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

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

(Received for publication, December 10, 1990)

In vivo labeling experiments of Forsythia intermedia plant tissue with \([8-^{13}\text{C}]\) and \([9,9-^{2}\text{H}_2,^{13}\text{C}^{13}\text{C}]\)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 \([\pm\text{-}[\text{Ar}-^{13}\text{C}]\text{secoisolariciresinol}\) 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 \(\text{NAD(P)}H\) and \(\text{H}_2\text{O}_2\), the cell-free extracts catalyzed the formation of \((-\))-secoisolariciresinol, with either \([8-^{13}\text{C}]\) or \([9,9-^{2}\text{H}_2,^{13}\text{C}^{13}\text{C}]\)coniferyl alcohols as substrates. The \((+\))-enantiomer was not formed. Finally, when either \((-\))[\text{Ar}-^{13}\text{C}]\) or \([\pm\text{-}[\text{Ar}-^{13}\text{C}]\text{secoisolariciresinol}\) were used as substrates, in the presence of \(\text{NAD(P)}\), 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 \(\text{NAD(P)}\)H and \(\text{H}_2\text{O}_2\) 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-dihydrobuthyrolactones (e.g. matairesinol 2, arctigenin 3, arctiiin 4), furofurans (e.g. pinoresinol 5, epipinoresinol 6), 1-phenylphtahalenes and tetralsins (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 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 \([\alpha]_D\) 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 aianthoides (12) has the \((-\)-enantiomer 5b. No satisfactory explanation has been proffered to account for this stereochrometric control leading to optical activity; other than that the reaction is somehow enzymatically mediated. Such control is \(\text{not possible via}\) intercession of a typical peroxidase/\(\text{H}_2\text{O}_2\)-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 elements of isolated lignans.

*This work was supported by NASA Grant NAGW-1277 and U. S. Department of Agriculture Grant 88-33521-4082. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

§ To whom correspondence should be addressed. Present address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6140. Tel.: 509-335-2682; Fax: 509-335-7643.
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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 conifer alcohol 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

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 conifer alcohol monomers 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-methoxyphenoxy)propionate was carried out in 38.7% yield using a reducing agent, made in situ from n-butyllithium and diisobutylalumium hydride, rather than Ca(BH₄)₂, which, in our hands, consistently gave low yields. Reduction of (±)-Secoisolariciresinols 1a/1b was obtained by LiAlH₄, reduction of (±)-matairesinol 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 HPLC

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.

FIG. 1. Representative lignans, monolignols, and hydroxycinnamic acids.
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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 [8-14C]coniferyl alcohol to F. intermedia. Note that unlabeled (-)-secoisolariciresinols 1a/1b were added as radiochemical carriers. Column, Chiralcel OD (Daicel) elution details: hexanes:EtOH (85:15); 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 (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 [8-14C]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.)

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,OC⁰⁰⁰H₃] 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 C₉ position of the monomer would contain either one or no deuterium atom.

[9,9-3H₂,OC⁰⁰⁰H₃] 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. 3. Chiral HPLC separations of matairesins. A, synthetic (unlabeled) (+)-matairesins 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, (-)-[14H]matairesinol obtained following administration of (+)-[14H]secoisolariciresinols to F. intermedia. E, and F, Matairesinol 2 fractions isolated after incubation of (-)-[14H] and (+)-[14H] secoisolariciresinols, respectively, with cell-free extracts of F. intermedia in the presence of NADP. Note that unlabeled (+)-matairesins 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.
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÷ + 10, less H2O), and 137 (which corresponds to a fragment derived from benzylic cleavage). On the other hand, the (−)-matairesinol 2b isolated from F. intermedia has two main signals at m/z 362 (M÷), 344 (M÷ + 10, less H2O) prove that the newly formed (−)-secoisolariciresinol lb 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 [9,9-2H2,OC2H5]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,OC2H5]coniferyl alcohol gave signals at m/z 366, 358, 140, and 137 (Table I). The peaks at m/z

### TABLE I

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<tr>
<th>m/z</th>
<th>Natural abundance (−)-matairesinol 2b isolated from F. intermedia</th>
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### TABLE II

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<td></td>
<td></td>
<td>ng Radioactivity mg−1 Protein</td>
<td>ng Radioactivity mg−1 Protein</td>
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<tr>
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</tr>
<tr>
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<td>NAD</td>
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</table>

H2,OC2H5)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 [9,9-2H2,OC2H5]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,OC2H5]coniferyl alcohol gave signals at m/z 366, 358, 140, and 137 (Table I). The peaks at m/z
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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 (−)-secoisolariciresinols 1b and (−)-matairesinols 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 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-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 [8,13C]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. H₂O₂ and NAD(P)H), it was found that secisoraliciresinol 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 (−)-secisoraliciresinol 1b, and not its (+)-antipode 1a.

The rate of formation of (−)-secisoraliciresinol 1b was 15.9 nmol h⁻¹ mg⁻¹ protein. Significantly, no formation of (−)-secisoraliciresinol 1b was observed when either cofactor was omitted (NADPH or H₂O₂) or when the enzyme was denatured (boiled 5 min). To further confirm that the enzymatic product was indeed (−)-secisoraliciresinol 1b, [9,9,13C₃OCH₃]coniferyl alcohol (6.97 mg) was incubated with cell-free extract, in the presence of NADPH and H₂O₂. The enzymatic product was confirmed to be (−)-[13C₃]secisoraliciresinol by comparison of its mass spectrum with that of natural abundance (±)-secisoraliciresinols 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 (−)-secisoraliciresinol 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 secisoraliciresinol 1 into matairesinol 2. Thus, cell-free extracts of F. intermedia were incubated individually with both (+)- and (−)-[Ar-1H]secisoraliciresinols (17 KBq mg⁻¹) for 1 h in the presence of NADP; both enantiomers of (±)-[Ar-1H]secisoraliciresinols 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, (−)-secisoraliciresinol 1b was converted into (−)-matairesinol 2b, but not its (+)-antipode 2a (Fig. 3E). We were unable to demonstrate the conversion of (+)-secisoraliciresinol 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 stereoelectronic control (or preference) of this enzymatic dehydrogenation. When (±)-[Ar-1H]secisoraliciresinols (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]secisoraliciresinol into [Ar-1H]matairesinol. Thus, (±)-[Ar-1H]secisoraliciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignan with CF₃CO₂H. The (±)-[Ar-1H]secisoraliciresinols, so obtained, were subjected to mass spectrometric analysis. As can be seen (Fig. 4C), the parent molecular ion (M⁺) for unlabeled secisoraliciresinol 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]secisoraliciresinols 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 (−)-secisoraliciresinol 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 stereocinetically controlled formation of (−)-secisoraliciresinol.
solin 1b from coniferyl alcohol 8 and the other in the conversion of lignan lb 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.

Acknowledgment—We wish to thank K. Harich for recording mass spectra.

REFERENCES


Chemical Syntheses. [8]-Secoisolariciresinol (23 kRg ml1) was prepared as previously described via reduction of the methyl ester of [8]-[14C]Secoisolariciresinol (obtained from vanillin and [1-14C]malonic acid (19) [8,9,10,11-D4C14]Coniferyl alcohol was synthesized in four steps as follows:

1. 4-Benzoyl-3-[14C]vanillin and C6H5-phenylacetaldehyde. To a stirred solution of 3-(4-benzoyl-3-[14C]vanillin (2.076 g) in 1.5 N sodium hydroxide (30 ml) under N2, were added benzylbromide (3.421 g) and K2CO3 (2.076 g). The reaction mixture was stirred at room temperature for 1.5 h, then cooled to 0 °C, washed with a saturated NaCl solution, dried (anhydrous Na2SO4) and evaporated in vacuo to give the desired aldehyde (8.25 g).

2. O-[N-Dimethylformamide]vanillin. A solution of 3-(4-benzoyl-3-[14C]vanillin (1.573 g) and 1H-NMR (CDCl3): 7.05 (d, J = 8 Hz, 1H, H4); 7.3-7.5 (m, 7H, Ar); 6.94 (s, 1H, CHO); 245, 253, 35.3, 99, 105; 94.

3. 4-Benzyol-3-[14C]methylbenzaldehyde. To a stirred solution of 3-(4-benzoyl-3-[14C]methylbenzaldehyde (1.67 g) and C6H5-phenylacetaldehyde (0.75 g) in CHCl3, were added dibenzyl ether (3 ml) and K2CO3 (2.076 g). The reaction mixture was stirred at room temperature for 1 h, and then extracted with CH2Cl2 (3 x 100 ml). The combined CH2Cl2 extracts were washed with a saturated NaCl solution, dried (anhydrous Na2SO4) and evaporated in vacuo to give a brownish solid (6.25 g).

4. O-[N-Dimethylformamide]vanillin. A solution of 3-(4-benzoyl-3-[14C]methylbenzaldehyde (1.66 g) in AcOH (10 ml), under N2, were added benzylbromide (3.421 g) and K2CO3 (2.076 g). The reaction mixture was stirred at room temperature for 1 h, then extracted with CH2Cl2 (3 x 75 ml). The combined CH2Cl2 extracts were washed with a saturated NaCl solution, dried (anhydrous Na2SO4), and the solvent removed in vacuo to give a yellow solid (4.09 g).

5. 4-Benzoyl-3-[14C]methylbenzaldehyde. A solution of 3-(4-benzoyl-3-[14C]methylbenzaldehyde (1.67 g) and C6H5-phenylacetaldehyde (0.75 g) in CHCl3, were added dibenzyl ether (3 ml) and K2CO3 (2.076 g). The reaction mixture was stirred at room temperature for 1 h, then extracted with CH2Cl2 (3 x 75 ml). The combined CH2Cl2 extracts were washed with a saturated NaCl solution, dried (anhydrous Na2SO4), and the solvent removed in vacuo to give a yellow solid (4.09 g).

6. O-[N-Dimethylformamide]vanillin. A solution of 3-(4-benzoyl-3-[14C]methylbenzaldehyde (1.66 g) in AcOH (10 ml) under N2, was added dibenzyl ether (3 ml) and K2CO3 (2.076 g). The reaction mixture was stirred at room temperature for 1 h, then extracted with CH2Cl2 (3 x 75 ml). The combined CH2Cl2 extracts were washed with a saturated NaCl solution, dried (anhydrous Na2SO4), and the solvent removed in vacuo to give a yellow solid (4.09 g).

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

(3) Ethyl 3-(2-hydroxyethoxymethoxy)phenylacetate Toluene at a stirred solution of (3)-secoisolariciresinol (10 mg) in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (2) (6.6 mg) was obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as a colorless crystal (mp 113-113.5°C). 

(−)-Matairesinol (12) (40 mg) was obtained and purified from F. intermedia shoots (10 cm long with 8-10 leaves) by means of a modified chromatographic technique described above in the case of (−)-secoisolariciresinol (2). The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-matairesinol (12) (6.5 mg) was obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as a colorless crystal (mp 113-113.5°C). 

(4) Pentadentate (84.7% 2H4,OCCH3) phenol carboxylic acid Toluene at a stirred solution of (−)-matairesinol (27 mg) in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-matairesinol (12) (6.6 mg) was obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as a colorless crystal (mp 113-113.5°C). 

(5) secoisolariciresinol (1) (29.7 mg) and matairesinol (12) (27 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (13 mg) and (−)-matairesinol (12) (9 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(6) (+)-Matairesinol (11) (2.9 mg) was obtained and purified from F. intermedia shoots (10 cm long with 8-10 leaves) by means of a modified chromatographic technique described above in the case of (−)-secoisolariciresinol (2). The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (+)-matairesinol (11) (1.4 mg) was obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as a colorless crystal (mp 113-113.5°C). 

(7) (−)-Secoisolariciresinol (1) (2.9 mg) and matairesinol (12) (2.9 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.4 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(8) (+)-Matairesinol (11) (2.6 mg) was obtained and purified from F. intermedia shoots (10 cm long with 8-10 leaves) by means of a modified chromatographic technique described above in the case of (−)-secoisolariciresinol (2). The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (+)-matairesinol (11) (1.3 mg) was obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as a colorless crystal (mp 113-113.5°C). 

(9) (−)-Secoisolariciresinol (1) (2.8 mg) and matairesinol (12) (2.8 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.3 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(10) (−)-Secoisolariciresinol (1) (2.9 mg) and matairesinol (12) (2.9 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.4 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(11) (−)-Secoisolariciresinol (1) (2.7 mg) and matairesinol (12) (2.7 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.4 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(12) (−)-Secoisolariciresinol (1) (2.6 mg) and matairesinol (12) (2.6 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.3 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C). 

(13) (−)-Secoisolariciresinol (1) (2.7 mg) and matairesinol (12) (2.7 mg) were dissolved in anhydrous MeOH (8 ml) and then water (2 ml) was added dropwise. The reaction mixture was dissolved in chloroform and then washed with water (5 ml) and then brine. The solvent was removed in vacuo and the product applied to a silica gel column (20 g). After elution with chloroform (200 ml), the (−)-secoisolariciresinol (1) (1.4 mg) and (−)-matairesinol (12) (1.5 mg) were obtained by preparative TLC on silica gel (2:1 MeOH-ChCl) as colorless crystals (mp 113-113.5°C).
(100 µl) were added as radiochemical carriers. The resulting adapt EqO solutions were dissolved in MeOH (250 µl, 10 µl of which were applied to a Waters Nova-pak 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 secoisolaricresinol 1 and matairesinol 2 were separately collected. Each fraction was further analyzed by chiral HPLC.

Administration of (9,9-2H2,OC2H3)comfrey alcohol (237 mg) was considered to be metabolized for 3 h as below. (-)-Matairesinol 2b was isolated as an above without addition of unlabelled carrier, and analyzed for mass spectroscopic examination. In a separate experiment, (9,9-2H2,OC2H3)comfrey alcohol (237 mg) was administered to four excised F. intermedia shoots, which were then allowed to metabolize for an additional 3 h. (+)-secoisolaricresinol 1b was isolated by successive purification as before but with no addition of unlabelled carrier, and subjected to mass spectroscopic analysis.

Administration of (9,9-2H2,OC2H3)proenoyl alcohol to F. intermedia (9-9-2H2,OC2H3)comfrey alcohol (337 mg) in 0.1 M potassium phosphate buffer pH 7.0, 528 µl) was administered to four excised F. intermedia shoots which were allowed to metabolize for 3 h as below. (+)-Matairesinol 2b was isolated as an above without addition of unlabelled carrier, and analyzed for mass spectroscopic examination. In a separate experiment, (9,9-2H2,OC2H3)comfrey alcohol (237 mg) was administered to four excised F. intermedia shoots, which were then allowed to metabolize for an additional 3 h. (+)-secoisolaricresinol 1b was isolated by successive purification as before but with no addition of unlabelled carrier, and subjected to mass spectroscopic analysis.

Enzymatic oxidation of (+)-[3H]secoisolaricresinol

The assay mixture contained 270 µl 0.1 M Tris-HCl buffer (pH 8.0, 30°C), 210 µl crude enzyme preparation and 10 µl 56 mCi NAD or NADP solution in 0.1 M potassium phosphate buffer (pH 7.3). To include the enzymatic reaction, (+)-(Ar,3H]secoisolaricresinol 1 (114.9 µg, 17 KBq µg−1) dissolved in MeOH (50 µl) and 0.1 M potassium phosphate buffer (pH 7.0) were added. Following incubation for 2 h at 3°C, the reaction mixture was incubated at 37°C for 1 h. Next, the reaction mixture was incubated at 30°C, EtOAc (2 ml) containing unlabelled (±)-matairesinol 2b was added, and the reaction was subjected to chiral HPLC and liquid scintillation counting (Table 2). Next, 50 µl pure samples of (+)-(Ar,3H]secoisolaricresinol 1 (45.1 µg, 7 KBq µg−1) and (+)-(Ar,3H]secoisolaricresinol 1 (895.5 µg, 6.55 KBq) were individually incubated exactly as above, with NADP as cosubstrate. Unlabelled (+)-matairesinol 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, subjected to chiral HPLC and liquid scintillation counting analyses.

Enzymatic oxidation of (+)-(Ar,3H]secoisolaricresinol

(+)-(Ar,3H]secoisolaricresinol (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 silica gel TLC, eluted with EI20 acetonitrile-water (1:1), developed three times in reversed phase HPLC and (ii) chiral HPLC, and then subjected to mass spectroscopic examination.