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

(Received for publication, December 10, 1990)

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In vivo labeling experiments of Forsythia intermedia plant tissue with [8,14C] and [9,9,2H2,OC3H3]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-14C]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,OC3H3]coniferyl alcohols as substrates. The (+)-antipode was not formed. Finally, when either (−)-[Ar-14C] or (±)-[Ar-2H]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 stereo-selectivity 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. matairesinol 2, arctigenin 3, arctiin 4), furfurans (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 lignans are not. It is perhaps significant that higher oligomeric forms of lignans (trimers, tetramers, etc.) typically have only very small [α] 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 5α, whereas Xanthoxylum australoides (12) has the (−)-enantiomer 5β. No satisfactory explanation has been proffered to account for this stereoochemical control leading to optical activity; rather than that the reaction is somehow enzymatically mediated. Such control is not possible via intervention of a typical peroxidase/H2O2-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...
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. For example, secoisolariciresinol 1, pinoresinol 5, and epipinoresinol 6 could arise via direct coupling of the two monolignol molecules, coniferyl alcohol 8.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

The first goal of our research was to identify the key enzymatic reaction affording entry into the specialized biosynthesis 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 diisobutylaluminum hydride, rather than Ca(BH4)2, which, in our hands, consistently gave low yielding reactions. (+)-Secoisolariciresinols 1a/1b were obtained by LiAlH4, reduction of (+)-matairesinols 2a/2b. Each racemic lignan 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 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 HPLC²

¹The "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.

²The abbreviations used are: HPLC, high performance liquid chromatography; m.p., melting point; lit.m.p., literature melting point; THF, tetrahydrofuran; TLC, thin layer chromatography.
Formation of (-)-Secoisolariciresinol and (-)-Matairesinol

**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 [8-14C]coniferyl alcohol to *F. intermedia*. Note that unlabeled (±)-secoisolariciresinols 1a/1b were added as radiochemical carriers. Column, Chiralcel OD (Daicel) elution details: hexanes:EtOH (79:21); 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, [8-14C]coniferyl alcohol (130 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 [8-14C]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, [8-14C]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) (±)-matairesinsols 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]secoisolariciresinols to *F. intermedia*. E, [1H]secoisolariciresinols, respectively, with cell-free extracts of *F. intermedia* in the presence of NADP. Note that unlabeled (±)-matairesinsols 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 aldehyde could have occurred prior to coupling. Clearly, this uncertainty could be resolved by administration of [9,9-2H₂,OC'H⁻] 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.

[9,9-2H₂,OC'H⁻] 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,
and then subjected to mass spectrometry. For comparison purposes, spectra were also recorded for synthetic (±)-unlabeled lignans, secoisolariciresins 1a/1b and matairesins 2a/2b. Thus, Fig. 4B shows the mass spectrum of synthetic (±)-secoisolariciresins 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 [9,9-²H₂,OC²H₆]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-


**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 [9,9-²H₂,OC²H₆]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>
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<td>0.1</td>
</tr>
<tr>
<td>363</td>
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</tr>
<tr>
<td>364</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>365</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>366</td>
<td>0.6</td>
<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 (±)-secoisolariciresins 1a/1b.

<table>
<thead>
<tr>
<th>Enzyme assay</th>
<th>Cofactor</th>
<th>Absolute incorporation of radioactivity into (−)-matairesinol 2b</th>
<th>(−)-Matairesinol 2b formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADP</td>
<td>2.0</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td>NAD</td>
<td>2.9</td>
<td>19.9</td>
</tr>
<tr>
<td>Controls</td>
<td>None</td>
<td>0.08</td>
<td>0.6</td>
</tr>
<tr>
<td>3 no cofactors</td>
<td>None</td>
<td>0.08</td>
<td>0.6</td>
</tr>
<tr>
<td>4 denatured NADP</td>
<td>None</td>
<td>0.0</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%.

3H₂,OC²H₆]coniferyl alcohol molecules without prior C₀ 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 methoxy 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 [9,9-²H₂,OC²H₆]coniferyl alcohol feeding to F. intermedia was also informative (see Table I). As shown in Fig. 4E 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 [9,9-²H₂,OC²H₆]coniferyl alcohol gave signals at m/z 366, 358, 140, and 137 (Table I). The peaks at m/
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 methoxyyl groups. The relatively low intensities of the deuterated peaks are a consequence of unlabeled (−)-matairesinol 2b which was 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-1H]secoisolariciresinols (17 KBq mg−1) were synthesized (from unlabeled synthetic material by exchange with CH3OD) 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-1H]secoisolariciresinols into matairesinol 2b was 0.94%. The isolated [Ar-1H]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 [8,14C]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 co-factors (i.e. H2O2 and NAD(P)H), it was found that secoisolariciresinol 1 formation only occurred in the presence of H2O2 (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 (−)-matairesinol 1b was observed when either cofactor was omitted (NAD(P)H or H2O2) or when the enzyme was denatured (boiled 5 min). To further confirm that the enzymatic product was indeed (−)-secoisolariciresinol 1b, [9,9-3H2,3O-1H]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 (−)-[3H1]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-1H]secoisolariciresinols (17 KBq mg−1) for 1 h in the presence of NADP; both enantiomers of (±)-[Ar-1H]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 1a 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 stereoselective control (or preference) of this enzymatic dehydrogenation. When (±)-[Ar-1H]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-1H]secoisolariciresinols into [Ar-1H]matairesinol. Thus, (±)-[Ar-1H]secoisolariciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignan with CF3CO2H. The (±)-[Ar-1H]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 1H NMR analysis. Following incubation of the (±)-[Ar-1H]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 stereochemically controlled formation of (−)-secoisolarici-
biosynthesis, raise obvious questions with respect to the lig-
vansion of lignan lb to (-)-matairesinol 2b. More needs to
spectra.
clarification.
leading to the optically active lignans and (purportedly op-
nification process. At what point (if any) does the pathway
be known about the coupling of the two phenylpropanoid
units, in terms of how this enzyme (or enzymes) differ from


Acknowledgment--We

Chemical Syntheses - [8-14C]Cinnamyl alcohol (23 KIq mg-1) was prepared as previously de-
cribed via reduction of the methyl ester of [8-14C]Cinnamic acid obtained from vahnil and [14C]phloroglucinol (1.9.9-H2). [14C]Cinnamyl alcohol was synthesized in four steps as follows:

1. 4-Benzoylxy-3-[14C]methoxybenzaldehyde. To a stirred solution of 3-4-benzoxaldehyde (2.076 g) in N-N dimethylformamide (30 ml) under N2, were added benzyl bromide (3.421 g) and K2CO3 (2.076 g). The reaction mixture was stirred for 4 h at room temperature. The resulting reaction mixture was poured into distilled water (100 ml), washed with a saturated NaCl solution, dried (anhydrous Na2SO4), and the solvent evaporated in vacuo. Recrystallization from EtOAc-hexanes afforded the product (2.31 g, 75%).

2. O-(Benzoyloxy)-4-[14C]methoxybenzaldehyde: A stirred solution of 4-benzoylxy-3-hydroxybenzaldehyde (1.573 g) and O-[14C]HCl (Aldrich 99 atom % 1H) in N-N dimethylformamide (15 ml) was added K2CO3 (3.76 g). After 3 h, the reaction mixture was filtered, washed with water (100 ml), and evaporated to dryness under reduced pressure. The residue was purified via silica gel TLC (eluting with hexane-EtOAc 90:10, Rf = 0.15). The resulting product (1.42 g, 84%) was subjected to acid hydrolysis (15 ml) in perhydrol (1:20). After 3 h, the mixture was washed with water, followed by ethanol and evaporated to dryness. The residue was purified via silica gel TLC (eluting with hexane-EtOAc 90:10, Rf = 0.15). The resulting product (1.04 g, 52%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.83 g, 39%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.52 g, 26%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.26 g, 13%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.13 g, 7%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.06 g, 3%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.03 g, 1.5%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.01 g, 0.6%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.005 g, 0.3%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.002 g, 0.1%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.001 g, 0.05%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0005 g, 0.02%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0001 g, 0.01%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00005 g, 0.005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00001 g, 0.001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000005 g, 0.0005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000001 g, 0.0001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0000005 g, 0.00005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0000001 g, 0.00001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00000005 g, 0.000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00000001 g, 0.000001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000000005 g, 0.0000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000000001 g, 0.0000001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0000000005 g, 0.00000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0000000001 g, 0.00000001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00000000005 g, 0.000000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.00000000001 g, 0.000000001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000000000005 g, 0.0000000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.000000000001 g, 0.0000000001%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10). The resulting product (0.0000000000005 g, 0.00000000005%) was subjected to silica gel flash chromatography (eluting with hexane-EtOAc 90:10).
Formation of (-)-Secoisolaricresinol and (-)-Matairesinol

(3) Ethyl 6-iodo-2-methoxybenzenesulfonate (977 mg, 21%).

1H NMR (CDCl3) δ 6.27 (s, 2 H, Ar-H), 6.63 (d, J = 9.5 Hz, 1 H, H-7), 7.29 (m, 4 H, 2 × CH2, 2 × Ar-H), 7.36 (m, 4 H, 2 × CH2, 2 × Ar-H), 7.84 (s, 1 H, H-5), 7.88 (s, 1 H, H-5), 8.12 (s, 1 H, H-5), 8.20 (s, 1 H, H-5), 9.81 (s, 1 H, H-5), 12.14 (s, 1 H, CO2H).

13C NMR (CDCl3) δ 156.8, 128.0, 128.5, 129.0, 129.5, 133.0, 140.0, 157.6.

MS m/z (%) 258 (M+ 1, 100), 224 (17), 197 (5), 181 (5), 154 (7), 135 (10), 117 (15), 99 (27), 83 (30), 71 (28), 59 (29), 47 (30), 35 (31), 23 (32), 11 (33), 9 (34).

The crude was purified by a column of silica gel (15 × 2 cm) chromatography, eluted with a gradient of 0% to 100% MeOH/CH2Cl2 (2:1). Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure ethyl 6-iodo-2-methoxybenzenesulfonate.

(-)-Secoisolaricresinol (1g) was obtained from ethyl 6-iodo-2-methoxybenzenesulfonate (977 mg, 21%) by a column of silica gel (15 × 2 cm) chromatography, eluted with a gradient of 0% to 100% MeOH/CH2Cl2 (2:1). Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-secoisolaricresinol.

(-)-Matairesinol (2b) was isolated from ethyl 6-iodo-2-methoxybenzenesulfonate (977 mg, 21%) by a column of silica gel (15 × 2 cm) chromatography, eluted with a gradient of 0% to 100% MeOH/CH2Cl2 (2:1). Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-matairesinol.

Isolation of (-)-secoisolaricresinol (2b) from ethyl 6-iodo-2-methoxybenzenesulphonate (977 mg, 21%).

The compound was obtained by preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-secoisolaricresinol.

Isolation of (-)-matairesinol (2b) from ethyl 6-iodo-2-methoxybenzenesulphonate (977 mg, 21%).

The compound was obtained by preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-matairesinol.

Application of (8,14C)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 l-ascorbic acid (100 mg, 23.2 mg/kg) in 0.1 M potassium phosphate buffer (200 ml, pH 7.0). Following uptake and metabolism, the leaves were removed and the stems frozen. The resulting dried material was homogenized with a Waring blender and solvents extracted with a mixture of petroleum ether and diethyl ether. The residue was weighed to determine yield.

(-)-Secoisolaricresinol (2b) was isolated from ethyl 6-iodo-2-methoxybenzenesulphonate (977 mg, 21%) by a column of silica gel (15 × 2 cm) chromatography, eluted with a gradient of 0% to 100% MeOH/CH2Cl2 (2:1). Fractions were collected and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-secoisolaricresinol. The (-)-secoisolaricresinol was then dissolved in ethanol (0.1 M, 2.5 ml) and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-secoisolaricresinol. The (-)-secoisolaricresinol was then dissolved in ethanol (0.1 M, 2.5 ml) and purified further as described in chromatography section. The fractions containing the desired compound were collected and subjected to preparative silica gel TLC (400 × 10 × 0.2 cm, silica gel 60F254, Merck) with elution with 100:1 MeOH/CH2Cl2. Fractions were evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH/H2O (1:1) to give pure (-)-secoisolaricresinol.
(100 μg) were added as radiomabeled carriers. The resulting dried EtOAc 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 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 fraction was further analyzed by chiral HPLC.

Administration of (9,9,9-H3) secoisolariciresinol to F. intermedia (9-9-9-H3 OCH3)coniferyl alcohol to F. intermedia was administered to four excised F. intermedia shoots which were allowed to metabolize for 3 h as before. (2b) Matairesinol 2b was isolated as above without addition of unlabelled carrier, and analyzed for mass spectroscopic examination. In a separate experiment (9,9,9-H3 OCH3)coniferyl alcohol (23.7 mg) was administered to four F. intermedia shoots which were then allowed to metabolize for an additional 3 h. (3b) secoisolariciresinol 1b was isolated by successive purification as before but with no addition of unlabelled carrier, and subjected to mass spectroscopic analysis.

Administration of (1)-[Ar-3H]secoisolariciresinol 1a to F. intermedia (1)-[Ar-3H]secosolariciresinol 1a (207 μg, 17 KBq mg−1) were dissolved in MeOH (23 μl) and 0.1 M potassium phosphate buffer (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 (3b) secoisolariciresinol 1b isolation. The EtOAc solutions 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 C18 column, and eluted with the gradient solvent system described in the Chromatography Materials and instrumentation section. Eluted fractions were collected every 30 s for t = 0 to 20 min and subjected to liquid scintillation counting. refinery aliquots (total 50 μl) were applied to the aborad 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 ± fresh weight) were cut into small pieces by hand (scissors), frozen (−20 °C) and crushed with a mortar and pestle. The powder so obtained was further ground for 9-17 min with polyvinyl alcohol (6.94 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 four layers of cheese cloth, and the filtrate (3 ml) centrifuged (15,000 g, 10 min). The resulting supernatant (2.7 ml) was again filtered (Whatman GFA glass fibre filter), and an aliquot (1.0 ml) of the filtrate was applied to a Sephadex G-25 column (18.7 x 1 cm). Phosphate buffer 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.

Enzymatic oxidation of (2)-[4H] and (2)-[Ar-2H]secosolariciresinols 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 7.0). To induce the enzymatic reaction, (2)[Ar-2H]secosolariciresinols 1a (114.9 μg, 17 KBq 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 (2)-matairesinols 2a-2b (15.8 μg) as radiochemical carriers was added, and the EIOAc solubles were removed. Following evaporation to dryness, the EIOAc extract was redissolved in MeOH (80 μl), with an aliquot (40 μl) then applied to the Waters Novapak C18 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 (2)-[Ar-3H]secosolariciresinols 1a (451 μg, 7.67 KBq) and (2)-[Ar-3H]matairesinols 2a (1135 μg, 65 KBq) were individually incubated exactly as above, but with NADP as co-factor. Unlabelled (2)-matairesinols 2b (55.8 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 (2)-[Ar-2H]secosolariciresinols (2b) (2)-[Ar-3H]secosolariciresinols (68.7 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 proportionally. The (2)-matairesinol 2b was isolated with no addition of unlabelled carrier, by silica gel TLC, eluted with EIOAc-heptanes (1:1), developed three times by reversed phase HPLC and by chiral HPLC, and then subjected to mass spectroscopic examination.