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THE *E*/*Z* ISOMERIZATION STEP IN THE BIOSYNTHESIS OF *Z*-CONIFERYL ALCOHOL IN *FAGUS GRANDIFOLIA*

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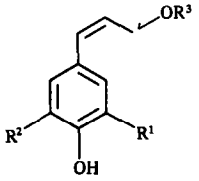
Key Word Index—*Fagus grandifolia*; Fagaceae; American beech; biosynthesis; *cis*/*trans* monolignols; lignin; *p*-hydroxycinnamyl alcohols.

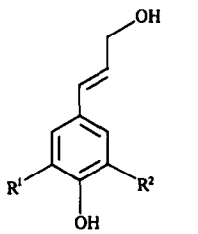
Abstract—Beech bark contains significant amounts of *Z*-coniferyl and *Z*-sinapyl alcohols but not the corresponding *E*-isomers. There are a number of steps along the cinnamate pathway where *E*/*Z* isomerism of the cinnamyl double bond could occur. Studies of the conversion, in beech bark, of ^{14}C-labelled *E*- and *Z*-ferulic acids, *E*-coniferylaldehyde and *E*- and *Z*-coniferyl alcohols indicate that this isomerism occurs at the level of the hydroxycinnamyl alcohols. This system differs, therefore, from that described for the isomerism of geraniol to nerol which proceeds via the corresponding aldehyde.

INTRODUCTION

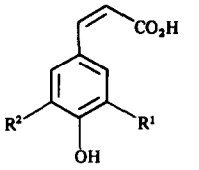
Methanol extracts of beech bark (*Fagus grandifolia Ehrh*) contain significant amounts of *Z* or *cis* monolignols **2** and **3**, but not the corresponding *E* or *trans* isomers **6** and **7** [1]. *Z*-Coniferyl alcohol (**2**), and its β-D-glucoside, faguside (**4**), have also been isolated from European beech (*F. sylvatica*) [2]. These are the only reported examples of naturally occurring *Z*-hydroxycinnamyl alcohols, although the existence of the related *Z*-acids **8**–**10** in grasses is well documented [3–6].



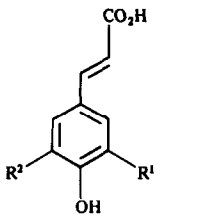
1 R^1, R^2, R^3 = H
2 R^1 = OMe, R^2, R^3 = H
3 R^1, R^2 = OMe, R^3 = H
4 R^1 = OMe, R^2 = H, R^3 = Glu



5 R^1, R^2 = H
6 R^1 = OMe, R^2 = H
7 R^1, R^2 = OMe



8 R^1, R^2 = H
9 R^1 = OMe, R^2 = H
10 R^1, R^2 = OMe



11 R^1, R = H
12 R^1 = OMe, R^2 = H
13 R^1, R^2 = OMe

The presence of these *Z*-alcohols pose interesting questions about their biosynthetic origin and possible role in lignification. Stereospecific enzymic deamination of phenylalanine [7, 8] and tyrosine [9], with concomitant loss of the pro-3-*S* hydrogen gives *E*-cinnamic or *E*-*p*-coumaric (**11**) acids respectively. In the pathway from phenylalanine, *E*-cinnamic acid is hydroxylated to give *E*-*p*-coumaric acid (**11**), this has been demonstrated with parsley cell cultures [10]. Hydroxylation and methylation of *E*-*p*-coumaric acid (**11**) give first *E*-ferulic acid (**12**), and subsequently *E*-sinapic acid (**13**) depending upon the plant species [11].

In grasses, grown in the presence of light, hydroxycinnamic acids, mainly cell wall bound, exist as mixtures of *E* and *Z* isomers, with the former predominating [4, 6]. The role of these acids is not well understood, although it has been postulated that they could be involved in the lignification process [12–14]. Experimental verification of this hypothesis has never been demonstrated. The reduction of *E*-acids **11**–**13** in various plant species occurs by activation of the acids via their AMP and CoA esters, followed by a two-step stereospecific reduction to the *E*-alcohols **5**–**7** [15].

In this regard, with soybean suspension cultures, it was demonstrated that *p*-coumarate:CoA ligase preferentially takes up the *E*-acid **11** as a substrate [16].

Our aim, in this study, was to identify at which step, in its biosynthesis from ferulic acid, *Z*-coniferyl alcohol (**2**) arises. It can be envisaged that *E*/*Z* isomerization could occur at the acid, aldehyde or alcohol stages in the hydroxycinnamate pathway.

RESULTS AND DISCUSSION

The biogenesis of bark lignin has not received much attention, although the lignin content of bark is

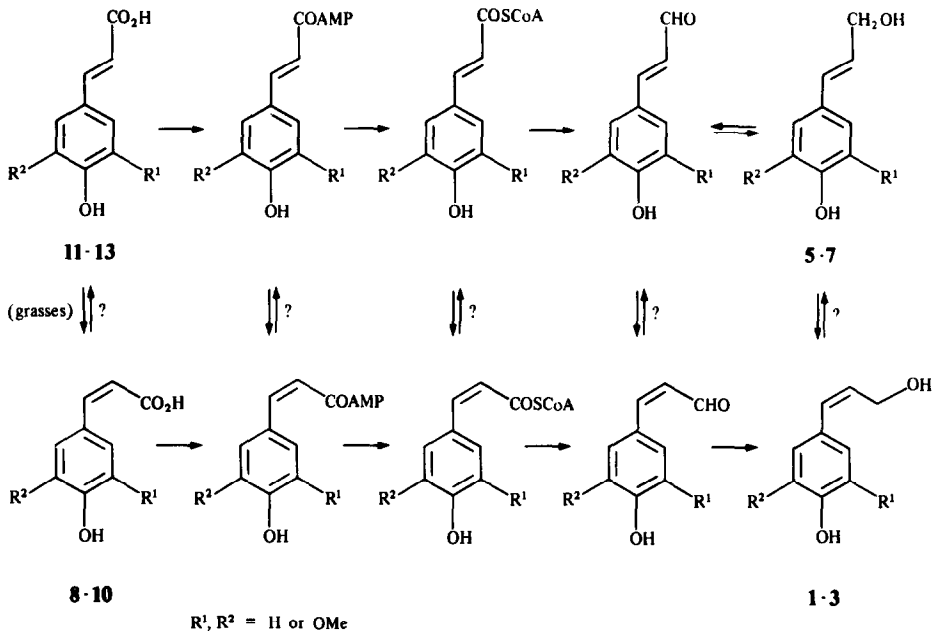


Fig. 1. Possible points of isomerization in the biosynthesis of Z-monolignols.

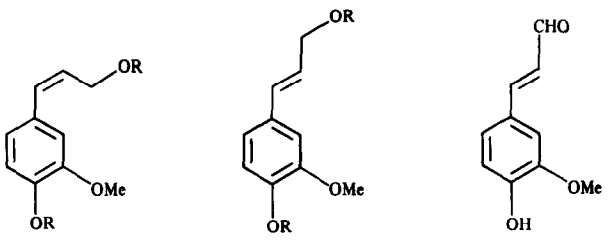
generally higher than that of woody tissue [17].

The upper portion of Fig. 1 shows the established biosynthetic route leading to the *E*-monolignols **5–7**. To account for the formation of the corresponding *Z*-isomers **1–3**, an isomerization could occur at any stage beyond hydroxycinnamic acid formation; the lower portion of Fig. 1 illustrates possible points of isomerization.

We first decided to examine the precursor relationship of both *Z*- and *E*-ferulic acids (**9** and **12**) in the biogenesis of *Z*-coniferyl alcohol (**2**) in beech bark. *E*-[2-^{14}C] Ferulic acid (**12**), prepared via base-catalysed condensation of vanillin and [2-^{14}C] malonic acid [18], was subjected to photochemically induced isomerization to give a mixture of [2-^{14}C] *E* and *Z* acids **12/9** [6]. The *Z* acid **9** was purified by chromatography of the mixture on cellulose plates in the absence of light. It should be noted, however, that all samples of the purified *Z* acid **9** used in these studies always contained about 2–3% of the corresponding *E* isomer **12** as determined by high performance liquid chromatography (HPLC).

E-**12** and *Z*-**9** acids were incubated individually with fresh bark tissue in the dark. Every effort was made to minimize the amount of cambial tissue in the bark (see Experimental). Following incubation, the bark tissue was removed, washed, and extracted with methanol in a Soxhlet apparatus containing *E*- and *Z*-coniferyl alcohols (**6** and **2**) as carrier. The alcohols were then purified (see Expt.) and converted to their respective 3,5-dinitrobenzoyl derivatives **15** and **14**. These derivatives were further purified by preparative HPLC and crystallization.

The incorporation data from several incubation experiments is shown in Table 1. As can clearly be seen, the *E* acid **12** was much more efficiently converted into both the *E*- and *Z*-coniferyl alcohols (**6** and **2**), than the corresponding *Z* acid **9**. Presumably the small amount of radioactivity in alcohols **2** and **6**, following the *Z* acid incubation, was due to the small quantity (< 2–3%) of *E* acid **12** as contaminant. This result strongly suggests that



R = 3,5 - dinitrobenzoyl

only *E*-ferulic acid (**12**) is involved in the biogenesis of *E*- and *Z*-coniferyl alcohols (**6** and **2**) in beech bark.

We next examined the precursor role of *E*-[2-^{14}C] coniferaldehyde (**16**) in the biosynthesis of alcohols **2** and **6**. [2-^{14}C] Coniferaldehyde (**16**) was prepared by conversion of *E*-[2-^{14}C] ferulic acid (**12**) to its corresponding methyl ester, which was then subsequently reduced to *E*-[2-^{14}C] coniferyl alcohol (**6**) by 'ATE' complex [1] and oxidized with dichlorodicyanobenzoquinone (DDQ) to give the required [2-^{14}C] coniferaldehyde (**16**) [19]. The role of *Z*-[2-^{14}C] coniferaldehyde was not examined. This was because (1) DDQ dehydrogenation of both *E*- and *Z*-coniferyl alcohols (**6** and **2**) affords only *E*-coniferaldehyde (**16**) and (2) irradiation (λ = 254 nm) of *E*-aldehyde **16** did not afford any measurable amounts of the corresponding *Z*-isomer.

The incorporation data obtained, following incubation of *E*-[2-^{14}C]-coniferaldehyde (**16**) with *F. grandifolia* bark tissue, is shown in Table 2. As a control experiment, *E*-[2-^{14}C]-ferulic acid (**12**) was also incubated with fresh bark tissue. As can clearly be seen (Table 2), *E*-[2-^{14}C] coniferaldehyde (**16**) was efficiently incorporated into both *E* and *Z* alcohols **6** and **2**.

Table 1. Incorporation of *E* and *Z*-[2-^{14}C]ferulic acids (**12** and **9**) into *E*- and *Z*-coniferyl alcohols (**6** and **2**) in *F. grandifolia* bark

Precursor	Radioactivity fed (dpm × 10^6)	Duration of incubation (hr)	Specific incorporation into 3,5-DNB* derivatives of *E* and *Z* alcohols **15** and **14** (%)	
			E-**15**	*Z*-**14**
1 *E*-Ferulic acid (**12**)	1.64	3	0.50	0.41
	1.72	6	0.64	0.66
	1.45	9	0.79	0.46
2 *E*-Ferulic acid (**12**)	2.68	12	1.88	0.12
Z-Ferulic acid (**9**)	3.00	12	<0.1	<0.01
3 *E*-Ferulic acid (**12**)	1.71	12	0.71	0.84
Z-Ferulic acid (**9**)	1.65	12	<0.11	<0.1
Boiled bark	(control)			
E-Ferulic acid (**12**)	1.00	12	<0.0001	<0.0001

*3,5-DNB = 3,5-dinitrobenzoyl.
All experiments in 1, 2 and 3 were conducted in parallel.

Table 2. Incorporation of *E*-[2-^{14}C]coniferaldehyde (**16**) into *E*- and *Z*-coniferyl alcohols (**6** and **2**) in *F. grandifolia* bark

Precursor	Radio-activity fed (dpm × 10^6)	Duration of incubation (hr)	Specific incorporation into 3,5 DNB* derivatives of **15** and **14** (%)	
			E-**15**	*Z*-**14**
1 *E*-[2-^{14}C] Coniferaldehyde (**16**)	1.68	12	2.76	1.33
2 *E*-[2-^{14}C] Coniferaldehyde (**16**)	1.71	12	0.69	0.9

*3,5-DNB = 3,5-dinitrobenzoyl.

These experiments raised the possibility that the point of isomerization might in fact be at the hydroxycinnamyl alcohol level. We therefore next incubated bark tissue with *E*-[2-^{14}C] coniferyl alcohol (**6**) in order to determine whether any activity would be found in *Z*-alcohol **2**. Again parallel *E*-[2-^{14}C] ferulic acid (**12**) incubations were carried out for control purposes. As can be seen from the results (Table 3), significant activity was found in the *Z*-isomer **14** indicating that the point of isomerization had occurred at the alcohol level.

We next turned our attention to the substrate specificity of the cinnamyl alcohol dehydrogenase from beech bark using *E*- and *Z*-coniferyl (**6** and **2**) alcohols as substrates. The crude enzyme was obtained from both lyophilized and fresh bark tissue as described in the Experimental section. In all cases, the cinnamyl alcohol dehydrogenase showed a strong substrate preference, with the ratio of V_{max} for *E*:*Z* isomers **6** and **2** being 15:1. This provides additional indirect evidence that the stage of *E*/*Z* isomerization occurs at the hydroxycinnamyl alcohol level.

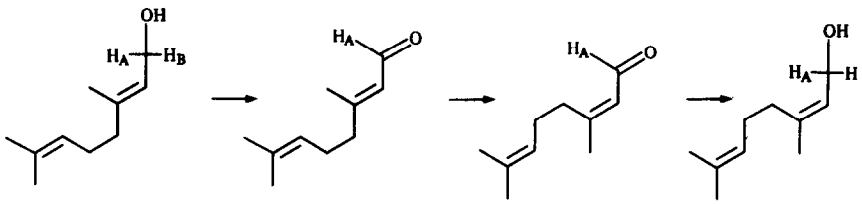
We also established that isomerization of the alcohols **2** and **6** did not occur as a consequence of the isolation procedure used or by photochemical means. In the latter case, this was established by irradiating pure samples of *Z* and *E* coniferyl alcohols (**2** and **6**) with either artificial or UV light (λ = 254 nm). No isomerization occurred, as evidenced by ^{1}H NMR and HPLC. These results therefore suggest that the interconversion is non-photochemical, and presumably enzyme-mediated.

It is interesting to note that the interconversion between the allylic alcohols, geraniol (**17**) and nerol (**20**) in *Menyanthes trifoliata* is known to be enzymatically mediated [20]. This occurs via stereospecific loss of the H_B proton of geraniol (**17**), presumably via an interconversion between the corresponding aldehydes **18**/**19** as shown in Fig. 2. However, for the hydroxycinnamyl

Table 3. Incorporation of *E*-[2-^{14}C]coniferyl alcohol (**6**) into *Z*-coniferyl alcohol (**2**) in *F. grandifolia* bark

Precursor	Radio activity fed (dpm × 10^6)	Duration of incubation (hr)	Specific incorporation into 3,5 DNB* derivatives of *E* and *Z* alcohols **15** and **14** (%)	
			E-**15**	*Z*-**14**
1 *E*-[2-^{14}C] Coniferyl alcohol	1.94	12	2.89	1.0
2 *E*-[2-^{14}C] Coniferyl alcohol	1.88	12	0.56	1.1

*3,5-DNB-3,5-dinitrobenzoyl.



17 **18** **19** **20**

Fig. 2. Biosynthetic sequence from geraniol (**17**) to nerol (**20**) [20].

alcohols in *F. grandifolia*, our incorporation and cinnamyl alcohol dehydrogenase data appear to exclude a similar biosynthetic sequence.

CONCLUSIONS

The incorporation and crude substrate specificity data, combined with our chemical studies, strongly suggest that the immediate precursor of *Z*-coniferyl alcohol (**2**) is the corresponding *E* isomer **6**. *Trans–cis* interconversions between allylic alcohols, geraniol (**17**) and nerol (**20**) have been reported to proceed through the respective aldehydes **18**/**19** [20]. Our results do not support such a mechanism in this case. However, in order to unambiguously answer this isomerization question in beech bark, isolation of the enzyme (or enzymes) specific for the *E*/*Z* conversion of these hydroxycinnamyl alcohols is necessary.

EXPERIMENTAL

(2Z)-3-(3-*methoxy*-4-*hydroxyphenyl*-[2-^{14}C]-2-*Propenoic acid* (**12**). Base-catalysed condensation of vanillin (437 mg, 2.87 mmol) with [2-^{14}C] malonic acid (300 mg, 2.88 mmol, 2.22×10^7 dpm/mg) was carried out based on the procedure of ref. [18]. Acid **12** (305 mg, 1.00×10^7 dpm/mg) was obtained in 55% yield. (mp 174°)

(2E)-3-(3-*methoxy*-4-*hydroxyphenyl*)-[2-^{14}C]-2-*Propenoic acid* (**9**). *E*-[2-^{14}C] Ferulic acid (**12**) (9.7 mg, 0.05 mmol, 1×10^7 dpm/mg) was dissolved in MeOH (1 ml) in a 100 × 15 mm Petri dish and irradiated with a Spectro line shortwave (254 nm, 60 Hz) UV lamp for 30 min at a distance of 2.5 cm [6]. The soln was then applied in the dark (safety light) to a prep. cellulose TLC plate. Following elution with H_2O–AcOH (47:3), the band corresponding to the *Z* isomer (R_f 0.69) was excised, suspended in MeOH, filtered and dried to give *Z*-ferulic acid (**9**) (1.32 mg, 13.5%, 1×10^7 dpm/mg). ^{1}H NMR (CH_3OD): δ3.85 (3H, *s*, OCH_3), 5.77 (1H, *d*, *J* = 12.9 Hz, CH=C<u>H</u>CO_2H), 6.75 (1H, *d*, *J* = 8.2 Hz, H-5'), 6.80 (1H, *d*, *J* = 12.9 Hz, C<u>H</u>=CHCO_2H), 7.09 (1H, *dd*, *J* = 8.26 Hz, J_2 = 1.97 Hz, H-6'), 7.73 (1H, *d*, *J* = 1.96 Hz, H-2'); MS *m/z*: 194 $[M]^+$ (100%), 179 $[M-Me]^+$ (18%).

(2E)-3-(3-*methoxy*-4-*hydroxyphenyl*)-[2-^{14}C]-2-*Propenol* (**6**). *E*-[2-^{14}C] ferulic acid (**12**) (71.7 mg, 0.37 mmol, 4.99×10^6 dpm/mg) was added to a stirred soln of MeOH (3 ml) and H_2SO_4 (0.23 ml, 36 M) under N_2. After solubilization, the temp. was raised to 35° and held for 60 min. The reaction mixture was then cooled to ~10° and carefully neutralized with 1 M $NaHCO_3$, and the MeOH removed *in vacuo*. The soln was then acidified to pH 3 with 1.5 M H_2SO_4 and then extracted with EtOAc (3 × 25 ml). The EtOAc extracts were combined, backwashed with water (50 ml), dried (Na_2SO_4), and the solvent removed *in vacuo* to give methyl [2-^{14}C] ferulate (75.2 mg) which was not further purified. ^{1}H NMR (CH_3OD) δ3.75 (3H, *s*, CO_2Me), 3.89 (3H, *s*, OMe), 4.89 (1H, *s*, OH), 6.36 (1H, *d*, *J* = 16 Hz, CH=C<u>H</u> CO_2Me), 7.06 (1H, *dd*, J_1 = 8.2 Hz, J_2 = 2.1 Hz, H-6'), 7.18 (1H, *d*, *J* = 2.0 Hz, H-2'), 7.61 (1H, *d*, *J* = 15.9 Hz, <u>C</u>H=CHCO_2Me); MS *m/z*: 208 $[M]^+$ (100%), 177 $[M-OMe]^+$ (69%); IR ν_{max}^{neat} cm^{-1}: 3400, 1703, 1591.

The crude methyl [2-^{14}C] ferulate (75.2 mg) was then reduced using 'ATE' complex as previously described [1] to afford, after purification, *E*-coniferyl alcohol (**6**) (29.6 mg, 45%, 5.1×10^6 dpm/mg) (mp 74–75°).

(2E)-3-(3-*methoxy*-4-*hydroxyphenyl*)-[2-^{14}C]*Prop*-2-*enal* (**16**). 2,3-Dichloro-5,6 dicyanobenzoquinone (DDQ) (18.9 mg, 0.083 mmol) was added to a stirred soln of *E*-[2-^{14}C]coniferyl alcohol (**6**) (15 mg, 0.083 mmol, 5.1×10^6 dpm/mg) in dry THF (10 ml) under N_2 at room temp. After 5 min., the solvent was

removed *in vacuo*, and the resulting residue washed with hot CH_2Cl_2 (4 × 5 ml). The CH_2Cl_2 solubles were combined, dried, and the crude product applied to a silica gel column carefully eluted with a hexane: EtOAc gradient. Fractions containing *E*-[2-^{14}C] coniferaldehyde (**16**) were combined and evaporated. Recrystallization afforded (**16**) (11.7 mg, 79%, 4.9×10^6 dpm/mg); ^{1}H NMR ($CDCl_3$) δ:3.95 (3H, *s*, OMe), 5.99 (1H, *s*, OH), 6.60 (1H, *dd*, J_1 = 8 Hz, J_2 = 7.8 Hz, CHCHO), 6.97 (1H, *d*, *J* = 8.2 Hz, H-5'), 7.07 (1H, *d*, *J* = 1.9 Hz, H-2'), 7.13 (1H, *dd*, *J* = 8.2 Hz, J_2 = 1.9 Hz, H-6'), 7.41 (1H, *d*, *J* = 15.8 Hz, CH=CHCHO), 9.66 (1H, *d*, *J* = 7.8 Hz, CHO); MS *m/z*: 178 $[M]^+$ (100%), 161 $[M-OH]^+$ (14%), 147 $[M-OMe]^+$ (27%), 135 $[M-CHO, Me]^+$ (29%); IR ν_{neat}^{max} cm^{-1}: 3400, 1650, 1600.

Procurement of bark tissue. The American beech (*Fagus grandifolia Ehrh*) bark used in these studies was either greenhouse grown (2-year-old seedlings) or obtained from its natural habitat. For the seedlings, only the bark from the main stem was used. For the naturally growing trees, two-year-old leaders from the upper crown were used as the tissue source. Immediately prior to incubation, bark tissue was carefully sliced parallel to the main axis of the stem, external to the cambium layer, thereby exposing as much of the secondary phloem as possible. Since the bark was very thin (< 2 mm thick), complete exclusion of all cambial cells was not possible. The sliced material was then immediately immersed in a 0.1 M Tris–HCl (pH 7.6) buffer soln, which was used as a rinse prior to incubation.

Incubation of E-[2-^{14}C] *ferulic acid* (**12**) *with* F. grandifolia *bark tissue.* *E*-[2-^{14}C] Ferulic acid (0.188 mg, 1.88×10^6 dpm, 0.9 μmol) was dissolved in 0.1 M Tris–HCl buffer (9 ml, pH 7.6), in a 4 ml vial. All manipulations were carried out under the illumination of a green safety light. Bark tissue (2.3 g net weight) was added to the incubation soln. The incubation flask was sealed, wrapped in Al foil, and then placed in a shaker bath (18 cycles per min agitation, 25°) in the dark for 12 hr. The incubation soln was decanted, and the bark tissue first washed with distilled H_2O (3 × 10 ml), pressed between filter paper to remove excess moisture and ground to a coarse powder in liq. N_2. The ground bark was extracted for 4 hr in a Soxhlet under N_2 with MeOH (25 ml) containing *cis*-(*Z*)-coniferyl (**2**, 5.1 mg) and *trans*-(*E*)-coniferyl (**6**, 5.1 mg) alcohols as carrier. The MeOH extract (233 mg) was then dried *in vacuo*, after which H_2O–MeCN (3.3 ml, 21:4) was added. The resulting suspension was transferred to a 15 ml centrifuge tube and the contents spun at 7720 *g* for 25 min. The supernatant was removed, and alcohols **2** and **6** isolated by preparative HPLC using a Waters μ-Bondapak C_{18} (7.8 × 300 mm) column, eluted with H_2O–MeCN (21:4, 2 ml/min flow rate). Eluant containing coniferyl alcohols **2** and **6** was combined, and extracted with Et_2O (4 × 30 ml) and dried (7.5 mg).

The mixture (7.5 mg) of *E*/*Z* coniferyl alcohols (**6**/**2**) was dissolved in C_5H_5N (2 ml) under N_2 at room temp. 3,5-Dinitrobenzoylchloride (55 mg, 0.24 mmol) was then added and the temp. was raised to 35° and held for 30 min. The mixture was then diluted with $CHCl_3$ (10 ml) and extracted with 1M HCl (3 × 5 ml), 1M $NaHCO_3$ (3 × 5 ml) and H_2O (2 × 5 ml). The $CHCl_3$ solubles were then dried (Na_2SO_4), and the solvent removed *in vacuo* to give crude products **15** and **14** (38.2 mg).

The crude derivatives were then applied to a silica gel TLC plate which was eluted with hexane–EtOAc–$CHCl_3$ (14:6:3). The bands corresponding to the derivatives were then excised, (*Z*, R_f 0.44; *E*, R_f 0.33), suspended in EtOAc, filtered, and dried. The *E* and *Z* derivatives were then further purified by preparative HPLC, using a Waters μ-Porasil Novapak column eluted with hexane–EtOAc (21:4) at a flow rate of 1 ml/min, to give pure *Z* derivative **14** (6.14 mg, 1279 dpm/mg), ^{1}H NMR ($CDCl_3$) δ:3.87 (3H, *s*, OCH_3), 5.26 (2H, *d*, *J* = 6.6 Hz, CH_2O), 6.03 (1H, *dd*, J_1 = 11.7 Hz, J_2 = 6.6 Hz, CHCH$_2$O), 6.84 (1H, *d*, *J* = 11.7 Hz, CH=CHCH$_2$), 6.95 (1H, *dd*, J_1 = 8.1 Hz, J_2 = 1.7 Hz, H-6'), 7.0 (1H, *d*, *J* = 1.6 Hz, H-2'), 7.22 (1H, *d*, *J* = 8.0 Hz, H-5'), 9.25–9.33 (6H, ArH); MS *m/z*: 568 $[M]^+$ (11.5%), 195 $[C_6H_3(CO)(NO_2)_2]^+$ (100%); IR ν_{max}^{KBr} cm^{-1}: 1735, 1540, 1340, 1250 and the *E*-derivative **15** (6.46 mg, 646 dpm/mg), ^{1}H NMR ($CDCl_3$) δ:3.87 (3H, *s*, OCH_3), 5.14 (2H, *d*, *J* = 6.6 Hz, CH_2O), 6.44 (1H, J_1 = 15.9 Hz, J_2 = 6.7 Hz, CH=CHCH$_2$O), 6.83 (1H, *d*, *J* = 15.8 Hz, CH=CHCH$_2$O), 7.08 (1H, *dd*, J_1 = 8.5 Hz, J_2 = 2.2 Hz, H-6'), 7.09 (1H, *d*, *J* = 2.2 Hz, H-2'), 7.16 (1H, *d*, *J* = 8.6 Hz, H-5'), 9.22–9.33 (6H, ArH); MS *m/z*: 568 $[M]^+$ (12%), 195 $[C_6H_3(CO)(NO_2)_2]^+$ (100%); IR ν_{max}^{KBr} cm^{-1}: 1735, 1540, 1340, 1250.

Cinnamyl alcohol dehydrogenase. Beech bark was removed from branches having cross-sectional diameters ranging from 8 to 20 mm, and placed in 25 mM potassium phosphate buffer (pH 7.5, 4°) containing 1 mM EDTA (Na_2 salt). After rinsing with distilled H_2O, the tissue was lyophilized, ground into a fine powder and then stored at −20° under N_2.

Protein extraction and purification. All procedures were conducted at 4°. Tris–HCl buffer (0.1 M, 50 ml) at pH 7.5 containing dithiothreitol (10 mM) and polyethylene glycol (0.5% w/v, M_r 8000), polyvinylpolypyrrolidone (3.4 g) and acid-washed quartz sand (1.7 g) were mixed together in a chilled mortar. The powdered bark (5 g) was added and the slurry homogenized with a pestle for 15 min. The slurry was filtered through 4 layers of cheese cloth and centrifuged (39 000 *g*) for 40 min. Bovine serum albumin (Sigma fraction V powder, 98–99% albumin) was added to the supernatant to give a concn of 0.1% w/v. The supernatant was fractionated with $(NH_4)_2SO_4$ in the range 40–80%, then centrifuged (27 000 *g*) for 30 min. The pellet was dissolved in Tris–HCl buffer (70 mM, 2 ml, pH 7.5) containing glycerol (30% v/v) and BSA (0.1% w/v). The resulting crude extract was desalted through a Sephadex G-25 (medium) column previously equilibrated with Tris–HCl buffer (70 mM, pH 7.5) containing dithiothreitol (10 mM) and glycerol (30%, v/v) and used for the enzyme assay.

Cinnamyl alcohol dehydrogenase activity. The enzyme activity was determined by monitoring the reduction of NADP spectrophotometrically at 340 nm. The reaction mixture (2.5 ml) contained the crude enzyme (50 μl), Tris–HCl (90 mM, pH 8.0) at 25°, Na-NADP (2 mM) and coniferyl alcohol (0.6 mM). The enzyme was preincubated at 25° for 5 min. and then coniferyl alcohol was added to the mixture to initiate the reaction. The enzyme from 1 g of lyophilized beech bark converted 0.306 mmol of *E*-coniferyl alcohol to the aldehyde per min under the assay conditions.

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