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A heartwood norlignan, (*E*)-hinokiresinol, is formed from 4-coumaryl 4-coumarate by a *Cryptomeria japonica* enzyme preparation

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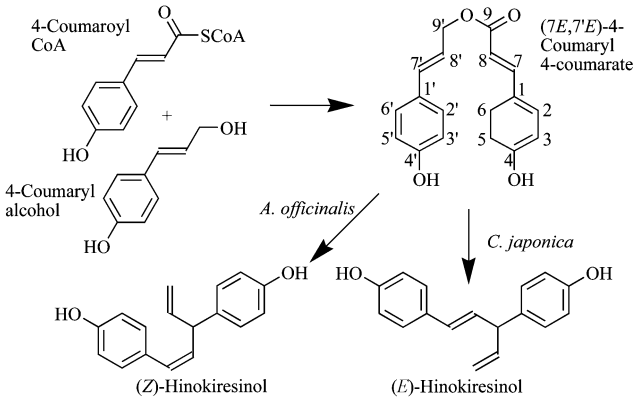
An enzyme preparation from the cultured cells of *Cryptomeria japonica* catalyses the formation of a heartwood norlignan, (*E*)-hinokiresinol, from two distinct phenylpropanoid monomers: 4-coumaroyl CoA and 4-coumaryl alcohol, and from a dimer: 4-coumaryl 4-coumarate.

Norlignans, which have the 1,3-diarylpentane structure, are found in coniferous trees (especially heartwood) and some monocotyledonous plants, including *Asparagus officinalis*.[1,2] Their biosynthesis is especially of interest in relation to heartwood formation, a metabolic event specific to woody plants.[1–3] Hinokiresinol is the simplest of the norlignans and therefore a good target for norlignan biosynthetic studies. Hinokiresinol has two geometrical (*E*)- and (*Z*)-isomers. (*E*)-Hinokiresinol is the source of the heartwood colour for *Chamaecyparis obtusa* (hinoki cypress),[4] and is typically found in conifer heartwood together with other norlignans,[5–8] in contrast to the distribution of (*Z*)-hinokiresinol in monocotyledonous herbs including *A. officinalis*.[2,3]

We have demonstrated for the first time the biosynthetic process and enzymatic formation of (*Z*)-hinokiresinol using the *A. officinalis* cell system (Scheme 1), the formation of 4-coumaryl 4-coumarate by the coupling of 4-coumaryl alcohol and 4-coumaroyl CoA, and the subsequent rearrangement to yield (*Z*)-hinokiresinol.[2,3] Our attention was next directed to the biosynthesis of (*E*)-hinokiresinol in relation to heartwood formation. Herein, we report for the first time, enzymatic (*E*)-hinokiresinol formation using the *Cryptomeria japonica* (Japanese cedar) cell system as an enzyme source.

Callus was initiated from the shoot tips excised aseptically from stems of *C. japonica* cv. Kumotooshi (collected at Wakayama University Forest, Kyoto University, in November 2002) on Murashige and Skoog (MS) gel medium (0.3% phytagel, 2% sucrose, 1×10^{-3} g l^{-1} of 2,4-dichlorophenoxyacetic acid, and 1×10^{-4} g l^{-1} of kinetin). A cell-suspension culture was obtained by transferring callus crumps to the MS liquid medium: 1% sucrose, 0.7 g l^{-1} of KCl, 0.93 g l^{-1} of KNO_3, free of NH_4NO_3, 1×10^{-3} g l^{-1} of 2,4-dichlorophenoxyacetic acid, and 1×10^{-4} g l^{-1} of kinetin.



Scheme 1 Proposed biosynthetic routes for (*E*)- and (*Z*)-hinokiresinols in *C. japonica* and *A. officinalis*.

The culture was maintained by continuous shaking (80 rpm) at 25 °C in the dark. The cells were maintained for over one year by repeated subculturing every 10 days.

Cell-free extracts were prepared as described previously.[3] Briefly, the cells incubated for 10 days after the last subculturing were frozen with liquid nitrogen and pulverized with a mortar and pestle. The powder obtained was ground in the presence of polyclar AT (30% of cell weight), sea sand, and 100 mM Tris-HCl buffer (THB) (pH 7.8) containing 15 mM 2-mercaptoethanol (ME), and the resultant slurry was filtered with gauze. The filtrate obtained was centrifuged, and the supernatant was applied to a Sephadex G-25 column, which had been pre-equilibrated and was eluted with 20 mM THB (pH 7.8) containing 15 mM ME. To the excluded fraction was added ammonium sulfate (0–70% saturation), and the resulting precipitates were recovered by centrifugation and desalted by passing through a Sephadex G-25 column as above. The effluent was used as the enzyme preparation. Two distinct phenylpropanoid monomers, 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA[3] were incubated with the enzyme preparation at 30 °C (Table 1, A-1) and then the product was analysed by GC-MS as previously described.[3]

The mass spectrum (Fig. 1*A*) is that of authentic (*Z*)-hinokiresinol TMS ether showing principal ions at *m*/*z* 396 (molecular ion, $[M]^+$) and 230. Although the mass spectrum is quite similar to that of (*E*)-hinokiresinol TMS ether (Fig. 1*B*), the (*Z*)- and (*E*)-isomers are distinguishable from each other by retention times (t_R), as shown in Fig. 2*A*. Fig. 1*C* is the spectrum of the (*E*)-hinokiresinol fraction obtained following incubation of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA with the enzyme preparation, and its t_R is shown in Fig. 2*B*. As can be seen in Fig 1*C*, in addition to the ions at *m*/*z* 396 and 230 which are derived from the unlabelled internal standard, ions at *m*/*z* 399 ($[M]^+ + 3$) and 233 are found, indicating the formation of (*E*)-[$^2H_3$]hinokiresinol. The slightly smaller t_R in the selected ion monitoring (SIM) chromatogram of *m*/*z* 399 of the fraction (16.54 min, Fig. 2*B*) compared to that of molecular ion (*m*/*z* 396) of unlabelled (*E*)-hinokiresinol (16.57 min, Fig. 2*A*), which is ascribed to the well-known isotope effect on GC retention time, further supports the formation of the deuterium labelled (*E*)-hinokiresinol. Proof that the formation of (*E*)-[$^2H_3$]hinokiresinol was enzymatic was obtained by control experiments (Table 1); the formation of (*E*)-[$^2H_3$]hinokiresinol from 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA was not observed when the denatured enzyme preparation was used, and when the enzyme preparation or the substrate(s) were omitted from the complete assay.

Next we incubated (7*E*,7′*E*)-4-[7′,9′,9′-$^2H_3$]coumaryl 4-coumarate[3] with the enzyme preparation at 30 °C. Like the result observed with the conversion of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA, (7*E*,7′*E*)-4-[7′,9′,9′-$^2H_3$]coumaryl 4-coumarate was found to be efficiently converted to (*E*)-[$^2H_3$]hinokiresinol (Figs. 1*D* and 2*C*, and Table 1, B-1).

To eliminate the involvement of (*Z*)-hinokiresinol as an intermediate in the formation of (*E*)-hinokiresinol, we incubated (*E*)-hinokiresinol and (*Z*)-hinokiresinol individually with the enzyme preparation. GC-MS analysis showed that neither

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Table 1 Enzymatic formation of (*E*)-[$^2H_3$]hinokiresinol

Assay[a]	Entry	Constituents of reaction mixture	(*E*)-[$^2H_3$]-Hinokiresinol/ nmol mg^{-1} protein	Reaction period/ min
Complete[b]	A-1	4-[7,9,9-$^2H_3$]Coumaryl alcohol, 4-coumaroyl CoA, and enzyme preparation	0.39	60
	A-2	4-[7,9,9-$^2H_3$]Coumaryl alcohol, 4-coumaroyl CoA, and enzyme preparation	0	<0.1
Control[c]	A-3	4-[7,9,9-$^2H_3$]Coumaryl alcohol and 4-coumaroyl CoA	0	60
	A-4	4-[7,9,9-$^2H_3$]Coumaryl alcohol and enzyme preparation	0	60
	A-5	4-Coumaroyl CoA and enzyme preparation	0	60
	A-6	Enzyme preparation	0	60
	A-7	4-[7,9,9-$^2H_3$]Coumaryl alcohol, 4-coumaroyl CoA, and denatured enzyme preparation	0	60
Complete[d]	B-1	4-[7′,9′,9′-$^2H_3$]Coumaryl 4-coumarate and enzyme preparation	4.3	60
	B-2	4-[7′,9′,9′-$^2H_3$]Coumaryl 4-coumarate and enzyme preparation	0.68	<0.1
Control[c]	B-3	4-[7′,9′,9′-$^2H_3$]Coumaryl 4-coumarate	0	60
	B-4	Enzyme preparation	0	60
	B-5	4-[7′,9′,9′-$^2H_3$]Coumaryl 4-coumarate and denatured enzyme preparation	0	60

[a] Protein content is 2.11 g l^{-1}. [b] Complete assay consisted of 90 μl of the enzyme preparation, 5 μl of 20 mM 4-[7,9,9-$^2H_3$]coumaryl alcohol in 10% MeOH–H_2O, and 5 μl of 20 mM 4-coumaroyl CoA in H_2O. [c] Control assays refer to the complete assay with the omission of substrate(s) or enzyme preparation, or with the denatured enzyme preparation (boiled for 10 min). [d] Complete assay consisted of 90 μl of the enzyme preparation and 10 μl of 20 mM 4-[7′,9′,9′-$^2H_3$]coumaryl 4-coumarate in MeOH.

(*Z*)- nor (*E*)-hinokiresinol was converted to the corresponding geometrical isomers under the conditions employed (data not shown), indicating that (*E*)-hinokiresinol was directly formed from 4-coumaryl 4-coumarate with the enzyme preparation, but not *via* (*Z*)-hinokiresinol. Taken together, our results have provided evidence for the (*E*)-hinokiresinol synthesizing system, which does not involve (*Z*)-hinokiresinol as an intermediate.

In addition, the present results, together with our previous report,[3] indicate that there are at least two hinokiresinol synthesizing systems in plants; one giving rise to the (*E*)-isomer, the other to the (*Z*)-isomer. They are probably two distinct hinokiresinol synthases, namely (*E*)- and (*Z*)-hinokiresinol synthases catalysing the direct conversion of (7*E*,7′*E*)-4-coumaryl 4-coumarate to (*E*)-hinokiresinol and (*Z*)-hinokiresinol, respectively. In fact, we have demonstrated that a purified *Asparagus* enzyme transformed (7*E*,7′*E*)-4-coumaryl 4-coumarate to (*Z*)-hinokiresinol (unpublished). However, it is still possible that (7*E*,7′*E*)-4-coumaryl 4-coumarate was first isomerised to (7*Z*,7′*E*)-4-coumaryl 4-coumarate which was then transformed to give rise to (*E*)-hinokiresinol by the *Cryptomeria* crude enzyme preparation.

In conclusion, the present study has demonstrated for the first time enzyme preparation catalysed (*E*)-hinokiresinol formation and this is the first report of the enzymatic formation of a heartwood norlignan.

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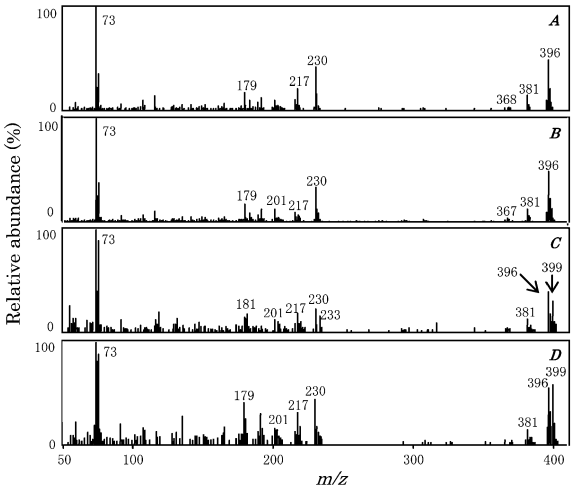

Fig. 1 Mass spectra of TMS ethers of (*Z*)- and (*E*)-hinokiresinols. *A*, unlabelled (*Z*)-hinokiresinol [isolated from *A. officinalis*[2]]. *B*, unlabelled (*E*)-hinokiresinol [isolated from *C. obtusa*[8]]. *C*, formed after incubation of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA with the enzyme preparation. *D*, formed after incubation of 4-[7′,9′,9′-$^2H_3$]coumaryl 4-coumarate with the enzyme preparation. Note that unlabelled (*E*)-hinokiresinol ([M]$^+$ = *m*/*z* 396) was added as an internal standard in the cases of *C* and *D*.

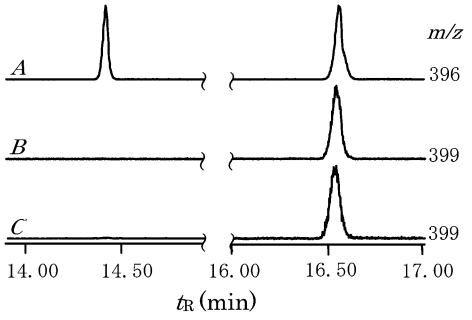

Fig. 2 SIM chromatograms of TMS ethers of (*Z*)- and (*E*)-hinokiresinols. *A*, mixture of unlabelled (*Z*)-hinokiresinol (t_R = 14.41) and (*E*)-hinokiresinol (t_R = 16.57) TMS ethers. *B*, formed after incubation of 4-[7,9,9-$^2H_3$]coumaryl alcohol and 4-coumaroyl CoA with the enzyme preparation. *C*, formed after incubation of 4-[7′,9′,9′-$^2H_3$]coumaryl 4-coumarate with the enzyme preparation. Note that *A* was monitored at *m*/*z* 396 ([M]$^+$), while *B* and *C* were monitored at *m*/*z* 399 ([M]$^+$ + 3).

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