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

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In vivo labeling experiments of Forsythia intermedia plant tissue with [8-14C]- and [9,9-2H2,OC2H5]-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-1H]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 NAD(P)H and H2O2, the cell-free extracts catalyzed the formation of (-)-secoisolariciresinol, with either [8-14C]- or [9,9-2H2,OC2H5]-coniferyl alcohols as substrates. The (+)-enantiomer was not formed. Finally, when either (-)[Ar-1H] or (±)-[Ar-2H2]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), furfurans (e.g. pinoresinol 5, epipinoresinol 6), 1-phenylnaphthalenes 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 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, appear to 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 5a, whereas Xanthoxylum ailanthoides (12) has the (-)-enantiomer 5b. No satisfactory explanation has been proffered to account for this stereochemical control leading to optical activity; rather than that the reaction is somehow enzymatically mediated. Such control is not possible via incorporation 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...
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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 affording 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 Dauget (21) with the following exception: reduction of methyl 2-carboxymethyl-3-(4-hydroxy-3-methoxyphenyl)propionate was carried out in 38.7% yield using a reducing agent, made in situ from n-butyllithium and diisobutyllithium aluminium hydride, rather than Ca(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 abbreviations used are: HPLC, high performance liquid chromatography; m.p., melting point; lit.m.p., literature melting point; THF, tetrahydrofuran; TLC, thin layer chromatography.

²The “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.
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 (+)-seciosolariciresinol 1a/1b (100 µg) added as radiochemical carriers. The lignans were isolated as described in the Miniprint. First, seciosolariciresinol 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 seciosolariciresinol 1 (0.3%) and matairesinol 2 (1.8%), respectively, based upon coincidence of radioactivity peaks with eluted lignans. Next, seciosolariciresinol 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 (+)-seciosolariciresinol 1b was detected in vivo. (Note that the UV elution profile shows the presence of both (+)- and (-)-forms since unlabeled (+)-seciosolariciresinol 1a/1b were added as radiochemical carriers.) In a similar manner to seciosolariciresinol 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,OC'H₃] coniferyl alcohol to F. intermedia plant tissue actively synthesizing the lignans, (+)-seciosolariciresinol 1b and (+)-matairesinol 2b. If intact incorporation of coniferyl alcohol 8 occurred, then the (+)-seciosolariciresinol 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, (+)-seciosolariciresinol 1b and (+)-matairesinol 2b were isolated from F. intermedia, but without addition of unlabeled carrier,
Formation of (−)-Secoisolariciresinol and (−)-Matairesinol

Table I

Relative intensities of molecular ion and base ion regions

<table>
<thead>
<tr>
<th>m/z</th>
<th>Natural abundance (−)-matairesinol 2b isolated from F. intermedia</th>
<th>(−)-Matairesinol 2b isolated following administration of [9,9-2H,OC2H3]coniferyl alcohol to F. intermedia</th>
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<tr>
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<tr>
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<tr>
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</table>

Table II

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

<table>
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<tr>
<th>Enzyme assay</th>
<th>Cofactor</th>
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<th>(−)-Matairesinol 2b formation</th>
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<td>2</td>
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<tr>
<td>3 no cofactors</td>
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<td>0.6</td>
</tr>
<tr>
<td>4 denatured</td>
<td>NADP</td>
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</tr>
</tbody>
</table>

Notes: *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%.

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 [9,9-2H,OC2H3]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-
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, which 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 2 was 99.4%. 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 [8,14C]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 cofactors (i.e. H2O 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 (−)-secoisolariciresinol 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 (−)-secoisolariciresinol 1b, [9,9,3H2,1OC3H7]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 (−)−[13C3]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-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 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 (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]secoisolariciresinol into [Ar-H]matairesinol. 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 1H 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 stereoselectively controlled formation of (−)-secoisolariciresinol 1b.
biosynthesis, raise obvious questions with respect to the lig-
against lignans. The current findings, as regards lignan
2b. More needs to
clarification.
leading to the optically active lignans and (purportedly) op-
formation of the more highly functionalized lignans, such as
units, in terms of how this enzyme (or enzymes) differ from
Acknowledgment—We
were maintained in VPI & SU greenhouse facilities and
obtained from Cox’s Nursery,
a campus of Virginia Polytechnic Institute and State University (VPI&SU), were used
Plant Material
were determined using
Radioactive samples were counted in Ecolure (CIN Biosciences) liquid scintillation
using a Beckman LS-250 Liquid Scintillation System. Counting efficiencies were
determined using n-14Hexadecane and 7-tricosene 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 (Virginia Tech), 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 VP&SU greenhouse facilities and used as a source of (-)-secoisolariciresinol 2b, and of enzyme preparations.

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

(3) Ethyl O-indurataethyloxyglycerol (OICr3H) 3. Fluoride to a stirred solution of OICr3H (100 mg) in dry THF (2 ml) following for an additional 1 h at the same temperature. The reaction mixture was filtered through silica gel (35 cm) over a period of 35 min at room temperature. Following stirring for 40 min at the same temperature, the resulting suspension was filtered (0.45 µm) and to this was added dropwise OICr3H (6 ml) to this, to the combined solution of the above methyl ester (6 ml) in vacua.

The sample was recrystallized in distilled water (ca. 50 ml) and the resulting crystals of coniferyl alcohol (70 mg) were redissolved and applied to a silica gel column (8 x 1 cm) which was eluted with ethyl acetate (35 ml) over a period of 35 min at room temperature. Following stirring for 40 min at the same temperature, the resulting suspension was filtered (0.45 µm) and the resulting crystals of coniferyl alcohol (70 mg) were redissolved and applied to a silica gel column (8 x 1 cm) which was eluted with ethyl acetate (35 ml) over a period of 35 min at room temperature. Following stirring for 40 min at the same temperature, the resulting suspension was filtered (0.45 µm) and the resulting crystals of coniferyl alcohol (70 mg) were redissolved and applied to a silica gel column (8 x 1 cm) which was eluted with ethyl acetate (35 ml) over a period of 35 min at room temperature.
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(100 μg) were added as radiochemical carriers. The resulting dried EtOAc solubles 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 then further analyzed by chiral HPLC.

Administration of [9,9-2H2]OC2H3-conifer alcohol to F. intermedia (9-9-2H2 OCH3-conifer alcohol (3.37 mg) in 0.1 M potassium phosphate buffer (pH 7.0, 526 μl) was administered to four excised F. intermedia shoots which were allowed to metabolize for 3 h as 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-conifer alcohol (23.7 mg) was administered to forty F. intermedia shoots which were then allowed to metabolize for an additional 3 h. (-)-Secoisolariciresinol 1b was isolated by successive purification as above but with no addition of unlabelled carrier, and subjected to mass spectroscopic analysis.

Administration of [9,9-2H2]Secoisolariciresinol 2a and [9,9,9-2H3]Secoisolariciresinol 2b to F. intermedia. (-)-[9,9-2H2]Secoisolariciresinol 2a (207 μg, 17 Kbg mg-1) and [9,9,9-2H3]Secoisolariciresinol 2b (84 μg, 17 Kbg mg-1) were dissolved in MeOH (23 μl) and 0.1 M potassium phosphate buffer (223 μl, pH 7.0) and administered to two shoots of F. intermedia. Following metabolism for 3 h, the leaves were removed and the stems freeze-dried. The resulting dried material was extracted in the same way as previously described for (-)-Secoisolariciresinol 1b isolation. The EtOAc solubles containing both secoisolariciresinol 1 and matairesinol 2 were dissolved in MeOH (250 μl). An aliquot of this solution (25 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 from 1 x 0 to 20 min and subjected to analysis by liquid scintillation counting. Next, aliquots (total 50 μl) were applied to the aforesaid column, and fractions corresponding to matairesinol 2 were collected, and subjected to chiral HPLC analysis.

Cell-free extract from F. intermedia

Young shoots (5-10 cm long) of F. intermedia were excised by means of a razor, then washed with both tap and distilled water, and the leaves removed. The resulting stems (27 g fresh weight) were cut into small pieces by hand (scissors), frozen (dry ice) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polyvinyl Alcohol (6% g), air-washed sea sand and 0.1 M potassium phosphate buffer (pH 7.0, 4 ml) containing 10 mM diothreitol. The slurry was filtered through four 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 GF A 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 diothreitol. 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 BioRad Protein Assay using bovine serum albumin as standard (25).

Enzymatic oxidation of (-)-(46) and (-)-(45-2H2)Secoisolariciresinol

The assay mixture contained 770 μl 0.1 M Tris-HCl buffer (pH 8.8, 30°C), 235 μl crude enzyme preparation and 10 μl 50 mM NAD or NADP solution in 0.1 M potassium phosphate buffer (pH 7.3). To include the enzymatic reaction, (-)-(45-2H2)Secoisolariciresinol 2a (114.9 μg, 1.7 Kbg mg-1) dissolved in MeOH (5 μl) and 0.1 M potassium phosphate buffer (pH 7.0, 4 ml) were added. Following enzymatic incubation for 1 h at 30°C, the reaction mixture was then collected by means of an inexhaustible (-)-Matairesinol 2b (358 μg, 5.5 Kbg) was isolated with no addition of unlabelled carrier, by (ii) silica gel TLC, eluted with EtOAc-heptanes (1:1), developed three times (vi) reversed phase HPLC and (vi) chiral HPLC, and then subjected to mass spectroscopic examination.

Enzymatic oxidation of (-)-(45-2H2)Secoisolariciresinol

(iii) Chiral HPLC mass spectroscopic examination