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First *in vitro* norlignan formation with *Asparagus officinalis* enzyme preparation

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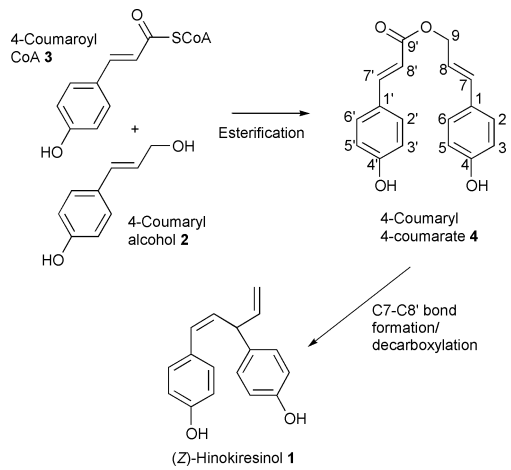
We report for the first time that an enzyme preparation from fungal-elicited *Asparagus officinalis* cultured cells catalyses the formation of a norlignan, (*Z*)-hinokiresinol, from two non-identical phenylpropanoid monomers, 4-coumaryl alcohol and 4-coumaroyl CoA, and from a dimer, 4-coumaryl 4-coumarate, without any additional cofactors.

Typical norlignans having the 1,3-diphenylpentane (C_6–C_2–C_3–C_6) structure occur in coniferous trees (especially in heartwood) and some monocotyledonous plants, including *Asparagus officinalis*.[1,2] Their biosynthesis is of interest especially in relation to heartwood formation, a metabolic event specific to woody plants. We have recently reported the first evidence that a norlignan, (*Z*)-hinokiresinol **1** (Scheme 1), originates from two non-identical phenylpropane units.[2] Administration of phenylpropanoid monomers labelled with ^{13}C and/or ^{2}H to fungal-elicited *A. officinalis* cells revealed that the C_6–C_3 moiety of (*Z*)-hinokiresinol **1** is derived from 4-coumaryl alcohol **2**, while the C_6–C_2 moiety is from a 4-coumaroyl compound such as 4-coumaroyl CoA **3** with loss of the C-9 (a carbon atom at the end of the side chain). Next we focused attention on the enzymes responsible for formation of the norlignan carbon framework from phenylpropanoid monomers. Herein we report for the first time the enzymatic formation of (*Z*)-hinokiresinol **1**.

A. officinalis cells were incubated and treated with a fungal elicitor for 21–22 h as previously described.[2] Ammonium sulfate precipitates (0–70% saturation) of cell-free extracts were prepared at 4 °C as previously reported.[3] The resulting precipitates were recovered and desalted by passing through a pre-equilibrated Sephadex G-25 column and were eluted with 0.05 M potassium phosphate buffer (pH 7.0). The effluent was used as the enzyme preparation. Two distinct phenylpropanoid monomers, 4-[7,9,9-$^{2}H_3$]coumaryl alcohol **2**-d_3† synthesized previously[2] and 4-coumaroyl CoA **3** prepared by the method of Stöckigt and Zenk,[4] were incubated with the enzyme preparation but without any additional cofactors. After incubating at 27 °C for 1 h, products were extracted with EtOAc containing unlabelled (*Z*)-hinokiresinol[2] as an internal standard. The EtOAc soluble fraction was submitted to GC-MS analysis after TMS derivatization as previously described.[2] The amount of formed (*Z*)-[$^{2}H_3$]hinokiresinol **1**-d_3 was calculated by comparing the relative intensity of the molecular ion (*m*/*z* 396) of unlabelled hinokiresinol TMS ether with that of (*Z*)-[$^{2}H_3$]hinokiresinol TMS ether (*m*/*z* 399).

The mass spectrum (Fig. 1*A*) of a GC peak of (*Z*)-hinokiresinol TMS ether shows ions at *m*/*z* 396, 230, and 217 which are derived from the unlabelled internal standard. In addition, this spectrum shows ions at *m*/*z* 399, 233, and 219, indicating the formation of (*Z*)-[$^{2}H_3$]hinokiresinol **1**-d_3 from 4-[7,9,9-$^{2}H_3$]coumaryl alcohol **2**-d_3 and 4-coumaroyl CoA **3**. Proof that the formation of (*Z*)-[$^{2}H_3$]hinokiresinol **1**-d_3 was enzymatic was obtained by control experiments as summarised in Table 1A. The formation of (*Z*)-[$^{2}H_3$]hinokiresinol **1**-d_3 from 4-[7,9,9-$^{2}H_3$]coumaryl alcohol **2**-d_3 and 4-coumaroyl CoA **3** did



Scheme 1 A putative mechanism for the formation of (*Z*)-hinokiresinol **1** with *A. officinalis* enzyme preparation.

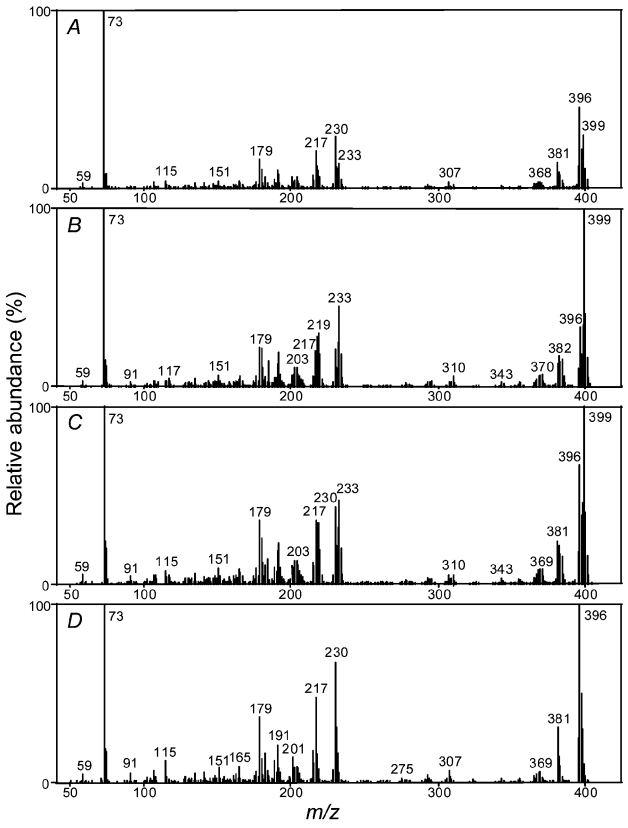


Fig. 1 Mass spectra of TMS ethers of deuterated and unlabelled (*Z*)-hinokiresinol. *A*, formed after incubation of 4-[7,9,9-$^{2}H_3$]coumaryl alcohol and 4-coumaroyl CoA with the enzyme preparation. *B*, formed after administration of 4-[7,9,9-$^{2}H_3$]coumaryl 4-coumarate to *A. officinalis* cultured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

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

Assay	Entry	Constituent(s) of reaction mixture	(*Z*)-[$^2H_3$]Hinokiresinol/ nmol mg^{-1}protein	Reaction period/min
A				
Complete[a]	A-1	4-[7,9,9-$^2H_3$]Coumaryl alcohol, 4-coumaroyl CoA, and enzyme preparation	3.4	60
	A-2	4-[7,9,9-$^2H_3$]Coumaryl alcohol, 4-coumaroyl CoA, and enzyme preparation	0	< 0.1
Control[b]	A-3	4-[7,9,9-$^2H_3$]Coumaryl alcohol and 4-coumaroyl CoA	0.2	60
	A-4	4-[7,9,9-$^2H_3$]Coumaryl alcohol and enzyme preparation	0.1	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
B				
Complete[c]	B-1	4-[7,9,9-$^2H_3$]Coumaryl 4-coumarate and enzyme preparation	63.9	60
	B-2	4-[7,9,9-$^2H_3$]Coumaryl 4-coumarate and enzyme preparation	4.7	< 0.1
Control[b]	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] Complete assay (total volume 235 µl) consisted of 220 µl of the enzyme preparation (protein content: 2.6-6.4 mg ml^{-1}), 5 µl of 25 mM 4-[7,9,9-$^2H_3$]coumaryl alcohol in MeOH, and 10 µl of 5 mM 4-coumaroyl CoA in 0.05 M potassium phosphate buffer (pH 7.0). [b] 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). [c] Complete assay (total volume 125 µl) consisted of 120 µl of the enzyme preparation (protein content: 4.8 mg ml^{-1}) and 5 µl of 5 mM 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate in MeOH.

not occur when the denatured (boiled for 10 min) enzyme preparation was used (Table 1A-7), and barely occurred when the enzyme preparation or the substrate(s) was omitted from the complete assay (Table 1A-3–6). On the other hand, incubation of 4-[7,9,9-$^2H_3$]coumaryl alcohol **2**-d_3 and unlabelled 4-coumarate (Tokyo Kasei Kogyo Co.) with the enzyme preparation did not afford (*Z*)-[$^2H_3$]hinokiresinol **1**-d_3 (data not shown). This eliminated the mechanism that 4-coumaroyl CoA **3** was first hydrolysed to 4-coumarate which then coupled with 4-coumaryl alcohol **2** to afford (*Z*)-hinokiresinol **1**. These results demonstrate for the first time that (*Z*)-hinokiresinol **1** was formed enzymatically from two distinct phenylpropanoid monomers.

Since esters are often biosynthesized by acyltransferase-catalysed condensation between the corresponding CoA esters and alcohol,[5] we next hypothesised that (*Z*)-hinokiresinol **1** was formed *via* the coupling of 4-coumaryl alcohol **2** and 4-coumaroyl CoA **3** to afford 4-coumaroyl 4-coumarate **4** followed by C7–C8′ bond formation and C9′ decarboxylation (Scheme 1). To test this hypothesis, we synthesised 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 {δ_H (acetone-d_6, carbon numbers are shown in Scheme 1): 6.21 (1H, s, 8-H), 6.37 (1H, d, J = 16.0, 8′-H), 6.81 (2H, d, J = 8.8, Ar-H), 6.89 (2H, d, J = 8.6, Ar-H), 7.33 (2H, d, J = 8.6, Ar-H), 7.55 (2H, d, J = 8.6, Ar-H), 7.63 (1H, d, J = 16.0, 7′-H)} by the similar method of Grabber *et al.*,[6] using 4-[7,9,9-$^2H_3$]coumaryl alcohol **2**-d_3[2] and unlabelled 4-coumarate as starting materials. Then 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 thus obtained was administered to the fungal-elicited *A. officinalis* cells. The mass spectrum (Fig. 1*B*) of (*Z*)-hinokiresinol **1** obtained following the administration shows ions at *m*/*z* 396, 230, and 217 which are ascribed to endogenous and unlabelled (*Z*)-hinokiresinol **1**. However, in addition significant ions at *m*/*z* 399, 233, and 219 are present, indicating the transformation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 into deuterium-labelled (*Z*)-[$^2H_3$]hinokiresinol **1**-d_3.

Furthermore, the conversion of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 to (*Z*)-[$^2H_3$]hinokiresinol **1**-d_3 was also demonstrated by an *in vitro* experiment; incubation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 with the enzyme preparation resulted in enzymatic (*Z*)-[$^2H_3$]hinokiresinol **1**-d_3 formation in a high yield (Fig. 1*C* and Table 1B). These *in vivo* and *in vitro* experiments strongly suggest that 4-coumaryl 4-coumarate **4** is the intermediate between the phenylpropanoid monomers (**2** and **3**) and (*Z*)-hinokiresinol **1** (Scheme 1).

Incubation of 4-[7,9,9-$^2H_3$]coumaryl 4-coumarate **4**-d_3 with horseradish peroxidase in the presence of H_2O_2 did not afford (*Z*)-[$^2H_3$]hinokiresinol **1**-d_3 (data not shown), indicating the peroxidase does not initiate the C7–C8′ bond formation and ultimate decarboxylation. This is in sharp contrast to C8–C8′ bond formation between hydroxycinnamyl alcohol units in lignan and lignin biosynthesis which is mediated by peroxidase or laccase.[7] Instead, 4-coumaryl 4-coumarate **4** might be converted to (*Z*)-hinokiresinol **1** *via* the ester enolate Claisen rearrangement (the [3,3]-sigmatropic rearrangement of allyl esters to γ,δ-unsaturated carboxylic acids)[8] followed by decarboxylation. However, the true mechanism awaits further experiments with purified enzymes.

In conclusion, the present study has demonstrated for the first time the enzymatic formation of (*Z*)-hinokiresinol **1** from 4-coumaryl alcohol **2** and 4-coumaroyl CoA **3**, and from 4-coumaryl 4-coumarate **4**.

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Notes and references

† d_3 in the compound numbers represents compounds labelled with three deuterium atoms.

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