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Formation of (-)-Secoisolariciresinol and (-)-Matairesinol

Chromatographic Materials and Instrumentation - Silica gel thin-layer chromatography (TLC) and silica gel column chromatography employed Kieselgel 60 F254 a (Merck, 70 x 200, 230-250 or 0.5 mm) and silica gel 60 (EM Science, 0.063-0.25 mm or 0.05 mm). 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 automatic injection module and a Model 990 photodiode array detector equipped with a NEC Power Wave 2, a Waters 990 Pump and a NEC personal computer. The lignans (-)-secoisolariciresinol 2a and (-)-matairesinol 2b were separated using a reversed phase column (Waters Nova-Pak C18 (150 x 3 mm), stainless steel) with detection at A254 nm. Elution details were as follows: a gradient solvent system (1 ml min-1) consisting of CH3CN:4%AcOH in H2O (95:05) at 0 min to 30:60 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 Dacel Chiralcel OD column (250 x 4 mm) at a flow rate of 0.5 ml min-1. The mixture of (S) and (R)-matairesinol 2a and 2b was separated following elution with hexane:EtOH (99:1) in hexanes (15:85) at a flow rate of 1 ml min-1. The mixture of (+) and (-)-matairesinol 2a and 2b was separated following elution with hexane:EtOH (20:30) at a flow rate of 0.5 ml min-1. The EHL used for chiral HPLC separations was demetalted with either MeOH (2%) and 2-propanol (4%) (Pierce, HPLC Spectrograde), or with MeOH (4%) and 2-propanol (4%) (Pierce, HPLC Spectrograde). 1H-NMR and 13C-NMR spectra were recorded with a Bruker 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 MA 212F-HF mass spectrometer equipped with a Meinke CR 210A mass filter. UV/Vis 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 Ecoum (ICN Biomedicals) liquid scintillation fluid using a Beckman LS-250 Liquid Scintillation System. Counting efficiencies were determined using 14C[3H][3H]-hexadecane and 14C[3H]-hexadecane 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 were used as a source of (-)-secoisolariciresinol 2a and of enzyme preparations.

Chemical Syntheses - [8-14C]Benzyloxy alcohol (23 kibq mg-1) was prepared as previously described via reduction of the methyl ester of [8-14C]benzoic acid obtained from and [3-14C]benzoic acid (ICN Biomedicals) liquid scintillation System Counting efficiencies were measured using a Perkin-Elmer 241 Polarimeter. UV spectra were obtained using a Perkin-Elmer Lambda 6 UV/VIS Spectrophotometer. Radioactive samples were counted in Ecoum (ICN Biomedicals) liquid scintillation fluid using a Beckman LS-250 Liquid Scintillation System. Counting efficiencies were determined using 14C[3H][3H]-hexadecane and 14C[3H]-hexadecane respectively for calibration. All melting points are uncorrected.

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Formation of (-)-Secoisolariciresinol and (-)-Matairesinol

(3) Ethyl O-toluene-sulfonate (O-TsCl)/1,3-dichloroacetone (DCl): To a stirred solution of [O-TsCl]/DCl (25 mmol), 2.0 ml of a 10% solution of NaH in DCl was added. After stirring at room temperature for 15 min, the reaction mixture was cooled to 0°C, and an ethereal solution of (+)-[O-TsCl]/DCl (25 mmol) was added. The resulting mixture was stirred at 0°C for 15 min, and the reaction was quenched with 10% NaHCl solution, followed by extraction with ethyl acetate. The extracts were washed with brine, dried over anhydrous sodium sulfate, and evaporated to dryness. The crude (-)-[O-TsCl]/DCl was obtained as a yellow solid.

(4) Pentadentate[Fe(II)/P4Cl4(OCH3)] complex: A solution of 1.0 mmol of (-)-[O-TsCl]/DCl in THF was added to a stirred solution of 1.0 mmol of pentadentate[Fe(II)/P4Cl4(OCH3)] in THF. The resulting mixture was stirred at room temperature for 1 h, and the solution was passed through a silica gel column (10 × 2 cm) to afford the (-)-[O-TsCl]/DCl complex as a yellow solid.

(5) (-)-Secoisolariciresinol (139 mg, 89%): A solution of (-)-[O-TsCl]/DCl (100 mg) in THF (5 ml) was added to a stirred solution of (-)-[O-TsCl]/DCl (100 mg) in DCl (25 mmol). The resulting mixture was stirred at room temperature for 1 h, and the solution was passed through a silica gel column (10 × 2 cm) to afford (-)-[O-TsCl]/DCl as a yellow solid.

(6) (-)-Matairesinol (144 mg, 79%): A solution of (-)-[O-TsCl]/DCl (100 mg) in THF (5 ml) was added to a stirred solution of (-)-[O-TsCl]/DCl (100 mg) in DCl (25 mmol). The resulting mixture was stirred at room temperature for 1 h, and the solution was passed through a silica gel column (10 × 2 cm) to afford (-)-[O-TsCl]/DCl as a yellow solid.

(7) Re-isolation of (-)-Secoisolariciresinol and (-)-Matairesinol: A solution of (-)-[O-TsCl]/DCl (100 mg) in THF (5 ml) was added to a stirred solution of (-)-[O-TsCl]/DCl (100 mg) in DCl (25 mmol). The resulting mixture was stirred at room temperature for 1 h, and the solution was passed through a silica gel column (10 × 2 cm) to afford (-)-[O-TsCl]/DCl as a yellow solid.
(100 μl) were added as radiolabeled carriers. The resulting mixed E2O solutions were dissolved in MeOH (250 μl, 10 μl of which were applied to a Waters Novapak C18 column and eluted with a gradient solvent system described in Chromatography Materials and instrumentation section. The resulting eluate was collected at 30 s

fractions corresponding to secoisolaricresinol 1 and matairesinol 2 were separated. Each fraction was further analyzed by chiral HPLC.

Administration of [9,9-2H2,OC2H3]comfrey alcohol to 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 (fg. 2H2) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polycar AT [64 g], and-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 as protein of a Bio-Rad Protein Assay using bovine serum albumin as standard.

Enzymatic oxidation of (+)-[2H]secoisolaricresinols

The assay mixture contained 770 μl 0.1 M Tris-HCl buffer (pH 8.0, 30°C), 310 μl crude enzyme preparation and 10 μl 56 mM NAD or NADP solution in 0.1 M potassium phosphate buffer (pH 7.3). To include the enzymatic reaction, (+)-[2H]secoisolaricresinols 1a, 1b (114.9 μg, 17 Kbez mg-1 dissolved in MeOH (5 μl) and 0.1 M potassium phosphate buffer (pH 7.0, 6 ml) were added. Following enzymatic incubation for 1 h at 30°C, EIOAc (2 ml) containing unlabelled (±)-matairesinols 2a, 2b (15.8 μg) as radioactive carriers was added, and the EIOAc solutions were removed. Following evaporation to dryness, the EIOAc extract was re-dissolved in MeOH (60 μl), and an aliquot (40 μl) then applied to the Waters Novapak C18 column. Following elution with the gradient solvent system described in the Chromatography Materials and instrumentation section, fractions were collected every 30 s for 1 x 5 to 20 min, and subjected to liquid scintillation counting (Table 2). Next, pbsy pure samples of (+)-[2H]secoisolaricresins 1a, 1b (114.9 μg, 17 Kbez mg-1 dissolved in MeOH (5 μl) and 0.1 M potassium phosphate buffer (pH 7.0, 6 ml) was isolated by the use of reversed phase HPLC, and subjected to chiral HPLC and liquid scintillation counting analyses.

Enzymatic oxidation of (+)-[2H]secoisolaricresinols

(+)-[2H]secoisolaricresins 1a, 1b (6.87 μg) 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. (+)-Matairesinol 2b was isolated with no addition of unlabelled carrier, by silica gel TLC, eluted with EIOAc-heptanes (1:1), developed three times in reversed phase HPLC and (chiral) HPLC, and then subjected to mass spectrometric examination.

Formation of (-)-Secoisolaricresinol and (-)-Matairesinol

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 (fg. 2H2) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polycar AT [64 g], and-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 as protein of a Bio-Rad Protein Assay using bovine serum albumin as standard.

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