Lignan Biosynthesis in Forsythia Species

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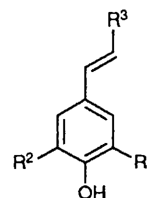
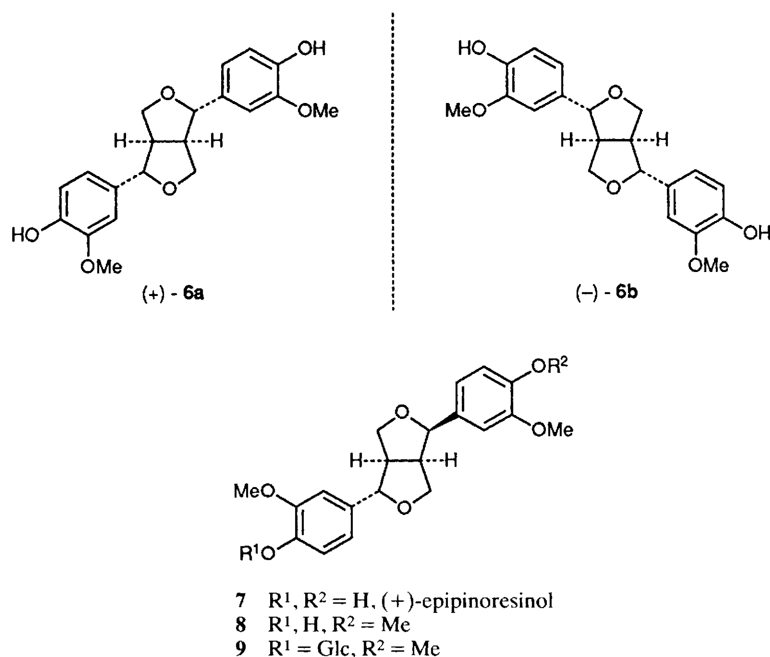
Both (+)-pinoresinol **6a** in *Forsythia suspensa* and (−)-secoisolariciresinol **14a** in *F. intermedia* are formed *via* a direct stereochemically-controlled coupling of coniferyl alcohol **2** derived moieties (*cf.* the typical peroxidase-catalysed reaction in the presence of H_2O_2), and the dibenzylbutyrolactone lignan, (−)-matairesinol **10a**, in *F. intermedia* is formed from a post-coupling modification of (−)-secoisolariciresinol **14a**; this transformation has been demonstrated *in vivo*, and *in vitro* with a crude enzyme preparation, and represents the first report of an enzyme specifically involved in lignan biosynthesis.

Lignins and lignans are ubiquitous constituents of terrestrial plants. They represent a major offshoot of the phenylpropanoid pathway, and hence are very abundant plant metabolites. In cell walls, lignin formation is viewed as occurring *via* an H_2O_2-requiring peroxidase-catalysed polymerization of monolignols **1–3**, the ratio of which can vary depending upon species, tissue and subcellular location.[1] This oxidative free-radical polymerization is believed to first afford mixtures of di-, tri- and higher oligomeric lignols which then undergo further reaction to give lignins.[2,3] Such a process would be expected to form a randomly-linked polymer with no measurable optical activity.

Numerous plant species also contain lignans (normally dimeric phenylpropanoids). These are of enormous structural diversity, and their formation has long been proposed to



1 $R^1, R^2 = H, R^3 = CH_2OH$
2 $R^1 = OMe, R^2 = H, R^3 = CH_2OH$
2a $R^1 = OCD_3, R^2 = H, R\text{-}3 = CD_2OH$
3 $R^1 = R^2 = OMe, R^3 = CH_2OH$
4 $R^1 = OMe, R^2 = H, R^3 = CHO$
4a $R^1 = OCD_3, R^2 = H, R^3 = CDO$
5 $R^1 = OMe, R_2 = H, R^3 = CO_2H$



7 R^1, R^2 = H, (+)-epipinoresinol
8 R^1, H, R^2 = Me
9 R^1 = Glc, R^2 = Me

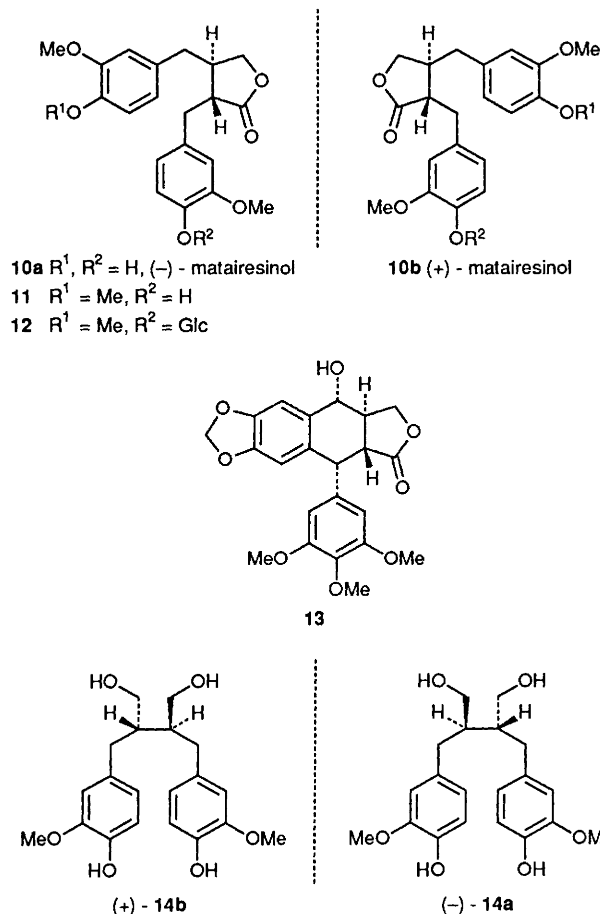
proceed in a manner analogous to that of lignin, *i.e. via* an H_2O_2-requiring peroxidase-catalysed oxidative coupling of monolignols.[4] Lignans are normally enantiomerically pure,[5] although the optical rotation of each may differ depending upon the plant species *e.g.* (+)-pinoresinol **6a** from *Forsythia suspensa*[6,7] or (−)-pinoresinol **6b** from *Xanthoxylum ailanthoides*.[8] No satisfactory explanation has been proffered to account for this stereochemical control affording optically active products, other than that the reaction is somehow enzyme mediated.

Several studies have been carried out in attempts to elucidate the biogenetic pathways to various lignans, *e.g.* podophyllum lignans such as podophyllotoxin **13**,[9–16] and the forsythia lignans **7–12**.[16,17] In spite of these substantive efforts, the chemical identity of the phenylpropanoid monomer (or monomers) that actually undergo coupling has remained speculative, and no evidence for a specific coupling enzyme has been obtained.

We herein report the first evidence for specific enzymatic reactions affording the lignans, (+)-pinoresinol **6a**, (−)-secoisolariciresinol **14a**, and (−)-matairesinol **10a**. We have established that coniferyl alcohol **2** is the specific precursor of (−)-secoisolariciresinol **14a**, and that a stereospecific dehydrogenation then occurs to afford (−)-matairesinol **10a**. We also report a convenient method for determining the enantiomeric purity of lignans.

The oxidative coupling of coniferyl alcohol **2** (20 mmol dm^{-3}) using horseradish peroxidase (HRP, EC 1.11.1.7; 0.09 units) in (Mes)–NaOH (Mes = 4-morphilineethanesulphonic acid) buffer (50 mmol dm^{-3}, pH 6.0) and H_2O_2 (1 mmol dm^{-3}) was carried out for 15 min at 30 °C, this being a reaction reported to produce racemic pinoresinol **6a** and **b**.[18] The resulting pinoresinol **6** was then foll enantiomeric purity was determined by application of the purified pinoresinol **6** to a Chiralcel OD column[20] (Daicel), under conditions previously optimized in our laboratory for the separation of (+)- and (−)-forms of synthetic pinoresinols **6a** and **6b**.

As shown in Fig. 1(a), essentially 1 : 1 formation of (+)- and (−)-pinoresinol, **6a** and **6b**, had occurred, in accordance with a non-specific coupling reaction catalysed by HRP and H_2O_2.



However, pinoresinol from *F. suspensa* (prior to recrystallization) exists exclusively as the (+)-enantiomer **6a** [Fig. 1(b)]. Thus, if a typical H_2O_2-requiring peroxidase was involved in pinoresinol formation, then any (−)-pinoresinol **6b** formed must either be rapidly assimilated into some other product(s),

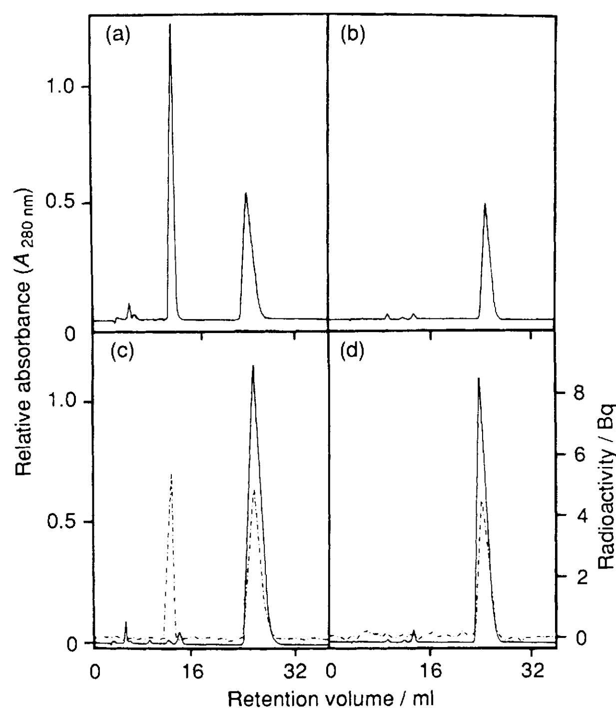


Fig. 1 HPLC chromatograms of purified pinoresinol in racemic **6a**, **6b** or enantiomerically pure **6a** form. (a) (±)-Pinoresinols **6a**, **6b** obtained following horseradish peroxidase/H_2O_2 treatment of coniferyl alcohol **2**; (b) (+)-Pinoresinol **6a** from *F. suspensa*; (c) (——) UV and (-·-·-) radiochromatograms of pinoresinols **6a**, **6b** isolated from *F. suspensa*, following [2-^{14}C]coniferyl alcohol administration to excised shoots for 3 h; (d) (——) UV and (-·-·-) radiochromatograms of pinoresinol **6a** from *F. suspensa*, following [U-^{14}C]phenylalanine administration to excised shoots for 3 h. (For radiochromatograms, the pinoresinol isolated was dissolved in 200 μl MeOH, and 10 μl was applied to the HPLC column)

or interconverted into the (+)-isomer **6a**. (−)-Matairesinol **10a** and (−)-secoisolariciresinol **14a** were also isolated from *F. intermedia* (data not shown), and the other enantiomeric forms were not detected.

A crude cell-free enzyme preparation[21] from *F. suspensa* in Hepes–NaOH [Hepes = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (50 mmol dm^{-3}, pH 7.5) was incubated with coniferyl alcohol **2** (20 mmol dm^{-3}) for 5 min at 30 °C, to test its ability to engender pinoresinol **6** formation. This preparation afforded pinoresinol **6** only when H_2O_2 (1 mmol dm^{-3}) was added, but the product was racemic.

The effect of the administration of [2-^{14}C]coniferyl alcohol **2** (5.7 μmol, 2.98 KBq μmol^{-1}) to excised shoots of *F. suspensa* was examined. Following precursor uptake, the plants were allowed to metabolise for 3, 9 and 24 h. In each case, radiolabelled racemic (±)-pinoresinols **6a**, **6b** were formed [*e.g.* 3.1% incorporation after 3 h metabolism, Fig. 1(c)], although the ratio of (+) to (−) increased with the length of the metabolic period. At a first glance, this result suggests that pinoresinol **6** formation occurs *via* non-specific coupling of two molecules of coniferyl alcohol **2**, as expected from an HRP–H_2O_2 catalysed reaction. The (−)-isomer **6b** could then be either selectively depleted by some other metabolic process, or slowly converted into its (+)-form **6a**, or both.

That this was not the case was concluded from the results of the following experiment. When [U-^{14}C]phenylalanine (6.06 μmol, 5.28 KBq μmol^{-1}) was administered to *F. suspensa* shoots for intervals of 3, 9 and 24 h, only radiolabelled

Table 1 Conversion of secoisolariciresinol **14** into matairesinol **10**[a]

F. intermedia plant material	Radiolabelled substrate: secoisolariciresinol **14** Optical purity	Radioactivity/ 10^5 dpm	Absolute incorporation of radioactivity into matairesinol (%)	
Stem	(±)-**14a**, **b**	9.46	0	0.94
Enzyme[b]	(±)-**14a**, **b**	1.17	0	2.0
	(±)-**14a**, **b**	1.17	0	2.9[c]
	(+)-**14b**	3.92	0	0
	(−)-**14a**	4.60	0	2.0

[a] All enzyme incubations were carried out in the presence of NADP (0.49 mmol dm^{-3}) for 1 h at 30 °C unless otherwise stated. [b] Crude enzyme preparation from stems. [c] 0.49 mmol dm^{-3} NAD

(+)-pinoresinol **6a** was formed, as in the 0.73% incorporation after metabolism for 3 h [see Fig. 1(d)]; no (−)-pinoresinol **6b** was detected.

These results can be interpreted as follows. When [2-^{14}C] coniferyl alcohol **2** was administered to excised *F. suspensa* shoots, the substrate was not properly compartmentalized in the plant and intercession of non-specific peroxidases (in the presence of H_2O_2) affords (±)-pinoresinol **6a**, **6b**. However, both phenylalanine and its subsequent metabolites are compartmentalized appropriately prior to oxidative coupling. This results in an organized entry (temporally and spatially) into the biochemical pathway affording monolignols, lignans, lignins, *etc.* (+)-Pinoresinol **6a** is then formed, presumably from coniferyl alcohol **2**, *via* a specific stereochemically controlled enzymatic process. The precise nature of this unusual enzyme is the subject of further investigation, but cannot be explained on the basis of a typical peroxidase H_2O_2 coupling reaction.

The formation of secoisolariciresinol **14** and matairesinol **10** in *F. intermedia* was studied. When [2-^{14}C]coniferyl alcohol **2** (7.2 μmol, 4.17 KBq μmol^{-1}) was administered to *F. intermedia* shoots for 3 h, the radiolabel was present only in (−)-secoisolariciresinol **14a** and (−)-matairesinol **10a** (absolute incorporations of 0.3 and 1.8% respectively); the corresponding radiolabelled enantiomeric forms were not detected. Next, [1-$^2H_2$, OC^2H_3]coniferyl alcohol **2a** was administered to *F. intermedia* shoots, and both (−)-secoisolariciresinol **14a** and (−)-matairesinol **10a** were isolated as before. Mass spectral analysis of (−)-secoisolariciresinol **14a** showed a new signal enhancement at 372 ($M^+ + 10$, corresponding to ~11% of the natural abundance molecular ion), indicative of direct coupling of two intact deuteriated coniferyl alcohol **2** units. In a similar manner, the (−)-matairesinol **10a** had a new molecular ion at 366 ($M^+ + 8$) showing that it was also derived from two coniferyl alcohol-derived units. (Comparable results were observed with [1-^{2}H, OC^2H_3]coniferaldehyde **4a**.)

We then established whether secoisolariciresinol **14** served as the precursor of matairesinol **10**. Thus, (±)-[Ar-^{3}H]secoisolariciresinols **14a**, **14b** were administered to *F. intermedia* shoots as a racemic mixture (see Table 1). After metabolism for 3 h the matairesinol **10** was isolated. Following dilution with unlabelled (±)-matairesinols **10a**, **10b**, and subsequent purification of each enantiomeric form, it was established that only (−)-matairesinol **10a** was radiolabelled.

Crude cell-free enzyme preparations from *F. intermedia* were analysed for their ability to synthesize (−)-secoisolariciresinol **14a** and (−)-matairesinol **10a** from [2-^{14}C]-coniferyl

alcohol **2**, in the presence or absence of H_2O_2. No conditions were identified where either (±)-secoisolariciresinols **14a**, **14b** or (±)-matairesinols **10a**, **10b** were formed. The possibility that secoisolariciresinol **14** was stereospecifically converted to matairesinol **10** was investigated. Thus crude enzyme preparations from *F. intermedia* were individually incubated with (±)-, (+)- and (−)-[Ar-^{3}H]secoisolariciresinols **14a**, **14b**, and **14b** and **14a** respectively, in the presence of 0.49 mmol dm^{-3} NAD/NADP (Table 1). Only the stereospecific conversion of (−)-secoisolariciresinol **14a** into (−)-matairesinol **10a** was observed. The (+)-isomer did not serve as a substrate for formation of either (+)- or (−)-matairesinols **10b**, **10a**. Note that this conversion did not occur when either NAD/NADP was absent or if the enzyme was denatured (boiled). Finally (±)-[Ar-^{2}H]secoisolariciresinols **14a**, **14b** were synthesized from unlabelled **14** by $CF_3CO_2{}^2H$ exchange; the resulting deuteriated substrate had an ion cluster centred at *m/z* 364 (M^+ + 2). When incubated with the crude enzyme preparation from *F. intermedia*, the (−)-matairesinol **10a** so formed gave an essentially identical ion cluster but now centred at *m/z* 360 (M^+ + 2), *i.e.* the (−)-[Ar-^{2}H]secoisolariciresinol **14a** was specifically converted into (−)-[Ar-^{2}H]matairesinol **10a**.

In summary, we have obtained conclusive evidence that representative forsythia lignans are not only enantiomerically pure, but that their formation cannot be rationalized on the basis of a typical HRP-catalysed coupling reaction. The results strongly suggest that both (+)-pinoresinol **6a** in *F. suspensa* and (−)-secoisolariciresinol **14a** in *F. intermedia* are formed *via* a direct stereochemically-controlled coupling of compartmentalized coniferyl alcohol **2** derived moieties. The precise nature of this enzymic process needs to be determined. The formation of (−)-matairesinol **10a** in *F. intermedia*, results from a subsequent post-coupling modification of (−)-secoisolariciresinol **14a** (or an oxidised equivalent thereof). It now needs to be established whether all forsythia and podophyllum lignans ultimately arise *via* enzymically-controlled chemical modifications of the coupling product of two coniferyl alcohol **2** units.

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