Note

Myricanol and myricanone biosynthesis in *Myrica rubra*: incorporation of two molecules of 4-coumaric acid

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ABSTRACT

Feeding experiments of *Myrica rubra* young shoot with 4-[8,9- $^{13}\text{C}_2$]coumaric acid revealed that the cyclic diarylheptanoids, myricanol and myricanone, were derived from two molecules of 4-coumaric acid by mass spectrometric analyses.

^{13}C -NMR of myricanol isolated after administration of 4-[8,9- $^{13}\text{C}_2$]coumaric acid demonstrated that C-8 and C-9 atoms of 4-coumaric acid are incorporated into C-8, C-9, C-11 and C-12 of corresponding myricanol.

Introduction

Diarylheptanoids comprise a class of natural products based on 1,7-diphenylheptane. Many kinds of diarylheptanoids have been isolated from Myricaceae, Betulaceae, Zingiberaceae, and Aceraceae plants.¹ The bark of *Myrica rubra* Sieb. et Zucc. has been used as an astringent, antidote and antidiarrheal in Japanese folk medicine and has also been used externally for burn and skin diseases in Chinese traditional medicine. In *M. rubra*, the cyclic diarylheptanoids, myricanol (**1**) and myricanone (**2**), are one of the major components.^{2,3} Recently, it has been reported that they have antitumor-promoting effects⁴ and anti-androgen activities.⁵

The biosynthesis of diarylheptanoids, especially phenylphenalenone derivatives,⁶⁻⁹ has been investigated and reported that two molecules of phenylpropanoids and one malonyl-CoA were concerned with the formation of phenylphenalenone skeleton. However, there is no evidence about the biosynthetic pathways for cyclic diarylheptanoids.

In this paper, we described *in vivo* feeding experiments using the ¹³C-labeled 4-coumaric acid (**3**). Biosynthetic MS and ¹³C-NMR studies indicated the involvement of two molecules of 4-coumaric acid (**3**) for the formation of acid to cyclic diarylheptanoids, myricanol (**1**) and myricanone (**2**), in *M. rubra*.

Materials and Methods

Analytical and preparative TLC were performed on silica gel (Merck Kieselgel 60 F₂₅₄). Silica gel column chromatography employed Kieselgel 60 (70 – 230 mesh, Merck). ¹H and ¹³C-NMR spectra were taken with JEOL JMM EX-270 and JEOL LAMBDA 500 FT-NMR spectrometers using tetramethylsilane as an internal standard. EI-MS analyses were performed with a

Shimadzu GCMS-QP 5050 gas chromatograph mass spectrometer (70 eV).

GC-MS analyses were carried out on a capillary column (TC-1; 30 m x 0.25 mm i.d.; film, 1 μ m; GL Sciences) at a rate of 5 $^{\circ}$ C min⁻¹ from 150 to 280 $^{\circ}$ C.

Extraction and isolation of authentic myricanol (**1**) and myricanone (**2**).

Branches of *Myrica rubra* Sieb. et Zucc. grown on the campus of Shizuoka University were harvested on June 2004. The xylem and bark were air dried and powdered. The powder (368 g) was extracted with hot MeOH for 23 h. The MeOH extracts were dissolved in EtOAc, and soluble part for EtOAc was partitioned with saturated NaHCO₃ and 1 N NaOH, successively. The NaOH layer was acidified with 6 N HCl to pH 2 and it was extracted with EtOAc. The EtOAc extracts were chromatographed on a column of silica gel (eluent: ethyl acetate/*n*-hexane, 1/2). The fractions containing myricanol (**1**) and myricanone (**2**) were combined, respectively. Myricanol (**1**) (36.4 mg) was further purified by TLC (solvent: MeOH/CHCl₃, 2/98) separation and analyzed by NMR and MS. Myricanone (**2**) was only identified by GC- and direct inlet (DI)-MS analysis.

Myricanol (**1**)

¹H-NMR (CDCl₃, 270 Hz) ¹⁰ δ (ppm): 1.50-1.60 (2H, m, 9-H, 10-H), 1.85-2.00 (3H, m, 8-H₂, 10-H), 1.65-1.75 (2H, m, 9-H, 12-H), 2.25-2.37 (1H, m, 12-H), 2.45-2.60 (1H, m, 7-H), 2.75-2.89 (1H, m, 7-H), 2.89-2.95 (2H, m, 13-H₂), 3.88 (3H, s, -OCH₃), 3.99 (3H, s, -OCH₃), 4.08 (1H, bt, *J*=9.9Hz, 11-H), 5.94 (1H, bs, 5-OH), 6.88 (1H, s, 19-H), 6.90 (1H, d, *J*=8.2Hz, 16-H), 7.08 (1H, dd, *J*=8.2, 2.3Hz, 15-H), 7.17 (1H, d, *J*=2.3Hz, 18-H), 7.68 (1H, bs, 17-OH).

¹³C-NMR (CDCl₃, 125 Hz) δ (ppm): 22.9 (9-C), 25.4 (7-C), 25.7 (8-C), 26.9 (13-C), 34.7 (12-C), 39.4 (10-C), 61.4 (-OCH₃ x 2), 68.6 (11-C), 116.8 (16-C),

122.6 (6-C), 123.4 (2-C), 124.7 (1-C), 129.4 (19-C), 130.0 (15-C), 130.7 (14-C), 133.1 (18C), 138.7 (4-C), 145.9 (3-C), 147.7 (5-C), 151.3 (17-C).

MS (DI) m/z (%): 136 (20), 257 (27), 271 (22), 297 (20), 358 (M^+ , 100), 359 (23); (TMS ether, GC) m/z (%): 73 (100), 145 (13), 427 (6), 453 (9), 574 (M^+ , 53).

Myricanone (**2**)

MS (DI) m/z (%): 128 (14), 143 (20), 271 (21), 285 (18), 356 (M^+ , 100), 357 (24); (TMS ether, GC) m/z (%): 73 (100), 75 (22), 87 (24), 207 (32), 500 (M^+ , 45).

Syntheses of labeled precursor

*Ethyl 4-[8,9- $^{13}C_2$]acetoxycinnamate (**4**)*

Ethyl 4-[8,9- $^{13}C_2$]acetoxycinnamate (**4**) was synthesized by Wittig-Horner reaction from 4-acetoxybenzaldehyde and ^{13}C -labeled triethyl phosphonoacetate (TEPA) by the modified method of Newman et al.¹¹

4-Hydroxybenzaldehyde was acetylated with acetic anhydride and pyridine (1/1) to give 4-acetoxybenzaldehyde. To a solution of 4-acetoxybenzaldehyde (271 mg, 1.65 mmol) and [1,2- $^{13}C_2$]TEPA (250 mg, 1.1 mmol, Aldrich, 99 atom% $^{13}C_2$) in toluene (2 ml), powdered KOH (85 %, 109 mg, 1.65 mmol) and 18-Crown-6 (349 mg, 1.32 mmol) were added under nitrogen atmosphere.¹² The resulting solution was stirred at ambient temperature for 30 min. The reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with water (twice) and saturated brine (twice), successively. It was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue (394 mg) was purified by column chromatography (eluent: ethyl acetate/*n*-hexane, 1/4) to give ethyl 4-[8,9- $^{13}C_2$]acetoxycinnamate (**4**) (161 mg, 62 % from TEPA).

^{13}C -NMR (CDCl_3 , 67.5 Hz) δ (ppm):

^{13}C -enriched-4: 118.4 (d, $J=75.7$ Hz, 8-C), 166.8 (d, $J=75.6$ Hz, 9-C).

Non-labeled authentic-4: 14.3 ($-\text{OCH}_2\text{CH}_3$), 21.1 ($-\text{OCOCH}_3$), 60.5 ($-\text{OCH}_2\text{CH}_3$), 118.4 (8-C), 122.1 (3,5- C_2), 129.1 (2,6- C_2), 132.2 (1-C), 143.4 (7-C), 152.0 (4-C), 166.8 (9-C), 169.1 ($-\text{OCOCH}_3$).

4-[8,9- $^{13}\text{C}_2$]coumaric acid (3)

To a solution of ethyl 4-[8,9- $^{13}\text{C}_2$]acetoxycinnamate (**4**) (161 mg, 0.68 mmol) in 80% ethanol (2 ml), powdered KOH (85 %, 360 mg, 5.4 mmol) was added under nitrogen atmosphere. The resulting solution was stirred at ambient temperature for 26 h. The reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with saturated brine (twice), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by TLC (solvent: $\text{MeOH}-\text{CHCl}_3$, 5/95) to give 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**) (95.5 mg, 84 %).

DI-MS m/z (%):

^{13}C -enriched-3: 65 (12), 92 (20), 119 (22), 120 (26), 149 (39), 163 (0.2), 164 (1.6), 165 (44), 166 (100), 167 (9).

None-labeled authentic-3: 65 (19), 91 (22), 118 (22), 119 (26), 147 (39), 163 (41), 164 (M^+ , 100), 165 (11), 166 (1.6), 167 (0.2).

^{13}C -NMR (CD_3OD , 67.5 Hz) δ (ppm):

^{13}C -enriched-3: 115.7 (d, $J=73.8$ Hz, 8-C), 171.0 (d, $J=73.2$ Hz, 9-C).

Non-labeled authentic-3: 115.6 (8-C), 116.8 (3,5- C_2), 127.3 (1-C), 131.0 (2,6- C_2), 146.6 (7-C), 161.2 (4-C), 171.0 (9-C).

Feeding experiment

4-[8, 9- $^{13}\text{C}_2$]coumaric acid (**3**) (25 mM in 0.1 % NaOH, 1 ml each) was administrated to excised *M. rubra* young shoots (harvested on October and November, 2005, shoot size: 20-30 cm), which were allowed to metabolize for 2 weeks at room temperature under continuous light. 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 hr. 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 (**1**) and myricanone (**2**) fractions were roughly separated from the extracts by TLC (solvent: ethyl acetate/*n*-hexane, 1/2), respectively. The both fractions were trimethylsilylated [TMSI-H (hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10, v/v))(GL Sciences)] and analyzed by GC-MS. The myricanol (**1**) and myricanone (**2**) fractions confirmed the incorporation of 4-[8,9- $^{13}\text{C}_2$]coumaric acids (**3**) by GC-MS analyses were combined, respectively, and further purified by TLC (solvent: MeOH- CHCl_3 , 2/95). The purified myricanol (**1**) (~ 3 mg) and myricanone (**2**) (< 1 mg) were analyzed by selected ion monitoring (SIM) of DI-MS, respectively. ^{13}C -NMR analysis of the myricanol (**1**) was also performed.

Results and Discussion

4-[8,9- $^{13}\text{C}_2$]Coumaric acid (**3**) was administrated to excised *M. rubra* young shoots and it was allowed to metabolize for 2 weeks. The shoots was grounded and extracted with hot MeOH. The myricanol (**1**) and myricanone (**2**) fractions, obtained by TLC separation, were analyzed by GC-MS, respectively. Comparison with the mass spectra of unlabeled authentic compounds (Fig. 1), the spectra of myricanol (**1**-TMS ether) and myricanone (**2**-TMS ether) obtained

following administration of 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**) clearly demonstrated that the molecular ions of unlabeled myricanol (**1**) at m/z 574 and myricanone (**2**) at m/z 500 are accompanied by extra ions at m/z 578, and 504, respectively, indicating the formations of [$^{13}\text{C}_4$]-myricanol and [$^{13}\text{C}_4$]-myricanone. The myricanol (**1**) and myricanone (**2**) fractions were further purified by TLC to confirm the incorporation rate of ^{13}C carbon into myricanol (**1**) and myricanone (**2**) using DI-MS with SIM mode. As shown in Table 1, the results indicated that ca. 6 % of myricanol (**1**) and ca. 9.5 % of myricanone (**2**) were biosynthesized from two molecules of 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**), respectively.

Furthermore, to determine the ^{13}C -enriched position in the side chains, ^{13}C -NMR analysis of myricanol (**1**) was conducted (Fig. 2). The NMR spectra of the isolated myricanol (**1**) displayed an enhancement of ^{13}C resonances at four positions. Due to ^{13}C - ^{13}C coupling, the resonances of C-8 (δ 25.7), C-9 (δ 22.9), C-11 (δ 68.6), and C-12 (δ 34.7) appear as pseudo triplet, indicating the occurrence of naturally-occurring isotopomers (singlet, central resonances of the pseudo triplets) and relatively high amounts of isotopomers containing two labeled carbons in these positions (doublets, C-8; $J=35.2$ Hz, C-9; $J=34.1$ Hz, C-11; $J=39.3$ Hz, C-12; $J=39.3$ Hz). It is clear that the doublets signals of this compound derived from 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**), and the relative intensities of the enhanced doublet signals based on the singlet signals were 5.32 for C-8, 5.34 for C-9, 4.46 for C-11 and 4.56 for C-12, respectively.

The possible biosynthetic pathway from 4-coumaric acid to myricanol (**1**) and myricanone (**2**) was shown in Fig. 3. The present investigations clearly demonstrated that two molecules of 4-coumaric acid (**3**) were concerned with the formation of diarylheptanoids skeleton in *M. rubra*.

In the biosynthesis of diarylheptanoids, the involvement with chalcone synthase related type III plant polyketide synthase (PKS III) is presumed.¹³ Very recently, Brand et al.¹⁴ has reported that a new PKS III gene has been cloned from *Wachendorfia thyrsiflora*, and the enzyme catalyzed the formation of a diketide, which is considered as a biosynthetic intermediate of phenylphenalenone derivatives. Furthermore, Ramirez-Ahumada et al.¹⁵ has detected the activity of curcuminoid synthase in turmeric, which required both 4-hydroxycinnamyl-CoA esters and malonyl-CoA for curcuminoid biosynthesis. Therefore, similar enzymes might be involved in the biosynthesis of myricanol derivatives in *M. rubra*. However, there are no carbon-carbon double bond in the heptane side chain of myricanol derivatives, (1) and (2), and it is unclear whether the saturated structure in myricanols was originated from dihydrocinnamic acid precursor(s) or from a cinnamic acid precursor followed by hydrogenation after condensation with a second cinnamate unit. Further feeding experiments with ¹³C-labeled 4-hydroxyphenylpropanoic acid are in progress for this question. The origin of the methoxyl groups in myricanol (1) and myricanone (2) also remains to be resolved. We are presently attempting to determine whether ¹³C-labeled ferulic acid is incorporated into myricanol derivatives (1) and (2).

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Table 1. Mass spectral data of molecular-ion region of myricanol (**1**) and myricanol (**2**) isolated from *M. rubra*

Myricanol (1)			Myricanone (2)		
Relative intensity (%)			Relative intensity (%)		
<i>m/z</i>	Unlabeled	Isolated after administration of 4-[8, 9- ¹³ C ₂]coumaric acid (3)	<i>m/z</i>	Unlabeled	Isolated after administration of 4-[8, 9- ¹³ C ₂]coumaric acid (3)
358	100	100	356	100	100
359	23.1	24.4	357	23.8	24.9
360	3.8	8.5	358	4.7	11.6
361	0.5	2.9	359	0.9	3.9
362	0.1	6.7	360	0.4	11.6

Figure legends

Fig. 1. Mass spectra of myricanol TMS ethers (**1**-TMS, **A**, **B**) and myricanone TMS ethers (**2**-TMS, **C**, **D**). **A**, **C**: unlabeled, **B**, **D**: formed after 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**) administration.

Fig. 2. ^{13}C -NMR spectra of myricanol (**1**). **A**: unlabeled, **B**: formed after 4-[8,9- $^{13}\text{C}_2$]coumaric acid (**3**) administration.

Fig. 3. Proposed biosynthetic pathways for myricanol (**1**) and myricanone (**2**) from 4-coumaric acid (**3**) in *M. rubra*.