Matairesinol is a central precursor in planta in the biosynthesis of numerous lignans, including that of the important antiviral and anticancer agent, podophyllotoxin. In this study, the ~32-kDa NAD-dependent secoisolariciresinol dehydrogenase, which catalyzes the enantiospecific conversion of (-)-secoisolariciresinol into (-)-matairesinol in Forsythia intermedia, was purified >6,000-fold to apparent homogeneity. The 831-base pair cDNA clone encoding this 277-amino acid protein was next obtained from a library constructed from F. intermedia stem tissue, whose fully functional recombinant protein, produced by expression of this cDNA in Escherichia coli, catalyzed the same enantiospecific conversion via the corresponding lactol intermediate. A homologous secoisolariciresinol dehydrogenase gene was also isolated from a Podophyllum peltatum rhizome cDNA library, whose 834-base pair cDNA clone encoded a 278-amino acid protein with a calculated molecular mass of ~32 kDa. Expression of this protein in E. coli produced a fully functional recombinant protein that also catalyzed the enantiospecific conversion of (-)-secoisolariciresinol into (-)-matairesinol via the intermediary lactol. Various kinetic parameters were defined and established conversion of the intermediary lactol to being rate-limiting. With this overall enzymatic conversion now unambiguously defined, the entire biochemical pathway to the lignans, secoisolariciresinol and matairesinol, has been elucidated. Last, both secoisolariciresinol and matairesinol are metabolized in the gut of mammals, following digestion of high fiber dietary grains, seeds, and berries, into the so-called “mammalian” lignans, enterodiol and enterolactone, respectively; these in turn confer significant protection against the onset of breast and prostate cancers.

The lignans are a structurally diverse class of vascular plant metabolites with a broad range of medicinal/health protective roles in addition to important physiological functions in planta (1). For example, podophyllotoxin 1 has antiviral properties, and etoposide 2 and teniposide 3 derivatives (2) (see Fig. 1) are representatives of only a handful of plant compounds extensively employed in cancer treatment; in addition, the structurally related trachelogenin 4 possesses anti-HIV properties (3). Furthermore, matairesinol 5 and secoisolariciresinol 6 confer dietary protection to humans, particularly against the onset of breast and prostate cancers (4). Both compounds 5 and 6 are present to different extents in various whole-grain cereal foods, seeds and berries, and are converted by intestinal microflora (5) during digestion to form the mammalian lignans, enterolactone 7 and enterodiol 8 (Fig. 1); the latter two compounds are considered as being specifically responsible for the observed reductions in these malignancies (6).

Lignans also have important physiological roles in planta, since many function as biocidal agents, feeding deterrents, antioxidants, and allelopathic chemicals (2, 7, 8). Additionally, certain plant species contain lignans with important roles in conferring or defining the quality, color, and durability of various heartwoods; e.g., about 20% of the dry weight of western red cedar (Thuja plicata) heartwood is composed of plicatic acid 9-derived lignans (9, 10).

In species such as Forsythia intermedia, formation of (+)-pinoresinol 10a, the entry point into its main (if not exclusive) lignan pathway, occurs by stereoselective coupling of two molecules of E-coniferyl alcohol 11 (Fig. 2). (+)-Pinoresinol 10a then undergoes sequential enantiospecific reduction to afford (+)-lariciresinol 12a and (+)-secoisolariciresinol 6a, with dehydrogenation of the latter occurring to give (-)-matairesinol 5a. Depending upon the species involved, matairesinol 5 is believed to be the precursor of bioactive molecules such as (-)-podophyllotoxin 1 in Podophyllum peltatum (11, 12), (-)-trachelogenin 4 in Ipomoea carica (3), and plicatic acid 9 in T. plicata (13) (Fig. 2).

In this study, the enzymology of formation of (-)-matairesinol 5a from (-)-secoisolariciresinol 6a in both F. intermedia and P. peltatum was investigated. This resulted in the purification to apparent homogeneity of (-)-secoisolariciresinol dehydrogenase, the cloning of the corresponding cDNAs, the expression of functional recombinant proteins in Escherichia coli, and determination of basic parameters (Km, Vmax). This research was conducted as a first step toward obtaining edible transgenic plants containing elevated levels of matairesinol 5 for health protection, as well as for attaining higher levels of medicinally active lignans such as podophyllotoxin 1.

EXPERIMENTAL PROCEDURES

Plant Materials—F. intermedia plants were either obtained from Bailey’s Nursery (var. Lynwood Gold, St. Paul, MN), and maintained in Washington State University greenhouse facilities or were gifts from
the local community. *P. peltatum* plants, propagated from rhizomes harvested in Virginia, were cultivated in the same greenhouse facilities.

**Synthesis of E-[9-3H2]Coniferyl Alcohol**

To a solution of coniferyl aldehyde in methanol (1.1 mmol, 5 ml) was added tritiated sodium borohydride (NaB\(\text{3H}_4\); 13.3 GBq/mmol, 3.7 GBq) at 0 °C. After stirring for 20 min, unlabeled sodium borohydride (NaBH\(_4\); 1.3 mmol) was added with the whole stirred for another 20 min. The pH was next adjusted to 6 (by the dropwise addition of 2 N HCl), and the reaction mixture was extracted with diethyl ether (50 ml). The ether solubles were extracted with water, dried (sodium sulfate), and evaporated to dryness. The residue was reconstituted in a small amount of ethyl acetate and applied to a short silica gel column (10 × 3 cm inner diameter) eluted with methylene chloride/ethyl acetate (4:1) to afford [9-3H]coniferyl alcohol (1 mmol, 1.21 GBq/mol).

**Synthesis of (6)-(9,9,9-3H]Secoisolariciresinols**

To [9-3H]coniferyl alcohol (1 mmol in acetone, 7 ml, 1.21 GBq/mol) was added iron (III) chloride hexahydrate (FeCl\(_3\) \(6\)H\(_2\)O; aqueous solution, 2.6 mmol, 24 ml) at room temperature. Following stirring for 10 min, the reaction mixture was extracted with diethyl ether (30 ml). The ether solubles were combined, extracted with water (20 ml), dried (sodium sulfate), and evaporated to dryness in vacuo. The residue was reconstituted in a minimum amount of methylene chloride and applied to a silica gel column (15 × 2.5-cm inner diameter) eluted with methylene chloride/diethyl ether (4:1) to give [9-3H]secoisolariciresinols (0.1 mmol, 2.42 GBq/mol, 70% yield).

**Synthesis of (2)-Lactol**

To (2)-matairesinol (120 mg, 0.34 mmol) in dry tetrahydrofuran (20 ml) was added dropwise 1 M lithium triethylborohydride (LiEt\(_3\)BH) in tetrahydrofuran solution (1.1 ml) at 0 °C, with the resulting suspension stirred for 1 h at this temperature. To quench the reaction, 2 N HCl was added slowly until the reaction mixture was of pH 6. To this was added ethyl acetate (150 ml), with the whole then washed with water (50 ml). The organic solubles were next dried (sodium sulfate) and evaporated to dryness in vacuo. The resulting residue was reconstituted in a minimum amount of ethyl acetate and applied to a silica gel column (20 × 2-cm inner diameter), eluted with ethyl acetate/hexanes (1:1 and 2:1) to afford (2)-lactol (72 mg, 60% yield in two isomers; 3:5 ratio). UV \(\lambda_{	ext{max}}\) (methanol): 229.7, 279.4 nm.; electron impact mass spectroscopy \(m/z\) (%): 360 (M\(^+\), 12.1), 205 (10.2), 163 (9.2), 137 (100), 122 (8.2); HRMS \(m/z\) found 360.1560 [M\(^+\)], calculated for C\(_{20}\)H\(_{24}\)O\(_6\): 360.1573; 1H NMR (CDCl\(_3\)): \(\delta\) 2.36–2.84 (6H, m, C\(_7\),7\(_9\)H, C\(_8\),8\(_9\)H), 3.53–4.16 (2H, m, C\(_9\)9\(_9\)H), 3.79, 3.85 (6H, s, OCH\(_3\)), 5.25 (1H, C9H), 6.42–6.84 (6H, m, Ar-H). 13C NMR (CDCl\(_3\)): \(\delta\) 31.30, 33.81, 38.69, 39.12, 39.58, 43.19, 46.05, 52.17, 53.18, 56.01, 56.18, 72.63, 72.92, 99.15, 103.68, 111.25, 111.29, 111.42, 111.75, 114.30, 114.38, 114.48, 114.55, 121.41, 121.80, 121.77, 131.67, 132.20, 132.52,

**FIG. 1.** Lignans with various pharmacological and physiological activities.
Secoisolariciresinol Dehydrogenase Assays—Assays with (±)-[9,9'-3H]secoisolariciresinols 6a/6b were carried out as reported elsewhere (12). Assays with (±)-lactol 13a as substrate at a final concentration of 55 μM were carried out as described for secoisolariciresinol 6, with matairesinol 9 formation being quantified using a previously established standard curve.

Chemical Conversion of Enzymatically Formed [9,9'-3H]Matairesinol 9 into [9'-H]Secoisolariciresinol 6—This chemical synthetic procedure was carried out as reported elsewhere (12).

General Procedures for Enzyme Purification—All manipulations were carried out at 4 °C with chromatographic eluents monitored at 280 nm, unless otherwise indicated. Protein concentrations, using γ-globulin as a standard, were determined by the method of Bradford (14).

Polyacrylamide gel electrophoresis was performed with Laemmi’s buffer system under denaturing or nondenaturing conditions, as well as with gradient gels (4–15%) (15); proteins were visualized by silver staining (16).

Preparation of Cell-free Extracts—F. intermedia stems (2 kg) were frozen (liquid N2) and pulverized in a Waring blender (model CB6). The resulting powder was homogenized with Tris-HCl buffer (50 mM, pH 7.5, 2 liters) containing 5 mM dithiothreitol (buffer A). The homogenate was filtered through four layers of cheesecloth into a beaker containing 5 mM dithiothreitol (buffer B) with the filtrate centrifuged at 10,000 g for 15 min and the resulting supernatant fractionated with ammonium sulfate. Proteins precipitating between 30 and 60% saturation were recovered by centrifugation (10,000 g, 30 min) with the pellet then reconstituted in a minimal amount of buffer A.

DEAE Chromatography—The crude enzyme preparation (445 mg) was applied to a DEAE-cellulose column (40 × 2.6-cm inner diameter) equilibrated in buffer A. The homogenate was filtered through four layers of cheesecloth into a beaker containing polyvinylpolypyrrolidone (10%, w/v), with the filtrate centrifuged at 10,000 g, 15 min) and the resulting supernatant fractionated with ammonium sulfate. Proteins precipitating between 30 and 60% saturation were recovered by centrifugation (10,000 g, 30 min) with the pellet then reconstituted in a minimal amount of buffer A.

Affinity (2',5'-ADP-Agarose) Chromatography—The active fractions from the DEAE-cellulose chromatography (201 mg, 14 nmol/h/mg protein) were applied to a 2',5'-ADP-agarose (10 × 1-cm inner diameter) column previously equilibrated in Tris-HCl buffer (25 mM, pH 7.5, 5 mM dithiothreitol) overnight.

Affinity (2',5'-ADP-Agarose) Chromatography—The active fractions from the DEAE-cellulose chromatography (201 mg, 14 nmol/h/mg protein) were applied to a 2',5'-ADP-agarose (10 × 1-cm inner diameter) column previously equilibrated in Tris-HCl buffer (25 mM, pH 7.5, 5 mM dithiothreitol) overnight. The column was first washed with 20 ml of the same buffer at a flow rate of 1 ml/min and then with 50 ml of buffer A containing 500 mM NaCl, and finally secoisolariciresinol dehydrogenase was eluted with NAD (10 mM) in buffer A. The active fractions were combined and dialyzed 15 h against buffer A.

MonoP (HR 5/20) Column Chromatography—Active protein (185 μg, 8.4 μmol/h/mg protein) from the preceding step was applied to a MonoP column equilibrated in buffer A, washed with buffer A (8 ml), and eluted with a linear NaCl gradient (0–2 M in 145 ml) in buffer A at a flow rate of 1 ml/min. The active fractions (74 μg, 17.7 μmol/h/mg protein) were combined, dialyzed against buffer A, and then subjected to a second round of MonoP column chromatography using the procedure described above. Secoisolariciresinol dehydrogenase (31 μg, 24.2 μmol/h/mg protein) obtained was next analyzed by SDS-polyacrylamide gel electrophoresis.

cDNA Library Synthesis—The amplified cDNA libraries individually prepared from young green stems of greenhouse grown F. intermedia plants (var. Lynwood Gold) and from rhizomes of greenhouse grown P. peltatum were constructed as described previously (12, 17). Both were screened for secoisolariciresinol dehydrogenase clones as detailed below.

Secoisolariciresinol Dehydrogenase DNA Probe Synthesis—The N-terminal and internal trypsin digest peptide amino acid sequences were used to construct degenerate oligonucleotide primers (see “Results and Discussion”). Purified F. intermedia cDNA library DNA (2 ng) (17) was used as template in 100-μl PCRs (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 0.2 mM each dNTP, and 2.5 units of Taq DNA polymerase) along with 2.5 pmol each of primer DEHYF26 and either primer DEHYF30RevA or primer DEHY30RevB (see “Results and Discussion”). PCR amplification was carried out in a thermocycler with 35 cycles of 94 °C denaturing for 1 min, 50 °C annealing for 2 min, and 72 °C extension for 3 min. PCR products were resolved in 1.5% agarose gels, where a single band of ~200 base pairs (bp) was obtained. The resulting PCR product was ligated into a pT7Blue T-vector and transformed into competent NovaBlue cells according to Novagen’s instructions. DNA sequence analysis indicated that the re-
combinant plasmid insert coded for the initial internal trypsin digest fragment IMSNASIGDPN (see “Results and Discussion”) obtained from the native plant protein. A BamHI/SpeI fragment of ~200 bp harboring this insert was excised from the plasmid preparation, purified by running on a 1% melting point agarose gel, and used as a probe with the cDNA library. The probe was prepared by boiling the purified DNA fragment (100 ng) for 10 min, followed by radiolabeling for 20 min at 37 °C using the Amersham Pharmacia Biotech QUICKPrime Kit and [α-32P]dCTP. Unincorporated nucleotides were removed from the radiolabeled probe by passing it through a CENTRIPIN®-20 spin column (Princeton Separations Inc.).

**TABLE I**

| Purification step | Protein | Specific activity | Purification factor |
|-------------------|---------|------------------|--------------------|
| Ammonium sulfate precipitation (30–60%) | 445 | 4 | 1 |
| DEAE-cellulose | 201 | 14 | 3.5 |
| ADP-agarose | 0.185 | 8405 | 2101 |
| MonoP 1 | 0.074 | 17,671 | 4418 |
| MonoP 2 | 0.031 | 24,272 | 6068 |

**RESULTS AND DISCUSSION**

The levels of the health-promoting lignans, matairesinol 5 and secoisolariciresinol 6, are typically very low in foodstuffs commonly used in the western diet. The overall purpose of this study was thus to obtain the gene(s) encoding the enzyme(s) catalyzing the conversion of secoisolariciresinol 6 into matairesinol 5, as a forerunner to designing strategies to obtain transgenic plants with elevated levels of these protective substances in staple western dietary foodstuffs. Indeed, this version represents the final step in the biochemical pathway from coniferyl alcohol 11 to matairesinol 5, since all the preceding steps from coniferyl alcohol 11 to secoisolariciresinol 6 have been fully defined, in terms of the proteins, enzymes, and genes involved (1, 17, 20–23). Moreover, since matairesinol 5 is also a precursor of the antiviral and antitumor lignan, podophyllotoxin 11 (11, 12), the opportunity also availed itself to obtain the corresponding gene(s) encoding secoisolariciresinol dehydrogenase from *Podophyllum* species; interestingly, *Podophyllum* sp. are difficult to cultivate, and the native species growing in the wild are being overharvested, particularly in Asia.

In earlier studies using crude cell-free extracts from *F. intermedia*, the enantiospecific conversion of (−)-secoisolariciresinol 6a into (−)-matairesinol 5a had been established, although whether one enzyme or more was involved was not known (Fig. 2) (24, 25). Accordingly, since *F. intermedia* also accumulated several matairesinol 5-derived substances, this plant species was selected initially as a suitable source of enzyme(s) involved in formation of the lignan, (−)-matairesinol 5a (26).

**Fig. 3.** SDS-polyacrylamide gel electrophoresis of purified (−)-secoisolariciresinol dehydrogenase.
purified (>6,000-fold) to apparent homogeneity using a combination of ammonium sulfate precipitation and DEAE-cellulose, ADP-agarose, and MonoP (HR 5/20) chromatographic steps, respectively (see Table I). Next, SDS-polyacrylamide gel electrophoretic analysis of the resulting secoisolariciresinol dehydrogenase gave an apparent molecular mass of 32 kDa (Fig. 3). As for the related pinoresinol/lariciresinol reductase (17), however, which catalyzes the two preceding biochemical steps, the corresponding secoisolariciresinol dehydrogenase was also present in very low abundance (37 μg from 2 kg of *F. intermedia* stems).

The enzyme assays employed for its detection utilized either (±)-[9,9′-3H]secoisolariciresinols 6a/6b or [Ar-2H]secoisolariciresinols 6a/6b as substrates; the (±)-[9,9′-3H]secoisolarici-

Fig. 4. Mass spectra of secoisolariciresinols 6 and matairesinols 5. *A*, synthetic (±)-[Ar-2H] secoisolariciresinols 6a/6b. *B*, [Ar-2H]matairesinol 5 obtained following incubation of (±)-[Ar-2H]secoisolariciresinols 6a/6b in the presence of the purified (−)-secoisolariciresinol dehydrogenase and 40 μM NAD for 2 h. *C*, unlabeled matairesinol 5.

The enzyme assays employed for its detection utilized either (±)-[9,9′-3H]secoisolariciresinols 6a/6b or [Ar-2H]secoisolariciresinols 6a/6b as substrates; the (±)-[9,9′-3H]secoisolariciresinols 6a/6b were obtained by iron chloride (FeCl₃)-catalyzed coupling of *E*- [9-3H]coniferyl alcohol 11 (1.21 GBq/mol) to afford the racemic (±)-[9,9′-3H]pinoresinols 10a/10b, with subsequent reduction (10% palladium on charcoal, H₂) generating the required (±)-[9,9′-3H]secoisolariciresinols 6a/6b (2.42 GBq/mg). [Ar-2H]Secoisolariciresinols 6a/6b, on the other hand, were prepared by acid-catalyzed deuterium exchange of the aromatic ring protons using deuterated trifluoroacetic acid (CF₃CO2D) as previously described (25); its mass spectrum displayed the expected molecular ion cluster centered at m/z 364 (Fig. 4A), this being indicative of two or three hydrogen atoms being replaced by deuterium.

Verification that the purified secoisolariciresinol dehydrogenase catalyzed the enantiospecific conversion of (−)-secoisolariciresinol 6a into (−)-matairesinol 5a was demonstrated in two different ways: first, via incubation of the enzyme with (−)-secoisolariciresinol 6a (2.8 mM, 3.4 kBq) in the presence of 40 μM NAD for 2 h, with unlabeled (−)-matairesinols 5a/5b (3 μg) being added as radiochemical carriers. The resulting radiolabeled [9,9′-3H]matairesinol 5a so formed was then purified by reversed-phase HPLC and subsequently reduced chemically with lithium aluminum hydride to regenerate secoisolariciresinol 6. This step was necessary, since both (−)- and (+)-matairesinols 5a and 5b are only partially resolved by chiral column HPLC. Fig. 5 shows the chiral column HPLC separations of both (−)- and (+)-secoisolariciresinol 6a/6b standards (Fig. 5A) as well as that of the product derived from enzymatic incubation (Fig. 5B). Thus, since only (−)-[9,9′-3H]secoisolariciresinol 6a was present, this demonstrated that the matairesinol 5 enzymatically produced was only in the (−)-form 5a; i.e., (−)-secoisolariciresinol 6b had not served as a substrate. Second, the purified NAD-dependent secoisolarici-

![Chiral HPLC analyses of synthetic and enzymatically generated secoisolariciresinols 6.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
resinol dehydrogenase converted [Ar-2H]secoisolariciresinol 6 into [Ar-2H]matairesinol 5 (Fig. 4B), i.e., the enzymatic product gave the expected molecular ion cluster at m/z 360, this being centered 2–3 mass units higher than that of natural abundance matairesinol 5 (m/z 358) (Fig. 4C) and thus demonstrating an intact conversion of substrate 6a into 5a. Together, both results unambiguously established the enantiospecificity and authenticity of the enzymatic conversion catalyzed by (-)-secoisolariciresinol dehydrogenase.

Gene Cloning, Functional Expression, and Characterization of Recombinant (−)-Secoisolariciresinol Dehydrogenase from F. intermedia—Attention was next directed toward obtaining the

![Resinol Dehydrogenase](http://www.jbc.org/Downloaded from)

**Fig. 6.** Microsequence data (left column) obtained from N-terminal and trypsin digestion oligopeptides of F. intermedia (−)-secoisolariciresinol dehydrogenase with cloning primers (right column) based on the regions underlined and labeled DEHYF26, DEHYF30RevA, and DEHYF30RevB.

**Fig. 7.** Forsythia intermedia (−)-secoisolariciresinol dehydrogenase (SDH Fi321) nucleotide and deduced amino acid sequences. The start Met is highlighted in boldface type, and the stop signal is indicated by an asterisk (*). N-terminal and trypsin digest amino acid sequences obtained from the original native protein are indicated by underlined letters, and the names of the primers derived from those regions are indicated above the sequence in italic type. Conserved amino acid residues associated with a NAD-binding motif are presented in boldface italic type, with the entire motif indicated by gray background-shaded italicized letters. A Kozak consensus DNA sequence is indicated by boldface underlined letters preceded by a few residues of a 5′-untranslated region indicated by italic type. Numbers to the right of the sequence indicate either the nucleotide or the amino acid residue.
encoding gene for (−)-secoisolariciresinol dehydrogenase. Thus, following the final column chromatographic step (Table I), the F. intermedia 32-kDa protein was subjected to SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane, this then being subjected to Edman degradation analysis (see Supplemental Material). Additionally, sequences of internal fragments were obtained by trypsin digestion of the purified secoisolariciresinol dehydrogenase (see Supplemental Material).

Fig. 6 shows the N-terminal and internal trypsin fragment sequences obtained; the 17-amino acid internal trypsin digestion sequence (VALITGGASGIGETTAK), which overlapped with the N-terminal sequence, had strong homology to the N-terminal sequences of other dehydrogenases when subjected to a BLAST homology search comparison (27), which included short-chain alcohol dehydrogenases from cowpea (Vigna unguiculata, 82% identity and 93% similarity) (28), and Nicotiana tabacum (82% identity, 87% similarity) (29). The other internal trypsin digestion sequence (LNIMFNSAGISDPNK), however, was also assumed to be in close proximity to the N-terminal sequence, as projected by comparison of its alignment with published sequences for alcohol dehydrogenases. Thus, forward (DEHYFP26) and reverse (DEHYFP30RevA and -B) degenerate oligonucleotide primers (Fig. 6) were next synthesized for sequences closest to, and furthest from, the N terminus, respectively.

Using the PCR-guided strategy described in the Supplemental Material, with the F. intermedia cDNA library as template (17), a prominent DNA band (∼200 bp) was obtained, this being cloned into a pT7 Blue T-vector (Novagen), and transformed into competent NovaBlue (Novagen) E. coli cells. The DNA sequence of this insert established that the internal trypsin digest fragment sequence IMFSAGISPDK (Fig. 6) was present in the PCR product cloned into the vector.

This 200-bp fragment was next used as a probe in an effort to screen the amplified F. intermedia cDNA library to obtain the complete cDNA clone. Numerous strong positive hybridization signals were detected, of which 11 were isolated and sequenced. One clone (SDH_Fi321, GenBank™ accession number AF352735) contained a start Met preceded by a 5′ untranslated region, and its 581-bp open reading frame, the longest of the sequences obtained, predicted a polypeptide of 277 amino acids in size and a calculated mass of 32 kDa (see Fig. 7). This is in close agreement with the estimated band size of the denatured protein as observed by SDS-polyacrylamide gel electrophoresis analysis of the purified native plant enzyme (see Fig. 3); the N-terminal sequence of SDH_Fi321 also matched the N terminus and trypsin fragment amino acid sequences obtained from the F. intermedia native protein originally isolated (Fig. 6).

5′ and 3′ primers were next designed to introduce NdeI restriction enzyme sites at both ends of the clone to allow for subsequent insertion into the SBET expression vector (30) for overexpression of the protein in E. coli. Deployment of the primers for PCR, with 2 ng of the previously obtained SDH_Fi321 clone plasmid DNA as template, gave a PCR product that was then cloned directly into the TA vector. The dehydrogenase clone was next excised from the construct using the NdeI sites at the 5′- and 3′-ends and cloned into the SBET vector; this construct was then transformed into the E. coli strain BL21(DE3) for heterologous overexpression.

Heterologous Expression of F. intermedia (−)-Secoisolariciresinol Dehydrogenase in E. coli—E. coli cells harboring the SDH_Fi321/SBET construct were grown until mid-log phase and then induced with isopropyl thio-β-D-galactoside, as described under “Experimental Procedures.” The resulting cell-free extract was incubated in the presence of (±)-[9,9-3H]secoisolariciresinols 6a/6b (2.8 μM, 3.4 kBq) and 40 μM NAD. After a 2-h incubation, unlabeled (±)-matairesinols 5a/5b (3 μg) were added as radiochemical carriers, with the enzyme extract then subjected to reversed-phase HPLC (Fig. 8). Two enzymatic products were observed. First, in terms of the enzymatically generated [9,9-3H]matairesinol 5, the enantiospecificity of its formation was established by subjecting it to a chemical reduction, using lithium aluminum hydride as before to afford secoisolariciresinol 6, with the latter being analyzed by chiral column chromatography (Chiralcel OD, Daicel). It was thus established that only (−)-secoisolariciresinol 6a was obtained following chemical reduction, and not the antipode 6b (Fig. 5C); i.e. as expected, only (−)-matairesinol 5a formation was catalyzed by the heterologously expressed (−)-secoisolariciresinol dehydrogenase. To further confirm the authenticity of this conversion, assays were also performed for 2 h with (±)-[Ar-3H]secoisolariciresinols 6a/6b (molecular ion cluster centered at m/z 364) as substrates (0.55 μmol) with 40 μM NAD as cofactor, in the presence of the cell-free extract. The enzymatically formed [Ar-3H]matairesinol 5 was then purified and subjected to mass spectrometric analysis. This gave the expected molecular ion cluster centered at m/z 362 (data not shown) as noted previously for the native protein (see Fig. 4B). Thus, both the F. intermedia native protein and the heterologously expressed (−)-secoisolariciresinol dehydrogenase catalyzed the same enantiospecific reaction. Note also that with a corresponding negative control (expression of an unrelated gene, phenylcoumaran benzylic ether reductase, cloned into the SBET vector (19)), no enzymatic activity of any type was detected using (±)-secoisolariciresinols 6a/6b as substrates (data not shown).

In addition to the expected enzymatic product (−)-matairesinol 5a, a second product was also noted during HPLC analysis with an elution volume of 17.5 ml. Attention was next directed
toward the identification of this unknown compound. This was found to have a molecular ion at m/z 360, with a base peak at m/z 137 and an UV absorption spectrum with maxima at λ 229.7 and 279.4 nm, respectively. Together, these spectroscopic data as well as its chromatographic behavior suggested that the compound might be the corresponding lactol 13. That this was indeed the case was established by the chemical synthesis of (−)-lactol 13a, obtained via reduction of (−)-matairesinol 5a with lithium triethylborohydride (LiEt₃BH); i.e. the resulting synthetic product displayed identical HPLC chromatographic behavior, as well as UV and mass spectra, to that of the enzymatic product. Furthermore, synthetic 13a was additionally characterized by 1H and 13C NMR spectroscopic analyses, as well as by HRMS (see “Experimental Procedures”). Thus, its identification unequivocally established that the enantiospecific conversion of (−)-secoisolariciresinol 6a to (−)-matairesinol 5a proceeded via the intermediary (−)-lactol 13a (see Fig. 9).

Cloning, Expression, and Characterization of (−)-Secoisolariciresinol Dehydrogenase Homologue from P. peltatum—As indicated earlier, both P. peltatum and Podophyllum hexandrum are sources of the important antiviral and antitumor lignan podophyllotoxin 1, and a recent study using radiolabeled substrates also demonstrated matairesinol 5 to be a precursor of podophyllotoxin 1 (12); i.e. the biochemical pathway to matairesinol 5 in Podophyllum sp. is the same as that for Forsythia. Accordingly, the next objective was to obtain the gene encoding the (−)-secoisolariciresinol dehydrogenase from a P. peltatum cDNA library.
erase primers (DEHYF26 forward and DEHYF30Rev reverse), was again employed, and this gave a 224-bp PCR band 70% similar and 50% identical to the homologous region of the F. intermedia secoisolariciresinol dehydrogenase gene. This was used to design the internal gene specific forward and reverse primers PDP7FOR and PDP7REV (see Fig. 10), which were then coupled with T7 or T3 primers, respectively, in a subsequent PCR using the P. peltatum rhizome cDNA library as template. The PCR products obtained thus provided the complete 5’ and 3’ segments of the gene, and these were next used to design the gene-specific 5’ forward and 3’ reverse primers PPDNTER and PPDCTER (see Fig. 10). The resulting PCR product yielded a clone encoding the entire P. peltatum secoisolariciresinol dehydrogenase sequence SDH_Pp7 (GenBank accession number AF352734; (Fig. 10), and this 834-bp gene encoded a 278-amino acid protein having 60% similarity and 51% identity to that of the F. intermedia secoisolariciresinol dehydrogenase. The P. peltatum gene was next cloned into an Invitrogen pTRCHIS2 TOPO TA vector and transformed into TOP10 E. coli cells. This system was used to obtain a more rapid cloning and expression of this gene compared with the previous SBET vector system used for the F. intermedia secoisolariciresinol dehydrogenase clone. Induction of a culture of transformed cells with isopropyl thio-D-galactoside (de-repress) of anionic and gel filtration chromatographic steps, and this preparation was used for determination of the pH and temperature optima, as well as kinetic parameters (K_m and V_max values). (-)-Lactol 13a was initially used as substrate, since only a single dehydrogenative step was involved.

The pH optimum was examined over the pH range 4–11.5 and reached a maximum at about pH 8.8. At this optimal pH, the apparent temperature optimum was established to be ~20 °C. Additionally, kinetic properties using racemic (-)-lactol 13a as a substrate were examined, with initial velocity studies using substrate concentrations ranging between 15 and 167 μM, while keeping the NAD concentration constant (100 μM). Apparent K_m values ~160 ± 0.8 μM with apparent maximum velocities ~7.1 ± 0.02 × 10^3 (expressed as mmol/ min/mg protein) were obtained from Lineweaver-Burk plots; i.e. typical Michaelis-Menten kinetics were observed.

Next, racemic (+)-secoisolariciresinol 6a/6b were used as substrates over the same range of concentrations as for the lactol 13. Given the potential for complexity due to the bifunctional nature of the enzyme (diol → lactol → lactone), kinetic parameters were studied as a function of secoisolariciresinol 6 depletion. At high substrate concentrations (≥75 μM), the reaction followed typical Michaelis-Menten kinetics. However, at lower substrate concentrations, there was a deviation from classical Michaelis-Menten kinetics, the basis of which will be investigated in the future. Of particular note, the overall conversion was stereospecific, using only the (−)-enantionmer, and the build-up of lactol 13 at high substrate concentrations (≥50 μM) strongly suggested that the second dehydrogenative step is rate-limiting.

**Sequence Homology Comparisons—Comparisons using the GAP program (31) revealed significant homology of the F. intermedia and P. peltatum secoisolariciresinol dehydrogenase genes to that of a drought-induced probable short-chain alcohol dehydrogenase of unknown function from cowpea (28) (i.e. 63 and 69% similarity and 55 and 49% identity, respectively). Interestingly, the F. intermedia SDH_Fi321 and P. peltatum SDH_Pp7 clones had an N-terminal initiation residue at a location similar to that of cowpea (CPRD12; Fig. 11), although differing by one amino acid. This association helped us to approximate the locations of the amino acid fragments that we had obtained from the native protein, which was instrumental in the approach to design degenerate primers. Moreover, the NAD-binding site is highly conserved for all of the proteins.
compared in Fig. 11 in accordance with previous observations with other NAD-dependent dehydrogenases. It was suggested that the preservation of both the structure and function of the NAD-binding domains, as is illustrated by the examples compared in Fig. 11, indicates an evolutionary relationship based on an ancestral NAD-binding protein (32). Indeed, there are now more than 100 different members of this NAD-dependent dehydrogenase family known (32), with enzymes of this type being commonly found in both plant and animal kingdoms. Interestingly, neither of the two secoisolariciresinol dehydrogenase sequences contain any secretory pathway signal sequences, in accordance with their presumed cytosolic character.

**Conclusion**—The isolation and characterization of the two secoisolariciresinol dehydrogenase genes from *F. intermedia* and *P. peltatum*, involved in the formation of (−)-matairesinol 5a, was of considerable interest for two reasons: the first is that this represents the final step in the biochemical pathway to the phytoestrogenic, health-protecting, lignan, matairesinol, and thus all steps from coniferyl alcohol 11 are fully characterized. The second reason is that we are now poised to manipulate the levels of these important compounds whether for disease prevention (i.e. matairesinol 5 and secoisolariciresinol 6) or for curative purposes (e.g. for enhanced podophyllotoxin 1 formation); i.e. future work will be directed to metabolic engineering of the levels of secoisolariciresinol 6 and matairesinol 5 in plant foodstuffs such as vegetables, grains, and fruits or as supplements for processed food items. As stated earlier, matairesinol 5 and secoisolariciresinol 6 are typically present at very low levels in these economically important crops; thus, engineering their biosynthetic pathways would provide a facile source of these beneficial lignans in staple dietary foodstuffs. Related studies will be directed also toward manipulating the levels of podophyllotoxin 1 in *Podophyllum* species or introducing the corresponding genes into organisms that can be more readily cultivated.

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