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Highly oxidized cuparene-type sesquiterpenes from a mycelial culture of *Flammulina velutipes*

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Abstract

Cuparene-type sesquiterpenes were isolated from a culture broth of *Flammulina velutipes* (Curt.:Fr.) Sing. Using spectroscopic methods (HR-MS, ^{1}H and ^{13}C NMR, and 2D NMR, spectroscopy), their structures were determined to be 2,3,4,5-tetrahydro-2,7-dihydroxy-5,8,10,10-tetramethyl-2,5-methano-1-benzoxepin and 5-methyl-2-(3-oxo-1,2,2-trimethylcyclopentyl)benzoquinone. Both showed antimicrobial activity against *Cladosporium herbarum* and *Bacillus subtilis*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Flammulina velutipes*; Tricolomataceae; Basidiomycota; Enokitake; Antimicrobial activity; Sesquiterpenoid; Cuparene-type; Enokipodin A; Enokipodin B

1. Introduction

Flammulina velutipes (Curt.:Fr.) Sing. (Enokitake in Japanese), which belongs to the family Tricolomataceae (Hymenomycetes, Basidiomycota), is a fresh edible mushroom frequently consumed in Japan. There are many reports confirming that compounds isolated from the fruit body of *F. velutipes* have antitumor and immunomodulatory activities. Most of the isolated compounds are proteins, polysaccharides, and glycoproteins (Wasser and Weis, 1999); lectin (Wang et al., 1998), sterol (Yaoita et al., 1998), and monoterpentriol (Hirai et al., 1998) compounds have also been isolated from this mushroom. A few reports describe antimicrobial activity of *F. velutipes*. Kozová and Rehácek (1967) investigated the antibiotic metabolites of *F. velutipes* active against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. Antagonism between *F. velutipes* and *Trichoderma harzianum* has also been reported (Tokimoto, 1985), although the compounds possessing antimicrobial activity have not been identified. In our search for antimicrobial compounds in edible mushrooms, we found two antimicrobial compounds accumulated in *F. velutipes* culture medium. The new compounds, named enokipodin A (**1**) and enokipodin B (**2**), are α-cuparene-type sesquiterpenoids. These being presumably linked through the intermediacy of intermediate (**3**). Similar compounds include the cuparenones (**4** and **5**) for *Thuja* sp. (Chetty and Dev, 1964; Matsuo et al., 1975) as well as the pigments helicobasidin (**6**) (Natori et al., 1964) and deoxyhelicobasidin (**7**) isolated from *Helicobasidium mompa* (Natori et al., 1967). The, lagopodins (**8**–**12**), are related compounds which include the violet pigments from *Coprinus lagopus* (**8**, **9** and **11**) and *C. macrorhizus* (**10** and **12**) (Bottom and Siehr, 1975; Bu'Lock and Darbyshire, 1976; Thomson, 1971), respectively and (**13**) from the red alga (*Laurencia okamurai*) (Suzuki and Kurosawa, 1978).

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Table 1
^{1}H and ^{13}C NMR spectral data of enokipodin A (**1**)

Position	^{13}C NMR	DEPT	δ_H[a] (*J*, Hz)	^{1}H–^{1}H COSY	HMBC	NOESY
1	146.1	C	–	–	H-2, H-5	–
2	116.9	CH	6.50 *s*	–	H_3-15	H_3-15
3	122.5	C	–	–	H_3-15, OH-4, H-5	–
4	147.4	C	–	–	H_3-15, H-5	–
5	111.0	CH	6.55 *s*	–	OH-4	H_3-14, OH-4
6	131.1	C	–	–	H_3-14, Hα-8, H-2	–
7	47.3	C	–	–	H-5, H_3-14, H_3-12, H_3-13	–
8	38.2	CH_2	α 1.78 *ddd* (3.7, 9.7, 12.7)	Hβ-8, Hα-9	H_3-14	Hβ-8, Hα-9,H_3-14
			β 1.90 *ddd* (6.5, 11.9, 12.7)	Hα-8, Hα-9, Hβ-9	–	Hα-8, Hα-9, H_3-12, H_3-14,
9	34.8	CH_2	α 2.17 *ddd* (6.5, 9.7, 14.3)	Hα-8, Hβ-8	–	Hα-8, Hβ-8, Hβ-9
			β 2.09 *ddd* (3.7, 11.9, 14.3)	Hβ-8		Hα-9, H_3-12, OH-10
10	109.6	C	–	–	H_3-12, H_3-13, OH-10	–
11	43.3	C	–	–	H_3-14, H_3-12, H_3-13	–
12	18.5	CH_3	1.09 *s*	13	H_3-13	Hβ-8, Hβ-9, H_3-13, H_3-14, OH-10
13	15.49	CH_3	0.80 *s*	12	H_3-12	H_3-12, H_3-14, OH-10
14	16.0	CH_3	1.24 *s*	–	–	H-5, Hα-8, Hβ-8, H_3-12, H_3-13
15	15.50	CH_3	2.16 *s*	–	H-2	H-2, OH-4
4-OH			4.30 *s*	–	–	H-5, H_3-15
10-OH			2.74 *s*	–	–	Hβ-9, H_3-12, H_3-13

[a] From HMQC.

2. Results and discussion

The antifungal activity of an ethyl acetate extract from the culture filtrate of mycelial *F. velutipes* grown for 30 days at 25°C was examined by TLC bioautography (Homans and Fuchs, 1970), using *Cladosporium herbarum* as the test fungus. Three antifungal spots were observed at R_f 0.07, 0.35 and 0.68. The major fungitoxins, enokipodin A (**1**) and enokipodin B (**2**), corresponding to the TLC spots at R_f 0.35 and 0.68, respectively, were isolated by preparative TLC. An aliquot of the crude extract equivalent to 0.25 ml of the culture filtrate produced a clear inhibitory zone (7 mm radius) against *B. subtilis* in the paper disk test.

2.1. Identification of 2,3,4,5-tetrahydro-2,7-dihydroxy-5,8,10,10-tetramethyl-2,5-methano-1-benzoxepin (enokipodin A (**1**))

HR-FIMS of compound **1** gave an $[M]^+$ ion at *m/z* 248.1444 corresponding to the formula $C_{15}H_{20}O_3$ (calculated 248.1407). The IR absorption at 3384 cm^{-1} indicated the presence of hydroxyl group(s). The UV data (λ_{max} at 207 and 299 nm) and signals in the ^{13}C NMR spectrum for six sp^2 carbons δ_C 147.4, 146.1, 131.1, 122.5, 116.9 and 111.0 suggested the presence of a benzene ring. The protons and proton-bearing carbons of **1** were assigned unambiguously by ^{1}H NMR, ^{1}H–^{1}H COSY, DEPT and HMQC experiments, as shown in Table 1.

The 15 carbons were classified as four methyls, two methylenes, two methines, and seven quaternary carbons. The remaining two protons, resonated at δ_H 4.30 and 2.74, were attributed to phenolic and alcoholic hydroxyl protons, respectively. Six of the 15 carbons were assigned to a benzene ring, including two methine carbons at δ_C 116.9 and 111.0, two oxygenated carbons at δ_C 147.4 and 146.1, and two alkylated carbons at δ_C 131.1 and 122.5. Two aromatic protons were located at the *para*-position, because they resonated at δ_H 6.55 and 6.50 as singlets.

The HMBC experiment revealed that C-3 (δ_C 122.5), one of the two alkylated aromatic carbons, was bearing a methyl group resonating at δ_H 2.16. HMBC and NOESY experiments determined a feasible substitution

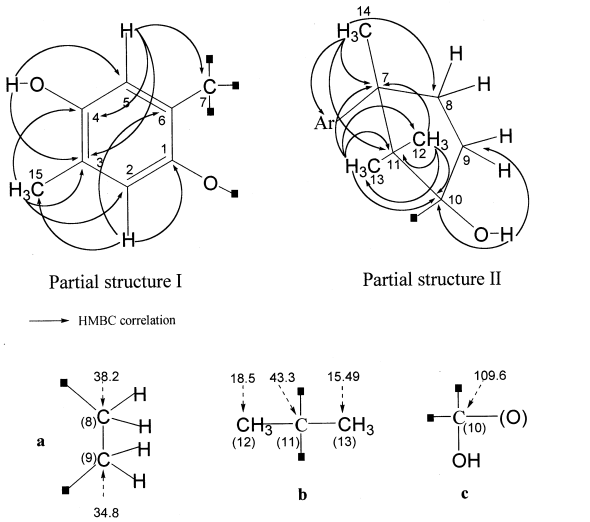


Fig. 1. Partial structures of enokipodin A obtained from HMBC and ^{1}H–^{1}H COSY correlations.

Table 2
^{1}H and ^{13}C NMR spectral data of enokipodin B (**2**)

Position	^{13}C NMR	DEPT	δ_H[a] (*J*, Hz)	^{1}H–^{1}H COSY	HMBC	NOESY
1	187.8	C			H-5	
2	135.3	CH	6.56 *s*	H-15	H-15	H-15
3	144.4	C			H-5, H-15	
4	188.2	C			H-2, H-15	
5	134.1	CH	6.69 *s*			Hα-8, Hβ-8, H-13, H-14
6	153.5	C			H-2, H-14	
7	49.0	C			H-5, Hα-8, Hα-9, H-12, H-13, H-14	
8	31.1	CH_2	α 2.27 *ddd* (9.8, 10.4, 12.8)	Hβ-8, Hα-9		H-5, Hα-8, Hβ-9, H-13
			β 1.88 *ddd* (2.4, 8.7, 12.8)	Hα-8, Hβ-9	Hβ-9, H-14	H-5, Hα-8, Hβ-9, H-12, H-14
9	33.7	CH_2	α 2.49 *ddd* (2.4, 9.8, 19.3)	Hα-8		Hα-8, Hβ-9,H-13
			β 2.44 *ddd* (8.7, 10.4, 19.3)	Hβ-8		Hβ-8, Hα-9, H-14
10	220.9	C			Hβ-8,Hα-9, Hβ-9, H-12, H-13	
11	52.3	C			H-12, H-13, H-14	
12	20.6	CH_3	1.23 *s*	H-13	H-13	Hβ-8, H-13, H-14
13	22.1	CH_3	0.76 *s*	H-12	H-12	H-5, Hα-8, Hα-9, H-12, H-14
14	23.1	CH_3	1.32 *s*		Hα-8	H-5, Hβ-8, Hβ-9, H-12, H-13
15	14.9	CH_3	2.04 *s*	H-2	H-2	H-2

[a] From HMQC.

pattern for the aromatic ring, shown as partial structure I (Fig. 1).

The aliphatic partial structure II shown in Fig. 1 was deduced from 2D NMR spectroscopic data. The ^{1}H–^{1}H COSY analysis revealed the presence of an isolated ethylene chain (four protons, all *ddd*; **a**) and geminal methyl groups (**b**), coupled to each other with a very small *J* value. The singlet methyl protons (C-14) were correlated with four carbons [C-6, δ_C 131.1 (aromatic); C-7, δ_C 47.3 (quaternary); C-8, δ_C 38.2 (methylene); and C-11, δ_C 43.3 (quaternary)] (see **a** and **b**). Therefore, the methyl group (C-14) was located on C-7, and the HMBC correlations connected C-7 to C-6, C-8 (**a**) and C-11 (**b**). The remaining quaternary carbon, resonated at an unusually low field (δ_C 109.6), was assigned as a hemiketal carbon (c) (Tahara et al., 1991; Hashidoko et al., 1993) i.e. bearing the aliphatic hydroxyl group and the ether oxygen directly bound to the benzene ring. As shown in structure I, H-5 (δ_H 6.55) was correlated with C-7 (δ_C 47.3) by HMBC experiments. Finally, partial structures of I and II were connected through a C–C bond between C-6 and C-7, and between C-1 and C-10 via an ether oxygen to give the single rigid structure shown as **1**.

Additional detailed assignments of two methyl groups and two methylene groups were obtained with NOESY experiments, and the results are shown in Fig. 2.

The established structure indicated that enokipodin A was a cuparene-type sesquiterpene. Similar sesquiterpenes have been isolated from the Basidiomycetes *Helicobasidium mompa* (**6** and **7**) (Natori et al., 1964, 1967), *Coprinus lagopus* and *C. macrorhizus* (**8**–**12**) (Thomson, 1971; Bottom and Siehr, 1975; Bu'Lock and Darbyshire, 1976), *Thuja* sp. (**4**, **5**) (Chetty and Dev, 1964; Matsuo et al., 1975), and the red alga *Laurencia okamurai* (**13**) (Suzuki and Kurosawa, 1978). Lagopodin B (**9**), which is analogous to the present metabolite, is known to tautomerize to a hemiketal-type isomer (**11**), presumed to be the structure found in the crystalline form (Bottom and Siehr, 1975).

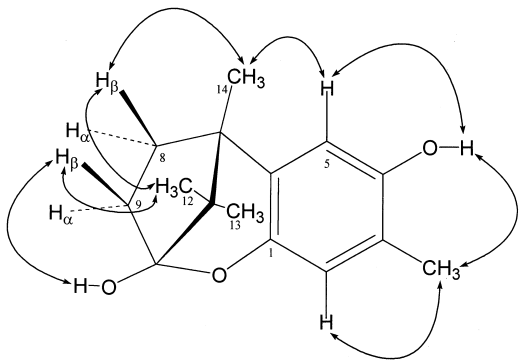


Fig. 2. Important NOESY correlations for enokipodin A.

2.2. 5-Methyl-2-(3-oxo-1,2,2-trimethylcyclopentyl)benzoquinone (enokipodin B (**2**))

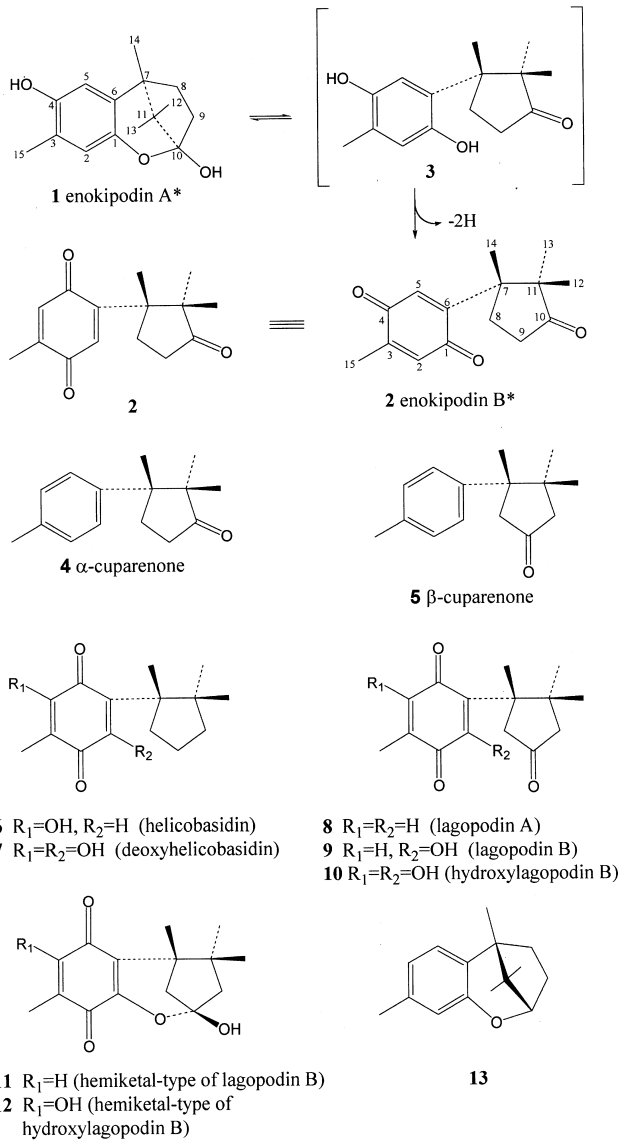
The second compound (**2**) from *F. velutipes*, which was detected at R_f 0.68 on silica gel thin-layer plates developed in $CHCl_3$:MeOH = 25:1, was less polar than **1**. The HR-FIMS revealed the molecular formula to be $C_{15}H_{18}O_3$ (analytical 246.1274, calculated 246.1251), two hydrogens less than **1**. The ^{13}C NMR spectrum revealed 15 carbons comprising four methyls, two methylenes, two methines, and seven quaternary carbons, which included an isolated ketone δ_C 220.9,

two benzoquinone carbonyls δ_C 187.8 and 188.2, and four sp^2 carbons. The IR spectrum exhibited strong absorptions at 1734 and 1652 cm^{-1} attributed to the carbonyl groups of cyclopentanone and benzoquinone. Unlike enokipodin A (**1**), enokipodin B (**2**) showed no hydroxyl group absorption. These spectroscopic characteristics indicated that the structure of enokipodin B was closely related to that of **1**, but was an oxocyclopentylbenzoquinone-type derivative and not a hemiketal-type. Furthermore, when dissolved in $CHCl_3$ or EtOAc, pure crystalline enokipodin A (**1**) slowly changed into enokipodin B (**2**).

Therefore, the structure of **2** was concluded to be 5-methyl-2-(3-oxo-1,2,2-trimethylcyclopentyl)benzoquinone (**2**), an autoxidized product of enokipodin A (**1**) via the keto-type tautomer illustrated as **3**, which has not yet been detected.

Full assignment of the ^1H and ^{13}C spectroscopic data for **2** was accomplished in a similar manner as with **1** (Table 2).

The oxidation pattern on the cyclopentane ring in the cuparane skeleton of **1** and **2** is noticeably different from those of **8**, **9** (**11**) and **10** (**12**). Enokipodins A (**1**) and B (**2**) are α-cuparenone-type derivatives, while lagopodins (**8**–**12**) are β-cuparenone-type derivatives. Both lagopodins [**8**, **9** (**11**), **10** (**12**)] and helicobasidins (**6**, **7**) possess a benzoquinone partial structure, instead of the hydroxybenzene ring in **1**.



3. Experimental

3.1. General

Spectroscopic analyses of the isolated compounds were conducted using the following apparatus. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on JEOL EX270 and Bruker AMX500 spectrometers, respectively. 2D NMR (^1H–^1H COSY, HMQC, HMBC and NOESY) was perfomed on a Bruker AMX500. The chemical shifts are relative to the TMS (^1H) and the solvent peak ($\delta = 77.0$ ppm; ^{13}C). EIMS and HR-EIMS spectra were recorded on a JEOL DX 500 mass spectrometer and the FDMS on a JEOL JMS-SX102A. The UV and IR spectra were recorded on Hitachi model U-3210 and Perkin Elmer System 2000 FT-IR spectrometers, respectively. The melting points were determined on a Yanako MP-30 micro-melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP-370.

3.2. Fungus and cultivation

The strain of *F. velutipes* Fv-4 used in this work was a gift from the Hokkaido Forest Products Research Institute, Asahikawa, Japan. The mycelia were cultured in 10, 300 ml, Erlenmeyer flasks containing 100 ml of malt peptone broth (3% Difco malt extract and 0.3% Merck peptone in distilled water, pH 4.5). Each flask was inoculated with five disks (7 mm i.d.) of the mycelia freshly grown on malt agar plates, and cultured for 30 days at 25°C under stationary conditions.

3.3. Isolation and purification

After the incubation period, 750 ml of culture broth was separated from the mycelia by filtration. The culture filtrate was extracted with 375 ml of EtOAc (3×). The combined EtOAc extracts were washed twice with a saturated solution of NaCl, dried ($MgSO_4$), and evaporated to give 490 mg of an oily residue, which was diluted with EtOAc. This crude extract was loaded on to preparative TLC plates (Silica Gel 60 F_{254} plates, 0.25 mm thick, Merck) and developed in $CHCl_3$:MeOH = 25:1. From the concentrated extract

corresponding to 150 ml of culture filtrate (100 mg of dried crude extract) about 40 mg of enokipodin A at R_f 0.35 and 2.2 mg of enokipodin B (**2**) at R_f 0.68 were obtained.

3.4. Enokipodin A (*1*): 2,3,4,5-tetrahydro-2,7dihydroxy-5,8,10,10-tetramethyl-2,5-methano-1-benzoxepin

^{1}H and ^{13}C NMR spectral data are given in Table 1; Colorless prisms; m.p. 138.5–138.9°C; $[\alpha]_D^{23}$ +48° (*c* 0.5, MeOH); FIMS *m/z* (rel. int.): 249 $[M + 1]^+$ (18), 248 $[M]^+$ (100); HR-FIMS *m/z*: 248.1444 ($C_{15}H_{20}O_3$, calculated 248.1407); EIMS *m/z* (rel. int.): 249 $[M + 1]^+$ (12), 248 $[M]^+$ (70), 178 (17), 177 (100), 175 (11), 164 (7), 163 (7), 162 (28), 151 (6), 71 (6); UV (MeOH) λ_{max}: 207, 236 and 299 nm; IR_{max}^{film}: 3384, 2975, 2876, 2359, 1505, 1393, 1307, 1189, 1164 and 1048 cm^{-1}.

3.5. Enokipodin B (*2*): 5-methyl-2-(3-oxo-1,2,2-trimethylcyclopentyl)benzoquinone

^{1}H and ^{13}C NMR spectral data are given in Table 2. Semisolid; $[\alpha]_D^{24}$ −63° (*c* 0.05, MeOH); FIMS *m/z* (rel. int.): 248 $[M + 2]^+$ (13), 247 $[M + 1]^+$ (25), 246 $[M]^+$ (100), 244 $[M - 2]^+$ (13); HR-FIMS *m/z*: 246.1274 ($C_{15}H_{20}O_3$, calculated 246.1254); EIMS *m/z* (rel. int.): 246 $[M]^+$ (21), 218 (40), 191 (33), 190 (74), 177 (23), 176 (23), 175 (100), 161 (20); UV (MeOH) λ_{max}: 254 nm; IR_{max}^{film}: 1734, 1651, 1090, 1068 and 931 cm^{-1}.

3.6. TLC bioautography

The culture broth was filtered and the filtrate was partitioned between ethyl acetate and water. The ethyl acetate extract equivalent to 0.25 ml of the culture broth was loaded onto TLC plates and developed in $CHCl_3$:MeOH = 25:1. A spore suspension of *C. herbarum* AHU9262 (Hyphomycetes) was sprayed over the developed TLC plates, which were incubated at 25°C under humid conditions for 3 days (Homans and Fuchs, 1970). The observed inhibitory zones were correlated with the spots seen on the TLC plates under UV 254 nm light.

3.7. Paper disk method

The ethyl acetate extract, equivalent to 0.25 ml of the culture broth, was aseptically loaded onto paper disks (8 mm i.d.), which were then dried to remove the solvent and put on a solid medium Brain Heart Infusion (Oxford) plate inoculated with about 10^5 CFU/ml of *B. subtilis*. The petri dishes were allowed to stand overnight at 4°C, so that the metabolites could diffuse into medium. The plates were then incubated at 37°C for 18 h. The antibacterial activity was determined by measuring the diameter of the clear inhibition zone around each paper disk.

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