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,OC-2H2]-coniferyl alcohols revealed that the lignans, (−)-secoisolariciresinol and (−)-matairesinol, were derived from two coniferyl alcohol molecules; no evidence for the formation of the corresponding (+)-enantiomers was found. Administration of (±)-[Ar-H]-secoisolariciresinols to excised shoots of F. intermedia resulted in a significant conversion into (−)-matairesinol; again, the (+)-antipode was not detected. Experiments using cell-free extracts of F. intermedia confirmed and extended these findings. In the presence of NAD(P)H and H2O2, the cell-free extracts catalyzed the formation of (−)-secoisolariciresinol, with either [8-14C]- or [9,9-2H2,OC-2H2]-coniferyl alcohols as substrates. The (+)-enantiomer was not formed. Finally, when either (−)-[Ar-H] 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 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, arctin 4), furufuranos (e.g. pinoresinol 5, epinoresinol 6), 1-phenylnapthalenes and tetralins (e.g. podophyllotoxin 7) (see Fig. 1).

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

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

Lignans and lignins, however, apparently differ in one fundamentally important aspect, namely optical activity. For the most part, dimeric lignans (e.g. secoisolariciresinol 1, pinoresinol 5, matairesinol 2, and podophyllotoxin 7) are optically active (1, 2), whereas isolated lignins are not. It is perhaps significant that higher oligomeric forms of lignans (trimers, tetramers, etc.) typically have only very small [α]0 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 stereochemical control leading to optical activity; other 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 neglected, even for medically 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, arctin 4, and podophyllotoxin 7, could be formed...
Formation of (−)-Secoisolariciresinol and (−)-Matairesinol

10211

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

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

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

Based on structural considerations, we rationalized that the initial coupling product was either secoisolariciresinol 1 or matairesinol 2, and both lignans were obtained in racemic (±)-form by total synthesis. (±)-Matairesinols 2a/2b were formed using the method of Brown and Daugan (21) with the following exception: reduction of methyl 2-carboxymethyl-3-(4-hydroxy-3-methoxypheyl)propanoate was carried out in 38.7% yield using a reducing agent, made in situ from Na-butyllithium and diisobutylaluminium hydride, rather than Ca(BH₄)₂, which, in our hands, consistently gave low yielding reactions. (±)-Secoisolariciresinols 1a/1b were obtained by LiAlH₄ 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.
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-EOH (98:2); flow rate, 0.5 ml min⁻¹.

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

These experiments did not, however, prove that coniferyl alcohol 8 had been incorporated intact into either lignan; enzymatic conversion of this alcohol to the acid or aldehyde could have occurred prior to coupling. Clearly, this uncertainty could be resolved by administration of [9,9-3H₂OC-H₃] coniferyl alcohol to F. intermedia plant tissue actively synthesizing the lignans, (-)-secoisolariciresinol 1b and (-)-matairesinol 2b. If intact incorporation of coniferyl alcohol 8 occurred, then the (-)-secoisolariciresinol 1b and (-)-matairesinol 2b formed de novo would contain 10 and 8 deuterium atoms, respectively. This could be proven by mass spectrometry. If, however, oxidation to the aldehyde or acid occurred prior to coupling, then the C₈ position of the monomer would contain either one or no deuterium atom.

[9,9-3H₂OC-H₃] Coniferyl alcohol obtained by total synthesis, as described in the Miniprint, was administered to excised F. intermedia shoots (0.59 mg/shoot) which were then allowed to metabolize for 3 h. Following this period, the lignans, (-)-secoisolariciresinol 1b and (-)-matairesinol 2b were isolated from F. intermedia, but without addition of unlabeled carrier.
that (-)-secoisolariciresinol lb was formed from two [9,9-\(\text{H}_2\)]coniferyl alcohol to \(F.\) \textit{intermedia}. B, synthetic (unlabeled) (\(\pm\))-secoisolariciresins 1a/1b. C, synthetic (\(\pm\))-\([\text{Ar}-\text{H}]\)secoisolariciresins. D, deuterated (-)-matairesinols obtained following incubation of (\(\pm\))-\([\text{Ar}-\text{H}]\)secoisolariciresins with cell-free extracts of \(F.\) \textit{intermedia} in the presence of NADP. E, unlabeled (-)-matairesinol ob-
tained following administration of [9,9-\(\text{H}_2\)]coniferyl alcohol to \(F.\) \textit{intermedia}.

Table I

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<th>(m/z)</th>
<th>Natural abundance (-)-matairesinol 2b isolated from (F.) \textit{intermedia}</th>
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Formation of (-)-Secoisolariciresinol and (-)-Matairesinol

and then subjected to mass spectrometry. For comparison purposes, spectra were also recorded for synthetic (\(\pm\))-unlabeled lignans, secoisolariciresins 1a/1b and matairesins 2a/2b. Thus, Fig. 4B shows the mass spectrum of synthetic (\(\pm\))-secoisolariciresins 1a/1b. As can be seen, there are three main signals at \(m/z\) 362 (\(M^+\)), 344 (\(M^+ - 18\), loss of \(\text{H}_2\text{O}\)), and 137 (which corresponds to a fragment derived from benzylic cleavage). In contrast, the (-)-secoisolariciresinol 1b obtained from \(F.\) \textit{intermedia} plant tissue (Fig. 4A), which had previously been administered [9,9-\(\text{H}_2\)]coniferyl alcohol, gave signals at \(m/z\) 372, 362, 354, 344, 140, and 137. The signals observed at \(m/z\) 362, 344, and 137 correspond to natural abundance (-)-secoisolariciresinol 1b already present in the plant tissue. The signals at \(m/z\) 372, 354 and 140 reveal that (-)-secoisolariciresinol 1b was formed from two [9,9-\(\text{H}_2\)]coniferyl alcohol molecules without prior \(C_6\) oxidation. This is because signals at \(m/z\) 372 (\(M^+\)) and 354 (\(M^+ + 10\), less \(\text{H}_2\text{O}\)) 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-\(\text{H}_2\)]coniferyl alcohol feeding to \(F.\) \textit{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 \(F.\) \textit{intermedia} previously treated with [9,9-\(\text{H}_2\)]coniferyl alcohol gave signals at \(m/z\) 366, 358, 140, and 137 (Table I). The peaks at \(m/
Formation of (-)-Secoisolariciresinol and (-)-Matairesinol

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

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 (+)-antiomer 2a (Fig. 3E). We were unable to demonstrate the conversion of (+)-secoisolariciresinol 2a into either (+)- or (-)-matairesinol 2a or 2b (Fig. 3F).

To confirm and extend these radiochemical observations, we next undertook to demonstrate the conversion of [Ar-1H]secoisolariciresinol 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 the 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 (-)-secoisolariciresinol 1b. Further investigation is required to elucidate the mechanism of this transformation.
sinol 1b from coniferyl alcohol 8 and the other in the conversion of lignan 1b to (-)-matairesinol 2b. More needs to be known about the coupling of the two phenylpropanoid units, in terms of how this enzyme (or enzymes) differ from typical peroxidase reactions. This is currently under investigation.

Research directed to the elucidation of the biosynthetic pathways (intermediates and enzymes) involved in the typical peroxidase reactions. This is currently under investigation. 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.

REFERENCES


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

Yoshimi Umezawa, Lawrence B. Davin and Norman G. Lewis

Chromatography Materials and Instrumentation - Silica gel thin-layer chromatography (TLC) and silica gel column chromatography employed Kieselgel 60 F254 (Merck, 200–230 mesh) and silica gel 60 (EM Science) respectively. All solvents and chemicals used were reagent grade, unless otherwise stated. High-performance liquid chromatography (HPLC) was performed on two Waters Model 510 solvent delivery systems fitted with a Model 722 spectrophotometric detector equipped with a Nucleosil 1000 A, a Waters 590 Filter and a Nucleon pump. The ligands, (-)-secoisolariciresinol 2a and (-)-matairesinol 2b were separated using a reversed-phase column (Waters Novapak C18 (150 × 3.9 mm) stainless steel) with detection at λ = 280 nm. Elution details were as follows: a gradient solvent system (1 ml min⁻¹) consisting of CH₃OH:0.5% ACOD in H₂O (90:10) at t = 0 min to 50:50 at t = 10 min, and which was held at that composition for an additional 10 min. Chromat column HPLC separations of lignans were performed using a Dacel Chromat OC2D column (250 × 4.6 mm). The mixture of (-)- and (+)-matairesinol 2a and 2b was separated following elution with ethyl acetate (EtOAc):0.1% ACOD in hexanes (15:85) at a flow rate of 1 ml min⁻¹. The mixture of (-)- and (+)-matairesinol 2a and 2b was separated following elution with hexanes (20:30) at a flow rate of 0.5 ml min⁻¹. The HPLC used for chiral HPLC separations was denatured with either MeOH (2%) and 2-propanol (4%) (Phree, HPLC Spectrograde; 19.5% MeOH and 15% 2-propanol), or with MeOH (7%) and 2-propanol (4%) (Fisher, HPLC Spectrograde; 19.5% MeOH). The HPLC and NMR spectra were recorded with a Bruker WP-270SYE spectrometer and a Varian Unity 400 spectrometer, respectively, using tetramethylsilane as an internal standard. Electron impact mass spectrometry was determined on a VG analytical Fisons TF200-HF mass spectrometer (voltage: 70 eV). Optical rotations were measured with a Perkin-Elmer 241 Polimeter. UV spectra were obtained using a Perkin-Elmer Lambda 6 UV-VIS Spectrophotometer. Radioactive samples were counted in Ecoline (ICN Biomedicals) liquid scintillation fluid using a Beckman LS-250 Liquid Scintillation System. Counting efficiencies were determined using [3H]-hexadecane and [7H]-urea respectively for calibration. All melting points are uncorrected.
Formation of (--)-Secoisolariciresinol and (--)-Mataresinol

(3) Ethyl O-1,2-dihydroxybenzyl ether (DCM, THF). To a stirred solution of 2-benzyl ether (1) (300 mg, 1.1 mmol) in DCM (10 ml) was added 3 ml of 0.1 M potassium phosphate buffer (pH 7.0). The reaction mixture was stirred at 52°C for 21 h, then cooled to ice-bath temperature and acidified to pH 2 with 1 HCl. The precipitated material was separated by filtration, washed thoroughly with water (10 ml) and then with EtOAc (2 x 5 ml). The combined EtOAc solutions were washed successively with a saturated NaCl solution until the washings became neutral, then a saturated NaHCO₃ solution (3 ml) and brine (2 ml). The solvent was removed in vacuo, and the product applied to a silica gel column (8 x 7 cm), eluted successively with EtOAc, CH₂Cl₂, and MeOH to give (--)-secoisolariciresinol (977 mg, 82%). 1HNMR (CDCl₃) δ 6.34 (1H, s, J = 7.8 Hz, CH₃OH), 4.26 (2H, q, J = 7 Hz, OCH₂CH₂), 6.29 (1H, d, J = 15 Hz, C₉), 6.82 (1H, d, J = 8 Hz, Ar H), 7.03 (1H, d, J = 15 Hz, C₉), 6.10 (1H, d, J = 8 Hz, Ar H), 6.26 (1H, d, J = 8 Hz, Ar H), 7.13 (1H, d, J = 15 Hz, C₉), 6.94 (1H, d, J = 8 Hz, Ar H), 7.06 (1H, d, J = 15 Hz, C₉), 6.21 (2H, m, C₉), 11.92 (1H, s, CO₂H), 11.12 (1H, s, CO₂H), 13.84 (1H, s, CO₂H).

Indicated conditions: 2-benzyl ether (1) (300 mg, 1.1 mmol) was added to a solution of 3 ml of 10% H₂O in MeOH and 3 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a 50 ml Erlenmeyer flask. The reaction mixture was stirred at room temperature for 2 h, then cooled to ice-bath temperature and acidified to pH 2 with 1 HCl. The precipitated material was separated by filtration, washed thoroughly with water (10 ml) and then with EtOAc (2 x 5 ml). The combined EtOAc solutions were washed successively with a saturated NaCl solution until the washings became neutral, then a saturated NaHCO₃ solution (3 ml) and brine (2 ml). The solvent was removed in vacuo, and the product applied to a silica gel column (8 x 7 cm), eluted successively with EtOAc, CH₂Cl₂, and MeOH to give (--)-secoisolariciresinol (977 mg, 82%). 1HNMR (CDCl₃) δ 6.34 (1H, s, J = 7.8 Hz, CH₃OH), 4.26 (2H, q, J = 7 Hz, OCH₂CH₂), 6.29 (1H, d, J = 15 Hz, C₉), 6.82 (1H, d, J = 8 Hz, Ar H), 7.03 (1H, d, J = 15 Hz, C₉), 6.10 (1H, d, J = 8 Hz, Ar H), 6.26 (1H, d, J = 8 Hz, Ar H), 7.13 (1H, d, J = 15 Hz, C₉), 6.94 (1H, d, J = 8 Hz, Ar H), 7.06 (1H, d, J = 15 Hz, C₉), 6.21 (2H, m, C₉), 11.92 (1H, s, CO₂H), 11.12 (1H, s, CO₂H), 13.84 (1H, s, CO₂H).

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

In order to obtain (-)-matairesinol, (9,9-2H2,OC2H3)coniferyl alcohol (2.37 mg) was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) and added as a radiochemical carrier to each assay mixture before extraction and analysis for mass spectroscopic examination. The assay mixture contained rat 3H][secosolariciresinol (385 pg, 6.55 KBq) were individually incubated exactly as above, but with NADP as co-factor. Unlabelled (+)-matairesinol (35.8 pg) was added as radiochemical carrier to each assay mixture before extraction and the matairesinol (2) was then separated by use of reversed phase HPLC, and subjected to chiral HPLC and liquid scintillation counting analyses.

Enzymatic oxidation of (9)-[3H]secosolariciresinol

The assay mixture contained 770 µl 0.1 M Tris-HCl buffer (pH 8.0, 30°C), 330 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, (9)-[3H]secosolariciresinol (36.9 µg, 17 KBq) of [9,9-2H2,OC2H3]coniferyl alcohol (2.37 mg) was isolated by means of chiral HPLC. Young shoots (5-10 cm long) of F. intermedia were excised by means of a razor, then washed with both tap and distilled water, and the leaves removed. The resulting stems (2.7 g fresh weight) were cut into small pieces by hand (scissors), frozen (dry ice) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polyclar AT [6.64 g], acid-washed sea sand and 0.1 M potassium phosphate buffer (pH 7.0, 4 ml) containing 10 mM dihydroshikimic acid. The slurry was filtered through four layers of cheesecloth, and the filtrate (3 ml) centrifuged (15,000 g for 5-7 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 56-150 µm, medium), pre-equilibrated in 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM dihydroshikimic acid. 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.

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