Formation of Lignans (-)-Seocoisolariciresinol 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 [9,9-2H2,0C2H3]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-3H]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 [9,9-2H2,0C2H3]coniferyl alcohols as substrates. The (+)-enantiomer was not formed. Finally, when either (-)-[Ar-3H] or (±)-[Ar-2H]secoisolariciresinols were used as substrates, in the presence of NAD(P)H, only (-) and not (+)-matairesinol formation occurred. The other antipode, (+)-secoisolariciresinol, did not serve as a substrate for the formation of either (+) or (-)-matairesinol. Thus, in F. intermedia, the formation of the lignan, (-)-secoisolariciresinol, occurs under strict stereochemical control, in a reaction or reactions requiring NAD(P)H and H2O2 as cofactors. This stereo-selectivity is retained in the subsequent conversion into (-)-matairesinol, since (+)-secoisolariciresinol is not a substrate. These are the first two enzymes to be discovered in lignin 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.

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

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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 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 PROCEDURES1

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-methoxyphenyl)propionate was carried out in 38.7% yield using a reducing agent, made in situ from n-butyllithium and diisobutyl 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

1 The “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.

2 The abbreviations used are: HPLC, high performance liquid chromatography; m.pt, melting point; lit.m.pt., literature melting point; THF, tetrahydrofuran; TLC, thin layer chromatography.
both radiochemical and UV detection. In this way, it was incorporated into (-)-matairesinol 2b, as evidenced by the radiopeaks with eluted lignans. Next, secoisolariciresinol 1 and unlabeled carriers. Column, Chiralcel OD (Daicel) elution details: hexanes:EtOH (70:30); flow rate, 0.5 ml min⁻¹.

The retention volume (mL) 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 [9,9-²H₂,0C₂H₃] 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:EtOH (85:15); flow rate: 1 ml min⁻¹.

Note that unlabeled (±)-secoisolariciresinols 1a/1b 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.

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Note that unlabeled (±)-secoisolariciresinols 1a/1b 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⁺ – 18, loss of H₂O), and 137 (which corresponds to a fragment derived from benzylic cleavage). In contrast, the (–)-secoisolariciresinol 1b obtained from F. intermedia plant tissue (Fig. 4A), which had previously been administered [9,9-²H₂,0C₂H₃]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-

Table I

| m/z | Natural abundance (–)-matairesinol 2b isolated from F. intermedia |
|-----|-------------------------------------------------------------------|
| 137 | 100.0                                                             |
| 138 | 26.5                                                             |
| 139 | 2.3                                                              |
| 140 | 0.3                                                              |
| 141 | 0.2                                                              |
| 142 | 0.0                                                              |
| 143 | 0.1                                                              |
| 144 | 0.0                                                              |
| 145 | 0.1                                                              |
| 146 | 0.0                                                              |
| 147 | 0.1                                                              |
| 148 | 0.0                                                              |
| 149 | 0.1                                                              |
| 150 | 0.0                                                              |
| 151 | 0.1                                                              |
| 152 | 0.0                                                              |
| 153 | 0.1                                                              |
| 154 | 0.0                                                              |
| 155 | 0.1                                                              |
| 156 | 0.0                                                              |
| 157 | 0.1                                                              |
| 158 | 0.0                                                              |
| 159 | 0.1                                                              |
| 160 | 0.0                                                              |
| 161 | 0.1                                                              |
| 162 | 0.0                                                              |
| 163 | 0.1                                                              |
| 164 | 0.0                                                              |
| 165 | 0.1                                                              |
| 166 | 0.0                                                              |
| 167 | 0.1                                                              |

Table II

| Enzymatic formation of (–)-matairesinol 2b from (±)-secoisolariciresinols 1a/1b |
|---------------------------------------------------------------------------------|
| Enzyme assay | Cofactor | Absolute incorporation of radioactivity into (–)-matairesinol 2b | (-)-Matairesinol 2b formation |
|--------------|----------|---------------------------------------------------------------|---------------------------------|
| 1 | NADP | 2.0 | 14.1 |
| 2 | NAD | 2.9 | 19.9 |
| Controls | None | 0.0 | 0.6 |
| 4 | denatured NADP | 0 | 0 |

The 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.

The 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%.

²H₂OOC₃H₃coniferyl 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 2 to that obtained following [9,9-²H₂,0C₃H₃]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 137 (derived from cleavage of the benzylic fragment). On the other hand, the (–)-matairesinol 2b isolated from F. intermedia previously treated with [9,9-²H₂,0C₃H₃]coniferyl alcohol gave signals at m/z 366, 358, 140, and 137 (Table I). The peaks at m/z/
z 358 and 137 again correspond to natural abundance (−)-matairesinol 2b, whereas the small signals at m/z 360 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 (−)-secoisolariciresinol 1b and (−)-matairesinol 2b molecules were derived from coniferyl alcohol 8, it was next of interest to determine whether (−)-matairesinol 2b was formed *in vivo* by direct dehydrogenation of (−)-secoisolariciresinol 1b. To answer this question, (±)-[Ar-1H]secoisolariciresinols (17 KBq mg⁻¹) were synthesized (from unlabeled synthetic material by exchange with 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 99.4%. 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 NADPH), it was found that secoisolariciresinol 1 formation only occurred in the presence of (−)-matairesinol 2b. When NAD(P) was omitted (Table II), it was established that the rate of formation of (−)-matairesinol 2b was 15.9 nmol h⁻¹ mg⁻¹ protein. Significantly, no formation of either (+)- or (−)-matairesinol 2a or 2b occurred when the enzyme was denatured (boiled 5 min) or when NAD(P) was omitted (Table II). This again demonstrates the strict stereochemical control (or preference) of this enzymatic dehydrogenation. When (±)-[Ar-1H]secoisolariciresinols (1.94 KBq) were incubated with the enzyme preparation in the presence of NADP and NAD (Table II), it was established that the rate of formation of (−)-matairesinol 2b was 14.1 and 19.9 nmol h⁻¹ mg⁻¹ protein, respectively.

To confirm and extend these radiochemical observations, we next undertook to demonstrate the conversion of (±)-[Ar-1H]secoisolariciresinols into [Ar-1H]matairesinol. Thus, (±)-[Ar-1H]secoisolariciresinols were prepared by deuterium exchange of aromatic protons of the unlabeled lignin with CF₃CO₂H. The (±)-[Ar-1H]secoisolariciresinols, so obtained, were subjected to mass spectroscopic analysis. As can be seen (Fig. 4C), the parent molecular ion (M⁺) for unlabeled secoisolariciresinol 1, previously noted at m/z 362 (Fig. 4B), was now shifted to an ion cluster centered at m/z 364, i.e. a partial aromatic substitution of H by D had occurred. This corresponds to the replacement of two to three aromatic hydrogens by deuterium. This observation was also confirmed by 1H NMR analysis. Following incubation of the (±)-[Ar-1H]secoisolariciresinols with the *F. intermedia* cell-free extract in the presence of NADP, the matairesinol 2b so obtained gave a cluster of ions now centered at m/z 360 (Fig. 4D). This cluster is centered two to three mass units higher than that of natural abundance (−)-matairesinol 2b (M⁺, 358; Fig. 4E) indicating the presence of two to three deuterium atoms in the enzymatically formed (−)-matairesinol. Thus, the stereoselective conversion of (−)-secoisolariciresinol 1b into (−)-matairesinol 2b had now been unequivocally demonstrated at the cell-free level.

In summary, we have detected enzymatic activities for lignan formation (Fig. 5), one of which is involved in the stereochemically controlled formation of (−)-secoisolariciresinol 1b and (−)-matairesinol 2b from coniferyl alcohol 8.
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 investi-
gation. Research directed to the elucidation of the biosyn-
thetic pathways (intermediates and enzymes) involved in
the formation of the more highly functionalized lignans, such
as arctin 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 li-
inification process. At what point (if any) does the pathway
leading to the optically active lignans and (or purportedly)
optically inactive lignins differ? This important point awaits
clarification.

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(3) Ethyl 6,10-dideuteromethyl 3,4,5-trimethoxybenzoate: To a stirred solution of 3,4,5-trimethoxybenzoic acid (280 mg) in dry THF (4 ml) at room temperature, under nitrogen, was added dropwise diethyl phosphorochloridate (0.63 ml) at room temperature. After stirring for 2 h, a solution of n-butyllithium (1 ml) in cyclohexane was added dropwise at -78°C. After 1 h, the reaction mixture was quenched with a 10% aqueous solution of ammonium chloride, then extracted with ethyl acetate and dried over sodium sulfate. The solvent was distilled under reduced pressure, and the residue was purified by column chromatography on silica gel using ethyl acetate-diethyl ether (1:1) as eluent. The pure product was obtained as a colorless oil (325 mg, 74%).

(4) Pentadecanoyl thio-β-D-galactoside: To a solution of the residual sodium thiolate (100 mg) in dimethylformamide (5 ml) at room temperature, a solution of 1,2-O-isopropylidene-β-D-galactose (100 mg) in dimethylformamide (5 ml) was added. The reaction mixture was stirred overnight at room temperature, then quenched with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate-diethyl ether (1:1) as eluent. The pure product was obtained as a colorless oil (150 mg, 75%).

(5) 3-Hydroxy-1,2,3,4,5-tetramethyl-1H-pyrazole: To a solution of 3-hydroxy-1,2,3,4,5-pentamethyl-1H-pyrazole (200 mg) in methanol (5 ml) at room temperature, hydrochloric acid (0.5 ml) was added. The reaction mixture was stirred overnight at room temperature, then quenched with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate-diethyl ether (1:1) as eluent. The pure product was obtained as a colorless oil (150 mg, 75%).

(6) 3-Hydroxy-1,2,3,4,5-pentamethyl-1H-pyrazole: To a solution of 3-hydroxy-1,2,3,4,5-pentamethyl-1H-pyrazole (200 mg) in methanol (5 ml) at room temperature, hydrochloric acid (0.5 ml) was added. The reaction mixture was stirred overnight at room temperature, then quenched with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate-diethyl ether (1:1) as eluent. The pure product was obtained as a colorless oil (150 mg, 75%).

(7) 3-Hydroxy-1,2,3,4,5-pentamethyl-1H-pyrazole: To a solution of 3-hydroxy-1,2,3,4,5-pentamethyl-1H-pyrazole (200 mg) in methanol (5 ml) at room temperature, hydrochloric acid (0.5 ml) was added. The reaction mixture was stirred overnight at room temperature, then quenched with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate-diethyl ether (1:1) as eluent. The pure product was obtained as a colorless oil (150 mg, 75%).
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Cell-free extract from F. intermedia

Young shoots (5-10 cm long) of F. intermedia were excised by means of a razor, then washed with both tap and distilled water, and the leaves removed. The resulting stems (2.7 g fresh weight) were cut into small pieces by hand (scissors), frozen (−80 °C) and crushed in a mortar and pestle. The powder so obtained was further ground for 5-7 min with polyethylene AT (0.54 g), acid-washed sea sand and 0.1 M potassium phosphate buffer (pH 7.0; 4 ml) containing 10 mM disodium. The slurry was filtered through 4 layers of cheese-cloth, and the filtrate (3 ml) centrifuged (15,000 g, 5 min). The resulting supernatant (2.7 ml) was again filtered (Whatman GFA

glass fibre filter), and an aliquot (1.5 ml) of the filtrate was applied to a Sephadex G-25 column (19.7 x 1 cm, Pharmacia, particle size 55-150 µm (medium)), pre-equilibrated in 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol. The fraction excluded from the gel (1.5 ml) was collected and used as the cell-free preparation. Protein content of the preparation was 2.0 mg ml⁻¹ on the basis of a Bio-Rad Protein Assay using bovine serum albumin as standard (26).

Enzymatic oxidation of (−)-(−)- and (−)-(+)Secoisolariciresinol

The assay mixture contained 770 µl 0.1 M Tris-HCl buffer (pH 8.8, 37°C), 230 µl crude enzyme preparation and 10 µl 50 mM NAD or NADP solution in 0.1 M potassium phosphate buffer (pH 7.0). To initiate the enzymatic reaction (−)-(−)-Secoisolariciresinol (33.7 mg) was dissolved in MeOH (20 µl) and 0.1 M potassium phosphate buffer (232 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 1β isolation. The EtOAc solution containing both secoisolariciresinol 1 and matairesinol 2 were dissolved in MeOH (210 µl). An aliquot of this solution (20 µl) was applied to the Waters-Novapak C18 column, and eluted with the gradient solvent system described in the Chromatography Materials and Instrumentation section. Eluted fractions were collected every 30 s from t = 0 to 20 min and subjected to 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.

Enzymatic oxidation of (−)-(−)-Secoisolariciresinol

(−)-(−)-Secoisolariciresinol (435 mg, 6.55 KBE) were individually incubated exactly as above, but with NADP (0.1 mM) and an aliquot (9 µl) of the Sephadex G-25 column. Following elution with the solvent system described in the Chromatography Materials and Instrumentation section, fractions were collected every 30 s from t = 0 to 20 min, and subjected to liquid scintillation counting (Table 2). Next, optical purity samples of (−)-(−)-Secoisolariciresinol 1β (451 µg, 7.67 KBE) and (+)-(−)-Secoisolariciresinol 1α (385 µg, 6.65 KBE) were individually incubated exactly as above, but with NADP as coenzyme (unlabelled (-)-matairesinol 2α (35.8 µg) was added as radioactive carriers to each assay mixture before extraction, and the matairesinol 2 was then isolated by the use of reversed phase HPLC, and subjected to chiral HPLC and liquid scintillation counting analyses.

Enzymatic oxidation of (−)-(−)-Secoisolariciresinol

(−)-(−)-Secoisolariciresinol (6.87 mg) were incubated with the crude enzyme preparation (11.5 ml) from F. intermedia, in the presence of NADP or NADH with volumes scaled up proportionately. The (+)-matairesinol 2α was isolated with no addition of an unlabeled carrier, by (i) silica gel TLC, mixed with EIAChromexanes (1:1), developed three times (ii) reversed phase HPLC and (iii) chiral HPLC, and then subjected to mass spectrometric examination.