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

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Abstract After feeding experiments of *Myrica rubra* young shoots with 4-[8,9-$^{13}C_2$]coumaric acid, mass spectrometric analyses revealed that the cyclic diarylheptanoids, myricanol and myricanone, were derived from two molecules of 4-coumaric acid. ^{13}C Nuclear magnetic resonance analysis of myricanol isolated after administration of 4-[8,9-$^{13}C_2$]coumaric acid demonstrated that the C-8 and C-9 atoms of 4-coumaric acid are incorporated into C-8, C-9, C-11, and C-12 of the corresponding myricanol.

Key words Cyclic diarylheptanoids · Myricanol biosynthesis · 4-Coumaric acid · ^{13}C-NMR · *Myrica rubra*

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 major components.[2,3] Recently, it has been reported that they have antitumor effects[4] and antiandrogen activities.[5]

The biosynthesis of diarylheptanoids, especially phenylphenalenone derivatives,[6–9] has been investigated and it has been reported that two phenylpropanoids and one malonyl-CoA involved the formation of the phenylphenalenone skeleton. However, there is no information about the biosynthetic pathways for cyclic diarylheptanoids.

In this report, we describe in vivo feeding experiments using ^{13}C-labeled 4-coumaric acid (**3**). Mass spectrometry (MS) and ^{13}C nuclear magnetic resonance (NMR) analysis 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*.

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Materials and methods

Analytical and preparative thin-layer chromatography (TLC) were performed on silica gel (Merck Kieselgel 60 F_{254}). Silica gel column chromatography employed Kieselgel 60 (70–230 mesh, Merck). ^1H and ^{13}C NMR spectra were recorded with JEOL JMM EX-270 and JEOL LAMBDA 500 FT-NMR spectrometers using tetramethylsilane as an internal standard. Electron-impact (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 × 0.25 mm i.d.; film, 1 μm; GL Sciences) at a rate of 5°C min^{-1} from 150° to 280°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 methanol (MeOH) for 23 h. The MeOH extracts were dissolved in ethyl acetate (EtOAc), and the EtOAc fraction was partitioned with saturated $NaHCO_3$ 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: EtOAc/*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/$CHCl_3$, 2/98) separation and analyzed by NMR and MS. Myricanone (**2**) was only identified by GC-MS and direct inlet (DI)-MS analysis.

*Myricanol (**1**)*

^{1}H NMR ($CDCl_3$, 270 Hz)[10] δ: 1.50–1.60 (2H, m, 9-H, 10-H), 1.85–2.00 (3H, m, 8-H_2, 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-H_2), 3.88 (3H, s, -OCH_3), 3.99 (3H, s, -OCH_3), 4.08 (1H, bt, J = 9.9 Hz, 11-H), 5.94 (1H, bs, 5-OH), 6.88 (1H, s, 19-H), 6.90 (1H, d, J = 8.2 Hz, 16-H), 7.08 (1H, dd, J = 8.2, 2.3 Hz, 15-H), 7.17 (1H, d, J = 2.3 Hz, 18-H), 7.68 (1H, bs, 17-OH).

^{13}C NMR ($CDCl_3$, 125 Hz) δ: 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 (-OCH_3 × 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 EtOAc 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: EtOAc/*n*-hexane, 1/4) to give ethyl 4-[8,9-$^{13}C_2$]acetoxycinnamate (**4**) (161 mg, 62% from TEPA).

^{13}C NMR ^{13}C-enriched **4** ($CDCl_3$, 67.5 Hz) δ: 118.4 (d, J = 75.7 Hz, 8-C), 166.8 (d, J = 75.6 Hz, 9-C).

^{13}C NMR nonlabeled authentic **4** ($CDCl_3$, 67.5 Hz) δ: 14.3 (-OCH_2CH_3), 21.1 (-$OCOCH_3$), 60.5 (-OCH_2CH_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 (-$OCOCH_3$).

*4-[8,9-$^{13}C_2$]Coumaric acid (**3**)*

To a solution of ethyl 4-[8,9-$^{13}C_2$]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 EtOAc 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: MeOH/$CHCl_3$, 5/95) to give 4-[8,9-$^{13}C_2$]coumaric acid (**3**) (95.5 mg, 84%).

^{13}C NMR ^{13}C-enriched **3** (CD_3OD, 67.5 Hz) δ: 115.7 (d, J = 73.8 Hz, 8-C), 171.0 (d, J = 73.2 Hz, 9-C).

^{13}C NMR nonlabeled authentic **3** (CD_3OD, 67.5 Hz) δ: 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).

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

DI-MS nonlabeled authentic **3** *m/z* (%): 65 (19), 91 (22), 118 (22), 119 (26), 147 (39), 163 (41), 164 (M^+, 100), 165 (11), 166 (1.6), 167 (0.2).

Feeding experiment

4-[8,9-$^{13}C_2$]Coumaric acid (**3**) (25 mM in 0.1% NaOH, 1 ml each) was administered to excised *M. rubra* young shoots (harvested in October and November, 2005, shoot size: 20–30 cm) that were allowed to metabolize for 2 weeks at room temperature under continuous light. After incubation, the leaves were removed from the resulting shoots; the 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 (**1**) and myricanone (**2**) fractions were roughly separated from the extracts by TLC (solvent: EtOAc/*n*-hexane, 1/2). Both fractions were trimethylsilylated [TMSI-H (hexamethyldisilazane and trimethylchlorosilane in pyridine; 2:1:10, v/v/v); GL Sciences] and analyzed by GC-MS. The myricanol (**1**) and myricanone (**2**) fractions confirmed the incorporation of 4-[8,9-$^{13}C_2$]coumaric acids (**3**), respectively, and fractions were 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-$^{13}C_2$]Coumaric acid (**3**) was administrated to excised *Myrica rubra* young shoots and it was allowed to metabolize

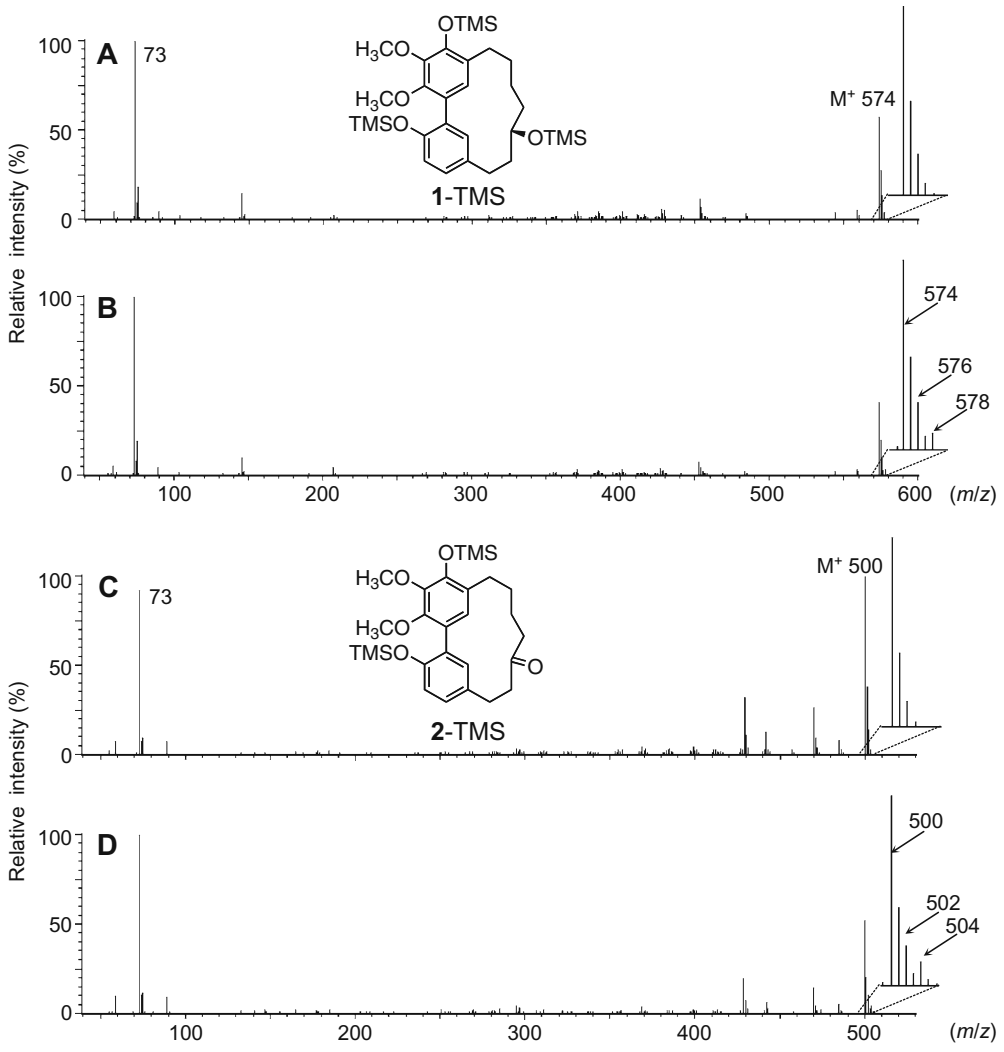

Fig. 1A–D. Mass spectra of myricanol trimethylsilyl (TMS) ethers (**1**-TMS, **A**, **B**) and myricanone TMS ethers (**2**-TMS, **C**, **D**). **A, C** Unlabeled, **B, D** formed after 4-[8,9-$^{13}C_2$]coumaric acid (**3**) administration

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

Myricanol (**1**)			Myricanone (**2**)		
	Relative intensity (%)			Relative intensity (%)	
m/z	Unlabeled	Isolated after administration of **3**[a]	*m/z*	Unlabeled	Isolated after administration of **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

[a] 4-[8,9-$^{13}C_2$]Coumaric acid

for 2 weeks. The shoots was ground and then extracted with hot MeOH. The myricanol (**1**) and myricanone (**2**) fractions, obtained by TLC separation, were analyzed by GC-MS, respectively. By 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-$^{13}C_2$]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. These indicate the formation of [$^{13}C_4$]-myricanol and [$^{13}C_4$]-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 myri-

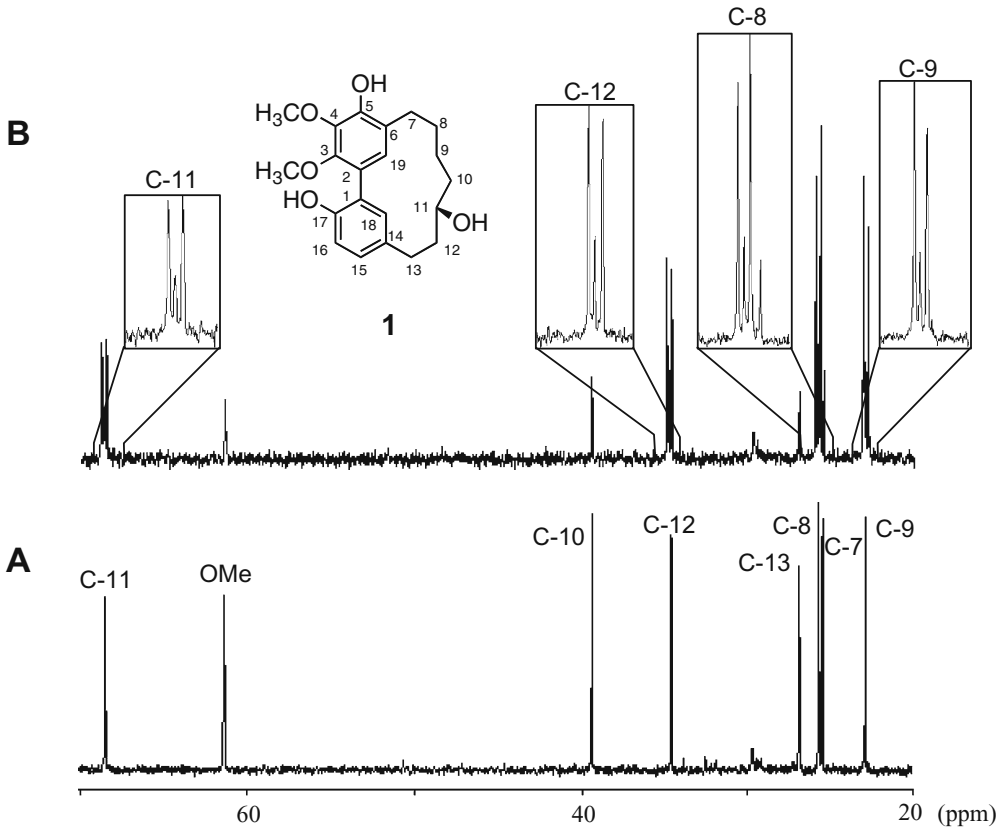

Fig. 2A, B. ^{13}C Nuclear magnetic resonance (NMR) spectra of myricanol (**1**). **A** Unlabeled, **B** formed after 4-[8,9-$^{13}C_2$]coumaric acid (**3**) administration

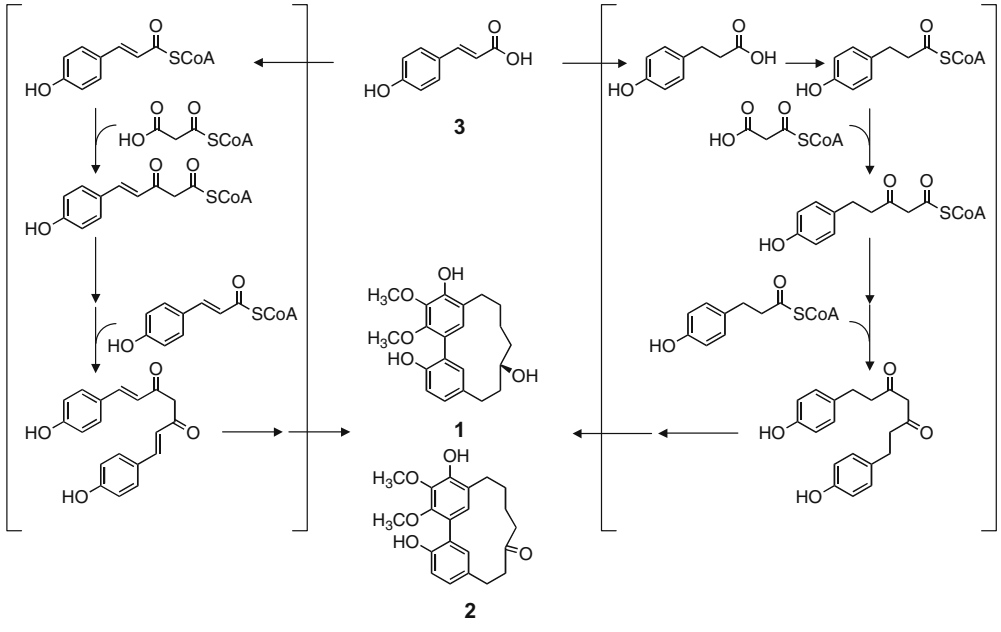

Fig. 3. Proposed biosynthetic pathways for myricanol (**1**) and myricanone (**2**) from 4-coumaric acid (**3**) in *Myrica rubra*

canol (**1**) and ca. 9.5% of myricanone (**2**) were biosynthesized from two molecules of 4-[8,9-$^{13}C_2$]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 triplets, 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 doublet signals of this compound were derived from 4-[8,9-

$^{13}C_2$]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**) is shown in Fig. 3. The present investigations clearly demonstrated that two molecules of 4-coumaric acid (**3**) were involved in the formation of the diarylheptanoid 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] reported that a new PKS III gene was 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] 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 is 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 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 ^{13}C-labeled 4-hydroxyphenylpropanic acid are in progress to address 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 ^{13}C-labeled ferulic acid is incorporated into myricanol derivatives (**1**) and (**2**).

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References

1. Inoue T (1993) Constituents of *Acer nikoense* and *Myrica rubra*. On diarylheptanoids (in Japanese). Yakugaku Zasshi 113:181–197
2. Begley MJ, Campbell RVM, Crombie L, Tuck B, Whiting DA (1971) Constitution and absolute configuration of meta,meta-bridged, strained biphenyls from *Myrica nagi*; X-ray analysis of 16-bromomyricanol. J Chem Soc C 3634–3642
3. Inoue T, Arai Y, Nagai M (1984) Diarylheptanoids in the bark of *Myrica rubra* Sieb. et Zucc. (in Japanese). Yakugaku Zasshi 104: 37–41
4. Ishida J, Kozuka M, Wang HK, Konoshima T, Tokuda H, Okuda M, Mou XY, Nishino H, Sakurai N, Lee KH, Nagai M (2000) Antitumor-promoting effects of cyclic diarylheptanoids on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. Cancer Lett 159:135–140
5. Matsuda H, Yamazaki M, Matsuo K, Asanuma Y, Kubo M (2001) Anti-androgenic activity of Myricae cortex – isolation for active constituents from bark of *Myrica rubra*. Biol Pharm Bull 24: 259–263
6. Hölscher D, Schneider B (1995) A diarylheptanoid intermediate in the biosynthesis of phenylphenalenones in *Anigozanthos preissi*. J Chem Soc Chem Commun 525–526
7. Hölscher D, Schneider B (2005) The biosynthesis of 8-phenylphenalenones from *Eichhornia crassipes* involves a putative aryl migration step. Phytochem 66:59–64
8. Schneider B, Gershenzon J, Graser G, Schmitt B, Hölscher D (2003) One-dimensional ^{13}C NMR and HPLC-^{1}H NMR techniques for observing carbon-13 and deuterium labeling in biosynthetic studies. Phytochem Rev 2:31–43
9. Kamo T, Hirai N, Tsuda M, Fujioka D, Ohigashi H (2000) Changes in the content and biosynthesis of phytoalexins in banana fruit. Biosci Biotechnol Biochem 64:2089–2098
10. Joshi BS, Pelletoer W (1996) Extensive 1D, 2D NMR spectra of some [7.0]metacyclophanes and X-ray analysis of (±)-myricanol. J Nat Prod 59:759–764
11. Newman J, Rej RN, Just G, Lewis NG (1986) Synthesis of (1,2-^{13}C), (1-^{13}C), and (3-^{13}C) coniferyl alcohol. Holzforschung 40:369–373
12. Kawai S, Jensen KA, Bao W, Hammel KE (1995) New polymeric model substrates for study of microbial ligninolysis. Appl Environ Microbiol 61:3407–3414
13. Schröder J (1997) A family of plant-specific polyketide synthase: facts and predictions. Trends Plant Sci 2:373–378
14. Brand S, Hölscher D, Schierhorn A, Svatos A, Schröder J, Schneider B (2006) A type III polyketide synthase from *Wachendorfia thyrsiflora* and its role in diarylheptanoid and phenylphenalenone biosynthesis. Planta 224:413–428
15. Ramirez-Ahumada M del C, Timmermann BN, Gang DR (2006) Biosynthesis of curcuminoids and gingerols in turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*): identification of curcuminod synthase and hydroxycinnammoyl-CoA thioesterases. Phytochemistry 67:2017–2029