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ORIGINAL ARTICLE

Shingo Kawai · Kyousuke Nakata · Hiroo Ichizawa
Tomoaki Nishida

3-(4-Hydroxyphenyl)propionic acid is involved in the biosynthesis of myricanol in *Myrica rubra*

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Abstract There is little evidence concerning the biosynthetic pathways for cyclic diarylheptanoids. We previously demonstrated that the cyclic diarylheptanoids myricanol and myricanone were biologically synthesized from two molecules of 4-coumaric acid by the feeding of 4-[8,9-$^{13}C_2$]coumaric acid to young shoots of *Myrica rubra*. In the present study, using a ^{13}C-labeled compound, we revealed that two molecules of 3-(4-hydroxyphenyl)propionic acid could also be a biosynthetic precursor of myricanol in *M. rubra*. These results indicated that both 4-coumaric acid and its dihydro-derivative were incorporated into myricanol. Competitive feeding experiments with 4-[8,9-$^{13}C_2$]coumaric acid and 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid were performed in *M. rubra* to determine the preferential incorporation of these two precursors. ^{13}C-NMR studies indicated that 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid was preferentially incorporated into myricanol. The data provided evidence for a biosynthetic sequence originating from 4-coumaric acid and leading to myricanol, through 3-(4-hydroxyphenyl)propionic acid, in *M. rubra*.

Key words *Myrica rubra* · Cyclic diarylheptanoids · Myricanol · Biosynthesis · 3-(4-Hydroxyphenyl)propionic acid · 4-Coumaric acid · ^{13}C-NMR

Introduction

Cyclic diarylheptanoids, myricanol (I) and myricanone (II), are two of the major components in *Myrica rubra* Sieb. et Zucc.,[1,2] and are reported to have antitumor-promoting effects[3] and to exhibit antiandrogen activities.[4] The bark of *M. rubra* was also used as an astringent, antidote, and antidiarrheal in Japanese folk medicine and has been used externally for burns and skin diseases in Chinese traditional medicine.

Diarylheptanoids comprise a class of natural products based on 1,7-diphenylheptane and have been isolated from Myricaceae, Betulaceae, Zingiberaceae, and Aceraceae plants.[5,6] The biosynthesis of diarylheptanoids, especially phenylphenalenone derivatives,[7–11] has been investigated, and two molecules of 4-coumaroyl-CoA and one of malonyl-CoA are reported to be concerned with the formation of their skeleton. However, there is little knowledge about the biosynthetic pathways for cyclic diarylheptanoids.

Our recent report on *M. rubra* young shoots fed with 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) revealed, through mass and ^{13}C-nuclear magnetic resonance (NMR) spectrometric analyses, that myricanol (I) and myricanone (II) were derived from two molecules of 4-coumaric acid (III).[12] In particular, the ^{13}C-NMR analyses of myricanol (I) isolated after administration of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) clearly demonstrated that C-8, C-9, C-11, and C-12 of myricanol (I) were derived from the C-8 and C-9 atoms of 4-coumaric acid (III). However, in a comparison of the chemical structures of myricanol (I) and 4-coumaric acid (III), the chain structures were apparently different, i.e., myricanol (I) had no unsaturated bond in the molecule. Therefore, it is conceivable that 3-(4-hydroxyphenyl)propionic acid (IV), which has a saturated chain and is the dihydro-analogue for 4-coumaric acid (III), could be a biosynthetic precursor for myricanol (I).

In this article, we reported on whether 3-(4-hydroxyphenyl)propionic acid (IV) was incorporated into myricanol (I). Furthermore, competitive feeding experiments on *M. rubra* with ^{13}C-labeled precursors of 4-coumaric acid (III) and 3-(4-hydroxyphenyl)propionic acid (IV) were conducted. Biosynthetic ^{13}C-NMR studies indicated that 3-(4-hydroxyphenyl)propionic acid (IV) was preferentially employed in the formation of myricanol (I) in *M. rubra*.

S. Kawai (✉) · K. Nakata · H. Ichizawa · T. Nishida
Laboratory of Forest Microbiology and Biochemistry, Department of Environment and Forest Resources Science, Faculty of Agriculture, Shizuoka University, Ohya, Suruga-ku, Shizuoka 422-8529, Japan
Tel. +81-54-238-4851; Fax +81-54-238-4851
e-mail: skawai@agr.shizuoka.ac.jp

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Materials and methods

General

Analytical and preparative thin-layer chromatography (TLC) was performed on silica gel (Merck Kieselgel 60 F_{254}). ^{1}H- and ^{13}C-NMR spectra were taken with a JEOL JMM EX-270 spectrometer using tetramethylsilane as an internal standard. Electron impact-mass spectroscopy (EI-MS) analyses were performed with a Shimadzu GCMS-QP 5050 gas chromatograph mass spectrometer.

Syntheses of labeled precursors and authentic compounds

The chemical structures of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) and 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) are illustrated in Fig. 1.

3-(4-Hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) was synthesized from [9-^{13}C]coumaric acid ([9-^{13}C]-III) prepared from 4-acetoxybenzaldehyde and [1-^{13}C]triethyl phosphonoacetate (Aldrich; 99 atom% ^{13}C) by the method as previously described.[12]

To a solution of [9-^{13}C]coumaric acid ([9-^{13}C]-III) (59.4 mg, 0.36 mmol) in methanol (2 ml) and acetic acid (0.1 ml), 10% palladium carbon (100 mg; Wako) was added, and the flask was evacuated and then filled with hydrogen gas. The resulting solution was stirred at ambient temperature for 60 min. The reaction mixture was filtered off and washed with methanol. The filtrate was evaporated under reduced pressure, and the residue was purified by TLC (solvent: MeOH-$CHCl_3$, 5/95) to give 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) (38.0 mg, 63%).

Direct-inlet mass spectroscopy (DI-MS) *m/z* (%): ([1-^{13}C]-IV) 77 (13), 91 (5.8), 107 (100), 108 (7.8), 120 (5.8), 166 (0.0), 167 (28), 168 (2.7), 169 (0.3); (IV) 77 (16), 91 (6.6), 107 (100), 108 (7.6), 120 (6.2), 166 (M^+, 23), 167 (2.4), 168 (0.2), 169 (0.0).

^{13}C-NMR (CD_3OD, 67.5 Hz) δ: ([1-^{13}C]-IV) 176.9 (C-1); (IV) 31.2 (C-3), 37.6 (C-2), 115.6 (C-8), 116.2 (C-3′, C-5′), 130.2 (C-2′, C-6′), 132.9 (C-1′), 156.7 (4′-C), 176.9 (C-1).

4-[8,9-$^{13}C_2$]Coumaric acid ([8,9-$^{13}C_2$]-III) was prepared as described previously.[12] Authentic myricanol (I) and myricanone (II) were previously isolated from the branches of *M. rubra*.[12]

Feeding experiment with 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV)

3-(4-Hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) (4.2 mg (25 μmol) in 1 ml 0.1 % NaOH) was administrated to excised *M. rubra* young shoots (harvested in October 2006; shoot size, 20–30 cm), which were allowed to metabolize for 2 weeks at room temperature under continuous light. The feeding experiments were duplicated. After incubation, the leaves were removed from the resulting shoots, and stems were frozen, powdered with a pestle and mortar, and extracted with hot MeOH for 12 h. The extracts were partitioned between EtOAc and water. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The myricanol (I) and myricanone (II) fractions were roughly separated from the extracts by TLC (solvent: ethyl acetate/*n*-hexane, 1/2), respectively, and analyzed by gas chromatography-mass spectroscopy (GC-MS). Then, the myricanol (I) and myricanone (II) fractions were combined, respectively, and further purified by TLC (solvent: MeOH-$CHCl_3$, 2/95). The purified myricanol (I) (~3 mg) and myricanone (II) (<1 mg) were analyzed by selected ion monitoring (SIM) of DI-MS, respectively. ^{13}C-NMR analysis of the myricanol (I) was also performed.

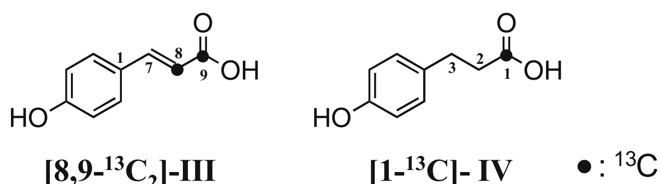

Fig. 1. Chemical structures of ^{13}C-labeled precursors. •, ^{13}C

Competitive feeding experiment with 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) and 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV)

Equal amounts of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) (2.1 mg, 12.5 μmol) and 3-(4-hydroxyphenyl)-[1-13C]propionic acid ([1-13C]-IV) (2.1 mg, 12.5 μmol) were combined and dissolved in 1 ml 0.1 % NaOH solution. The mixed solution was administrated to excised M. rubura young shoots (harvested in October 2006 and 2007; shoot size, 20–30 cm), which were allowed to metabolize for 2 weeks at room temperature under continuous light. The feeding experiments were duplicated. After incubation, the myricanol (I) and myricanone (II) fractions were separated and analyzed as already mentioned.

Results and discussion

3-(4-Hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) was administered to excised *M. rubra* young shoots, and it was allowed to metabolize for 2 weeks. The myricanol (I) and myricanone (II) fractions, obtained by TLC separation, were analyzed by GC-MS (data not shown), respectively, and the formation of [$^{13}C_2$]-myricanol (I) and [$^{13}C_2$]-myricanone (II) was determined by comparison with unlabeled authentic compounds. These fractions were further purified by TLC to confirm the incorporation rate of ^{13}C carbon into myricanol (I) (Fig. 2B) and myricanone (II) (Fig. 2E) using DI-MS with SIM mode. As shown in Table 1, the results indicated that ~3% of myricanol (I) and ~3% of myricanone (II) were biosynthesized from two molecules of 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV), respectively.

^{13}C-NMR analysis of myricanol (I) was conducted to determine the ^{13}C-enriched position in the heptane chains (Fig. 3). The NMR spectra of the isolated myricanol

Table 1. Mass spectral data of molecular-ion region of myricanol (**I**) and myricanone (**II**) isolated from *Myrica rubra*

Myricanol (**I**)				Myricanone (**II**)			
m/z	Relative intensity (%)			*m/z*	Relative intensity (%)		
	Unlabeled	Isolated after administration of			Unlabeled	Isolated after administration of	
		[1-^{13}C]-IV	**[8,9-$^{13}C_2$]-III** and **[1-^{13}C]-IV**			**[1-^{13}C]-IV**	**[8,9-$^{13}C_2$]-III** and **[1-^{13}C]-IV**
358	100	100	100	356	100	100	100
359	23.1	25.3	24.9	357	23.8	26.0	26.5
360	3.8	7.1	7.7	358	4.7	7.9	11.4
361	0.5	1.5	2.5	359	0.9	1.5	5.1
362	0.1	0.5	1.4	360	0.4	0.4	3.1

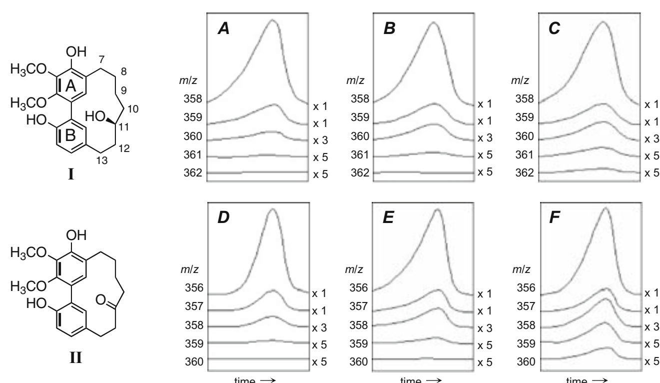

Fig. 2. Mass chromatograms of molecular ion regions of myricanol (I) and myricanone (II) using Direct-inlet mass spectroscopy (DI-MS) with selected ion monitoring (SIM) mode. *A*, *D* Unlabeled. *B*, *E* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) administration. *C*, *F* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) and 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) administration

(I) after administration of 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) (Fig. 3B) displayed enhancement of ^{13}C resonances at C-9 (δ 22.9) and C-11 (δ 68.6) positions. The magnified figures of each heptane-chain carbon (C-7–C-13) in the ^{13}C-NMR spectra of myricanol (I) are illustrated in Fig. 4, and the relative integral intensities of the heptane-chain carbons, based on that of C-10, were calculated as shown in Table 2. The relative intensities of the ^{13}C-signals were enhanced 3.6 and 3.2 times (i.e., intensity of singlet peak/intensity of naturally occurring singlet peak) for C-9 and C-11, respectively (Fig. 4B). These results clearly indicated that two molecules of 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) were incorporated into myricanol (I).

We have recently reported that two molecules of 4-coumaric acid (III) were precursors of myricanol (I) and myricanone (II) in *M. rubra*.[12] In extending our feeding experiments, we have obtained more detailed data indicating that 3-(4-hydroxyphenyl)propionic acid (IV) also behaves as an intermediate. The heptane chain of myricanol (I) is saturated, whereas 4-coumaric acid (IV) has an unsaturated propane chain. Therefore, it is conceivable that 3-(4-hydroxyphenyl)propionic acid (IV), which is the dihydro-analogue for 4-coumaric acid (III), could be a biosynthetic precursor for myricanol (I).

A competitive feeding experiment was conducted with two ^{13}C-labeled 4-coumaric acid ([8,9-$^{13}C_2$]-III) and single ^{13}C-labeled 3-(4-hydroxyphenyl)propionic acid ([1-^{13}C]-IV) to examine which is the downstream metabolism precursor for myricanol biosynthesis. The myricanol (I) and myricanone (II) fractions were purified and analyzed by DI-MS (Fig. 2C,F). As shown in Table 1, the results proved that ^{13}C derived from labeled precursor(s) was incorporated into both myricanol (I) and myricanone (II), but the results could not determine which precursor, ([8,9-$^{13}C_2$]-III) or ([1-^{13}C]-IV), was preferentially incorporated into (I) and (II). Consequently, the ^{13}C-NMR spectrum of myricanol (I) formed by this feeding experiment was measured (Fig. 3C).

If 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) was incorporated into myricanol (I), the resonances of C-8 (δ 25.7), C-9 (δ 22.9), C-11 (δ 68.6), and C-12 (δ 34.7) would appear as pseudo-triplets, thus indicating the presence of naturally occurring isotopomers (a singlet, central resonances of the pseudo-triplets) and isotopomers derived from two labeled carbons of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) in these positions (a doublet, due to ^{13}C-^{13}C coupling with adjacent ^{13}C). However, if 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) was incorporated, the singlet resonances of C-9 and C-11 would be stronger than the signals of naturally occurring isotopomers.

As shown in Fig. 4C, the ^{13}C-NMR spectrum of myricanol (I) isolated after administration of ^{13}C-labeled 4-coumaric acid ([8,9-$^{13}C_2$]-III) and 3-(4-hydroxyphenyl) propionic acid ([1-^{13}C]-IV) is very complex. As the C-9 and C-11 singlet signals were enhanced, it is clear that two molecules of 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) were incorporated into myricanol (I). On the other hand, doublet signals were also detected at C-8, C-9, C-11, and C-12; thus, incorporation of two molecules of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) to myricanol (I) was also confirmed. However, the incorporation ratios were different. In comparison with the doublet signals of C-9 and C-11, the stronger singlet signals of C-9 and C-11 revealed a higher degree of incorporation of 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) compared to 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III). Based on the intensities of doublets (i.e., sum of doublet intensities) and singlets (i.e., intensity of singlet peak – intensity of naturally occurring singlet) (see Table 2), the incorporation ratios of

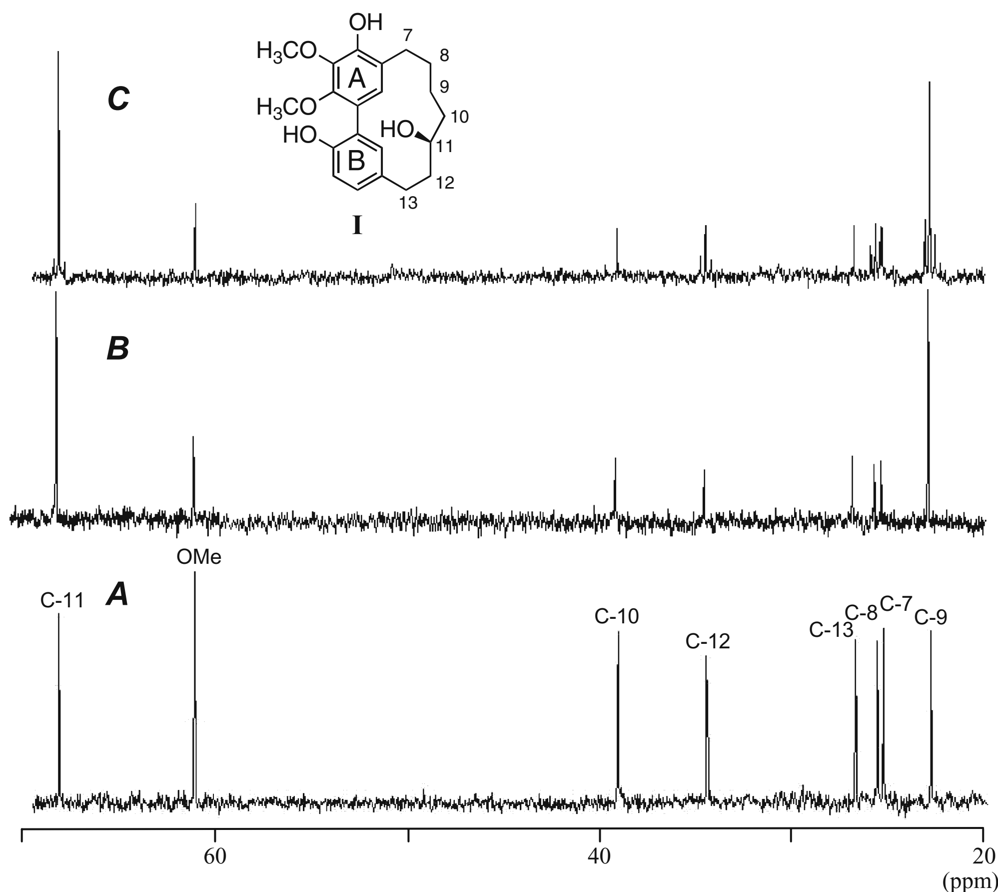

Fig. 3. ^{13}C-Nuclear magnetic resonance (NMR) spectra of myricanol (I). *A* Unlabeled. *B* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) administration. *C* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) and 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) administration

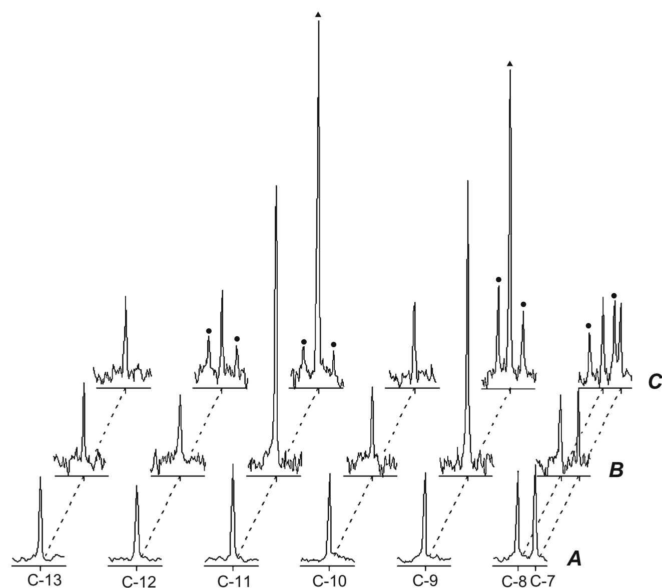

Fig. 4. Detailed resonances of side-chain carbons in ^{13}C-NMR spectra of myricanol (I). *A* Unlabeled. *B* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) administration. *C* Formed after 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) and 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) administration. ●, Signals derived from [1-^{13}C]-IV; ▲, signals derived from [8,9-$^{13}C_2$]-III

Table 2. Relative absorption intensities of heptane-chain carbons signal based on the intensity of C-10 in ^{13}C-NMR of myricanol (**I**) isolated from *M. rubra*

	Unlabeled	Isolated after administration of	
		[1-^{13}C]-IV	**[8,9-$^{13}C_2$]-III** and **[1-^{13}C]-IV**
C-7	1.01	0.97	1.03
C-8 (doublet)	–	–	1.67[a]
C-8 (singlet)	0.95	0.90	1.11
C-9 (doublet)	–	–	2.09[a]
C-9 (singlet)	1.01	3.65	4.28
C-10	1.00	1.00	1.00
C-11 (doublet)	–	–	0.46[a]
C-11 (singlet)	1.11	3.58	4.98
C-12 (doublet)	–	–	0.55[a]
C-12 (singlet)	0.86	0.82	1.08
C-13	0.95	1.03	1.07

[a] Sum of doublet intensities

labeled precursors ([8,9-$^{13}C_2$]-III) and ([1-^{13}C]-IV) were estimated to be ~1:1.6 for C-9 [doublet (2.09):singlet (3.27; 4.28 − 1.01)] and ~1:8.4 for C-11 [doublet (0.46):singlet (3.87; 4.98 − 1.11)], respectively.

In our present studies, 3-(4-hydrixyphenyl)propionic acid (IV) could be a biosynthetic precursor of myricanol (I) and preferentially incorporated into myricanol (I) rather than 4-coumaric acid (III). Under the in vivo conditions

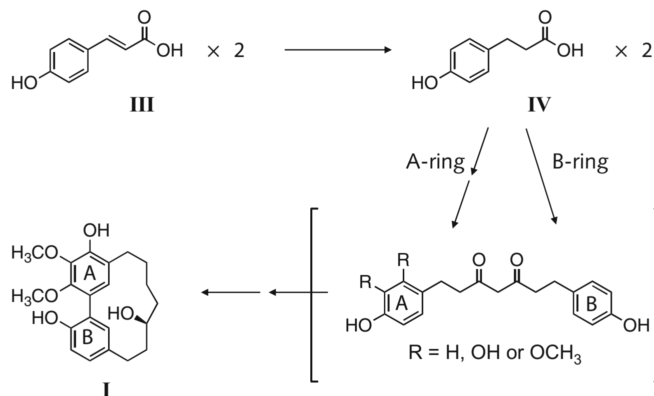

Fig. 5. Proposed biosynthetic pathways for myricanol (I) from 4-coumaric acid (III) in *Myrica rubra*

used in this study, multiple factors, such as solubility of the precursors, their absorption by the plant, and so on, are affecting the incorporation of precursors. However, when 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) or 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) was administered alone into *M. rubra*, their incorporation rates into myricanol (I) were relatively similar (3%–6%). Therefore, the present results suggest that direct incorporation of dihydrophenylpropanoid-type precursors into myricanol (I) and the dehydrogenation of a side chain of 4-coumaric acid (III) occurs before the formation of a diarylheptanoid skeleton (Fig. 5).

Similar results were reported by Kindl and coworkers[13,14] regarding the biosynthesis of 9,10-dihydrophenanthrenes (stilbenoids) of Orchidaceae. They demonstrated that 3-(3-hydroxyphenyl)propionic acid, as a dihydro-phenylpropanoid precursor, was incorporated into orchinol, and that 3,3′,5-trihydroxybibenzyl, obtained through the operation of bibenzyl synthase, and not stilbene synthase, was an intermediate compound of 9,10-dihydrophenanthrenes. However, the possibility of the interconversion between 4-coumaric acid (III) and 3-(4-hydroxyphneyl)propionic acid (IV)[15] in *M. rubra* remains to be investigated.

In the biosynthesis of diarylheptanoids, the involvement with chalcone synthase-related type III plant polyketide synthase (PKS III) was presumed.[16] Brand et al.[17] reported a new PKS III gene cloned from *Wachendorfia thyrsiflora*, and the enzyme catalyzed the formation of a diketide, which is considered to be a biosynthetic intermediate of phenylphenalenone derivatives. Furthermore, Ramirez-Ahumada et al.[18] detected the activity of curcuminoid synthase in turmeric, which required both 4-coumaroyl-CoA esters and malonyl-CoA for curcuminoid biosynthesis. Katsuyama et al.[19] recently demonstrated the in vitro synthesis of curcuminoids by PKS III from *Oryza sativa*. Therefore, a similar PKS III might be involved in the biosynthesis of myricanol derivatives in *M. rubra*. However, in the case of myricanol (I), it is likely that another diphenylheptane synthase, which uses a dihydro-phenylpropanoid precursor as a substrate, could be involved.

Furthermore, the findings that the incorporation ratios of 4-[8,9-$^{13}C_2$]coumaric acid ([8,9-$^{13}C_2$]-III) and 3-(4-hydroxyphenyl)-[1-^{13}C]propionic acid ([1-^{13}C]-IV) differ between C-9 (1:1.6) and C-11 (1:8.4) in myricanol (I) is an interesting result. Myricanol (I) has two different types of aromatic rings; the A-ring has two methoxyl and one phenolic hydroxyl groups, whereas the B-ring has only one phenolic hydroxyl group. If the diarylheptanoid skeleton is built from the same two precursors, for example, 3-(4-hydroxyphenyl)propionyl-CoA, the incorporation ratios at C-9 and C-11 may be indistinguishable as a consequence of symmetry. These results strongly suggest that hydroxylation (and/or methylation) of the A-ring may occur before building of the diarylheptanoid skelton. We are presently attempting to determine whether ^{13}C-labeled caffeic acid, ferulic acid, dihydro-caffeic acid, and/or dihydro-ferulic acid are incorporated into myricanol (I).

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