Stereospecificity of (+)-Pinoresinol and (+)-Lariciresinol Reductases from Forsythia intermedia*

(Received for publication, June 7, 1993, and in revised form, August 26, 1993)

Alex Chu, Albena Dinkova, Laurence B. Davin, Diana L. Bedgar, and Norman G. Lewis†
From the Institute of Biological Chemistry, Washington State University, Pullman, Washington 99163-6340

Pinoresinol/lariciresinol reductase catalyzes the first known example of a highly unusual benzylic ether re-
duction in plants; its mechanism of hydride transfer is
described. The enzyme was found in Forsythia interme-
dia and catalyzes the presumed regulatory branch-
points in the pathway leading to benzylaryl tetrahy-
drofurans, dibenzylbutane, dibenzylbutyrolactones, and
aryltetrahydrodiphenylalkan lignans. Using [7,7'-3H]pi-
noresinol and [7,7'-3H]lariciresinol as substrates, the
hydride transfers of the highly unusual reductases were
demonstrated to be completely stereospecific (>99%).
The incoming hydrides were found to take up the pro-R
position at C-7 (and/or C-7) in lariciresinol and secoiso-
lariciresinol, thereby eliminating the possibility of ran-
don hydride delivery to a planar quinone methide in-
termediate. As might be expected, the mode of hydride
abstraction from NADPH was also stereospecific: using
[4R-3H] and [4S-3H]NADPH, it was found that only the 4
pro-R hydrogen was abstracted for enzymatic hydride
transfer.

Lignans are a structurally diverse family of phenylpropanoid
metabolites found throughout the plant kingdom, principally in
woody gymnosperms and angiosperms; they are most fre-
quently found as dimers (1), although higher oligomers exist
(2). Based on their known properties, various physiological
roles in plants have been proposed. These include antioxidant
(3, 4), bactericidal (5), fungicidal (6), antiviral (7), insect anti-
feedant (8), and cytokinin-like (10) functions. Although direct
evidence is lacking, it has been proposed that lignans are involved in
nification (11). Many lignans exhibit pharmacologically impor-
tant effects: for example, podophyllotoxin (as its etoposide and
arctigenin (9), the furofu-
rans (e.g. pinoresinol 4, medioloxin 5), the arylnaphthalenes
(e.g. chinenin 6) and the aryldihydrodiphenylalkanes (e.g.
podophyllotoxin 7). In terms of biosynthesis, the enzymatic
steps leading to formation of the C4-C9 monomeric units (mono-
lignols) have been firmly established and recently reviewed (17,
18). Surprisingly, the subsequent enzymatic transformations
involved in monomeric coupling and post-coupling modifica-
tions are only now being delineated (16, 19-25). Thus, it has
been established that solinol enzyme preparations from For-
sythia sp. catalyze the formation of (+)-pinoresinol 4a from two
achiral molecules of E-coniaryl alcohol 8 (22). (More recent
studies targeted toward purification of this enzyme have revealed
that O2 is required as a cofactor.)

Once formed, (+)-pinoresinol 4a undergoes highly enantiospecific NADPH-depen-
dent reductions to first afford the benzylaryl tetrahydro-
drofuran lignan, (+)-lariciresinol 9a (24, 25) and then the diben-
zylbutane lignan, (-)-secoisolariciresinol 1b (23-25); to our
knowledge, these are the first examples of benzylic ether re-
ductions in plants. (-)-Secoisolariciresinol 1b can next be ste-
reospecifically oxidized by a NADP+-dependent dehydrogenase to
give the (-) antipode of matairesinol 2 (20, 21), thereby
providing entry into the dibenzylbutyrolactone subgroup (16).

Matairesinol 2 has also been proposed to serve as a precursor of
aryltetrahydrodiphenylalkane lignans, such as podophyllotoxin 7
(26).

Thus, the sequential reductive fission of (+)-pinoresinol 4a to
give (+)-lariciresinol 9a and (-)-secoisolariciresinol 1b permits
a rational and hitherto unexpected entry into the various lig-
nan subgroups and, indeed, may represent key regulatory
points in lignan pathway branching. Moreover, these highly
unusual reductive steps raise intriguing questions regarding
enzyme-catalyzed hydride transfer mechanisms. Using (+)-pi-
noresinol 4a as an example (Fig. 2), three possible hydride
transfer scenarios can be envisaged, resulting in either the
original C-H bond geometry of the benzylic C-7' carbon being
retained, inverted, or undergoing "racemization" as shown.
(Note that this is the case, regardless whether a pentacoordi-
ate S2O or a quinone methide enzyme-bound transition state
is involved.) This report describes delineation of the stereo-
chemical course of these unusual benzylic ether reductions,
affording (+)-lariciresinol 9a and (-)-secoisolariciresinol 1b,
respectively.

EXPERIMENTAL PROCEDURES

Plant Materials

Forsythia intermedia (var. Lynwood Gold) plants, obtained from Bai-
ley's Nursery (St. Paul, MN), were maintained in Washington State
University greenhouse facilities.

†To whom correspondence should be addressed. Tel.: 509-335-2682;
Fax: 509-335-7643.

*This work was supported by National Science Foundation Grant
MCB9219586 and United States Department of Agriculture Grant
91371036638. The costs of publication of this article were defrayed in
part by the payment of page charges. This article must therefore be
hereby marked "advertisement" in accordance with 18 U.S.C. Section
1734 solely to indicate this fact.

1 P Paré, L. B. Davin, D. L. Bedgar, and N. G. Lewis, unpublished
results.
**Stereospecificity of Benzylic Ether Reductases**

1a: (+)-secoisolariciresinol  
1b: (-)-secoisolariciresinol  
2: R = H, matairesinol  
3: R = CH₃, arctigenin  
4a: R = H, (+)-pinoresinol  
4b: R = H, (-)-pinoresinol  
5a: R = OCH₃, (+)-medi resinol  
5b: R = OCH₃, (-)-medi resinol

6: chinensin  
7: podophyllotoxin  
8: E-coniferyl alcohol  
9a: (+)-lariciresinol  
9b: (-)-lariciresinol

R¹ = Ribose-diphosphate-ribose-adenine

**Fig. 1.** Common lignan structural classes, E-coniferyl alcohol, and [³H]NADP⁺/NADPH.

R² = 4-Hydroxy-3-methoxyphenyl

**Fig. 2.** Possible stereochemical consequences during hydride transfer by (+)-pinoresinol reductase from *F. intermedia*. Designations Hₐ and Hₐ refer to pro-R and pro-S, respectively.

Ar = 4-Hydroxy-3-methoxyphenyl

H = C-7/C-7' protons of (+)-pinoresinol and (+)-lariciresinol

(+)-Lariciresinol 9a

**Materials**

Sodium borodeuteride (98 atom % ²H) and MeODH (99.5 atom % ²H) were purchased from Aldrich and deuterated gas (99.8 atom % ²H) from Isotec Inc. n-(1-²H)Glucose (222 GBq mmol⁻¹) was obtained from New England Nuclear. Yeast Glc-6-P dehydrogenase (Type I, 6.2 pkat mg⁻¹ protein) and yeast hexokinase (Type F300, 4.2 pkat mg⁻¹ protein) were purchased from Sigma and dihydrofolate reductase (Lactobacillus casei, 9.3 pkat mg⁻¹ protein) from Biopure Co.

**Instrumentation and Chromatography Materials**

Silica gel thin layer and column chromatography were performed on Kieselgel 60 F₂₅₄ (0.25 mm) and Silica Gel-60 (EM Science, 230–400 mesh), respectively. DEAE-cellulose (fine mesh) was purchased from Sigma, and Affi-Gel Blue Gel (100–200 mesh) from Bio-Rad. All solvents and chemicals used were reagent or HPLC² grade; tetrahydrofuran was distilled over sodium/benzophenone immediately prior to use. ¹H Nuclear magnetic resonance spectra (300 and 500 MHz) were recorded on Bruker AMX300 and Varian VXRS00S spectrometers, respectively, using CDCl₃ as solvent with chemical shifts (δ ppm) reported downfield from tetramethylsilane (internal standard). IR and UV spectra

²The abbreviations used are: HPLC, high performance liquid chromatography; ppm, parts/million; 2D-NOESY, two-dimensional nuclear Overhauser effect; 2D-COSY, two-dimensional correlation spectroscopy.
were obtained on Perkin-Elmer 1720-X infra-red Fourier Transform and Lambda 6 UV/VIS spectrometers, respectively. Mass spectra were recorded on a VG 7070E spectrometer (ionizing voltage 70 eV). All melting points are uncorrected. High performance liquid chromatography was performed as described (23). HPLC separations of lignans were carried out using either reversed-phase (Waters, Nova-pak C18, 150 x 3.9 mm, 3 μm) or silica gel HPLC elution conditions for enzyme reaction mixtures containing secoisolariciresinol 1, lariciresinol 9, and pinoresinol 4 consisting of MeOH/3% AcOH in H2O (30:70) with a flow rate of 0.4 ml min⁻¹. Chiral column HPLC separations of (+)- and (−)-secoisolariciresinol 1a and 1b was achieved using a Chiral OD column eluted with MeOH/SiO₂ (50:50) as eluant, flow rate of 0.8 ml min⁻¹. Analytical HPLC separations, the EtOH was denatured with 2-PrOH (5%, Al₂O₃). The resulting foam (105 mg) was re-crystallized from EtOH/xylene (3:7) suspension stirred for 18 h under N₂. The resulting crystals were filtered (ACRO LC3A disposable filter, 0.45 pm, Gelman Science) prior to recrystallization from EtOH/Me₂CO (1:1) and evaporation under reduced pressure. Recrystallization from EtOH/Me₂CO afforded the amorphous powder. 'H NMR (300 MHz) (C₂DCl₃): δ 8.3 Hz, ArH), 7.72 (1H, dd, J₈.₅,₇.₆ = 8.0 Hz, C₆H), 5.71 (1H, brs, OH), 5.89 (1H, brs, OH), 6.53-6.57 (4H, m, C₆H), 229, 281; MS (m/z): 360 (M⁺, 3.48), 359 (M⁺ + 1, 6.6), 358 (M⁺, 3.0), 345 (1.5), 329 (3.5), 328 (4.8), 235 (2.2), 223 (2.2), 221 (1.9), 207 (1.5), 206 (1.2), 205 (1.4), 154 (27.4), 153 (42.0), 152 (24.9), 151 (100), 138 (50.1), 137 (46.4), 125 (20.8); High resolution MS: calculated for C₉₀H₁₄₂O₆, 360.1542; found, 360.1542.

**Chemical Syntheses**

(a),7,7'-H₃Pinoresinols 4a/4b and (±)-7,7'-H₃Lariciresinols 9a/9b—These were synthesized as described (25).

**Partial Purification of Pinoresinol/Lariciresinol Reductase**

All steps were carried out at 4 °C. F. intermedia stems (20–25 cm long, 100 g) were cut into 1-cm sections, frozen in liquid N₂, and pulverized (Waring Blender). The resulting powder was further homogenized in a Waring blender with potassium phosphate buffer (0.1 M, pH 7.0, 300 ml) containing 10 mM dithiothreitol. The homogenate was filtered through four layers of cheesecloth into a beaker of alcohol (14% (v/v)), and the resulting suspension stirred for 18 h under N₂ at room temperature. The suspension was diluted with diethyl ether (200 ml) and filtered through glass gel (5 g) with the filtrate evaporated to dryness in vacuo (3.2 g). The whole was reconstituted in a minimum amount of EtOAc and applied to a silica gel column (5 x 20 cm) eluted with EtOAc/ethane (1:9). Fractions containing the reaction products were combined and evaporated to dryness in vacuo to afford (±)-7,7'-H₃Lariciresinols 9a/9b (52 mg, 32%) as an amorphous powder.

**(+)-Lariciresinols 9a/9b—These were synthesized as described (25).**

**High pressure liquid chromatography (HPLC)**

Chemical syntheses of (+)-7,7'-H₃Pinoresinols 4a/4b and (±)-7,7'-H₃Lariciresinols 9a/9b were prepared as described (25, 28) from the known diastereomers 13a/13b.(1). Fraction evaporation of the reaction mixture was chromatographed on four prepacked PD-10 columns, (Pharmacia Biotech Inc., Uppsala, Sweden) to afford (±)-7,7'-H₃Lariciresinols 9a/9b (32 mg, 32%) as an amorphous powder.
buffer A. The eluted fraction (14.0 ml) was next applied to an Affi-Gel Blue Gel column (1 x 15 cm) equilibrated in buffer A (29, 30). After rinsing the column (buffer A, 20 ml), pinosinol/lariciresinol reductase was eluted with a NaCl gradient (0.5 - 5 M in 100 ml) in buffer A at a flow rate of 0.2 ml min⁻¹. Proteins were detected at 280 nm and each fraction (1 ml) was assayed for pinosinol and lariciresinol reductase activities (see below). The fractions were monitored by TLC and used as enzyme preparations for stereospecificity studies. Protein contents were determined using the method of Bradford (31) using Bio-Rad dye reagent and bovine γ-globulins as standard.

**Enzyme Assays**

**Pinosinol Reductase**—Pinosinol reductase activity was assayed by monitoring the formation of [1H]lariciresinol 9 and secoisolariciresinol 1. Each assay, conducted in quadruplicate, consisted of (a)-pinosinols 4a/4b (5 mM in MeOH, 40 µl), the partially purified enzyme preparation (200 µl) and buffer (0.1 M, Tris-Cl, pH 8.0, 120 µl). The reaction mixture was incubated with EtOAc (400 ml) containing (α)-lariciresinol 9a/9b (40 μg) and (+)-lariciresinol 1a/1b (40 μg) as radiochemical carriers. After reaction (15,800 × g, 5 min), the EtOAc solutions were removed and the extraction procedure was repeated with EtOAc (400 ml). For each assay, the EtOAc solutions were combined, evaporated to dryness in vacuo, and reconstituted in MeOH/3% AcOH in H₂O (50:50, 100 ml) containing ferulic acid (15 mg, 0.2 ml) and distilled water (0.5 ml). An aliquot (1 ml) from one assay was subjected to reversed-phase HPLC analysis with fractions collected every minute (t = 0-30 min) and analyzed by liquid scintillation counting. The remaining assays were divided into 60-µl aliquots and individually subjected to reversed-phase HPLC, with (α)-lariciresinol 9a/9b and (+)-lariciresinol 1a/1b (as radioactive carriers) eluted coincidentally during the NaCl gradient. Indeed, this was also observed to be the case even when the reductase was incubated with the enzyme preparation under identical conditions as described above to give (−)-[7,7'S-2H₄]secoisolariciresinol 1b as an amorphous powder. H NMR (300 MHz, CDCl₃): δ 6.19 (2H, m, C₉H₄), 3.74 (2H, d, J = 1.7 Hz, C₉H₂), 3.68 (2H, d, J = 1.7 Hz, C₆H₂). A high-resolution MS calculated for C₉H₄O₄H₂₄N₄: 362.1710; found, 362.1706.

**Enzymatic Formation of (−)-[7,7'S-2H₄]Secoisolariciresinol**—The solution containing buffer (20 mM Tris-HCl, pH 8.0, and 5 mM dithiothreitol, 43 ml), NADPH (20 ml in H₂O, 7.6 ml) and (−)-[7,7'S-2H₄]lariciresinol 9a (5.0 mM in MeOH, 1.9 ml), was added the partially purified enzyme preparation (38 ml). After incubating at 30°C with shaking for 1 h, the reaction mixture was extracted with EtOAc (3 × 25 ml). The EtOAc solutions were combined, washed with saturated NaCl (25 ml), dried (Na₂SO₄), and evaporated to dryness in vacuo. The resulting foam was reconstituted in a minimum amount of EtOAc, applied to a silica gel column (0.5 x 7 cm) and eluted with EtOAc/hexanes (1:1). Fractions containing the desired product were combined and evaporated to dryness in vacuo to give (−)[7,7'S-2H₄]secosolariciresinol 1b (1.3 mg, 37.7%), as an amorphous powder. H NMR (300 MHz, CDCl₃): δ 5.83 (2H, m, C₉H₄ + C₆H₂), 2.70 (1H, d, J = 1.7 Hz, C₉H₂), 3.54 (2H, dd, J₁ = 1.7 Hz, J₂ = 11.7 Hz, C₆H₂), 3.73-3.78 (8H, m, 2 × OCH₃ + C₆H₂), 6.56 (2H, d, J = 2.0 Hz, C₉H₂ + C₆H₂), 6.68 (2H, dd, J₁ = 2.0 Hz, J₂ = 8.0 Hz, C₆H₂ + C₆H₂), 6.78 (2H, d, J = 8.0 Hz, C₆H₂ + C₆H₂); MS m/z (%): 365 (M + 3, 15.2), 364 (M + 2, 2.0), 363 (M + 1, 0.7), 362 (M - O₂, 0.3), 347 (0.3), 346 (2.3), 345 (0.4), 344 (1.3), 328 (138.93), 137 (16.3); High resolution MS calculated for C₉H₄O₄H₂₄N₄: 363.1918; found, 363.1932.

**Enzymatic Formation of (−)-[7,7'S-2H₄]Secessolariciresinol**—The solution containing buffer (20 mM Tris-HCl, pH 8.0, and 5 mM dithiothreitol, 43 ml), NADPH (20 ml in H₂O, 7.6 ml) and (−)-[7,7'S-2H₄]lariciresinol 9a/9b (5.2 mM) was incubated with the enzyme preparation under identical conditions as described above to give (−)-[7,7'S-2H₄]secosolariciresinol 1b as an amorphous powder. H NMR (300 MHz, CDCl₃): δ 6.19 (2H, m, C₉H₄ + C₆H₂), 2.70 (2H, d, J₁ = 7.5 Hz, C₉H₂ + C₆H₂), 3.54 (2H, dd, J₁ = 7.5 Hz, C₉H₂), 3.54 (2H, dd, J₂ = 11.7 Hz, C₆H₂), 3.73-3.78 (8H, m, 2 × OCH₃ + C₆H₂), 6.56 (2H, d, J = 2.0 Hz, C₉H₂ + C₆H₂), 6.68 (2H, dd, J₁ = 2.0 Hz, J₂ = 8.0 Hz, C₆H₂ + C₆H₂), 6.78 (2H, d, J = 8.0 Hz, C₆H₂ + C₆H₂); MS m/z (%): 365 (M + 3, 15.2), 364 (M + 2, 2.0), 363 (M + 1, 0.7), 362 (M - O₂, 0.3), 347 (0.3), 346 (2.3), 345 (0.4), 344 (1.3), 328 (138.93), 137 (16.3); High resolution MS calculated for C₉H₄O₄H₂₄N₄: 363.1918; found, 363.1932.

### RESULTS AND DISCUSSION

The stereospecificity of the pinosinol/lariciresinol reductase-catalyzed hydride transfer was investigated from two perspectives, namely (i) the stereochemical consequences of hydride addition during enzymatic product formation and (ii) the mode of hydride abstraction from the NADPH cofactor. In order to investigate these stereochemical questions, (−)-pinosinol (+)-lariciresinol reductase was partially purified (50-fold) by affinity chromatography (Affi-Gel Blue Gel eluted with a NaCl gradient; see "Experimental Procedures"), and used as the enzyme preparation for this study. It should be noted, however, that both pinosinol and lariciresinol reductase activities eluted coincidently during the NaCl gradient. Indeed, this was also observed to be the case even when the reductase was purifed ~1,000-fold via a combination of hydroxyapatite and affinity yellow chromatography.

With partially purified pinosinol/lariciresinol reductase in hand, attention was first directed toward defining the stereospecificity of hydride transfer. As can be seen in Fig. 3A, the native stereochemical change during the reductive cleavage of the furan rings of 4a and 9a is destruction of the chiral centers at C-7/C-7'. But in order to address the stereospecificity of hydride transfer, it was necessary to unabiguously distinguish the C-7/C-7' pro-R and pro-S methylenic protons in the enzymatic products, (+)-lariciresinol 9a and (+)-lariciresinol 1b. This was achieved via a combination of ¹H, 13C, and ²D-NMR spectroscopic experiments aided by molecular modeling. Using (−)-lariciresinol 9a/9b as an example, the ¹H and 13C-NMR spectra revealed that the C-7' methylenic protons were magnetically non-equivalent, with each individually observed as a pair of doublet of doublets at 62.55 (Jᵥᵥ = 10.8 Hz, Jᵥᵥ = 13.5 Hz) and 2.92 (Jᵥᵥ = 5.2 Hz, Jᵥᵥ = 13.5 Hz), respectively (Fig. 4A). Based on the magnitude of the vicinal coupling constants (5.2 and 10.8 Hz) between the C-7' methylenic protons and the adjacent C-8' proton, two possible conformers A and B (as shown by their Newman projections in Fig. 3B) bearing the requisite dihedral angles could be envisioned (32). But inspection of Dreiding molecular models suggests that only conformer A was favorable, since conformer B is apparently more sterically hindered via unfavorable interactions between the aromatic ring and the pendant C-9 hydroxymethyl group (Fig. 3B). That this was indeed the case was...
established by a 2D-NOESY experiment (500 ms mixing time), which reveals correlations between protons spatially located within 5 Å from each other (33). Thus the detection of a cross-peak between the C-2' proton (δ 8.67) of the aromatic ring and the C-9β proton (δ 8.75) established that both protons were in close proximity to one another, as in conformer A (data not shown). By contrast, no cross-peak was observable between the C-2' aromatic proton at δ 8.67 and the C-9 hydroxymethylene protons at δ 3.78 and 3.92, indicating that conformer B was not favored. This result agreed with those obtained from a molecular modeling routine, using the program Macromodel with MM2 force field calculations for energy minimizations. This again revealed unfavorable steric interactions between the aromatic ring and the hydroxymethylene group (C-9) in conformer B (data not shown) which were not evident with conformer A. Thus, based on the results from both 2D-NOESY experiments and energy minimization calculations, A was the preferred conformer (Fig. 3B). With the conformation so established, it was possible to assign the C-7 and C-7' protons in unlabeled (+)-lariciresinol 9a and lariciresinol 9b (data not shown) which were not evident with conformer A. The pro-S and pro-R protons at C-7 were assigned based on observed coupling constants and MM2 force field calculations for energy minimization.

Having established a NMR spectroscopic method to distinguish the pro-S and pro-R protons at C-7' of (+)-lariciresinol 9a and C-7/C-7' of (-)-secoisolariciresinol 1b, attention was directed to defining the stereochemistry of hydride transfer. This presented the new problem of differentiating the incoming hydride from that of the existing C-7 or C-7' proton present in the substrates 4a and 9a. One means to differentiate both protons was to employ selective deuterium labeling, either involving deuteride transfer from NADP+ or via selective deuteration at the C-7 and C-7' sites of pinoresinol 4 and lariciresinol 9; the second option was employed because of readily available synthetic methodology (25, 28). Thus, β-ketolactones 13a/13b were obtained from the known diastereomeric alcohols 12a/12b following treatment with pyridinium chlorochromate; these were then reduced with sodium borodeuteride to give the deuterated diols 14a/14b (Fig. 5). Subsequent LiAlH₄ reduction afforded tetrals 15a/15b, which were treated with bromotrimethylsilane to give a ~4:1 mixture of (±)-O,O'-dibenzyl-[7,7'-(2H₂)₂]pinoresinols 16a/16b and (±)-O,O'-dibenzyl-[7,7'-(2H₂)₂]epipinoresinols (25). Debenzylation via hydrogenation over Pd-C afforded the required (±)-[7,7'-(2H₂)₂]pinoresinols 4a/4b. Subsequent comparison of the ¹H NMR and mass spectra of deuterated 4a/4b with its unlabeled analogue unequivocally established the positions of deuteration within the molecule. Thus, both ¹H NMR spectra were essentially identical, except for the absence of a 2H doublet at δ 8.74 in the deuterated 4a/4b sample; this resonance had previously been assigned to the C-7/C-7' protons in unlabeled (±)-pinoresinols 4a/4b (25, 28). Analysis of the mass spectra of both deuterated and unlabeled (±)-pinoresinols 4a/4b confirmed and extended the ¹H NMR findings. With unlabeled (±)-pinoresinols 4a/4b, a molecular ion at m/z 358 was evident together with ion fragments at m/z 206, 152, and 137 corresponding to [ArCHO]+, [ArCHO]-, and [ArCH₂]+, respectively, where Ar = 4-hydroxy-3-methoxyphenyl (25). By contrast, the deuterated analogue had a molecular ion at m/z 360, corresponding to the presence of two deuterium atoms, with ion fragments at m/z 207, 153, and 139.
and 138 due to [M-ArC=CH+, [ArC=CH]+, and [ArCH2H]+.

Thus, it was unequivocally established that the (d-pinore- 
sinol), as before. By comparison, the deuterated analogue had a

deuteration over 10% Pd-C. Comparison of the 'H NMR

corresponding to the C-8 pro-S proton is observed, whereas the C-7' pro-S proton signal at 82.92 is essentially absent (>99% reduction).

Note also that the C-7' pro-R proton resonance at 82.51 is only a doublet (J = 10.6 Hz) due to the absence of geminal coupling and that its chemical shift is moved upfield by 0.04 ppm; this shift is consistent with the reported shielding effects caused by replacing a proton with deuterium in a methylene or methyl group (34). Mass spectroscopic analyses of the enzymatic product further supported this finding; the enzymatically formed deuterated lariciresinol 9a had a molecular ion at (m/z) 362, corresponding to the presence of two deuterium atoms. That these deuterium atoms remained at C-7 and C-7' was established from the ion fragmentation pattern; thus, the enzymatic (deuterated) product 9 gave ion fragments at (m/z) 237, 153, and 138 corresponding to [M-ArH]+, [ArC=CH]+, and [ArCH2H]+ where Ar = 4-hydroxy-3-methoxyphenyl. Chiral HPLC column analysis of the deuterated product revealed that only (+)-lariciresinol 9a was formed, in accordance with the known enantiomeric specificity of this transformation (25). Thus, it was concluded that the reductase-catalyzed hydride transfer was >99% stereospecific, where the incoming hydride exclusively took up the pro-R position with the existing carbon-deuterium bond geometry at C-7' undergoing inversion to assume the pro-S position, i.e. the enzymatic product is (+)-[7,7'S-2H2]lariciresinol 9a.

The possibility that lariciresinol 9 reduction proceeded in an analogous manner was next investigated. Thus, (+)-[7,7'-2H3]lariciresinol 9a/9b (0.11 mM) were incubated for 1 h at 30 °C with the partially purified reductase in the absence of 1.7 mM NADPH. As before, the secoisolariciresinol 1 formed was purified, with its 'H NMR and mass spectra and chiral HPLC profiles subsequently recorded and compared to authentic unlabeled (+)-secoisolariciresinol 1a/1b. The 'H NMR spectroscopic findings are shown in Fig. 6, A and B, where only the pertinent regions of the 'H NMR spectra corresponding to the C-7' pro-R, C-7' pro-S, and C-7 pro-S, pro-R, and C-7 pro-S, respectively, in unlabeled (±)-secoisolariciresinol 1a/1b (Fig. 6A). Thus, given the small expected upfield proton resonance shift of ~0.04–0.07 ppm due to deuterium substitution (34), it can again be proposed that hydride transfer is stereospecific (>99%) resulting in replacement of the C-7 pro-S proton by deuterium. The mass spectrum of the enzymatically synthesized (deuterated) secoisolariciresinol 1 confirmed the presence of three deuterium atoms, as evidenced by the molecular ion at (m/z) 365. That these deuterium atoms remained at C-7 and C-7' positions was again established from the ion fragmentation patterns with ion fragments (m/z) at 139 and 138 corresponding to [ArC=CH]+ and [ArCH2H]+, respectively, where Ar = 4-hydroxy-3-methoxyphenyl. Chiral HPLC analysis of the deuterated product revealed that only (±)-secoisolariciresinol 1b synthesis had occurred, again in accordance with the known enantiomeric specificity of the conversion (24, 25).

Thus, as for (+)-[7,7'S-2H2]pinoresinol 4a reduction, the conversion of (+)-[7,7'S-2H2]lariciresinol 9a into (±)-[7,7'S-2H2] secoisolariciresinol 1b was stereospecific. But, in the latter case, a degree of uncertainty remained regarding the assignment of the 62.70 resonance to the C-7' pro-R proton, since it relied exclusively upon a 0.06 ppm upfield chemical shift from...
Stereospecificity of Benzylic Ether Reductases

PCC = Pyridinium chlorochromate
BrTMSi = Bromotrimethylsilane

Fig. 5. Chemical synthesis of (+)-[7,7'-2H2]pinoresinols 4a/4b and (+)-[7,7'-2H2]lariciresinols 9a/9b.

The enzymatically synthesized, deuterated, (-)-secoisolariciresinol 1b formed was purified and subjected to 1H NMR and mass spectral analyses. As can be seen in Fig. 6C, a 2H doublet at 82.70 (J = 7.5 Hz) was again evident in the 1H NMR spectrum. Since it had already been established that the hydride transferred during conversion of (+)-pinoresinol 4a into (+)-lariciresinol 9a becomes the C-7 pro-R proton, and that the 1H NMR spectrum of (-)-deuterated 1b shows only a single 2H doublet, it follows that its subsequent conversion into (-)-secoisolariciresinol 1b has proceeded in an analogous manner, i.e. in both reduction steps, the incoming hydride takes up the pro-R position.

Lastly, it was also instructive to determine whether the 4 pro-R or 4 pro-S hydrogen on the nicotinamide ring of NADPH was abstracted during hydride transfer. [4R-3H] and [4S-3H]NADPH 10b and 10a were conveniently prepared by modification of the procedure by Moran et al. (27). Each tritiated cofactor was individually incubated for 1 h at 30 °C with the partially purified reductases in the presence of (a) 0.5 mM (+)-pinoresinols 4a/4b and (b) 0.5 mM (+)-lariciresinols 9a/9b, respectively. (Note that following incubation, unlabeled (*)-lariciresinols 9d9b (40 pg) and (+)-secoisolariciresinols 1d1b (40 pg) were added as radiochemical carriers, with each lignan individually purified and resolved by chiral column HPLC.) The results obtained are summarized in Table 1. With (+)-pinoresinols 4a/4b as substrates, radiolabeled lariciresinol 9 was only formed (1.37 pkat mg⁻¹ protein) when [4R-3H]NADPH 10b was employed as cofactor. Moreover, chiral column HPLC analysis again revealed that only the (+)-enantiomer 9a was radiolabeled. The reduction of lariciresinol 9 to (-)-secoisolariciresinol 1b proceeded in an analogous manner: radiolabeled secoisolariciresinol 1 was only synthesized (2.19 pkat mg⁻¹ protein) when [4R-3H]NADPH 10b was added as a cofactor; chiral
column HPLC chromatographic analysis established that only the (−)-antipode 1b was enzymatically formed. Thus, both (+)-pinosinol and (+)-lariciresinol reductases abstract the 4 pro-R hydrogen from NADPH. This mode of hydride abstraction is comparable in action to cinnamyl alcohol dehydrogenases (EC 1.1.1.195) from Forsythia (35, 36) and soybean (Glycine max) (37), which catalyze the reduction of E-coniferaldehyde to E-coniferyl alcohol 8. But this mode of hydride abstraction is opposite to that of the preceding step in monolignol synthesis affording E-coniferaldehyde, i.e. p-hydroxycinnamoyl CoA: NADP+ oxidoreductase (EC 1.2.1.44) which catalyzes abstraction of the 4 pro-S hydrogen in Forsythia and soybean (G. max) during E-coniferaldehyde synthesis (38).

In summary, the (+)-pinosinol and (+)-lariciresinol reductases function in a highly stereospecific manner. Both abstract the 4 pro-R hydrogen of NADPH, in such a manner that the incoming hydride adopts the pro-R position (>99%) at C-7′,C-7′′ in the lignan product. These findings, therefore, rule out the possibility of a S_{s}1 mechanism involving a planar transition state with random hydride delivery from either side of the molecule (Fig. 2). The next phases of our research will be to establish whether the enzyme-bound transition state is in either the furano or quinone methide form, and the regulatory roles of these enzymes in lignin biosynthesis.

Acknowledgments—We thank Drs. Jaroslav Zajicek (Washington State University NMR Spectroscopy Center) for obtaining the 2D-NOESY spectrum of lariciresinol, William Siems for recording the mass spectroscopy spectra and Susan Johns (Washington State University Visualization Analysis and Design in Molecular Sciences Customer Services) for performing MM2 calculations.

REFERENCES

1. Ayres, D. C., and Leike, J. D. (1990) Lignans: Chemical, Biological and Clinical Properties, 402 pages Cambridge University Press, Cambridge, United Kingdom
2. Sakakibara, A., Sasaya, T., Miki, K., and Takahashi, H. (1987) Holzforschung 41, 1-11
3. Faure, M., Lissi, E., Torres, R., and Videla, L. A. (1990) Phytochemistry 29, 3773-3775
4. Osaawa, T., Nagata, M., Namiki, M., and Fukuda, Y. (1985) Agric. Biol. Chem. 49, 3351-3352
5. Hattori, M., Hads, S., Watahiki, A., Ibara, H., Shu, Y.-Z., Kakiuchi, N., Minuno, T., and Namba, T. (1998) Chem. Pharm. Bull. 46, 3868-3893
6. Belmares, H., Berrera, A., Castillo, E., Ramos, L. F., Hernandez, F., and Hernandez, V. (1979) Ind. Eng. Chem. Prod. Res. Dev. 18, 220-226
7. Markkanen, T., Makinen, M. L., Maunukela, E., and Himanen, P. (1981) Drug Exp. Clin. Res. 7, 711-718
8. Havaux, J., and Navrot, J. (1984) Biochim. Biophys. Acta 791, 95-98
9. Elakovitch, S., and Stenvena, K. L. (1986) J. Chem. Soc. 11, 27-33
10. Bionte, A. N., Chen, R. H., Wood, H. N., and Lynn, D. G. (1987) Proc. Natl. Acad. S. A. 84, 980-984
11. Rahman, M. A., Dewick, P. M., Jackson, D. E., and Lucas, J. A. (1990) Phytochemistry 29, 1841-1846
12. Adlerecrutz, H. (1984) Gastroentrology 98, 761-764
13. Adlerecrutz, H. (1991) in Nutrition, Toxicity and Cancer (Rowland, I. R., ed.). pp. 137-195 CRC Press, Boca Raton, FL
14. Borsello, S. F., Setchell, K. D. R., Axelton, M., and Lawson, A. M. (1985) J. Appl. Bacteriol. 68, 37-43
15. Schroeder, H. C., Herr, U., Stenvena, W. E. G., Sarin, P. S., Trumon, S., Schultz, J., and Eich, E. (1990) Z. Naturforsch. 45c, 1221-1225
16. Osawa, S., Davin, L. B., and Lewis, N. G. (1990) Phytochemistry 38, 643-652
17. Lewis, N. G., and Yamamoto, E. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 455-496
18. Davin, L. B., and Lewis, N. G. (1992) Rev. Adv. Phytochem. 28, 335-375
19. Umezawa, T., Davin, L. B., Yamaomoto, E., Kingston, D. G. I., and Lewis, N. G. (1990) J. Chem. Soc. Chem. Commun. 1406-1408
20. Umezawa, T., Davin, L. B., and Lewis, N. G. (1990) Biochem. Biophys. Res. Commun. 171, 1009-1014
21. Umezawa, T., Davin, L. B., and Lewis, N. G. (1991) J. Biol. Chem. 266, 10210-10217
22. Davin, L. B., Bedgar, D. L., Katayama, T., and Lewis, N. G. (1992) Phytochemistry 31, 3869-3874
23. Katayama, T., Davin, L. B., and Lewis, N. G. (1992) Phytochemistry 31, 3875-3881
24. Lewis, N. G., Davin, L. B., Katayama, T., and Bedgar, D. L. (1992) Bull. Liaison Groupe Polycéphales 16, 98-103
25. Katayama, T., Davin, L. B., Chu, A., and Lewis, N. G. (1995) Phytochemistry 40, 1791-1795
26. Kaml, W. M., and Dewick, P. M. (1986) Phytochemistry 25, 2093-2102
27. Moren, R. G., Sartori, P., and Reicher, Y. (1984) Anal. Biochem. 138, 196-204
28. Fujimoto, H., Nakatsuko, F., and Higuchi, T. (1982) Biochem. Biophys. Res. Commun. 102, 555-562
29. Bielmann, J.-F., Samama, J.-P., Branden, C. I., and Eklund, H. (1979) Eur. J. Biochem. 102, 107-110
30. Prochaska, H., and Tsalad, P. (1984) J. Biol. Chem. 261, 1372-1376
31. Bradford, M. M. (1976) Anal. Chem. 47, 248-254
32. Slessor, K. N., and Tracey A. S. (1971) Can. J. Chem. 49, 2074-2084
33. Kumar, A., Ernst, R. R., and Wuthrich, K. (1980) Biochim. Biophys. Res. Commun. 86, 1-6
34. Bovey, F. A. (1969) Nuclear Magnetic Resonance Spectroscopy, p. 86, Academic Press, New York
35. Massel, B. L., Gross, G. G., Stockigt, J., Franke, H., and Zenk, M. H. (1974) Phytochemistry 13, 2427-2435
36. Kischies, M., Stockigt, J., and Zenk, M. H. (1978) Phytochemistry 17, 1523-1525
37. Grisebach, H., Wenzgennayer, H., and Wyrambik, D. (1977) in Pyridine Nucleotide Dependent Dehydrogenases (Sund, H., ed.), pp. 458-471, de Gruyter, Berlin.
38. Grisebach, H. (1981) in The Biochemistry of Plants: A Comprehensive Treatise (Conn, E. E., ed.) Vol. 7, pp. 457-478, Academic Press, New York