PERKIN 1

Norlignan biosynthesis in *Asparagus officinalis* L.: the norlignan originates from two non-identical phenylpropane units†

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Little is known about the biosynthetic mechanism(s) of norlignans with C_6–C_5–C_6 skeletons in spite of their important contributions to heartwood formation in conifers. To clarify the mechanism(s), we have established cell-suspension cultures of *Asparagus officinalis* that produce a norlignan, (*Z*)-hinokiresinol, after fungal elicitor treatment. Feeding experiments with ring- or side chain-^{13}C- and/or ^{2}H-labelled phenylpropanoid monomers show that two units of L-phenylalanine, cinnamic acid, 4-coumaric acid, or 4-coumaryl alcohol are efficiently incorporated into the norlignan. ^{13}C NMR of (*Z*)-hinokiresinols isolated after individual administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid conclusively demonstrate that the side chain 7-C, 8-C, and 9-C atoms of cinnamic acid are incorporated into C-1 and C-3, C-2 and C-4, and C-5 of (*Z*)-hinokiresinol, respectively. Thus, ring- and side-chain-labelled tracer results indicate that all carbon atoms of (*Z*)-hinokiresinol are found to originate from C_6–C_3 (phenylpropanoid) monomers, and this compound is formed with a loss of one carbon atom at the 9-position of one of the coupling monomers. Furthermore, a competitive tracer experiment with simultaneous administration of 4-[*ring*-$^{13}C_6$]coumaric acid and 4-[7,9,9-$^{2}H_3$]coumaryl alcohol indicates that the C_6–C_3 moiety of (*Z*)-hinokiresinol is derived from 4-coumaryl alcohol, while the C_6–C_2 moiety originates from a 4-coumaroyl compound such as 4-coumaroyl CoA and not directly from 4-coumaryl alcohol.

Introduction

Norlignans are a class of natural phenolic compounds with diphenylpentane carbon skeletons (C_6–C_5–C_6) found in many conifers and some Leguminosae trees as well as monocotyledonous plants.[1–6] Typical norlignans having the 1,3-diphenylpentane [C_6–C_3(C_2)–C_6] structure {*e.g.*, hinokiresinol [(*E*)-hinokiresinol] **2**, agatharesinol **3**, and sequirin-C **4**, Fig. 1} occur in coniferous trees (especially in heartwood) of Cupressaceae, Taxodiaceae, and Araucariaceae,[2–4,7] while γ-lactonized 1,3-diphenylpentane norlignans (*e.g.*, pueroside A and B) were isolated from two Leguminosae trees (*Pueraria lobata* and *Sophora japonica*).[8–10] Some monocotyledonous Liliaceae and Hypoxidaceae plants are also good sources of 1,3-diphenylpentane and 1,5-diphenylpentane norlignans. For instance, (*Z*)-hinokiresinol (= nyasol **1**) (Fig. 1) which is the geometrical isomer of a coniferous heartwood norlignan, (*E*)-hinokiresinol, was isolated from *Asparagus* and *Anemarrhena*.[11–13]

Biosynthesis of norlignans and related compounds is receiving widespread interest in many aspects. First, they have various biological activities: *e.g.*, antifungal activity,[14] antiprotozoal activity,[11] estrogen-like activity,[15] inhibitory effect on cyclic adenosine monophosphate (cAMP) phosphodiesterase,[12,16] and inhibitory activity of Leukotriene B_4 binding to human neutrophils.[17] In addition, some norlignans are known as phytoalexins {(*Z*)-hinokiresinol in *Asparagus officinalis*[18] and hinokiresinol [= (*E*)-hinokiresinol] in *Cryptomeria japonica*[19]}.

Second, probably related to the nature of phytoalexins,

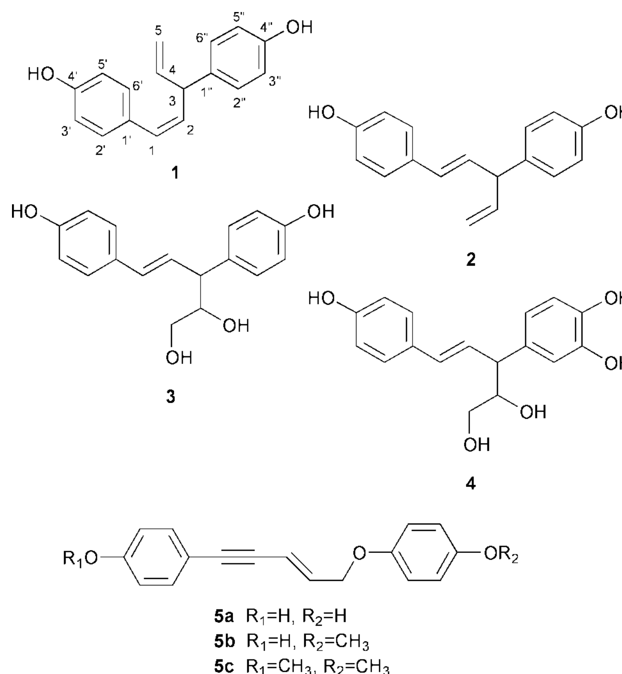

Fig. 1 Chemical structures of norlignans and related compounds. **1**, (*Z*)-hinokiresinol; **2**, (*E*)-hinokiresinol; **3**, agatharesinol; **4**, sequirin-C; **5a**, asparenydiol; **5b**, asparenyol; **5c**, asparenyn.

norlignans are deposited in significant amounts in the heartwood region of woody plants. In addition, heartwood coloration of the conifers *C. japonica* (Japanese cedar)[20] and *Chamaecyparis obtusa* (hinoki cypress),[21] which account for 31 and 40% of total artificially afforested area in Japan, respectively,[22] is due to norlignans. Therefore, their biosynthesis is involved in heartwood formation that is the

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metabolic event specific to woody plants, but not to herbaceous plants.

During the last decade, metabolic engineering of trees has been developed significantly with the aid of the knowledge of herbaceous plant molecular biology. For example, a transgenic aspen (*Populus tremuloides*) with less lignin content and higher cellulose content has been produced by Chiang and co-workers[23] Now, exploiting knowledge of molecular mechanisms involved in metabolic events specific to woody plants is necessary to accelerate metabolic engineering of trees. However, nothing is known about the molecular mechanisms specific to woody plants. Fortunately, the fact that norlignan biosynthesis is involved in heartwood formation implies that the molecular mechanisms can be a clue to help us elucidate mechanisms of metabolisms specific to woody plants.

Several hypothetical biosynthetic pathways have been proposed based on the chemical structures of norlignans,[4,5,24–29] but without any experimental evidence. Recently, Takasugi[18] reported that a herbaceous plant, *Asparagus officinalis*, inoculated with a phytopathogen produced (*Z*)-hinokiresinol **1** as a phytoalexin. Terada *et al.*[30] reported that the cell cultures of the plant produced norlignan-related C_6–C_5–O–C_6 compounds, asparenydiol **5a** and its methylated compounds (asparenyol **5b** and asparenyn **5c**) (Fig. 1), without elicitor treatment. Later, they demonstrated that asparenyol **5b** was derived from two units of phenylalanine with a loss of one carbon atom at the 9-position of phenylalanine based on ^{13}C tracer experiments,[31] and assumed hinokiresinol **1** or **2** as a putative precursor of asparenyol **5b**, although without any experimental evidence.[32]

These reports[18,30–32] stimulated us to examine whether fungal treatment of *Asparagus* cell culture would elicit norlignan production, and to seek the possibility of using this system as a model to elucidate norlignan biosynthesis in conifers. Using a fungal-elicited *A. officinalis* cell system, we report here the first evidence that (*Z*)-hinokiresinol **1** is formed from the coupling of two units of phenylpropanoid monomers with the loss of one carbon atom at the 9-position of one of the monomers. In addition, the evidence indicating that two non-identical phenylpropane units, probably 4-coumaryl alcohol and a 4-coumaroyl compound, are the immediate precursors of (*Z*)-hinokiresinol **1** was obtained based on simultaneous administration of two distinct phenylpropanoid monomers to the cultured cells.

Results and discussion

Asparagus officinalis cell suspension culture producing (*Z*)-hinokiresinol 1 after elicitor treatment

First, we induced *Asparagus* cell suspension culture according to Terada *et al.*,[30] and submitted it to treatment with three fungal elicitors. Preliminary GC-MS analysis (data not shown) revealed that the amount of (*Z*)-hinokiresinol in the cultured cells before the elicitor treatment was negligible, but increased significantly 24–45 h after any one of the elicitor treatments, indicating that all three fungal elicitors worked similarly.

Next, to confirm the production of (*Z*)-hinokiresinol unequivocally, it was isolated chromatographically from MeOH extracts of the elicitor-treated cells following their β-glucosidase treatment. The compound was identified by comparing the mass spectrum of its trimethylsilyl (TMS) ether, retention time on GC, and the ^{1}H NMR and ^{13}C NMR spectral data with those of (*E*)-hinokiresinol **2** isolated from *Chamaecyparis obtusa*[33] and with the literature data of (*Z*)-hinokiresinol.[13,15] The yield was 0.01% based on dry cell weight. GC-MS analysis MeOH extracts also showed the presence of trace amounts of (*E*)-hinokiresinol, which was identified by comparing the mass spectrum and retention time on GC with those of an authentic sample.[33]

Table 1 Mass spectral data of molecular-ion region of unlabelled and labelled (*Z*)-hinokiresinols (TMS ether) formed by *A. officinalis*

	Relative intensity (%)[a]			
		Administered cinnamic acids (CAs)		
m/*z*	Unlabelled	[7-^{13}C]CA	[8-^{13}C]CA	[9-^{13}C]CA
395	27.3			
396	100	100	100	100
397	39.9	67.1	59.6	100.4
398	15.8	114	55.7	39.9

[a] Relative intensity was calculated on the basis of peak intensity at *m*/*z* 396, which is the molecular ion of unlabelled (*Z*)-hinokiresinol. The values are the average of triplicated measurements.

All carbon atoms of (*Z*)-hinokiresinol 1 are derived from phenylpropanoid monomers

We first administered L-[*ring*-$^{13}C_6$]phenylalanine (Icon, 98 atom% ^{13}C) to the elicitor-treated *A. officinalis* cells, and the β-glucosidase-treated MeOH extract was submitted to GC-MS analysis to examine the incorporation of ^{13}C. Compared with the mass spectrum of unlabelled (*Z*)-hinokiresinol TMS ether (Fig. 2*A*), the enhanced ion peak at *m*/*z* 408 ($[M]^+ + 12$) was observed, indicating unequivocally that the two aromatic rings of (*Z*)-hinokiresinol were derived from L-phenylalanine (Fig. 2*B*).

Similarly, we next administered cinnamic acids labelled with ^{13}C in the side chain to the *Asparagus* cells individually, and quantified ^{13}C incorporation into (*Z*)-hinokiresinol. Table 1 shows relative intensities of molecular-ion region of the mass spectra of unlabelled (*Z*)-hinokiresinol TMS ether and ^{13}C-enriched (*Z*)-hinokiresinol TMS ethers formed after the individual administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid.‡ High intensities at *m*/*z* 398 were observed when we administered [7-^{13}C]cinnamic acid and [8-^{13}C]cinnamic acid, clearly indicating that two ^{13}C atoms were incorporated into (*Z*)-hinokiresinol from [7-^{13}C]cinnamic acid and [8-^{13}C]cinnamic acid, respectively. On the other hand, in the case of [9-^{13}C]cinnamic acid administration, the ion intensities at *m*/*z* 397 and 396 were almost equal, indicating the enrichment by one ^{13}C atom. At the same time, the intensity at *m*/*z* 398 was 39.7% of that at *m*/*z* 397, nearly identical to the ion intensity ratio between *m*/*z* 398 and 397 of the unlabelled compound, and revealing that incorporation of two ^{13}C atoms into (*Z*)-hinokiresinol from [9-^{13}C]cinnamic acid did not occur.

In order to confirm the mass spectral analysis and to determine the ^{13}C-enriched position in the side chain of (*Z*)-hinokiresinol, we next isolated ^{13}C-enriched (*Z*)-hinokiresinols formed after administration of the three [^{13}C]cinnamic acids in separate experiments, and then they were submitted to ^{13}C NMR measurements. As shown in Table 2, when [7-^{13}C]-cinnamic acid was administered, specific ^{13}C enrichments at C-1 (11.7 atom% excess) and C-3 (10.6 atom% excess) of (*Z*)-hinokiresinol were observed. Similarly, ^{13}C enrichments at C-2 (32.3 atom% excess) and C-4 (31.2 atom% excess) occurred when [8-^{13}C]cinnamic acid was fed. As for the feeding of [9-^{13}C]cinnamic acid, significant ^{13}C enrichment at only C-5 (26.3 atom% excess) was observed. ^{13}C Enrichments at other positions were negligible ($-0.27 \sim 0.56$ atom% excess). Although the signal of C-5 of (*Z*)-hinokiresinol appears close to those of C-3″, C-5″, C-3′, and C-5′, a C–H correlation in HMQC spectra indicates that the enhanced signal in the [9-^{13}C]cinnamic acid administration was assigned to C-5 (data not shown). Thus, these ring (Fig. 2*B*) and side chain (Tables 1

‡ The non-systematic numbering scheme for the labelling used in this paper is shown in Fig. 3. Strictly speaking, '[9-^{13}C]cinnamic acid' should read 'cinnamic [^{13}C] acid' throughout.

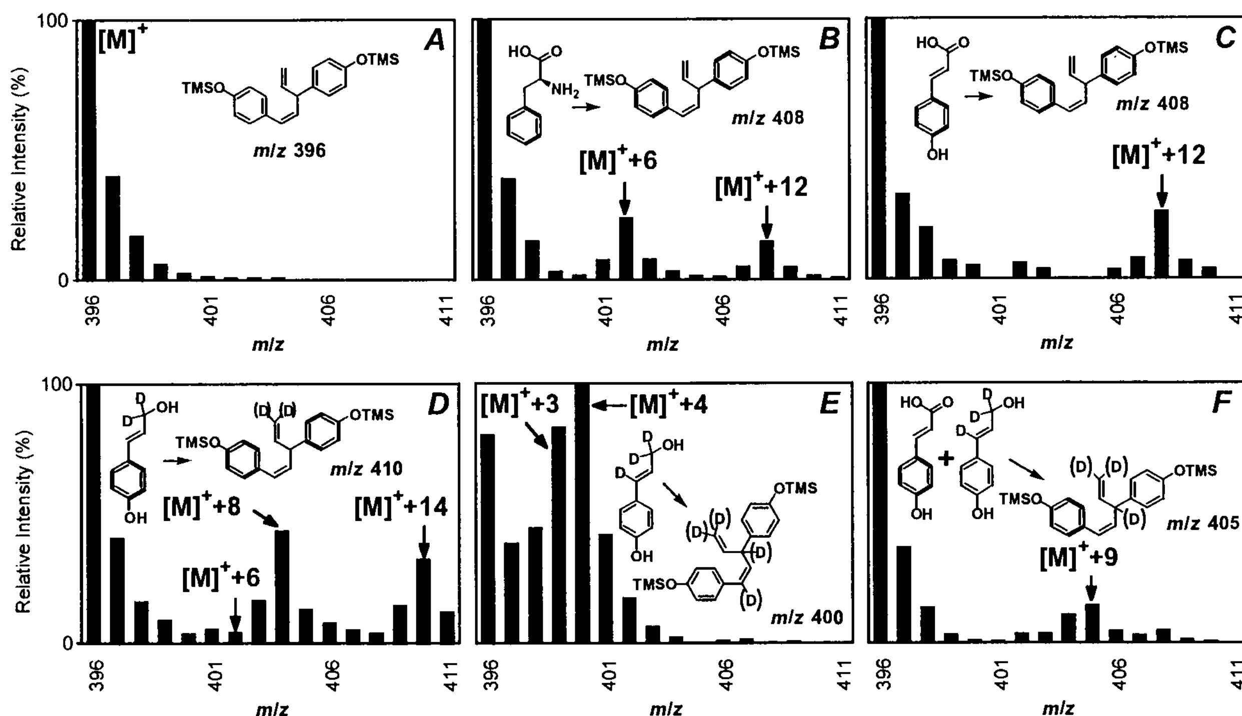

Fig. 2 Mass spectra of molecular-ion region of (*Z*)-hinokiresinol TMS ethers. *A*, unlabelled. *B*, formed after L-[*ring*-$^{13}C_6$]phenylalanine administration. *C*, formed after 4-[*ring*-$^{13}C_6$]coumaric acid administration. *D*, formed after 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol administration. *E*, formed after 4-[7,9,9-$^2H_3$]coumaryl alcohol administration. *F*, formed after simultaneous administration of 4-[*ring*-$^{13}C_6$]coumaric acid and 4-[7,9,9-$^2H_3$]-coumaryl alcohol. ^{13}C Atoms are shown as bold lines in aromatic rings, and D in the chemical structures represents ^2H.

Table 2 ^{13}C Enrichments of carbons in (*Z*)-hinokiresinol isolated following administration of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid in the elicited *A. officinalis* cells

		Atom% ^{13}C excess^a		
		Administered cinnamic acids (CAs)		
Carbon number (*N*)	δ_C ($CDCl_3$)	[7-^{13}C]CA	[8-^{13}C]CA	[9-^{13}C]CA
4′	154.6	0	0	0
4″	154.1	0.04	−0.02	0.03
4	140.8	−0.10	31.2	−0.01
1″	135.7	−0.27	0.21	0.07
2	131.8	−0.19	32.3	0.20
2′, 6′	130.1	−0.21	0.39	−0.01
1′	129.9	0.41	0.55	0.55
2″, 6″	128.9	−0.02	0.29	0.15
1	128.7	11.7	−0.08	0.18
3″, 5″	115.4	0.04	0.56	−0.04
3′, 5′	115.2	0.07	0.36	0.00
5	115.1	0.17	−0.17	26.3
3	46.8	10.6	0.25	0.05

^{a} Atom% ^{13}C excess = $\{(R_N/R_{N(UL)}) \times 1.1\} - 1.1$, where R_N is the ratio of the peak intensity at *N*-position in labelled (*Z*)-hinokiresinol calculated on the basis of the peak intensity at the 4′-position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at *N*-position in unlabelled (*Z*)-hinokiresinol. The value 1.1 is the theoretical ^{13}C natural abundance (atom%).

and 2) ^{13}C-tracer experiments unequivocally established that all 17 carbon atoms of (*Z*)-hinokiresinol are derived from phenylpropanoid monomers. Also, it was conclusively demonstrated that the side chain, 7-C, 8-C, and 9-C atoms, of cinnamic acid were incorporated into C-1 and C-3, C-2 and C-4, and C-5 of (*Z*)-hinokiresinol, respectively (Fig. 3). Thus, intramolecular rearrangement of the side chain carbon atoms of the monomers did not occur in (*Z*)-hinokiresinol formation.

The immediate C_6–C_3 precursors of (*Z*)-hinokiresinol

Our attention was next focused on the immediate C_6–C_3 (phenylpropanoid monomer) precursor(s) of (*Z*)-hinokiresinol. We synthesized the following ^{13}C- and/or ^2H-labelled compounds: 4-[*ring*-$^{13}C_6$]coumaric acid, 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]-coumaryl alcohol, 4-[7,9,9-$^2H_3$]coumaryl alcohol, and 4-[9-^2H, *ring*-$^{13}C_6$]coumaraldehyde,§ and administered the compounds individually to the elicited *Asparagus* cells.

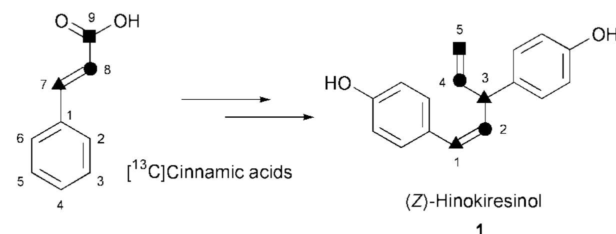

Fig. 3 ^{13}C-Labelling patterns of (*Z*)-hinokiresinol incorporating [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, or [9-^{13}C]cinnamic acid. ▲ = 7-^{13}C; ● = 8-^{13}C; ■ = 9-^{13}C.

When 4-[*ring*-$^{13}C_6$]coumaric acid was fed, GC-MS analysis of the formed (*Z*)-hinokiresinol showed significant enhancement of the ion peak at *m*/*z* 408 ([M]$^+$ + 12) (Fig. 2*C*), indicating that 4-coumaric acid was on the metabolic pathway leading to (*Z*)-hinokiresinol. When 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol was fed to the cells, great enhancement of the ion peak at *m*/*z* 410 ([M]$^+$ + 14) was observed (Fig. 2*D*). This result indicated that two units of 4-coumaryl alcohol were converted ultimately to (*Z*)-hinokiresinol with the loss of the two 9-positioned deuterium atoms from one of the monomers, but did not imply two units of the alcohol are directly involved in dimerization giving rise to (*Z*)-hinokiresinol. Importantly, when 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol was administered, enhancement at *m*/*z* 404 ([M]$^+$ + 8) (Fig. 2*D*) was also observed, which was assigned to (*Z*)-[$^2H_2$, $^{13}C_6$]hinokiresinol TMS ether, *i.e.*, the product of coupling of one unit of exogenous 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol with an endogenous unlabelled phenylpropane unit. This endogenous precursor-induced dilution effect is rather common in feeding

§ Structures for unlabelled coumaric acid **8a**, coumaryl alcohol **9** and coumaraldelhyde **8c** are presented in Fig. 4.

experiments, and, in fact, also occurred in the case of L-[*ring*-$^{13}C_6$]phenylalanine administration (Fig. 2*B*). In addition to the significant enhancement of the ion peak at *m*/*z* 408 ($[M]^+ + 12$), due to the incorporation of two [$^{13}C_6$]phenylalanine units into (*Z*)-hinokiresinol, great enhancement was also observed at *m*/*z* 402 ($[M]^+ + 6$), and may be ascribed to coupling of one [$^{13}C_6$]phenylalanine unit and one endogenous unlabelled phenylpropane unit. Also, in the case of 4-[9-^{2}H, *ring*-$^{13}C_6$]-coumaraldehyde feeding, the ion peak at *m*/*z* 403 ($[M]^+ + 7$) was increased in addition to enhancement at *m*/*z* 409 ($[M]^+ + 13$) (data not shown). Interestingly, however, the ion peak at *m*/*z* 402 {$[M]^+ + 6$, (*Z*)-[$^{13}C_6$]hinokiresinol TMS ether} (Fig. 2*D*) after 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol administration was not significant. If one such labelled 4-coumaryl alcohol unit and one endogenous unlabelled 4-coumaryl alcohol unit are directly involved in the dimerization, both $[M]^+ + 8$ and $[M]^+ + 6$ ions must appear with equal intensity. This suggests that two 4-coumaryl alcohol units were not involved directly in coupling, and implies the coupling of one 4-coumaryl alcohol unit and another phenylpropane unit which can be formed from 4-coumaryl alcohol.

It is established that the reduction of cinnamaldehyde and cinnamoyl CoA by cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl CoA reductase (CCR), respectively, is reversible.[34–36] Hence, we hypothesized that some of the exogeneously administered 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol was converted to 4-[9-^{2}H, *ring*-$^{13}C_6$]coumaraldehyde and 4-[*ring*-$^{13}C_6$]coumaroyl CoA, which in turn coupled with 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol to afford (*Z*)-[$^2H_2$, $^{13}C_{12}$]-hinokiresinol.

To test this hypothesis we carried out the simultaneous administration of two distinct, possible precursors. Thus, equal molar amounts of 4-[*ring*-$^{13}C_6$]coumaric acid and 4-[7,9,9-$^2H_3$]-coumaryl alcohol were administered to elicited cells in a single flask, and the results were compared with those obtained after individual administration of the two precursors as positive controls. Again, as shown in Fig. 2*C*, administration of only 4-[*ring*-$^{13}C_6$]coumaric acid resulted in the enhanced ion peaks of $[M]^+ + 12$ {(*Z*)-[$^{13}C_{12}$]hinokiresinol TMS ether}. Similarly, administration of 4-[7,9,9-$^2H_3$]coumaryl alcohol alone resulted in formation of (*Z*)-[$^2H_4$]hinokiresinol TMS ether ($[M]^+ + 4$) and (*Z*)-[$^2H_3$]hinokiresinol TMS ether ($[M]^+ + 3$) (Fig. 2*E*) which corresponded to (*Z*)-[$^2H_2$, $^{13}C_{12}$]hinokiresinol TMS ether ($[M]^+ + 14$) and (*Z*)-[$^2H_2$, $^{13}C_6$]hinokiresinol ($[M]^+ + 8$), respectively, in 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol administration (Fig. 2*D*). In sharp contrast, the simultaneous administration of the two precursors (Fig. 2*F*) provided no significant evidence for coupling products of two units of 4-[7,9,9-$^2H_3$]coumaryl alcohol {$[M]^+ + 4$, (*Z*)-[$^2H_4$]hinokiresinol TMS ether}. In addition, the ion peak at *m*/*z* 408 {$[M]^+ + 12$, (*Z*)-[$^{13}C_{12}$]hinokiresinol TMS ether} showed only a small increase, compared with the unlabelled one (Fig. 2*A*). The ion peak at *m*/*z* 405 ($[M]^+ + 9$) was prominent, and was derived by the coupling of one 4-[7,9,9-$^2H_3$]coumaryl alcohol unit and with 4-[*ring*-$^{13}C_6$]coumaric acid unit, confirming our hypothesis that (*Z*)-hinokiresinol is not formed by the direct dimerization of two units of 4-coumaryl alcohol. Instead, the C_6–C_3 moiety of (*Z*)-hinokiresinol is derived from a 4-coumaryl alcohol unit, while the C_6–C_2 moiety is from a 4-coumaroyl compound (HO–C_6H_4–CH=CH–CO–R) such as 4-coumaric acid, 4-coumaroyl CoA, or 4-coumaraldehyde (Fig. 4).

Furthermore, the incorporation of four deuterium atoms into (*Z*)-hinokiresinol from 4-[7,9,9-$^2H_3$]coumaryl alcohol (Fig. 2*E*) indicates that the hydrogen atom at the 7-position of 4-coumaryl alcohol is retained in (*Z*)-hinokiresinol, which therefore eliminates the possibility of oxidation at the 7-position of the monomer to give a C-7 carbonyl group, and the intermediacy of 3-(4-hydroxyphenyl)-3-oxopropionic acid derivatives **10** as precursors in the formation of (*Z*)-hinokiresinol (Fig. 5). Also, the results suggest that 1,3-bis(4-hydroxyphenyl)pent-4-en-1-one **11**, which was isolated from *Anemarrhena asphodeloides* together with (*Z*)-hinokiresinol,[37] is not a prerequisite intermediate of (*Z*)-hinokiresinol biosynthesis (Fig. 5), since this compound does not have a proton at the 1-position.

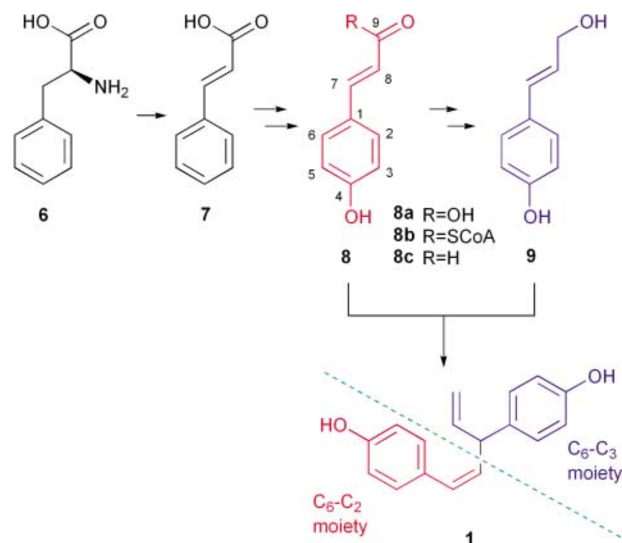

Fig. 4 Proposed biosynthetic pathway for (*Z*)-hinokiresinol. **6**, L-phenylalanine; **7**, cinnamic acid; **8a**, 4-coumaric acid; **8b**, 4-coumaroyl CoA; **8c**, 4-coumaraldehyde; **9**, 4-coumaryl alcohol. The C_6–C_3 moiety of (*Z*)-hinokiresinol (blue) was derived from 4-coumaryl alcohol **9**, while the C_6–C_2 moiety (red) was from a 4-coumaroyl compound **8**.

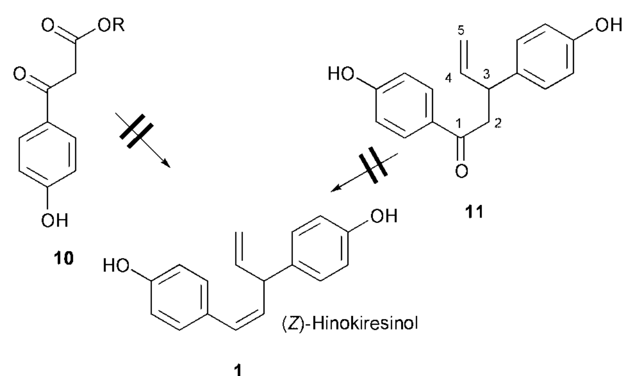

Fig. 5 The involvement of 3-(4-hydroxyphenyl)-3-oxopropionic acid derivatives **10** and 1,3-bis(4-hydroxyphenyl)pent-4-en-1-one **11** was not prerequisite in *Z*-hinokiresinol biosynthesis.

In conclusion, it has been shown for the first time that all carbon atoms of a norlignan, (*Z*)-hinokiresinol, are derived from phenylpropanoid monomers with the loss of one carbon atom at the 9-position of one of the monomers. The C_6–C_3 moiety of (*Z*)-hinokiresinol originates from 4-coumaryl alcohol, while the C_6–C_2 moiety was from a 4-coumaroyl compound. The biogenesis of the C_6–C_2 moiety awaits further evidence from enzymatic studies, which are underway in our laboratory.

Experimental

Plant material

Seeds of *Asparagus officinalis* cv. Akuseru (Takii Seed Co., Japan) were germinated aseptically on Linsmaier & Skoog agar medium (0.8% agar, 3% glucose, 4×10^{-4} g l^{-1} of 2,4-dichlorophenoxyacetic acid and 2×10^{-4} g l^{-1} of benzyl-adenine).[38] Callus was formed directly from the seedlings. The cell-suspension culture was obtained by transferring pieces of callus to the same medium but without agar, followed by incubation under the same condition (96–98 rpm at 27 °C in the

dark) as reported by Terada *et al.*[30] The cells were subcultured every 4 weeks.

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a JNM-LA400MK FT-NMR System (JEOL). Chemical shifts and coupling constants (J) are given in δ and Hz, respectively. GC-MS, DI-MS and HR-MS were performed on a JMS-DX303HF mass spectrometer (JEOL) equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system. GC-MS measurement conditions were as follows: electron-impact mode, 70 eV; gas-chromatographic column, Shimadzu Hicap CBP-10M25-025 (5 m × 0.2 mm); temperature, 40 °C at t = 0–2 min, then to 190 °C at 20 °C min^{-1}; carrier gas, He; splitless injection. Samples dissolved in *N,O*-bis(trimethylsilyl)acetamide were subjected to GC-MS measurement after heating at 60 °C for 45 min.

Synthesis of labelled precursors

Synthesis of ^{13}C-labelled cinnamic acids. [7-^{13}C]Cinnamic acid was prepared by Knoevenagel condensation of unlabelled malonic acid and [*carbonyl*-^{13}C]benzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C). [7-^{13}C]Cinnamic acid (98 atom% excess): δ_H ($CDCl_3$; carbon numbers are shown in Fig. 3) 6.46 (1 H, dd, J 16.0 and 1.1, 8-H), 7.40–7.42 (3 H, m, ArH), 7.54–7.57 (2 H, m, ArH) and 7.80 (1 H, dd, J 156.6 and 15.9, 7-H); *m*/*z* (EI) 149.0549 (M$^+$, 85%. $C_8{}^{13}CH_8O_2$ requires *M*, 149.0558), 148 (100), 132 (23), 121 (6), 105 (11), 104 (53), 103 (21), 92 (18), 78 (24), 77 (21), 52 (13) and 51 (18).

[8-^{13}C]Cinnamic acid was prepared from [2-^{13}C]malonic acid (Cambridge Isotope Laboratories, 99 atom% ^{13}C) and unlabelled benzaldehyde by a method similar to that for [7-^{13}C]-cinnamic acid. [8-^{13}C]Cinnamic acid (98 atom% excess): δ_H($CDCl_3$) 6.46 (1 H, dd, J 162.8 and 16.1, 8-H), 7.38–7.42 (3 H, m, ArH), 7.53–7.57 (2 H, m, ArH) and 7.80 (1 H, dd, J 15.9 and 2.9, 7-H); *m*/*z* (EI) 149.0546 (M$^+$, 79%), 148 (100), 132 (21), 121 (5), 105 (10), 104 (48), 103 (17), 91 (11), 78 (20), 77 (20), 52 (10) and 51 (16).

[9-^{13}C]Cinnamic acid was prepared similarly from [1,3-$^{13}C_2$]-malonic acid (Cambridge Isotope Laboratories, 99 atom% ^{13}C) and unlabelled benzaldehyde. [9-^{13}C]Cinnamic acid (98 atom% excess): δ_H ($CDCl_3$) 6.46 (1 H, dd, J 15.9 and 2.7, 8-H), 7.40–7.41 (3 H, m, ArH), 7.54–7.57 (2 H, m, ArH) and 7.80 (1 H, dd, J 15.9 and 6.8, 7-H); *m*/*z* (EI) 149.0546 (M$^+$, 85%), 148 (100), 132 (22), 120 (6), 104 (6), 103 (44), 102 (15), 91 (14), 77 (27) and 51 (20).

Synthesis of 4-[*ring*-$^{13}C_6$]coumaric acid. 4-[*ring*-$^{13}C_6$]-Coumaric acid was prepared from unlabelled malonic acid and 4-hydroxy[*ring*-$^{13}C_6$]benzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C) by a method similar to that for [7-^{13}C]cinnamic acid. 4-[*ring*-$^{13}C_6$]Coumaric acid (99 atom% excess): δ_H (acetone-d_6) 6.33 (1 H, dd, J 16.0 and 5.2, 8-H), 6.66–7.11 (2 H, m, ArH), 7.31–7.77 (2 H, m, ArH) and 7.60 (1 H, ddt, J 16.0, 5.3 and 1.8, 7-H); *m*/*z* (EI) 170.0677 (M$^+$, 100%. $C_3{}^{13}C_6H_8O_3$ requires *M*, 170.0675), 169 (44), 168 (4), 167 (0.6), 166 (0.3), 165 (0.2), 164 (0.1), 153 (45), 152 (7), 125 (27), 124 (20), 113 (9), 96 (15) and 68 (6).

Synthesis of 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol. 4-Hydroxy[*ring*-$^{13}C_6$]benzaldehyde (Cambridge Isotope Laboratories, 99 atom% ^{13}C) was condensed with ethyl hydrogen malonate to give ethyl 4-[*ring*-$^{13}C_6$]coumarate, which was ethoxyethylated [ethyl vinyl ether, (±)-camphor-10-sulfonic acid, CH_2Cl_2, 0 °C] to give ethyl 4-[*ring*-$^{13}C_6$]coumarate ethoxyethyl ether. This ether was reduced with $LiAl^2H_4$ (Aldrich, 98 atom% ^{2}H) to yield 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol ethoxyethyl ether, which was hydrolyzed with 0.1 M HCl–acetone (1 : 9, v/v) to afford 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol (99 atom% excess ^{2}H and 99 atom% excess ^{13}C): δ_H (acetone-d_6) 6.17 (1 H, br d, J 16, 8-H), 6.49 (1 H, m, 7-H), 6.55–6.99 (2 H, m, ArH) and 7.02–7.48 (2 H, m, ArH); *m*/*z* (EI) 158.1007 (M$^+$, 75%. $C_3{}^{13}C_6H_8{}^2H_2O_2$ requires *M*, 158.1008), 157 (10), 156 (3), 155 (1), 154 (1), 153 (0.5), 152 (0.1), 151 (0.1), 150 (0.1), 139 (12), 138 (7), 114 (30), 113 (100), 112 (10), 101 (21), 100 (27), 82 (10), 70 (5) and 56 (6).

Synthesis of 4-[9-^{2}H, *ring*-$^{13}C_6$]coumaraldehyde. 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]Coumaryl alcohol ethoxyethyl ether was oxidized (activated MnO_2, CH_2Cl_2, room temperature) to afford 4-[9-^{2}H, *ring*-$^{13}C_6$]coumaraldehyde ethoxyethyl ether, which was hydrolyzed with HCl in acetone to give rise to 4-[9-^{2}H, *ring*-$^{13}C_6$]-coumaraldehyde (>99 atom% excess ^{2}H and 99 atom% excess ^{13}C): δ_H (acetone-d_6) 6.59 (1 H, dd, J 16.0 and 4.8, 8-H), 6.69–7.15 (2 H, m, ArH), 7.37–7.83 (2 H, m, ArH) and 7.58 (1 H, ddt, J 15.9, 5.3 and 1.1, 7-H); *m*/*z* (EI) 155.0802 (M$^+$, 100%. $C_3{}^{13}C_6H_7{}^2HO_2$ requires *M*, 155.0788), 154 (85), 153 (21), 152 (2), 151 (0.4), 150 (0), 149 (2), 148 (0.8), 145 (0), 138 (29), 127 (27), 126 (24), 125 (20), 114 (7), 110 (6), 101 (17), 97 (25), 96 (17), 94 (6), 69 (11), 68 (9) and 54 (5).

Synthesis of 4-[7,9,9-$^2H_3$]coumaryl alcohol. Methyl 4-hydroxybenzoate was benzylated with benzyl bromide and K_2CO_3 to give methyl 4-hydroxybenzoate benzyl ether, which was reduced with $LiAl^2H_4$ in anhydrous THF at room temperature to yield 4-benzyloxy[α-$^2H_2$]benzyl alcohol. 4-Benzyloxy[α-$^2H_2$]benzyl alcohol was oxidized (activated MnO_2, benzene, 50 °C) to afford 4-benzyloxy[α-^{2}H]benzaldehyde, which was hydrolyzed (conc. HCl in AcOH, refluxing temperature) to give 4-hydroxy[α-^{2}H]benzaldehyde. Then, 4-[7,9,9-$^2H_3$]coumaryl alcohol was prepared similarly to the preparation of 4-[9,9-$^2H_2$, *ring*-$^{13}C_6$]coumaryl alcohol, but with 4-hydroxy-[α-^{2}H]benzaldehyde as a starting material. 4-[7,9,9-$^2H_3$]-Coumaryl alcohol (99 atom% excess): δ_H ($CDCl_3$) 6.17 (1 H, s, 8-H), 6.76–6.80 (2 H, m, ArH) and 7.24–7.27 (2 H, m, ArH); *m*/*z* (EI) 153.0861 (M$^+$, 72%. $C_9H_7{}^2H_3O_2$ requires *M* 153.0869), 152 (5), 151 (3), 150 (1), 136 (9), 109 (25), 108 (100), 95 (28), 94 (19), 78 (11), 66 (5) and 52 (5).

Preparation of fungal elicitors

An unidentified filamentous fungus was separated from the experimental field of the Wood Research Institute, Kyoto University, by repeated streaking on a potato dextrose agar (Difco Laboratories). The isolated fungus was cultivated in a liquid medium (K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, Fe(III)-EDTA 20 mg, yeast extract 1 g, D-glucose 30 g, and L-asparagine 2 g l^{-1} of distilled water[39]) at 27 °C for 2 weeks. *Fusarium oxysporum* f. sp. *asparagi* IFO31382 and *Fusarium solani* IFO5892 were obtained from the Institute for Fermentation, Osaka. They were cultured in the same liquid media as described above. Mycelia of each fungus were collected by filtration, washed with distilled water, freeze-dried and powdered with pestle and mortar. The powdered mycelia thus obtained were suspended in distilled water (5 g l^{-1}), autoclaved (121 °C, 20 min), and used as fungal elicitors, individually.

Isolation of (*Z*)-hinokiresinol

A. officinalis cells, harvested 19 days after subculture, were collected by filtration through a tea strainer. The cells (222 g, fresh weight) thus obtained were resuspended in the fungal elicitor suspension (40 ml). After incubation (120 rpm) with shaking for an additional 42 h at 27 °C in the dark, the cells were collected by filtration and freeze-dried. The resulting dried material (12.9 g) was powdered with a pestle and mortar, and extracted with hot MeOH. The extract was suspended in 42 ml of 0.1 M AcONa buffer (pH 5.0) solution of β-glucosidase

(SIGMA, 3.8 units mg^{-1}, 0.175 g), and kept for 24 h at 37 °C. Next, the reaction mixture was extracted with EtOAc, and the solvent was evaporated off. The resultant residue was purified with silica gel column chromatography and silica gel TLC, giving rise to (*Z*)-hinokiresinol **1** (1.5 mg, 0.01% based on dry cell weight): δ_H ($CDCl_3$) 4.48 (1 H, dd, *J* 9.5 and 6.0, 3-H), 4.75 (1 H, br s, OH), 4.87 (1 H, br s, OH), 5.13–5.17 (2 H, m, 2 × 5-H), 5.67 (1 H, dd, *J* 11.3 and 10.1, 2-H), 5.96–6.04 (1 H, m, 4-H), 6.51 (1 H, d, *J* 11.4, 1-H), 6.76–6.79 (4 H, m, ArH), 7.09 (2 H, br d, *J* 8.6, ArH) and 7.16 (2H, br d, *J* 8.6, ArH); *m/z* [EI, (*Z*)-hinokiresinol TMS ether] 396 (M^+, 100%), 382 (15), 381 (27), 230 (59), 217 (43) and 179 (38); δ_C ($CDCl_3$) data are shown in Table 2.

Administration of labelled precursors

The following is the typical administration procedure for GC-MS analysis. After subculturing, *A. officinalis* cell-suspension culture was incubated for 19–30 days. Then, the fungal elicitor suspension (1 ml) was aseptically added to the cells (3–5 g). The culture was incubated (120 rpm at 27 °C in the dark) for an additional 3 h. Next, aqueous solutions of labelled precursors (3 mg, dissolved in the minimal amount of 0.1 M KOH, then made up to 0.5 ml with distilled water) were added aseptically. After incubation under the same condition for an additional 35 h, the cells were collected and freeze-dried. The resulting dried material (0.3–0.4 g) was powdered with a pestle and mortar, and extracted with hot MeOH. The extract was treated with β-glucosidase by a method similar to that for (*Z*)-hinokiresinol isolation, but scaled down proportionately. An aliquot of EtOAc extracts thus obtained was submitted to GC-MS analysis after TMS derivatization.

In separate experiments, three batches of *A. officinalis* cell-suspension culture were incubated for 24, 35, and 21 days, respectively. Then, the fungal elicitor suspensions (200, 200, and 80 ml) were aseptically added to the fresh cells (573, 595, and 615 g). The cultures were incubated (120 rpm at 27 °C in the dark) for an additional 3 h. Next, aqueous solutions of labelled precursors (247, 282, and 224 mg of [7-^{13}C]cinnamic acid, [8-^{13}C]cinnamic acid, and [9-^{13}C]cinnamic acid, respectively, were dissolved in the minimal amount of 0.1 M KOH, then made up to 30, 26, and 18 ml with distilled water, respectively) were added aseptically. After incubation under the same condition for an additional 35 h, the cells were collected and freeze-dried. The resulting dried materials (31.7, 22.1, and 45.7 g) were powdered with and pestle and mortar, and extracted with hot MeOH. The extracts were treated with β-glucosidase, individually. Aliquots of the reaction products were subjected to GC-MS analysis, individually, as above, and the remainders were purified by a similar method to that for (*Z*)-hinokiresinol isolation. The purified (*Z*)-hinokiresinols were submitted to ^{13}C NMR measurements.

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