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Research Paper

Use of P450 cytochrome inhibitors in studies of enokipodin biosynthesis

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Abstract

Enokipodins A, B, C, and D are antimicrobial sesquiterpenes isolated from the mycelial culture medium of *Flammulina velutipes*, an edible mushroom. The presence of a quaternary carbon stereocenter on the cyclopentane ring makes enokipodins A-D attractive synthetic targets. In this study, nine different cytochrome P450 inhibitors were used to trap the biosynthetic intermediates of highly oxygenated cuparene-type sesquiterpenes of *F. velutipes*. Of these, 1-aminobenzotriazole produced three less-highly oxygenated biosynthetic intermediates of enokipodins A-D; these were identified as (*S*)-(-)-cuparene-1,4-quinone and epimers at C-3 of 6-hydroxy-6-methyl-3-(1,2,2-trimethylcyclopentyl)-2-cyclohexen-1-one. One of the epimers was found to be a new compound.

Key words: Antimicrobial compound, cuparene-1,4-quinone, edible mushroom, enokitake, *Flammulina velutipes*.

Introduction

Flammulina velutipes (Curt. Fr.) Sing. (Enokitake in Japanese), in the family Physalacriaceae (Agaricales, Agaricomycetes), is one of the most popular edible mushrooms in Japan. Many bioactive metabolites have been isolated from this fungus, including proteins (Komatsu *et al.*, 1963, Lin *et al.*, 1974, Tsuda, 1979, Ko *et al.*, 1995, Tomita *et al.*, 1998), glycoproteins (Ikekawa *et al.*, 1985), polysaccharides (Yoshioka *et al.*, 1973, Leung *et al.*, 1997, Yaoita *et al.*, 1998, Wasser and Wess, 1999, Smiderle *et al.*, 2006), sterols (Yaoita *et al.*, 1998), and monoterpenetriol (Hirai *et al.*, 1998). In a previous screen for antimicrobial secondary metabolites from edible mushrooms, we identified four highly oxygenated cuparene-type sesquiterpenes, enokipodins A-D (compounds 1-4), from *F. velutipes* (Ishikawa *et al.*, 2000, 2001). Enokipodins A-D demonstrated antimicrobial activity against the fungus *Cladosporium herbarum* (Ishikawa *et al.*, 2000, 2001) and the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Ishikawa *et al.*, 2005). Following our report, several research groups synthesized these compounds (Srikrishna and Rao, 2004, Saito and Kuwahara, 2005, Srikrishna *et al.*, 2006, Secci *et al.*, 2007, Yoshida *et al.*, 2009, Luján-Montelongo and Ávila-Zarraga, 2010, Srikrishna and Rao, 2010, Leboeuf et al., 2013). The influence of mycelial culture conditions on biosynthetic production by *F. velutipes* was also studied (Ishikawa *et al.*, 2005, Melo *et al.*, 2009). We speculated that the antimicrobial activity of enokipodins A-D correlates to a highly oxygenated cuparene nucleus. The involvement of cytochrome P450s in many complex bioconversion processes, including detoxification reactions and the production of secondary metabolites, has been established in fungi (van den Brink *et al.*, 1998). Although these enzymes carry out a wide range of biocatalytic conversions, the general equation for all of these reactions may be summarized as RH + NAD(P)H + $H^+ + O_2 \rightarrow$ ROH + $NAD(P)^+ + H_2O$ (van den Brink *et al.*, 1998). The presence of a quaternary carbon stereocenter on the cyclopentane ring has made enokipodins A-D attractive synthetic targets. However, considering the absence of biosynthetic studies involving these sesquiterpenes, the aim of the present study was to trap the biosynthetic intermediates of highly oxygenated cuparene-type sesquiterpenes of *F. velutipes* using cytochrome P450 inhibitors.

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Materials and Methods

General notes

Merck Kieselgel 60 F_{254}, 0.25-mm thick TLC plates were used to purify the metabolites, while the spots were viewed under UV light (254 and 365 nm). IR spectra were recorded on a PerkinElmer 2000 FTIR, while mass spectra were recorded on a JEOL JMS-SX 102 mass spectrometer. ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra were recorded on a Bruker AMX-500 spectrometer. Conformation analysis was assisted by MM2 calculations using the ChemBio3D molecular modeling program in ChemOffice (CambridgeSoft).

Cultivation of the fungus

Flammulina velutipes (Fv-4) was cultivated in a 300 mL volume in 22 Erlenmeyer flasks containing 100 mL of malt peptone broth (3% Difco malt extract and 0.3% Merck peptone in distilled water, pH 4.5; the medium was sterilized by autoclaving at 121 °C for 15 min). Each flask was inoculated with five disks (7 mm i.d.) of freshly grown mycelia on malt agar plates, and cultured for 30 days at 25 °C under stationary conditions.

Incubation with cytochrome P450 inhibitors

On day 20 of fermentation, a 1 mM ethanolic solution (1 mL) of each inhibitor was passed through a Millipore membrane filter (0.22 nm pore size) and added to two flasks under aseptic conditions. To investigate the mechanism of enokipodin oxygenation, the fungus was inoculated with nine cytochrome P450 inhibitors: 1-aminobenzotriazole, α-naphthoflavone, ancymidol, 1-benzylimidazole, chlorocholine chloride, ketoconazole, miconazole, SKF-525A, and xanthotoxin. Of these, 1-aminobenzotriazole produced three less highly oxygenated metabolites (compounds 5-7). The carbon atoms in compounds 5-7 were numbered on the basis of biosynthetic considerations. Two flasks inoculated with ethanol (1 mL each) and two uninoculated flasks were used as a negative control. Fermentation was carried out at 25 °C for an additional 10 days. The mycelia were filtered, washed with water and ethyl acetate (EtOAc), and the broth thus obtained was extracted with EtOAc (600 mL each). The extracts were concentrated in a vacuum and the crude extracts thus obtained were spotted on TLC plates in parallel with an aliquot of enokipodins A-D as references. The analysis suggested that 1-aminobenzotriazole produced two less-polar new spots (B-1 and -2). In this test, the Rf values using toluene-acetone (4:1), in order of polarity, were: enokipodin C (Rf 0.09), enokipodin D (Rf 0.23), compound B-2 (Rf 0.30), enokipodin A (Rf 0.43), enokipodin B (Rf 0.75), and compound B-1 (Rf 0.87). The experiment was therefore scaled up to 5 L and repeated using 1-aminobenzotriazole. Part (567 mg) of the gum (810 mg) thus obtained was chromatographed on a silica gel (toluene: acetone = 6:1) to give two fractions containing B-1 and -2, respectively. The fractions containing B-1 were purified by TLC using hexane-EtOAc (20:1) as a mobile phase to obtain compound 5 (6.1 mg). Those fractions containing B-2 were purified by preparative TLC using toluene-acetone (15:1) and hexane-EtOAc (3:1) to give compounds 6 and 7 (14.0 mg) as a 3.7:1 mixture of epimers (^{1}H-NMR analysis).

Compound 5

M.p.: 68-75 °C (lit. 72-73 °C) (Matsuo et al., 1977). $[\alpha]_D^{24}$: -7º (*c* 0.01, $CHCl_3$), +10º for (*R*)-enantiomer (Matsuo et al., 1977). IR max (film) 2959, 1642, 1370 cm^{-1}. EIMS *m/z* (rel. int.): 233 (M+1+, 6), 232 (M+, 36), 217 (M+-15, 32), 202 (8), 189 (43), 164 (100), 150 (34), 149 (19), 137 (18), 95 (22), and 69 (28). HREIMS *m/z* 232.1486 ($C_{15}H_{20}O_2$ requires 232.1464). For ^{1}H and ^{13}C spectral analysis, see Table 1.

A 3.7:1 mixture of compounds 6 and 7

$[\alpha]_D^{24}$: -61º (*c* 0.01, $CHCl_3$), IR max (film) 3445, 1645 cm^{-1}. EIMS *m/z* (rel. int.): 237 (M++1, 9), 237 (M+, 50), 218 (M+-H_2O, 16), 203 (15), 180 (34), 135 (38), 121 (52), 109 (100), 91 (77), 79 (40), and 43 (81). HREIMS *m/z* 236.1770, ($C_{15}H_{24}O_2$ requires 236.1772). For ^{1}H and ^{13}C spectral analyses of the major diastereomer compound 6, see Table 2.

Compound 7

^{1}H NMR ($CDCl_3$, 500 MHz): (Apparent signals were selected.) 1.97 (1H,ddd, Hβ-2), 2.10 (1H, ddd, Hα-2), 2.42 (1H, dddd, Hα-1), 2.59 (1H, ddd, Hβ-1), 3.63 (1H, s, OH), 6.00 (1H, d, H-5). ^{13}C NMR δ ($CDCl_3$, 125 MHz) 19.3, 22.3, 24.0, 24.9, 26.3, 27.8, 35.7, 36.7, 40.6, 44.6, 52.7, 72.3, 122.2, 172.6, and 202.7.

Results and Discussion

1-Aminobenzotriazole inhibited the biosynthesis of enokipodins A-D (1-4) to produce two less-highly oxygenated metabolites (compounds 5-7) by inhibiting the activity of the fungal cytochrome P450 enzymes.

The EIMS of compound 5 showed a molecular ion peak at *m/z* 232, which was confirmed by recording the FDMS. HREIMS of the metabolite showed the precise molecular mass to be 232.1486, corresponding to the molecular formula $C_{15}H_{20}O_2$, and hence proved that the compound contained one less oxygen and two more protons than enokipodin B. The ^{1}H-NMR, ^{13}C-NMR, and HMQC spectra of compound 5 exhibited the presence of four methyl, three methylene, two methane, and six quaternary carbons. Two quaternary carbons resonated at δ 188.2 and 188.5 due to the carbonyls of the quinone moiety. A quaternary olefinic and an olefinic methine carbon were featured at δ 143.6 and 135.5, respectively. Assignments of all proton and carbon signals were made based on HMQC, HMBC,

Table 1 - ^{1}H and ^{13}C NMR spectral data of compound 5 in $CDCl_3$.

Position	δC[a]		δH[a] (*J*, Hz)	^{1}H-^{1}H COSY	HMBC	NOESY[b]
1	188.2	C	-	-	-	-
2	135.5	CH	6.50 d (2)	15	4, 6, 15	15
3	143.6	C	-	-	-	-
4	188.5	C	-	-	-	-
5	133.8	CH	6.65 s	-	1, 3, 7	8α(s), 8β(w), 12(w), 13(w), 14(w)
6	154.9	C	-	-	-	-
7	51.4	C	-	-	-	-
8	38.6	CH_2	α2.24 m	8β, 9	-[c]	5(s), 8β, 9, 13(w)
			β1.60 m	8α, 9	10	8α, 14
9	19.9	CH_2	αβ ca. 1.7 m	8, 10	-[c]	8αβ, 10α, 12, 13, 14
10	41.6	CH_2	α1.54 m	9, 10β	-[c]	10β, 13
			β1.73 m	9, 10α	-[c]	8β, 10α, 12
11	44.1	C	-	-	-	-
12	25.3	CH_3	1.12 s	-	7, 10, 11, 13	5(w), 13(s), 14(s)
13	27.9	CH_3	0.74 s	-	7, 10, 11, 12	5(w), 8α(w), 10α, 12
14	23.0	CH_3	1.29 s	-	6, 7, 8, 11	5(w), 8β, 10β
15	14.9	CH_3	2.01 d (2)	2	2, 3, 4	2

[a]From HMQC. [b]w; weak cross peak, s: strong cross peak. [c]Accumulation time was not enough.

Table 2 - ^{1}H and ^{13}C NMR spectral data of compound 6 in $CDCl_3$.

Position	δC[a]		δH[a] (*J*, Hz)	^{1}H-^{1}H COSY	HMBC	NOESY[b]
1	27.1	CH_2	α2.39 dddd (2.5, 4, 13, 16)	1β, 2αβ	6	1β, 2αβ, 12 (s), 13, 15(s)
			β2.65 ddd (2, 4, 16)	1α, 2β	3, 5, 6	1α, 2αβ, 8α, 12, 13(w), 14 (w)
2	36.7	CH_2	α2.13 ddd (2, 4, 12)	1αβ, 2β	3, 4, 6, 15	1α, 2β, 15
			β1.95 ddd (4, 12, 13)	1αβ, 2α	1, 3, 4, 6, 15	1αβ, 2α
3	72.3	C	-	-	-	-
4	202.7	C	-	-	-	-
5	122.1	C	5.98 d (2.5)	-	1, 3, 7	8αβ(s), 13(w), 14, 15(w)
6	172.5	C	-	-	-	-
7	52.7	C	-	-	-	-
8	36.1	CH_2	α2.22 m	8β, 9	14	8β, 9, 13
			β1.53 m	8α, 9	7, 10, 14	8α, 14
9	18.9	CH_2	αβ ca. 1.69 m	8, 10	10	8α(s), 8β(w), 10α, 12, 13(w), 14
10	40.2	CH_2	α ca. 1.56 m	9, 10β	9, 11, 13	8α 10β, 12 13, 14
			β ca. 1.69 m	9, 10α	9	8β, 10α, 12, 13(w), 14
11	44.3	C	-	-	-	-
12	24.3	CH_3	1.08 s	-	7, 10, 11, 13	1α(s), 1β(w), 5, 13(s)
13	26.1	CH_3	0.82 s	-	7, 10, 11, 12	1α(s), 1β(w), 5(w), 8α(s), 12, 15(s)
14	22.3	CH_3	1.10 s	-	6, 7, 8. 11	1α(w), 1β(s), 5(w), 8β, 10β
15	23.9	CH_3	1.31	-	2, 3, 4	1α, 2α, 5(w), 13(s)
OH			3.65	-	2, 3, 4	15

[a]From DEPT. [b]w; weak cross peak, s: strong cross peak.

^{1}H-^{1}H COSY, and NOESY spectra (Table 1) to give the structure of compound 5 as shown. Compound 5 was previously isolated from the liverworts *Jungermannia rosulans* (Matsuo *et al.*, 1977), *Radula javanica* (Asakawa *et al.*, 1991), *Lejeunea aquatic* (Toyota *et al.*, 1997), and *Lejeunea flava* (Toyota *et al.*, 1997). The ^{1}H and ^{13}C spectral data for compound 5 were identical to those for synthesized racemic 5 (Paul *et al.*, 2003). Thus, we report here for the first time the complete ^{1}H- and ^{13}C-NMR assignments of compound 5. The NOE data for compound 5 revealed the conformation of the main or averaged rotamer as shown in Figure 1. The ring current in quinone shows a deshielding effect on Hα-8 (δ 2.24) and shielding effect on H-13 (δ 0.74).

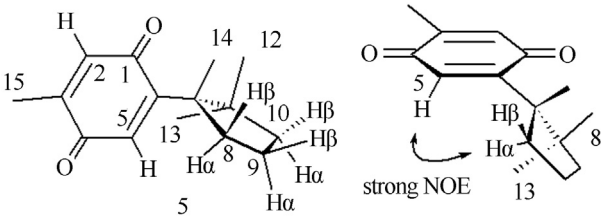

Figure 1 - A stable conformation of (*S*)-(-)-cuparene-1,4-quinone (5). Important NOE correlations is shown by arrow.

Compounds 6 and 7 were difficult to separate; therefore, they were analyzed as a mixture. The ^{1}H spectrum of the mixture of compounds 6 and 7 revealed that the chemical shift and coupling pattern corresponding to each signal in compounds 6 and 7 were quite similar; the ratio was 3.7:1. The carbon signal patterns for those compounds were also similar. Since they seemed to be epimers, the major one (compound 6) was examined first (Table 2). The molecular formula for compounds 6 and 7 was determined to be $C_{15}H_{24}O_2$ by HREIMS. The DEPT spectra of compound 6 showed the presence of 12 aliphatic carbons containing 4 methyl, 5 methylene, and 3 quaternary carbons. The re-

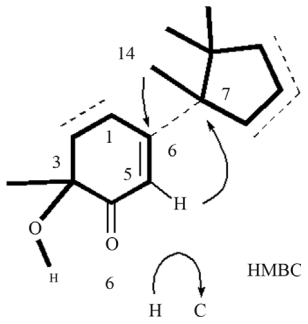

Figure 2 - Planar structure of compound 6, with ^{1}H-^{1}H connectivities represented by dotted lines, HMBC correlations indicated by bold-faced bonds and those of H-4 and H-14 indicated by arrow.

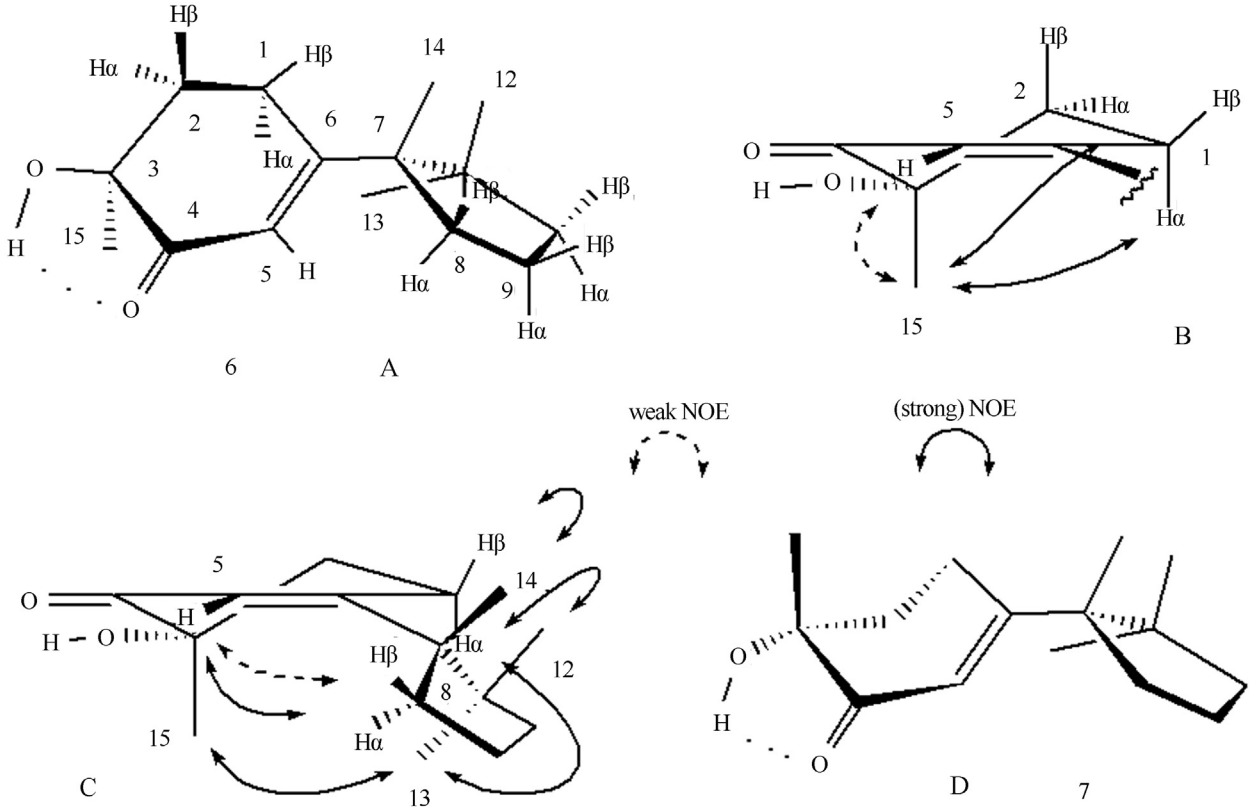

Figure 3 - (A) A stable conformation for compound 6. (B) Key NOEs observed of the cyclohexenone moiety in 6. (C) Key NOEs observed between the protonse of the cyclohexenone moiety and those of the cyclopentane moiety in 6. (D) A possible conformation for compound 7.

maining three carbons (122.1, 172.5, and 202.7) may form an α, β-unsaturated ketone moiety. An IR absorption at 3445 cm^{-1}, dehydration ion at *m/z* 218 by EIMS, and the presence of a tertiary carbonyl carbon resonating at δ 72.3 in the ^{13}C-NMR spectrum indicated compound 6 to be a tertiary alcohol. A sharp signal corresponding to a hydroxy proton was observed at δ 3.65 in the ^{1}H-NMR spectrum, indicating the existence of intramolecular hydrogen bonding between the hydroxy proton and carbonyl oxygen. The HMBC spectrum revealed five- and six-membered rings in compound 6 (Figure 2). The HMBC correlations of H-5 to C-7 and H-14 to C-6 led to the assignment of a cuparene skeleton for compound 6. The relative stereochemistry of compound 6 as shown in Figure 3A is derived from several lines of data: i) the observed NOEs of H-15/Hα-1 (1,3-diaxial), H-15/H-5, and H-15/Hα-2; ii) an allyl coupling (2.5 Hz) between H-5 and Hα-1 (pseudoaxial) in Figure 3B; and iii) the observed NOEs of H-15/H-13, H-5/Hαβ-8, Hαβ-1/H-12,13, and H-14/Hβ-1 in Figure 3C.

In light of the model of biogenesis shown in Figure 4, the configuration at C-7 in compound 6 must be *S*, as indicated. The IUPAC name for compound 6 will be, therefore, (*S*)-6-hydroxy-6-methyl-3-[(*S*)-1,2,2-trimethylcyclopentyl]-2-cyclohexen-1-one. Compound 7 is deduced, tentatively, to be an epimer of compound 6 at C-3 and a novel compound. Very recently, compounds 5 and 6 and related compounds were isolated from a solid culture of *F. velutipes* growing on cooked rice (Wang *et al.*, 2012a, 2012b). The ^{1}H and ^{13}C spectral data for compound 6 are identical to those reported for flamvelutpenoid C (Wang *et al.*, 2012a). Compound 5 showed weak antibacterial activity against *B. subtilis* (Wang *et al.*, 2012a). Flamvelutpenoid C showed weak antibacterial activity against *Escherichia coli*, *B. subtilis*, and methicillin-resistant *S. aureus* (Wang *et al.*, 2012b). This means that appropriate strains of the fungus can produce a series of cuparene-type sesquiterpenes under suitable culture conditions. The precise assignment of ^{1}H signals for flamvelutpenoid C is reported here.

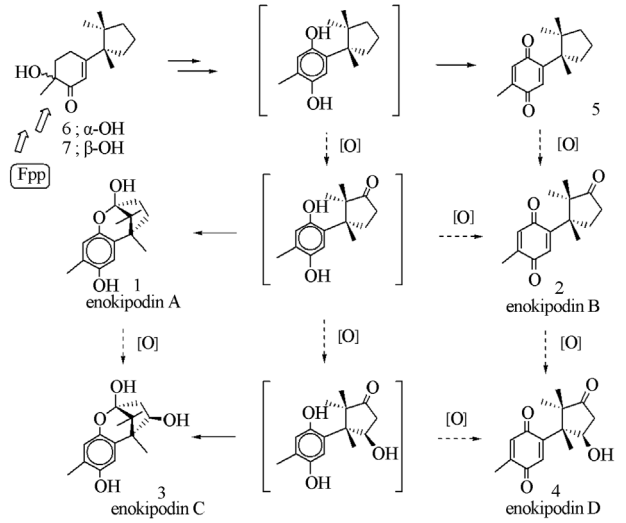

Figure 4 - Hypothetical biogenesis scheme for enokipodins A~D (1~4) in *Flammulina velutipes*. Fpp = farnesyl pyrophosphate.

Three intermediates, compounds 5-7, were isolated using 1-aminobenzotriazole as a cytochrome P450 inhibitor in this study. Of these, compounds 6 and 7 are likely key precursors of the phenolic ring in cuparene-type sesquiterpenes, including enokipodins A-D (1-4).

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