Pergamon

Tetrahedron Letters, Vol. 35, No. 27, pp. 4731-4734, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)00921-X

(+)-Pinoresinol Synthase: A Stereoselective Oxidase Catalysing 8,8'-Lignan Formation in *Forsythia intermedia*

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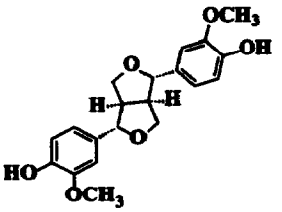
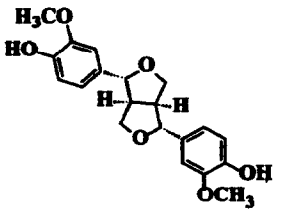
Key Words: Stereoselective coupling, oxidase, (+)-pinoresinol synthase, lignans.

Abstract: 8,8'-Lignan skeletal formation is engendered by (+)-pinoresinol synthase, an oxidase catalysing the stereoselective coupling of two achiral *E*-coniferyl alcohols to give (+)-pinoresinol in >97% enantiomeric excess.

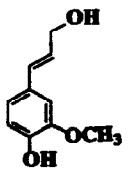
Lignans are a diverse class of phenylpropanoids most frequently found as optically active 8,8'-linked dimers[1, 2] although higher oligomers have been reported.[3, 4] Certain representatives are involved in lignin synthesis, e.g., pinoresinol **1**,[5] whereas others have potent antioxidant, e.g., sesaminol,[6] allelopathic, e.g., nordihydroguaiaretic acid,[7] antimicrobial, e.g., polygamain,[8] and insecticidal, e.g., haedoxan,[9] properties, strongly suggesting a major role in plant defense.



(+)-Pinoresinol **1a**



(-)-Pinoresinol **1b**



Coniferyl alcohol **2**

The mechanism of formation of optically pure lignans has long been enigmatic[10] given the fact that all previously described enzymatically catalysed phenolic coupling steps (i.e., with O_2-laccase or H_2O_2-peroxidase) afford only racemic products[11, 12] e.g., (±)-pinoresinols **1a/1b** from *E*-coniferyl alcohol **2**.

By contrast, the presence of optically pure lignans in the plant kingdom[1] raised the intriguing possibility that an hitherto uncharacterised mode of stereoselective phenylpropanoid coupling was operative.[2] Preliminary evidence in support of this contention had been obtained when we established that *F. suspensa* "cell-wall residues", following removal of readily soluble proteins, catalysed the conversion of *E*-[8-^{14}C]coniferyl alcohol **2** into [8,8'-^{14}C]pinoresinol **1** without addition of exogenously supplied co-factors.[13] Significantly, the (+)-antipode **1a** was apparently preferentially formed (65:35) over its (-)-enantiomer **1b**, and time-course studies implied that two distinct coupling enzymes, one stereoselective and the other non-stereoselective, were present; the non-stereoselective enzyme has since been dissolved and purified to apparent homogeneity and found to be a laccase (Mw ~ 100,000).

In this report, we describe the discovery of (+)-pinoresinol synthase, the first example of a stereoselective phenylpropanoid coupling oxidase in lignan formation, and which has been dissolved from *F. intermedia* "cell wall residues". (+)-Pinoresinol synthase assays were carried out by incubation of [9-^{3}H]coniferyl alcohol **2** with the partially purified enzyme preparation at pH 5.[14] At the end of each assay, unlabelled (+)- and (-)-pinoresinols **1a/1b** (7.5 μg) were added to the incubation mixture as radiochemical carrier. This was then purified by reversed phase HPLC, and subsequent chiral HPLC analyses revealed that essentially only (+)-[9,9'-^{3}H] pinoresinol **1a** (>97% enantiomeric excess) was formed, as evidenced by coincidence of radiochemical and UV absorbance elution profiles (Fig. 1A).

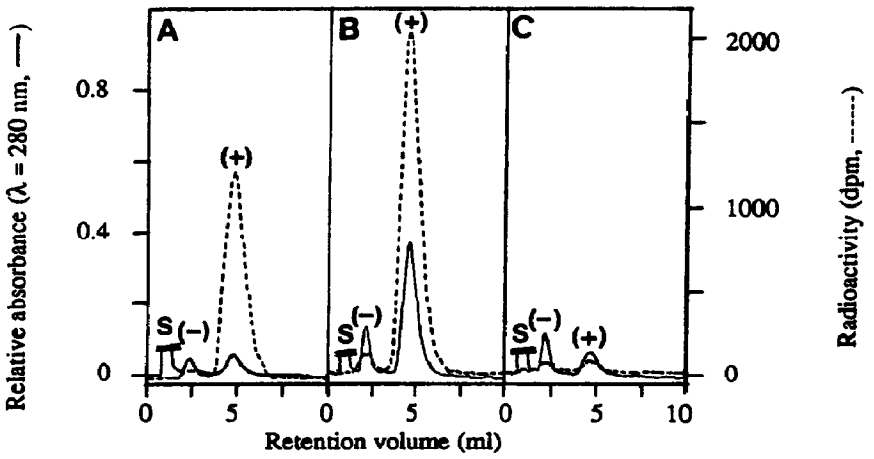


Fig. 1. (+)-Pinoresinol synthase catalysed stereoselective coupling of *E*-[9-^{3}H]coniferyl alcohol **2** into (+)-[9,9'-^{3}H]-pinoresinol **1a**; A) at 30°C and B) at 21°C in air and C) at 21°C under argon. (—) UV trace of unlabelled carriers **1a/1b** designated (+) and (-); (----) radiochemical trace; S = solvent.

To confirm and extend these observations, deuterated [9-$^2H_2$,OC^2H_3]coniferyl alcohol **2** was next prepared[15] as substrate, and incubated with partially purified (+)-pinoresinol synthase. The enzymatically synthesized (+)-[9,9'-$^2H_2$,OC^2H_3]pinoresinol **1a** obtained was recovered and subjected to mass spectroscopic analysis giving a molecular ion [M^++10] at m/z 368, with a base peak fragment ion at m/z 154 corresponding to [ArCO]$^+$ where Ar = 4-hydroxy-3-OC^2H_3 phenyl. This established that ten deuterium atoms had been introduced into (+)-pinoresinol **1a** and, hence, unequivocally demonstrated the authenticity of the enzyme catalysed conversion (Fig. 2A). The mass spectrum of unlabelled (±)-pinoresinols **1a/1b** is included for comparison purposes (Fig. 2B).

Having established the integrity of the enzymatic transformation, a time-course analysis of the reaction was carried out, this being *approximately* linear over the first hour and reaching a maximum (plateau) level by *ca* 2 hr. Assays were next conducted at various pH values (ranging from pH 3.0 to 8.0), and revealed an optimum from pH 4.4 to 5.0. The temperature dependence of the reaction was also investigated with maximum activity at 30°C being observed. An oxygen requirement was determined by conducting assays at 21°C for 30 min, at pH 5.0, under argon; this was achieved by freeze-thawing (2X) the partially purified protein in a Warburg flask

under high vacuum (5 torr) and replacing the atmosphere with argon. The assay, initiated by mixing the [9-^{3}H]coniferyl alcohol 2 with the degassed enzyme/buffer solution, was carried out as previously described.[14] For comparison purposes, controls were carried out exactly as above but in an atmosphere of air. As before, unlabelled (±)-pinoresinols **1a/1b** (7.5 μg) were added as radiochemical carrier to the assay mixture, with the pinoresinol 1 isolated by reversed-phase HPLC and subjected to chiral HPLC analyses. The results are shown in Figs. 1B and 1C, respectively. As can be seen in Fig. 1C, when the assays were carried out under argon, the radiochemical elution profile revealed that formation of (+)-[9,9'-^{3}H]-pinoresinol **1a** was suppressed (>96%) thereby confirming the requirement for O_2 (cf. Fig. 1B).

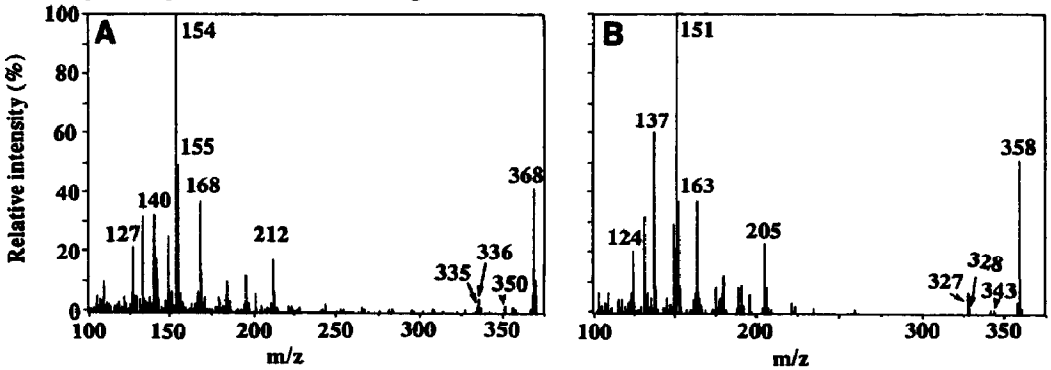


Fig. 2. Electron impact mass spectrum of A) (+)-[9,9'-$^2H_2$, OC^2H_3]pinoresinol **1a** following incubation of *E*-[9-$^2H_2$, OC^2H_3]coniferyl alcohol **2** with (+)-pinoresinol synthase and B) unlabelled (±)-pinoresinols **1a/1b**; fragmentation pattern interpretation of **1a** has been described elsewhere.[15]

Our current working hypothesis for the mode of action of this unusual stereoselective oxidase is as follows. Two molecules of *E*-coniferyl alcohol **2** are bound to the enzyme active site, with each undergoing one-electron oxidation as shown (Fig. 3). The resulting enzyme-bound intermediates are then aligned in such a way as to permit *only* 8,8'-coupling. Although, there are four possible orientations for coupling [i.e., where the reactive species approach each other from their *re-re*, *re-si*, *si-re* or *si-si* faces], only coupling between the *si-si* faces gives the required [8*R*, 8'*R*]pinoresinol **1a** chirality.

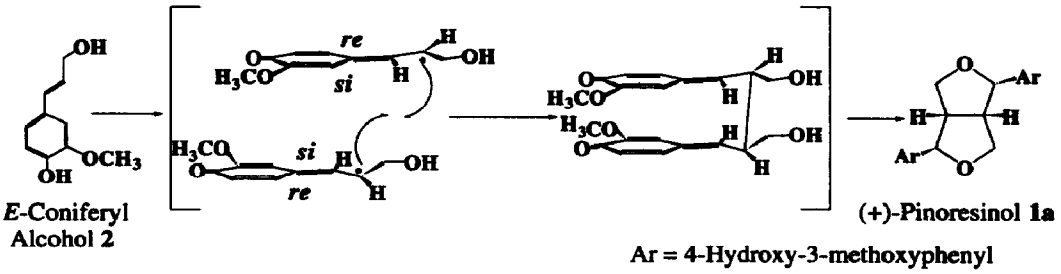
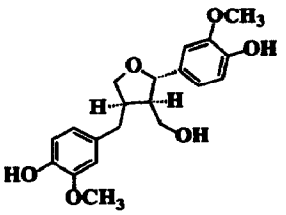
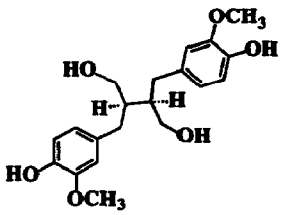


Fig. 3. Proposed mechanism of (+)-pinoresinol synthase showing *si-si* coupling.

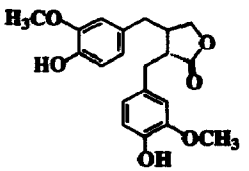
Previous studies in our laboratory established that in *Forsythia intermedia*, the furofuran lignan, (+)-pinoresinol **1a**, undergoes sequential enantioselective NAD(P)H-dependent benzylic ether reductions[15, 16] [via transfer of the 4-pro*R* H of NAD(P)H] to afford (+)-lariciresinol **3** and then (-)-secoisolariciresinol **4**,[17] with dehydrogenation of the latter giving (-)-matairesinol **5**.[18, 19] Thus (+)-pinoresinol **1a** formation serves as a key entry point to the lignans, the metabolism of which apparently gives rise to the majority of 8,8'-linked lignan skeleta encountered in the plant kingdom, e.g., the furofurans, dibenzylfurans, dibenzylbutanes and dibenzylbutyrolactones. The precise nature of this hitherto uncharacterised oxidase, and its mechanism of stereoselective coupling, will be the subject of future study.



(+)-Lariciresinol **3**



(-)-Secoisolariciresinol **4**



(-)-Matairesinol **5**

The authors wish to thank the National Science Foundation (MCB9219586) for financial support.

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(*Received in USA* 6 *April* 1994; *revised* 4 *May* 1994; *accepted* 6 *May* 1994)