PERGAMON

Phytochemistry 55 (2000) 537–549

PHYTOCHEMISTRY

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Dirigent-mediated podophyllotoxin biosynthesis in *Linum flavum* and *Podophyllum peltatum*

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Received 9 May 2000

Abstract

Given the importance of the antitumor/antiviral lignans, podophyllotoxin and 5-methoxypodophyllotoxin, as biotechnological targets, their biosynthetic pathways were investigated in *Podophyllum peltatum* and *Linum flavum*. Entry into their pathways was established to occur via dirigent mediated coupling of *E*-coniferyl alcohol to afford (+)-pinoresinol; the encoding gene was cloned and the recombinant protein subsequently obtained. Radiolabeled substrate studies using partially purified enzyme preparations next revealed (+)-pinoresinol was enantiospecifically converted sequentially into (+)-lariciresinol and (−)-secoisolariciresinol via the action of an NADPH-dependent bifunctional pinoresinol/lariciresinol reductase. The resulting (−)-secoisolariciresinol was enantiospecifically dehydrogenated into (−)-matairesinol, as evidenced through the conversion of both radio- and stable isotopically labeled secoisolariciresinol into matairesinol, this being catalyzed by the NAD-dependent secoisolariciresinol dehydrogenase. (−)-Matairesinol was further hydroxylated to afford 7′-hydroxymatairesinol, this being efficiently metabolized into 5-methoxypodophyllotoxin. Thus much of the overall biosynthetic pathway to podophyllotoxin has been established, that is, from the dirigent mediated coupling of *E*-coniferyl alcohol to the subsequent conversions leading to 7′-hydroxymatairesinol.

Keywords: *Podophyllum peltatum*; *Linum flavum*; Berberidaceae; Linaceae; Dirigents; 7′-Hydroxymatairesinol; 5-Methoxypodophyllotoxin; Podophyllotoxin; Pinoresinol/lariciresinol reductase; Secoisolariciresinol dehydrogenase

1. Introduction

Podophyllotoxin (**1**) (Scheme 1) is a very important antitumor and antiviral agent isolated from *Podophyllum* species. Its pharmacological usage dates back many centuries, when may apple (*P. peltatum*) alcoholic extracts, obtained from rhizomes and roots, were employed first as a poison and later, in smaller doses, for treatment of various pathological conditions (Ayres and Loike, 1990). It was subsequently demonstrated that the cytotoxic effect of these extracts was due to the lignan, podophyllotoxin (**1**). However, direct application/administration of podophyllotoxin (**1**) was accompanied with severe toxicity, which precluded its direct use in cancer treatment. Podophyllotoxin (**1**) has since found direct medicinal usage in an effective treatment of venereal warts (Beutner and von Krogh, 1990). Today, etoposide (**2a**), etopophos (**2b**) and teniposide (**3**), semisynthetic derivatives of podophyllotoxin (**1**) which do not display the same high levels of cytotoxicity, are widely used clinically for treatment of Hodgkin's and non-Hodgkin's lymphoma, testicular/small cell lung cancers and acute leukemia (O'Dwyer et al., 1985; Williams et al., 1987; Young, 1992; Schacter, 1996; Canel et al., 2000).

Because of the limited supply of *Podophyllum* rhizomes, due to their intensive collection in the wild, there is considerable interest in both defining and biotechnologically exploiting the podophyllotoxin (**1**) biosynthetic pathway, thereby making this compound more generally available (Lewis and Davin, 1999). This need is driven in part because studies, using both cell suspension and root tissue cultures of *Podophyllum* and *Linum* species, have had limited success thus far in attaining significantly elevated levels of either podophyllotoxin (**1**) or its 5-methoxy analogue (**4**) (van Uden et al., 1990a; 1990b). Accordingly, we are deciphering the biosynthetic pathway to the medicinally important lignan,

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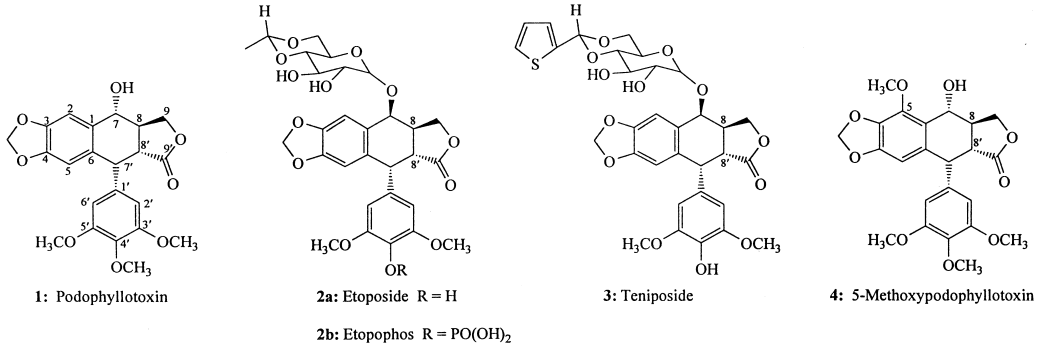
0031-9422/00/$ - see front matter
PII: S0031-9422(00)00242-9

podophyllotoxin (**1**), as a first step towards the biotechnological exploitation of its biochemical pathway. This paper describes recent progress towards meeting these goals.

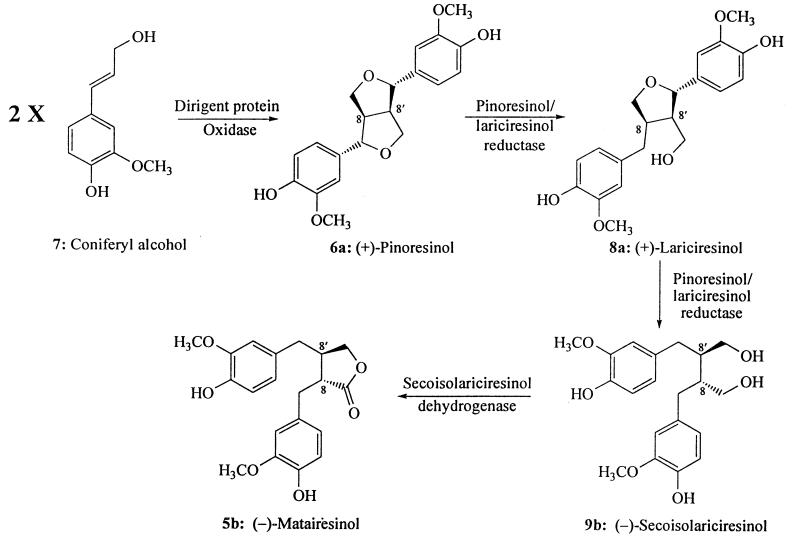
2. Results and discussion

Previous work had suggested the possibility of a biochemical precursor relationship between matairesinol (**5**) and podophyllotoxin (**1**) (Broomhead et al., 1991) although, based on incorporation levels for *Podophyllum* species, it was not possible to conclude that this was a direct intermediate in the pathway. A biosynthetic pathway to matairesinol (**5**) had, however, been rigorously established in *Forsythia* species (Scheme 2): this occurred through the action of the dirigent protein affording (+)-pinoresinol (**6a**) from *E*-coniferyl alcohol (**7**) (Davin et al., 1997; Gang et al., 1999a), as well as that of pinoresinol/lariciresinol reductase (Chu et al., 1993; Katayama et al., 1993; Dinkova-Kostova et al., 1996) and secoisolariciresinol dehydrogenase (Umezawa et al., 1991; Xia et al., 2000) in downstream metabolism. All of these proteins and enzymes were purified from *F. intermedia* with the corresponding genes cloned and fully functional recombinant proteins expressed. Accordingly, with both *P. peltatum* and *L. flavum* plants available for study, where the former accumulates podophyllotoxin (**1**) and the latter, its 5-methoxy analogue (**4**), we wished to establish their biochemical pathway(s). Of particular interest was whether matairesinol (**5**) was an intermediate and, if so, the steps involved in the biochemical pathway to matairesinol (**5**) from *E*-coniferyl alcohol (**7**).

We sought first to establish whether the biochemical pathway to matairesinol (**5**) in *Linum flavum*, which accumulates 5-methoxypodophyllotoxin (**4**), was as for *Forsythia* sp. That is, it was established that, following dirigent protein mediated (+)-pinoresinol (**6a**) formation (Davin et al., 1997), this then undergoes sequential enantiospecific reduction to give (+)-lariciresinol (**8a**) and (−)-secoisolariciresinol (**9b**) (Chu et al., 1993;



Scheme 1. Podophyllotoxin (**1**), its derivatives (**2a**, **2b** and **3**) and an analogue (**4**).



Scheme 2. Biosynthetic pathway from *E*-coniferyl alcohol (**7**) to matairesinol (**5**) in *Forsythia intermedia*. Note: compounds designated with an "**a**" indicate the (+)-antipode, whereas those with a "**b**" depict the (−)-enantiomer. [In each case, only one enantiomeric form is illustrated.]

Katayama et al., 1993; Dinkova-Kostova et al., 1996), followed by enantiospecific dehydrogenation to afford (−)-matairesinol (**5b**) (Umezawa et al., 1991). Thus, cell-free extracts from *L. flavum* roots were first incubated with racemic (±)-pinoresinols (**6a/6b**) in the presence of [4*R*-^{3}H]-NADPH. Following 2 h incubation, the resulting assay mixture (see Experimental) was extracted with EtOAc containing both racemic (±)-lariciresinols (**8a/8b**) and (±)-secoisolariciresinols (**9a/9b**) as radiochemical carriers. The resulting EtOAc solubles were then applied to a C18 reversed phase HPLC column eluted with MeOH–3%HOAc in H_2O (30:70), with fractions corresponding to lariciresinol (**8**) and secoisolariciresinol (**9**) individually collected and evaporated to dryness. Reconstitution of each sample in EtOH–hexanes (1:1), followed by chiral HPLC analysis (Figs. 1A–D), revealed that radioactivity was only associated with (+)-lariciresinol (**8a**) (Fig. 1C) and (−)-secoisolariciresinol (**9b**) (Fig. 1D). As noted, *no* radioactivity was detectable in either (−)-lariciresinol (**8b**) or (+)-secoisolariciresinol (**9a**), even with unlabeled racemic lignans having been added as radiochemical carriers (Figs. 1A and B). Moreover, the sequential reductive steps abstracted only the [4*R*-^{3}H]-NADPH hydride at the 4*R*-position and not that from the corresponding 4*S*-position (data not shown). Thus the biosynthetic pathway from *E*-coniferyl alcohol (**7**) to secoisolariciresinol (**9**) in *L. flavum* root tissue was as for *F. intermedia*. This result could not, however, have been predicted a priori since lignans are found in different plant species with differing degrees of enantiomeric purity (Lewis and Davin, 1999).

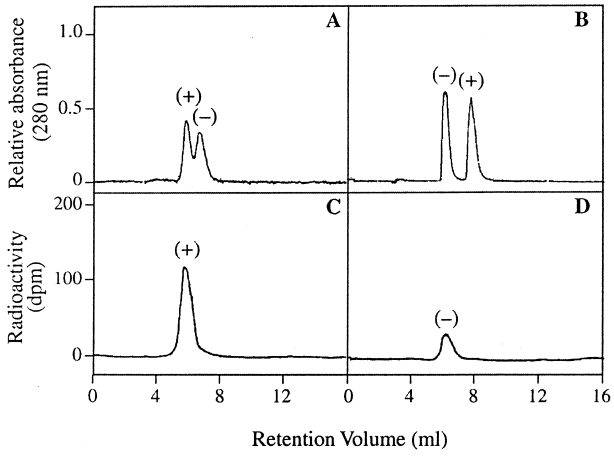


Fig. 1. A and B: Chiral column HPLC separations of (**A**) synthetic (±)-lariciresinols (**8a/8b**) and (**B**) (±)-secoisolariciresinols (**9a/9b**) on Chiralcel OD and OC columns, respectively; these racemates were used as radiochemical carriers. C and D: Chiral HPLC analyses with radiochemical detection of enzymatic products, (**C**) lariciresinol (**8**) and (**D**) secoisolariciresinol (**9**) following incubation of a crude pinoresinol/lariciresinol reductase preparation from *L. flavum* with (±)-pinoresinols (**6a/6b**) (0.4 mM) in the presence of [4*R*-^{3}H]-NADPH (0.8 mM). Elution conditions: Chiralcel OD: hexanes–EtOH (70:30); flow rate, 0.5 ml min^{-1}. Chiralcel OC: hexanes–EtOH (20:80); flow rate, 0.2 ml min^{-1}.

It was next instructive to ascertain whether secoisolariciresinol (**9**) would be converted into matairesinol (**5**) when incubated in the presence of NAD with *L. flavum* cell-free extracts. In order to investigate this question, both (±)-[Ar-^{2}H]-secoisolariciresinols (**9a/9b**) and (±)-[9,9′-$^3H_2$]-secoisolariciresinols (**9a/9b**) were synthesized and individually employed as potential substrates. Thus, 0.55 μmol (±)-[Ar-^{2}H]-secoisolariciresinols (**9a/9b**) were incubated in the presence of 40 μM NAD, with a partially purified enzyme preparation from *L. flavum* roots, obtained via consecutive DEAE cellulose and ADP-Sepharose column chromatographic steps. After 2 h, the resulting assay mixture was extracted with EtOAc, with the EtOAc solubles combined and applied to a C18 reversed phase HPLC column eluted with CH_3CN–3% HOAc in H_2O. The fraction corresponding to matairesinol (**5**) was collected and subjected to electron impact mass spectroscopic analysis (see Fig. 2). For comparative purposes, Fig. 2A shows the mass spectrum of unlabeled matairesinol (**5**), whose molecular ion [M^+] was clearly observed at *m/z* 358, together with its benzylic base peak ion at *m/z* 137 (Umezawa et al.,

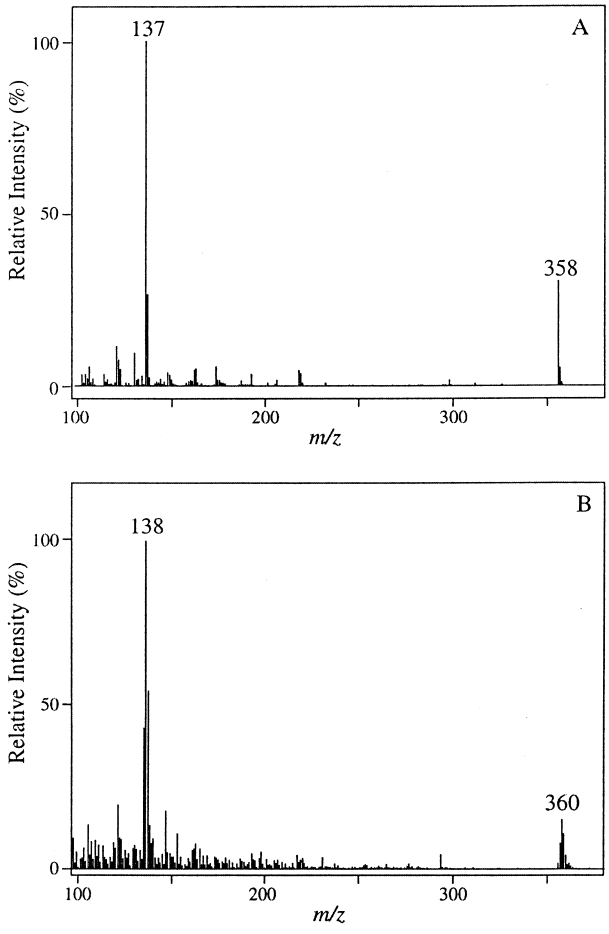


Fig. 2. Mass spectral fragmentation pattern (EI mode) of (**A**) natural abundance matairesinol (**5**) and (**B**) [Ar-^{2}H]-matairesinol (**5**) obtained following incubation of [Ar-^{2}H]-secoisolariciresinol (**9**) with a partially purified *L. flavum* secoisolariciresinol dehydrogenase preparation.

1991). The mass spectrum of the enzymatic product obtained following incubation with [Ar-^{2}H]-secoisolariciresinols (**9a/9b**) confirmed the product to be [Ar-^{2}H]-matairesinol (**5**) (Fig. 2B). That is, there was a molecular ion cluster centered at m/z 360 [$M^+ + 2$] corresponding to deuterated matairesinol (**5**), with additional confirmation that the deuterium was in the aromatic ring being the base peak ion fragment centered at m/z 138 [$Ar^+ + 1$]. Thus, [Ar-^{2}H]-secoisolariciresinol (**9**) had been intactly converted into [Ar-^{2}H]-matairesinol (**5**).

To determine the enantiospecificity of this conversion, the partially purified secoisolariciresinol dehydrogenase preparation was next incubated for 2 h with 2.8 μM (±)-[9,9′-$^3H_2$]-secoisolariciresinols (**9a/9b**) in the presence of excess NAD (40 μM). Following incubation, the corresponding radiolabeled matairesinol (**5**) was isolated using C18 reversed phase HPLC as before, where it was subsequently demonstrated that only (−)-[9,9′-$^3H_2$]-secoisolariciresinol (**9b**) was converted into (−)-[9-$^3H_2$]-matairesinol (**5b**), i.e. the corresponding (+)-enantiomer (**9a**) was not utilized. The enantiospecificity of the conversion was demonstrated by chiral HPLC analysis: however, since chiral column HPLC (Chiralcel OD) does not completely separate both (+)- and (−)-antipodes of matairesinol (**5a**) and (**5b**) (data not shown), the matairesinol (**5**) that had been enzymatically produced was chemically reduced with $LiAlH_4$ (see Experimental) to afford secoisolariciresinol (**9**). Chiral HPLC analysis revealed that only the (−)-antipode (**9b**) was radiolabeled (data not shown) and that the conversion was enantiospecific. This observation was in agreement with previous results with *F. intermedia* (Umezawa et al., 1991; Xia et al., 2000). Thus, taken together, these data established that, in the podophyllotoxin-forming species, the biochemical pathway to (−)-matairesinol (**5b**) was exactly the same as in *Forsythia* (see Scheme 2).

Attention was next focused on the metabolism of matairesinol (**5**) in *L. flavum* root tissues, and whether it served as a precursor of 5-methoxypodophyllotoxin (**4**) or not. Thus, administration of a solution containing 0.28 mmol unlabeled matairesinol (**5**) to *L. flavum* root segments was carried out for 6 h, following which the root tissue was homogenized and then extracted with MeOH under conditions of reflux. The resulting MeOH solubles were next treated with β-glucosidase, with the resulting EtOAc extract subjected to silica gel chromatography and C18 reversed phase HPLC, successively. HPLC analysis revealed the presence of a new metabolite at an elution volume of 14.3 ml (Fig. 3A), the EI mass spectrum of which gave a molecular ion at m/z 374 [$M^+ + 16$] (Fig. 4B). This metabolite was tentatively identified as 7′-hydroxymatairesinol (**16**), based on analysis of its mass spectral fragmentation pattern. This interpretation was subsequently established as being correct through comparison with an authentic sample

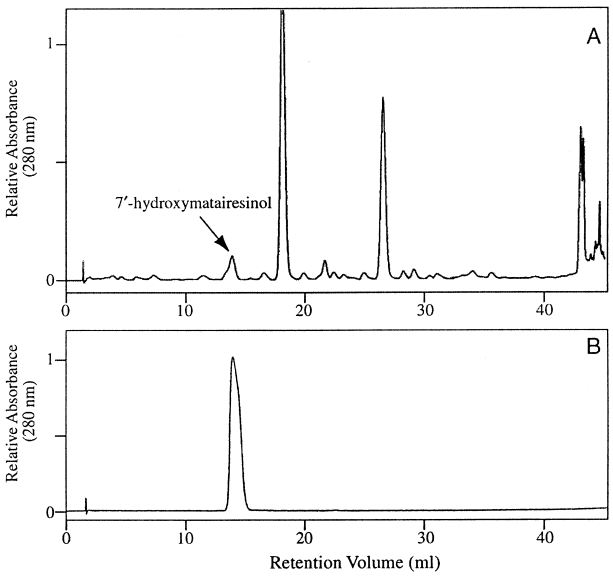


Fig. 3. C18 reversed phase HPLC chromatogram of (**A**) a MeOH extract obtained after incubation of matairesinol (**5**) with *L. flavum* root tissue, and (**B**) authentic 7′-hydroxymatairesinol (**16**). Elution conditions: linear gradient CH_3CN–3%HOAc in H_2O from 10:90 to 30:70 between 0 and 35 min; flow rate 1 ml min^{-1}.

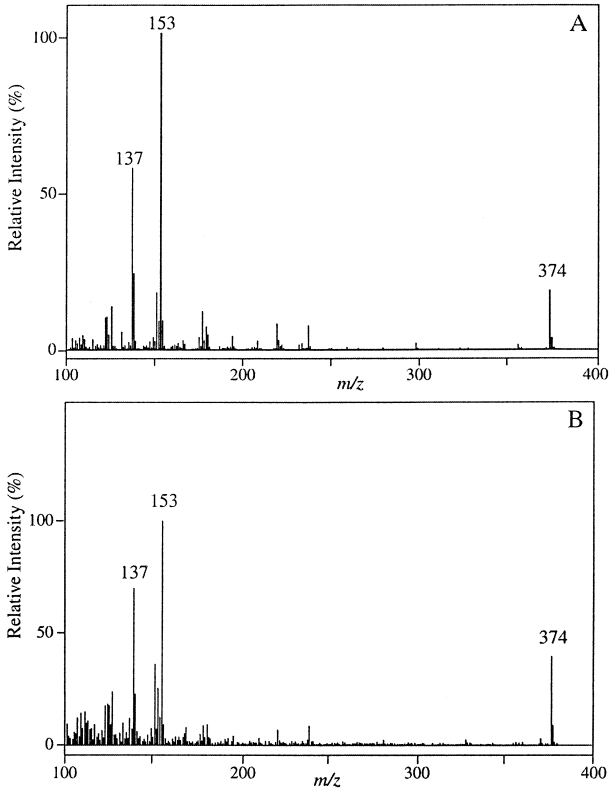
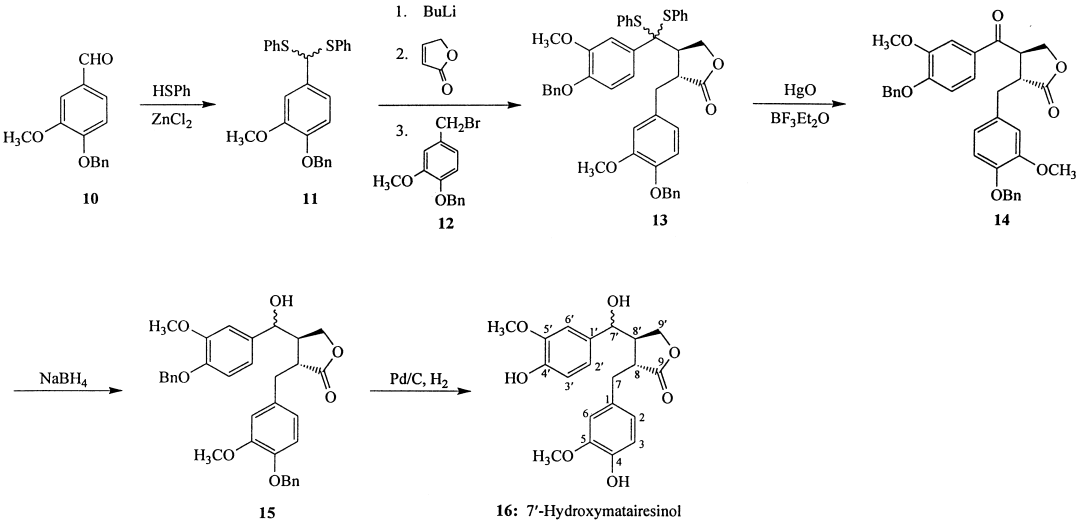


Fig. 4. Mass spectral fragmentation pattern (EI mode) of (**A**) synthetic 7′-hydroxymatairesinol (**16**), and (**B**) the putative 7′-hydroxymatairesinol (**16**) product obtained after administration of matairesinol (**5**) to *L. flavum* root tissue.



Scheme 3. Total synthesis of 7′-hydroxymatairesinol (**16**).

obtained by total synthesis (Scheme 3 and Fig. 3B). As can be seen in Scheme 3, authentic 7′-hydroxymatairesinol (**16**) was synthesized from an *O*-benzylic derivative of vanillin (**10**). In this one-pot reaction, the protected vanillin (**10**) was first converted into its bisphenylthioacetal derivative (**11**) and then into the corresponding anionic form with *n*-BuLi. Subsequent nucleophilic attack with 2(5H)furanone and the bromo-derivative (**12**) afforded the desired butyrolactone (**13**) which was then converted into the corresponding ketone (**14**) via the action of HgO/BF_3Et_2O (Ziegler and Schwartz, 1978). Consecutive reductions, using $NaBH_4$, and deprotection (Pd/C, H_2), respectively, afforded authentic 7′-hydroxymatairesinol (**16**).

The synthetic 7′-hydroxymatairesinol (**16**) had both an identical retention volume of 14.3 ml (Fig. 3B) to that of the *L. flavum* matairesinol-derived metabolite (Fig. 3A), as well as an identical UV spectrum (284, 228 and 202 nm). Furthermore, mass spectral comparison of the synthetic product to that of the *Linum* metabolite further confirmed its identity (see Figs. 4A and B). The authentic 7′-hydroxymatairesinol (**16**) (Fig. 4A) gave the expected parent molecular ion at *m*/*z* 374 together with two fragment ions at *m*/*z* 153 and 137 corresponding to both 7′-hydroxylated benzylic and benzylic ion fragments, respectively. These data thus clearly established that the hydroxyl group was at position 7′. EI mass spectral analysis of the matairesinol-derived metabolite gave an essentially identical fragmentation pattern (Fig. 4B), thereby confirming its identity. Thus, matairesinol (**5**) had been converted into 7′-hydroxymatairesinol (**16**) in *L. flavum*. Note also that this compound had previously been reported in western hemlock (*Tsuga heterophylla*) (Krahmer et al., 1970), suggesting its involvement in the biosynthesis of the lignan, α-conidendrin (Lewis and Davin, 1999).

Next, we wished to establish if 7′-hydroxymatairesinol (**16**) served as an intermediate in the biosynthesis of 5-methoxypodophyllotoxin (**4**). Thus, [7′-^{3}H]-7′-hydroxymatairesinol (**16**) was synthesized from ketone (**14**), via deployment of NaB^3H_4 as the reducing reagent (see Scheme 3). Following deprotection, the [7′-^{3}H]-7′-hydroxymatairesinol (**16**) was administered to *L. flavum* root tissue for 6 h. The resulting methanol extract was again treated with β-glucosidase, at which time it was demonstrated that this precursor had been converted into radiolabeled [7-^{3}H]-5-methoxypodophyllotoxin (**4**) [1.3% total incorporation] (see Fig. 5).

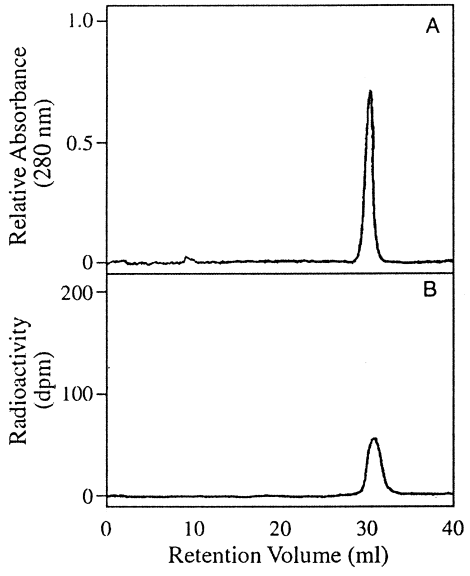


Fig. 5. HPLC analysis of 5-methoxypodophyllotoxin (**4**) isolated from *L. flavum* roots following administration of [7′-^{3}H]-7′-hydroxymatairesinol (**16**). (**A**) UV absorbance at 280 nm and (**B**) radioactivity elution profiles.

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          .         .         .         .         .
.............................FDDPITLDNNFHSPPVGRA 106
                                |||||||||| ||||||||
YHNATSAIVGSPQWGNKTAMAVPFNYGDLVVFDDPITLDNNLHSPPVGRA 99
          .         .         .         .         .
QGLYVYDRKDTFHSWLSFSFTLNTTMHQGTLIFMGADPILIKNRDITVVG 156
||:| ||.|.|:..|| |||  |.| : ||| | ||||:| | |||.|:|
QGMYFYDQKNTYNAWLGFSFLFNSTKYVGTLNFAGADPLLNKTRDISVIG 149
          .         .         .
GTGDFFMARGIATIATDAFEGDVYF.............. 181
||||||||||:||: ||||||||||
GTGDFFMARGVATLMTDAFEGDVYFRLRVDINLYECW*  187
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Fig. 6. GAP comparison of the PCR DNA fragment isolated from the *P. peltatum* rhizome cDNA library (upper sequence) and the corresponding dirigent sequence from *F. intermedia* (lower sequence), indicating 80.2% similarity and 72.5% identity. Numbers to the right of the sequences indicate the amino acid residue locations in the respective sequences. Boldface characters indicate the residues from which degenerate dirigent primers were designed to obtain the *P. peltatum* dirigent homologue sequence. A pipe character (|) between two sequence symbols indicates an identical residue match, a colon indicates a comparison value greater than or equal to the average positive non-identical comparison value in the scoring matrix, and a period signifies a comparison value greater than or equal to 1.

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GGAATTCGGCACCAGATAGAGAGTACCATTTTCCACAAACCAAGCATGGGAGGAGAAAAA 15
                                                M  G  G  E  K   5

GCTTTCAGTTTCATTTTCCTCCTCTTCTTGTGCTTCTTCCTGGCCAACCTCTCTGCTTCT 75
A  F  S  F  I  F  L  L  F  L  C  F  F  L  A  N  L  S  A  S   25

TCAGCTCACCCCCCTCGTCAGAAGCTCAAGCAACGCATACCATGTAAACAATTAGTCCTA 135
S  A  H  P  P  R  Q  K  L  K  Q  R  I  P  C  K  Q  L  V  L   45

TACTTCCATGATGTAGTTTACAATGGTCACAACAAGGCTAATGCAACAGCATCCATTGTG 195
Y  F  H  D  V  V  Y  N  G  H  N  K  A  N  A  T  A  S  I  V   65

GGTGCACCACAAGGCGCAGACCTTGTAAAATTAGCAGGGGAAAACCATTTTGGCAATGTG 255
G  A  P  Q  G  A  D  L  V  K  L  A  G  E  N  H  F  G  N  V   85

GTTGTGTTCGACGACCCAATTACTCTAGACAACAATTTTCACTCCCCACCTGTTGGTCGT 315
V  V  F  D  D  P  I  T  L  D  N  N  F  H  S  P  P  V  G  R   105

GCACAAGGGTTGTATGTTTATGACAAGAAGGACACATTCCACTCATGGCTAAGTTTCTCA 375
A  Q  G  L  Y  V  Y  D  K  K  D  T  F  H  S  W  L  S  F  S   125

TTTACTCTTAATACTACTATGCATCAAGGTACCCTTATTTTCATGGGAGCTGACCCTATT 435
F  T  L  N  T  T  M  H  Q  G  T  L  I  F  M  G  A  D  P  I   145

TTAATCAAGAATAGGGATATCACAGTTGTCGGTGGTACAGGGGATTTCTTCATGGCTCGA 495
L  I  K  N  R  D  I  T  V  V  G  G  T  G  D  F  F  M  A  R   165

GGAATTGCAACTATAGCAACTGATTCATACGAAGGGGAGGTCTATTTTCGACTTAAAGTT 555
G  I  A  T  I  A  T  D  S  Y  E  G  E  V  Y  F  R  L  K  V   185

GATATCAAGTTGTATGAGTGTTGGTAATTTCTTTTACTTTGTGAGTGGTAACTGGTAACT 615
D  I  K  L  Y  E  C  W  *                                     194
GAGATGAGATGTTATATTATGTAATGGGTTTATTTATCCATACTTTATGGCTGAATAAAT 675
TTGAGGCTTTGGAAGACCTCATTTGTTTGTAATTTATTTGACAAGGTTAAGCAAAGTTGT 735
CAATTAAAAGTTTTTAATTGAACCAAAAAAAAAAAAAAAAAAACTCGAGGGGGGGGCCGG 795
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Fig. 7. Complete cDNA and amino acid sequence of *P. peltatum* dirigent homologue *psd-Pp1*. The numbers to the right indicate the DNA base or amino acid sequence residue locations, respectively. A Kozak consensus DNA sequence is identified by underlined letters; the translation initiation methionine and the stop codon are in bold type; a termination consensus signal is indicated by bold italicized letters, and the polyA tail is presented in underlined bold type. Three potential *N*-glycosylation sites are indicated by gray background italicized amino acid residues. Underlined bold, italicized amino acid residue characters indicate locations where degenerate primers PS6F and PS2R annealed to upon the initial PCR screening.

Therefore, taken together, these results demonstrated that the biochemical pathway to matairesinol (**5**) in *L. flavum* was equivalent to that in *Forsythia*. Furthermore, in the latter species, the matairesinol (**5**) so formed is next hydroxylated to 7′-hydroxymatairesinol (**16**), which is then converted into 5-methoxypodophyllotoxin (**4**). The remaining conversions to this important lignan thus involve aryltetrahydronaphthalene ring formation, additional hydroxylation and *O*-methylation steps, as well as formation of the methylenedioxy bridge. The latter can be assumed to result from a NADPH-dependent cytochrome P-450 catalyzed ring closure as demonstrated previously for the sesame (*Sesamum indicum*) lignans (Jiao et al., 1998). Investigation of the actual enzymology involved in these latter steps to 5-methoxypodophyllotoxin (**4**) will be the subject of additional studies.

Lastly, with the general pathway to podophyllotoxin (**1**) and 5-methoxypodophyllotoxin (**4**) so defined, it was next instructive to obtain the gene encoding the dirigent forming (+)-pinoresinol (**6a**) from *P. peltatum*, since this represents the entry point into the overall pathway and thus a promising candidate for genetic manipulation.

A rhizome cDNA library from *P. peltatum* was first constructed and screened for the corresponding dirigent gene(s) as follows. Thus, a band of approximately 300 bp size was obtained by screening the *P. peltatum* rhizome-derived cDNA library, using a polymerase chain reaction (PCR), with consensus primers designed from dirigent homologs from 12 plant species (Davin and Lewis, 2000). This 300 bp fragment was next cloned into a TOPO TA vector (Invitrogen), with plasmid preparations being procured from clones having the correct size insert, and the plasmid DNA then sequenced. The amino acid sequence corresponding to the 300 bp fragment was found to be 72% identical and 80% similar to the homologous region in the *F. intermedia* dirigent (Fig. 6). The locations of the amino acid residues corresponding to the forward and reverse degenerate primers used to obtain this fragment are indicated in Fig. 7 by bold, italicized, underlined characters. This fragment was excised from the plasmid, labeled with ^{32}P-dCTP, and used as a probe for screening approximately 3×10^5 pfu of the cDNA library, at a hybridization temperature of 55°C. After two rounds of screening, six positive clones were excised into pBluescript phagemids using ExAssist helper phage and transformed into SOLR cells (Stratagene). Of these six isolates, five translated into an identical amino acid sequence (Fig. 8, PSD-Pp1) and the sixth was similar except for two amino acid residues (Fig. 8, PSD-Pp2). The 579 bp complete gene sequence (Fig. 7) encodes a protein of 193 amino acid residues, including a 40 amino acid secretory signal peptide; the calculated mass of the secreted protein is ~17 kDa. This amino acid sequence has 68.4% similarity and 60.4% identity to the *F. intermedia* dirigent (Fig. 8, PSD-Fi1) (*psd-Fi1,* GenBank accession # AF210061) involved in a stereoselective phenoxy free-radical coupling reaction initiating the lignan biosynthesis pathway (Davin et al., 1997; Gang et al., 1999a). Comparison of the peptides excluding the signal peptide sequence shows an even greater similarity between the two proteins (Fig. 8). Three potential *N*-glycosylation sites are present (Fig. 7), similar in number to the four sites in the *Forsythia* dirigent gene (*psd-Fi1*).

The corresponding dirigent was next obtained in an insect cell expression system capable of undergoing posttranslational modification. Primers were constructed to produce a PCR fragment containing the clone for insertion into a DES TOPO vector (Invitrogen) for transfection into S2 *Drosophila melanogaster* cells. Selection of the transfected insect cells in hygromycin medium yielded a

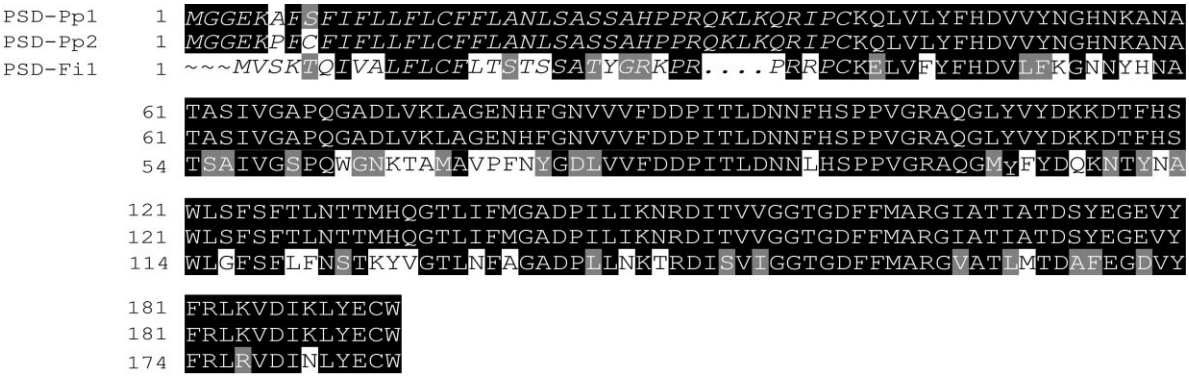

Fig. 8. BOXSHADE comparison between entire *P. peltatum* dirigent homologues (PSD-Pp1 and PSD-Pp2) and *F. intermedia* dirigent (PSD-Fi1), showing 68.4% similarity and 60.4% identity. Numbers to left of sequences indicate the amino acid residue locations. Black shading indicates that the residue is identical to the column consensus; gray shading indicates that the residue is not identical but at least similar to the column consensus; and no shading indicates that the residue is neither identical nor similar to the consensus (BOXSHADE version 3.2, written by Kay Hofmann and Michael D. Baron). The presumed leader peptides are indicated by italicized characters.

population of cells that expressed the homologous dirigent: the gene produced the expected ~26 kDa glycosylated dirigent, which cross-reacted with *F. intermedia* dirigent polyclonal antibodies (data not shown).

3. Concluding remarks

In this study, it was demonstrated that the biochemical pathway leading to formation of (−)-matairesinol (**5b**) in *L. flavum* parallels that occurring in *F. intermedia*. Moreover, following its formation, this intermediate is hydroxylated at the 7′-position to afford 7′-hydroxymatairesinol (**16**), which is subsequently converted into 5-methoxypodophyllotoxin (**4**). Thus, the biosynthetic pathway to this important lignan involves dirigent mediated coupling to afford (+)-pinoresinol (**6a**), and subsequent metabolism to give podophyllotoxin (**1**) and its derivatives. Additionally, heterologously expressed *P. peltatum* dirigent cross-reacted with *F. intermedia* dirigent antibodies, and thus is expected to also control coniferyl alcohol (**7**) derived coupling to give (+)-pinoresinol (**6a**), the initial step in the biosynthesis of podophyllotoxin (**1**).

4. Experimental

4.1. General procedures

^{1}H NMR spectra were obtained on a Bruker AMX300 spectrometer and chemical shifts are given in δ ppm relative to TMS. Electron impact (EI) mass spectra were recorded on a Waters Integrity HPLC/MS system at an ionization voltage of 70 eV, whereas high resolution mass spectra were obtained on a VG 7070 EHF mass spectrometer. UV spectra and RNA/DNA quantification employed a Lambda 6 UV/VIS spectrophotometer (Perkin-Elmer). Silica gel thin layer and column chromatographic procedures were performed using Kieselgel 60 F_{254} and silica gel 60 (EM Science, 230–400 mesh), respectively, whereas high performance liquid chromatography utilized a Waters HPLC system as described (Davin et al., 1992) using either reversed-phase (Waters, NovaPak C18, 150×3.9 mm inner diameter), or chiral (Chiral Technologies Inc., Chiralcel OD or Chiralcel OC, 240×4.6 mm inner diameter) columns, with detection at 280 nm. Radioactive samples were analyzed in ScintiVerse (Fischer Scientific) and measured using a liquid scintillation counter (Packard, Tricarb 2000 CA).

An Amplitron II Thermolyne thermocycler (Barnstead/Thermolyne Corporation) was used for PCR amplification. Plasmid DNA for probes and for sequencing was purified from *E. coli* host cells using Promega's Wizard™ *Plus* SV Minipreps DNA Purification System. DNA sequences were determined using an Applied Biosystems model 373A automated sequencer.

4.2. Chemicals and enzymes

DEAE cellulose, 2′,5′-ADP Sepharose and almond β-glucosidase were purchased from Sigma. *FisherBiotech* brand of Thermostable *Taq* DNA polymerase was obtained from Fisher Scientific and restriction endonuclease enzymes were purchased from Promega. TOPO TA, pTrcHis2 TOPO TA, and DES TOPO TA cloning kits, TOP10 competent *E. coli* cells, and *Drosophila melanogaster* S2 cells were all obtained from Invitrogen. Radiolabeled nucleotides [α-^{32}P]-dCTP and [α-^{32}P]-dATP were purchased from DuPont NEN. Oligonucleotide primers used for polymerase chain reaction (PCR) experiments and for sequencing were synthesized by Life Technologies, Inc. Purification of PCR fragments was accomplished using low melting point (L.M.P.) agarose from Life Technologies, Inc., followed by enzymatic digestion with Promega's Agar-*ACE*® according to manufacturer's instructions.

4.3. Plant material

L. flavum plants, obtained from Hillview Gardens Products, Kennewick, WA, were maintained in Washington State University greenhouse facilities. *P. peltatum* plants, propagated from rhizomes harvested in Virginia, were cultivated in the same greenhouse facilities.

4.4. Chemical syntheses

4.4.1. 4,4′-Dibenzyl-7-oxo-matairesinol (*14*)

To a soln of thioacetal (**11**) (Katayama et al., 1993) (1.0 g, 2.25 mmol) in dry THF under Ar at −78°C, was added dropwise *n*-BuLi (1.6 M in hexanes, 1.4 ml, 2.24 mmol) over 30 min. The resulting suspension was allowed to stir for 1 h, following which a soln of 2(5*H*)-furanone (0.16 ml, 2.26 mmol) in THF (10 ml) was added dropwise. After stirring for another 60 min, compound (**12**) (Brown and Daugan, 1987) (700 mg, 2.27 mol) in dry THF (10 ml) was subsequently added. After stirring for 1 h, the suspension was allowed to warm to room temp. with EtOAc (100 ml) slowly added. The suspension was washed with H_2O (50 ml) and satd brine (50 ml), then dried (Na_2SO_4), filtered and evaporated to dryness in vacuo to give crude (**13**) (1.8 g). The resulting residue was dissolved in 15% aq THF solution (60 ml), whereupon HgO (2 g) was added followed by dropwise addition of BF_3Et_2O (1 ml). The reaction mixture was allowed to stir for 20 min, following which $CHCl_3$ (120 ml) was added, with the whole washed twice with H_2O (60 ml). The $CHCl_3$ solubles

were dried (Na_2SO_4) and evaporated to dryness with the resulting residue subjected to silica gel column chromatography (30×2 cm) eluted with EtOAc–hexanes (1:2) to give compound (**14**) (420 mg, 34% yield). UV λ_{max} MeOH nm: 314, 280, 230 and 209.; MS *m*/*z* (rel. int.): 552 $[M]^+$ (51), 284 (28), 241 (100), 225 (31), 194 (25), 181 (48), 167 (24), 151 (68), 136 (46), 121 (25), 107 (30). HRMS *m*/*z*: found 552.2123 $[M]^+$, calculated for $C_{34}H_{32}O_7$: 552.2148; ^1H NMR ($CDCl_3$): δ 2.98–3.25 (2H, *m*, H-7), 3.49–3.58 (1H, *m*, H-8), 3.71 (3H, *s*, OMe), 3.90 (3H, *s*, OMe), 4.04–4.16 (2H, *m*, H-9′), 4.30–4.41 (1H, *m*, H-8′), 5.04 (2H, *s*, CH_2), 5.22 (2H, *s*, CH_2), 6.51–7.44 (16H, *m*, Ar-H). ^{13}C NMR (CD_3COCD_3): δ 35.13, 45.48, 47.04, 55.30, 55.55, 68.56, 70.56, 70.80, 110.93, 112.20, 113.46, 114.06, 121.71, 123.20, 127.86, 128.17, 128.50, 128.66, 129.46, 131.07, 136.96. 149.78, 149.92, 153.30 and 177.02.

4.4.2. 7′-Hydroxydibenzyl matairesinol (*15*)

To compound (**14**) (200 mg, 0.36 mmol) in MeOH (20 ml) was slowly added $NaBH_4$ (30 mg, 0.79 mmol). The resulting reaction mixture was stirred for 20 min and acidified to pH 4.0 with 2 N HCl. To this was added EtOAc (100 ml), with the whole then washed with H_2O (40 ml). The organic solubles were next dried (Na_2SO_4), and evaporated to dryness in vacuo. Following reconstitution of the residue in a minimum amount of EtOAc, the resulting solution was applied to a silica gel column (20×2 cm), eluted with EtOAc–hexanes (1:1 and 2:1) to afford (**15**) (190 mg, 95% yield). UV λ_{max} MeOH nm:280, 230 and 209; MS *m*/*z* (rel. int.): 554 $[M]^+$ (5), 465 (4), 243 (42), 227 (50), 225 (41), 181 (40), 153 (74), 137 (100), 131 (61), 105 (39). HRMS *m*/*z*: found 554.2282 $[M]^+$, calculated for $C_{34}H_{34}O_7$: 554.2304; ^1H NMR ($CDCl_3$): δ 2.5–2.66 (1H, *m*, H-8), 2.84–3.09 (3H, *m*, H-7, H-8′), 3.82 (3H, *s*, OMe), 3.84 (3H, *s*, OMe), 3.84–3.96 (2H, *m*, H-9′), 4.31–4.64 (1H, *m*, H-7′), 5.11 (2H, *s*, CH_2), 5.15 (2H, *s*, CH_2), 6.52–7.46 (16H, *m*, Ar-H). ^{13}C NMR (CD_3OD): δ 29.57, 36.33, 43.84, 46.61, 56.25, 71.14, 72.01, 72.10, 74.52, 110.68, 114.20, 114.85, 115.04, 118.88, 122.60, 128.70, 128.72, 129.32, 132.28, 136.91, 138.64, 138.70, 148.07, 148.46, 150.65, 150.70 and 181.05.

4.4.3. 7′-Hydroxymatairesinol (*16*)

To a solution of (**15**) (190 mg, 0.34 mmol) in EtOAc (40 ml), was added 10% Pd/C (100 mg). The resulting suspension was stirred for 1 h at room temp. under an H_2 atmosphere, following which the whole was applied to a short silica gel column (4×2 cm) eluted with EtOAc to remove the Pd/C catalyst. The EtOAc solubles were combined and evaporated to dryness, with the resulting residue reconstituted in a minimum amount of Me_2CO, and applied to a silica gel column (20×2 cm), eluted with EtOAc–hexanes (2:1) to give (**16**) (96 mg, 75% yield). UV λ_{max} MeOH nm: 284, 228 and 202; MS *m*/*z* (rel. int.): 374 $[M]^+$ (23), 177 (20), 153 (100), 137 (54), 125 (17). HRMS *m*/*z*: found 374.1353 $[M]^+$, calculated for $C_{20}H_{22}O_7$: 374.1365; ^1H NMR ($CDCl_3$): δ 2.55–2.65 (1H, *m*, H-8), 2.8–3.03 (3H, *m*, H-7, H-8′β), 3.77 (3H, *s*, OMe), 3.81 (3H, *s*, OMe), 3.86–4.02 (2H, *m*, H-9′), 4.32–4.64 (1H, *m*, H-7′), 5.71 (1H, *s*, OH-Ar), 5.84 (1H, *s*, OH-Ar), 6.54–6.85 (6H, *m*, Ar-H). ^{13}C NMR (CD_3OD): δ 30.65, 35.99, 44.23, 46.46, 56.11, 56.14, 70.60, 74.74, 110.38, 113.80, 115.80, 119.46, 123.10, 130.37, 135.19, 146.10, 146.80, 148.68, 148.85 and 182.22.

4.4.4. [7′-^{3}H]-7-Hydroxymatairesinol (*16*)

To (**14**) (50 mg, 0.09 mmol) in MeOH (5 ml), was slowly added NaB^3H_4 (925 MBq, 7.6 GBq $mmol^{-1}$). The resulting reaction mixture was stirred for 20 min, to which was added unlabeled $NaBH_4$ (5 mg, 0.13 mmol), with the whole stirred for an additional 20 min. The reaction mixture was next acidified to pH 4.0 with 2 N HCl. To this was added EtOAc (25 ml) with the whole then washed with H_2O (10 ml). The organic solubles were dried (Na_2SO_4), and evaporated to dryness in vacuo. Following reconstitution of the residue in a minimum amount of EtOAc, the resulting solution was applied to a silica gel column (10×2 cm), eluted with EtOAc–hexanes (1:1 and 2:1) to afford [7-^{3}H]-(**15**) (48 mg, 120 MBq, 2.5 MBq/mg, 95% yield). To a solution of [7-^{3}H]-(**15**) (48 mg, 120 MBq) in EtOAc (10 ml), was added 10% Pd/C (20 mg). The resulting suspension was then stirred for 1 h at room temp. under an H_2 atmosphere, following which the reaction mixture was applied to a short silica gel column (4×2 cm), eluted with EtOAc. The EtOAc solubles were combined and evaporated to dryness in vacuo. The resulting residue was reconstituted in a minimum amount of Me_2CO, then applied to a silica gel column (10×2 cm), and eluted with EtOAc–hexanes (2:1) to yield [7′-^{3}H]-7′-hydroxymatairesinol (**16**) (14.6 mg, 54 MBq, 3.7 MBq mg^{-1}, yield 45%).

4.4.5. Syntheses of (±)-pinoresinols (*6a*/*6b*), (±)-secoisolariciresinols (*9a*/*9b*), (±)-[9,9′-$^3H_2$) secoisolariciresinols (*9a*/*9b*) and (±)-matairesinols (*5a*/*5b*)

These were prepared as previously reported (Umezawa et al., 1991; Chu et al., 1993; Gang et al., 1999b).

4.5. Crude soluble protein preparation from L. flavum

Whole plant material (300 g) was frozen (liquid N_2), and pulverized in a Waring blender for 20 sec. The resulting powder was homogenized in Tris–HCl buffer (50 mM, pH 7.5) containing 5 mM dithiothreitol (buffer A, 500 ml). The corresponding homogenate was next filtered through four layers of cheesecloth into a beaker containing 10% (wt./vol.) polyvinylpolypyrrolidone. The filtrate was centrifuged (12,000×*g*, 15 min), with the resulting supernatant employed for both enzyme assays and protein purification, respectively.

4.6. Partial purification of L. flavum secoisolariciresinol dehydrogenase

The above crude enzyme preparation (150 mg protein in buffer A) was applied to a DEAE cellulose column (20×2.6 cm) equilibrated in buffer A. After washing the column with buffer A (25 ml), secoisolariciresinol dehydrogenase was eluted with a linear NaCl gradient (0–2 M in 500 ml) in buffer A at a flow rate of 2.5 ml min^{-1}. Active fractions were combined, concentrated via Amicon ultrafiltration (PM10 membranes) to ca 20 ml and dialyzed against Tris–HCl buffer (25 mM, pH 7.5 containing 5 mM DTT, buffer B). The dialyzed enzyme solution (13 mg protein in buffer B) was next applied to a 2′,5′-ADP-Sepharose column (10×1 cm) equilibrated in buffer B. After washing with buffer B, the column was next eluted with 10 mM NAD in buffer B. The active fractions were combined, concentrated to 5 ml, and assayed for matairesinol (**5**) formation.

4.7. Protein and enzyme assays

4.7.1. Pinoresinol/lariciresinol reductase

Pinoresinol/lariciresinol reductase activity was assayed as previously reported (Dinkova-Kostova et al., 1996). In brief, each assay consisted of (±)-pinoresinols (**6a**/**6b**) (5 mM in MeOH, 20 μl), the enzyme extract (100 μl), [4*R*-^{3}H]-NADPH (10 mM, 6.79 kBq $mmol^{-1}$, 20 μl) and Tris–HCl buffer (20 mM, pH 8.0, 110 μl). The enzymatically formed lariciresinol (**8**) and secoisolariciresinol (**9**) were separated by reversed phase HPLC, individually collected, freeze dried and further analyzed using chiral HPLC (Chiralcel OC and OD columns).

4.7.2. Secoisolariciresinol dehydrogenase

*4.7.2.1. Enzymatic formation of [9′-^{3}H]-matairesinol (**5**).* Secoisolariciresinol dehydrogenase activity was assayed by monitoring the formation of [9′-^{3}H]-matairesinol (**5**). Each assay consisted of (±)-[9,9′-$^3H_2$]secoisolariciresinols (**9**) (1.4 nmol in EtOH, 5 μl, 3.4×10^3 Bq, 2.42 GBq $mmol^{-1}$), NAD (4 mM in 0.1 M KPi buffer, pH 7.5, 5 μl) and buffer (50 mM Tris–HCl, pH 8.8, 440 μl). The enzymatic reaction was initiated by addition of the enzyme preparation (50 μl). After 2 h incubation at 30°C with shaking, the mixture was extracted with EtOAc (500 μl) containing unlabeled (±)-matairesinols (**5a/5b**) (3 μg) as radiochemical carriers. After centrifugation (13,800×*g*, 5 min), the EtOAc solubles were removed, and the extraction procedure was repeated with EtOAc (500 μl) but without addition of unlabeled radiochemical carriers. For each assay, the EtOAc solubles were combined, evaporated to dryness in vacuo, reconstituted in MeOH–3% HOAc in H_2O (1:1, 200 μl), with an aliquot (20 μl) subjected to reversed-phase HPLC analysis with the following elution conditions: linear gradient CH_3CN–3% HOAc in H_2O from 10:90 to 30:70 between 0 and 35 min; then to 5:95 in 5 min with this being held at this composition for an additional 5 min, at a flow rate of 1 ml min^{-1}. Fractions corresponding to matairesinol (**5**) were individually collected, with aliquots removed for liquid scintillation counting, and the remainder freeze-dried for chiral HPLC analysis (Chiralcel OD column) as described below.

The optical activity of enzymatically formed [9′-^{3}H]-matairesinol (**5**) was determined by chiral HPLC analysis after its chemical conversion to [9′-^{3}H]-secoisolariciresinol (**9**). The freeze-dried enzymatically formed [9′-^{3}H]-matairesinol (**5**) (0.5 kBq, 0.18 MBq mg^{-1}) purified above was mixed with unlabeled matairesinol (**5**) (0.5 mg) in dry THF (2 ml), and reduced with $LiAlH_4$ (1 mg) for 1 h. The reaction was quenched with HCl (2 N, 20 μl), and the resulting mixture was directly applied to silica gel TLC. The TLC was performed in EtOAc–hexanes–MeOH (10:10:1) with the secoisolariciresinol (**9**) band (R_f 0.25) eluted with EtOAc (3 ml). The EtOAc solubles were evaporated in vacuo to give [9′-^{3}H]-secoisolariciresinol (**9**) (0.25 mg, 0.25 kBq) which was then subjected to chiral HPLC analysis (Chiralcel OD column) as previously described (Dinkova-Kostova et al., 1996).

*4.7.2.2. Enzymatic formation of [Ar-^{2}H]-matairesinol (**5**).* (±)-[Ar-^{2}H]-Secoisolariciresinols (**9a**/**9b**) (5.5 μmol), synthesized as previously described (Umezawa et al., 1991), were incubated with the partially purified secoisolariciresinol dehydrogenase (ca 2 μg protein), Tris–HCl buffer (50 mM, pH 8.8, 10 ml) and NAD (40 μmol). After 2 h incubation at 30°C with shaking, the mixture was extracted twice with EtOAc (2×10 ml). The EtOAc solubles were combined, evaporated to dryness in vacuo, reconstituted in MeOH–3% HOAc in H_2O (1:1, 600 μl) and an aliquot subjected to HPLC analysis. The enzymatically formed matairesinol (**5**) (0.8 mg) was collected, freeze-dried, and further submitted to mass spectral analysis. MS *m*/*z* (rel. int.) 363 (0.6) 362 (2.9), 361 (9.2), 360 ($M^+ + 2$) (15.1), 359 (8.7), 358 (1.7), 139 (51), 138 (benzylic group with one deuterium) (100), 137 (44), 123 (22.3), 122 (9.2).

4.8. Administration of matairesinol (**5**) to L. flavum roots

Root pieces (200 g fresh weight, 2 cm sections) from 5-month old *L. flavum* plants were incubated with a soln of matairesinol (**5**) [0.28 mmol, 100 mg in EtOH–H_2O (1:10, 5.5 ml)] for 6 h. The tissues were then extracted for 2 h with MeOH (2×100 ml) under conditions of reflux. After cooling to room temp., the MeOH solubles were combined and evaporated to dryness, with the residue incubated with β-glucosidase (20 mg, 2.85 units/mg^{-1}) in NaAc–HOAc buffer (100 mM, pH 5.5, 30 ml) for 2 h. The resulting mixture was extracted with EtOAc (2×100 ml), with the organic solubles combined, dried (Na_2SO_4) and evaporated to dryness. The residue so

obtained was reconstituted in a minimum amount of Me_2CO, and applied to a silica gel column (30×2 cm), eluted with EtOAc–hexanes (1:2, 1:1 and 2:1). Fractions eluting after those containing matairesinol (**5**) were combined, evaporated to dryness and further analyzed by HPLC with the elution conditions previously described in Section 4.7.2. The peak corresponding to 7′-hydroxy-matairesinol (**16**) (elution volume: 14.3 ml), formed following administration of matairesinol (**5**) to *L. flavum* roots, was collected, freeze-dried and subjected to mass spectral analysis. MS *m*/*z* (rel. int.):374 $[M]^+$ (40), 177 (16), 153 (100), 137 (62), 125 (18).

4.9. Administration of [7′-^{3}H]-7′-hydroxymatairesinol (*16*) to L. flavum roots

[7′-^{3}H]-7′-Hydroxymatairesinol (**16**) (110 μg, 0.41 MBq, 3.7 MBq mg^{-1}) in EtOH–H_2O (1:12.5, 540 μl) was incubated for 6 h with 2 g of *L. flavum* roots as described above. The resulting tissues were extracted for 2 h with MeOH (2×4 ml) under conditions of reflux. The MeOH solubles were combined, evaporated to dryness, and the residue was next incubated with β-glucosidase (1 mg, 2.85 units mg^{-1}) in NaAc–HOAc buffer (100 mM, pH 5.5, 10 ml) for 2 h. The mixture was extracted with EtOAc (2×20 ml), with the organic solubles combined, dried (Na_2SO_4) and evaporated to dryness. The resulting residue was purified by HPLC as described above. The peak corresponding to 5-methoxypodo-phyllotoxin (**4**) (elution volume: 30.5 ml) was collected, freeze-dried and submitted to HPLC analysis with fractions collected every min for radioactivity determination using liquid scintillation counting.

4.10. Dirigent cloning and heterologous expression

4.10.1. Podophyllum peltatum rhizome cDNA library synthesis

Total RNA was extracted from fresh young rhizome tissues using the LiCl precipitation procedure of Dong and Dunstan (1996). A Promega PolyATtract® mRNA Isolation System was used to isolate poly(A)$^+$ mRNA from the total RNA. A *P. peltatum* rhizome cDNA library, yielding a primary library titer of 6.5×10^5 pfu, was constructed using 5 μg of the mRNA with Stratagene's ZAP-cDNA synthesis kit, Uni-ZAP® XR vector, and Gigapack® III Gold Packaging Extract. The library was amplified once immediately, according to Stratagene's protocol, and gave a final titer of 4.35×10^8 pfu ml^{-1}.

4.10.2. Dirigent DNA probe synthesis

Degenerate oligonucleotide primers used for dirigent PCR screening were designed from the consensus regions of 18 dirigent homologs obtained from 12 plant species (Davin and Lewis, 2000). The primers used for PCR were: 5′-KGTGTTYGAYGAYCCYATTACYBTWGA-CAAC-3′ (= forward primer PS6F) and 5′-GAAAT-AAACATCTCCYTCAWATGMATCRGT-3′ (=reverse primer PS2R), where K = T/G, Y = C/T, B = C/T/G, W = A/T, R = A/G, and M = A/C, S = C/G, and X = A/C/T/G.

To screen for dirigent homologues, 3×10^6 pfu of the *P. peltatum* cDNA library were used as template combined with 2.5 pmol each of PS6F forward and PS2R reverse primers. The PCR experiments were performed in a final volume of 50 μl, containing a Fisher Company buffer supplied with the *Taq* DNA polymerase, which provided a reaction mixture of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% vol/vol Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.0 unit of *Taq* DNA polymerase. Thermocycler settings for PCR amplifications were 35 cycles of 1 min at 96°C, 2 min at 48°C, and 3 min at 70°C, with a 5 min 72°C final extension cycle. PCR products were resolved on 1% low melting point agarose gels. Nucleotide sequence determination of the amplified bands was accomplished by cloning the *AgarACE*®-digested isolated agarose PCR bands into a TOPO TA vector and transforming into competent TOP10 *E. coli* cells according to Invitrogen's instructions. Bacterial colonies were screened for inserts using a PCR screening technique of colony selection and inoculation directly into 25 μl of PCR mix containing M13 Forward and M13 Reverse primers (Invitrogen). These primer sequences border the cloning site of the TOPO TA vector. Clones containing the predicted insert size, determined by the locations of the degenerate primer sequences to consensus regions of known dirigents, were grown in a 4 ml Luria-Bertani (LB) culture medium (Sambrook et al., 1994) containing carbenicillin (100 μg ml^{-1}), processed into plasmid DNA, and used for DNA sequence analysis. One clone containing a sequence homologous to dirigent genes previously isolated from other species was next selected and used as a radiolabeled probe for cDNA library screening. The probe was prepared by excising the desired fragment out of the TOPO TA vector using the *Eco* RI restriction enzyme sites, providing a DNA fragment of 302 bp which included 11 bp of the TOPO vector on each end of the fragment.

4.10.3. cDNA library screening

A total of 3×10^5 pfu of the *P. peltatum* amplified cDNA library was plated with XL1-Blue MRF competent *E. coli* cells for primary screening according to Stratagene's instructions. Phage plaques were lifted onto Magna Nylon 137 mm membranes (Osmonics, Inc.), air dried for at least 10 min, then fixed and denatured on the membranes using a one-step autoclaving procedure for 2 min at 100°C. The membranes containing denatured plaques were next washed by gently shaking for 30 min at 37°C in 6 X standard saline citrate

(SSC) (Sambrook et al., 1994) and then prehybridized for 6 h with gentle shaking at 55°C in a covered crystallization dish containing 300 ml of a hybridization solution consisting of 6 X SSC, 0.5% SDS and 5 X Denhardt's reagent. The probe was prepared by boiling 100 ng of the LMP-purified DNA fragment, previously digested from the TOPO TA vector plasmid preparation, for 10 min and then labeling for 20 min at 37°C using Pharmacia's ^{T7}QuickPrime Kit and [α-^{32}P]-dCTP. The radiolabeled probe was purified from unincorporated nucleotides using a CENTRI·SPIN™-20 spin column (Princeton Separations, Inc) according to the manufacturer's instructions. To the labeled probe was added 0.5 mg sheared salmon sperm DNA (Sigma); the sample was boiled again for 10 min, placed on ice for 5 min, then added to 300 ml of fresh hybridization solution which was preheated in a crystallization dish. The membranes were transferred to this dish and allowed to hybridize at 55°C with gentle shaking. After 24 h, membranes were washed in 4 X SSC, 0.5% SDS for 10–20 min at room temp. At this point, the membranes were washed briefly in 6 X SSC at room temp., individually wrapped with plastic wrap, and exposed to Kodak X-OMAT AR film for 24 h at −80°C in a metal cassette with intensifying screens. Positive plaques from the first screening were isolated through a second screening using hybridization conditions similar to the above.

4.10.4. In vivo excision and sequencing of P. peltatum dirigent-containing phagemids

Purified cDNA clones were rescued from phage using the in vivo excision protocol outlined by Stratagene. Both strands of the excised cDNAs were sequenced completely.

4.10.5. Sequence analysis

DNA and amino acid sequence analyses were performed using the Unix-based GCG Wisconsin Package (Program Manual for the Wisconsin Package, 1997; Rice, 1996). A homology search against the non-redundant peptide database at the National Center for Biotechnology Information was conducted using a BLAST search program (Altschul et al., 1990).

4.10.6. Heterologous expression of dirigent in Drosophila melanogaster cells

Using gene-specific N- and C-terminal primers which included the native start and stop codons, respectively, a PCR product was generated using the pBluescript phagemid vector containing the dirigent clone as a template. The PCR product, which included a putative native secretory signal peptide, was then cloned into Invitrogen's DES TOPO TA vector downstream of the inducible metallothionine promoter. *D. melanogaster* S2 cells were next transfected with the DES TOPO TA dirigent construct, along with the selection vector pCoHYGRO, according to Invitrogen's instructions (*Drosophila* Expression System, Version A). After 4 weeks selection in 300 μg ml^{-1} hygromycin B (GIBCO BRL) in HyQ®SFX-Insect™ Serum-Free Insect Cell Culture Medium (HyClone Company), a small-scale 20 ml preparation of transfected cells was induced to express the heterologous protein with $CuSO_4$ at a final concentration of 1 mM. Cells were harvested 36 h post-induction, centrifuged gently at 600×*g* for 15 min at 4°C to pellet the cells without damage, since this provides a supernatant containing the secreted dirigent while minimizing contamination by internal cytoplasmic proteins. The supernatant was then centrifuged at 15,000×*g* for 30 min at 4°C to remove any remaining debris. Ten micrograms of the final supernatant were used for Western analysis (Burlat et al., 2000) for detection of the secreted dirigent protein.

Acknowledgements

We gratefully acknowledge the National Science Foundation (MCB9976684), the NSF REU Program, and McIntire Stennis for financial support.

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