Antimicrobial Cuparene-Type Sesquiterpenes, Enokipodins C and D, from a Mycelial Culture of *Flammulina velutipes*

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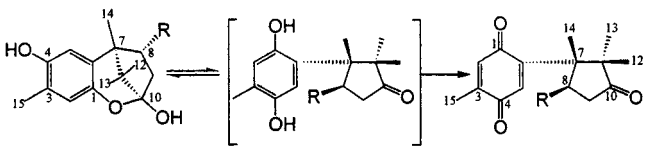
Received December 8, 2000

Two new cuparene-type sesquiterpenes, enokipodins C (**1**) and D (**2**), were isolated from culture medium of an edible mushroom, *Flammulina velutipes*, along with enokipodins A (**3**) and B (**4**). The structures of **1** and **2** were determined using spectroscopic methods (HRMS, ^{1}H and ^{13}C, and 2D NMR). The absolute configuration of enokipodin C was determined from the observed ^{1}H NMR chemical shifts and NOEs in NOESY experiments after conversion into the corresponding esters with the chiral reagent 2-(2′-methoxy-1′-naphthyl)-3,4-dichlorobenzoic acid. All the metabolites showed antimicrobial activity against a fungus, *Cladosporium herbarum*, and Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*.

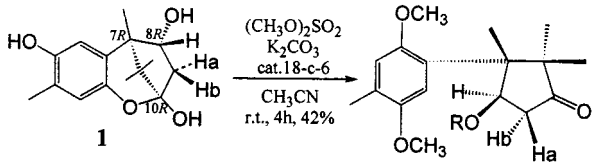
Global antibacterial resistance is an increasing public health problem, because bacterial resistance has developed to almost all available antibiotics.[1] Although fungi are well known for the production of important antibiotic compounds (e.g., penicillins, streptomycins, rifamycins, and others), the occurrence of antibiotics in mushrooms is less well documented.[2] *Flammulina velutipes* (Curt.:Fr.) Sing. (enokitake in Japanese), which belongs to the Tricolomataceae (Hymenomycetes, Basidiomycota), is an edible mushroom frequently consumed in Japan. Compounds with medicinal properties have been isolated from this mushroom,[3−8] including proteins, glycoproteins, and polysaccharides with antitumor and immunomodulatory activities, lectins, sterols, and monoterpenetriols. We report here the isolation, structure elucidation including the absolute configuration, and antimicrobial activities of two new cuparene-type sesquiterpenes, named enokipodins C (**1**) and D (**2**), from culture medium of *F. velutipes*. In our search for antimicrobial compounds in edible mushrooms, we had reported enokipodins A (**3**) and B (**4**) from *F. velutipes*.[9] Compounds **1** and **2** are cuparene-type sesquiterpenes that are more highly oxygenated than **3** and **4**, respectively. The carbon atoms in **1** and **2** are numbered as indicated in Scheme 1 on the basis of biosynthetic considerations.

Scheme 1



1 R = OH (enokipodin C)
3 R = H (enokipodin A)
5 R = OH
6 R = H
2 R = OH (enokipodin D)
4 R = H (enokipodin B)

Scheme 2



7 R = H
8 R = (a*S*)-MNBC
9 R = (a*R*)-MNBC

Results and Discussion

The molecular formula of enokipodin C (**1**) was established as $C_{15}H_{20}O_4$ by HREIMS. The absorption at 3384 cm^{-1} in the IR spectrum and a dehydration ion at *m*/*z* 246 as a base peak in the MS of **1** indicated the presence of a hydroxyl group. A methine carbon corresponding to the carbinyl carbon appeared at δ 77.2, indicating that **1** was a secondary alcohol. UV absorption (λ_{max} at 206 and 299 nm) and signals in the ^{13}C NMR for six sp^2 carbons (4×C+2×CH) suggested the presence of a tetrasubstituted benzene ring. The remaining nine carbons were classified as four methyls, one methylene, one methine, and three quaternary carbons. Comparison of ^{13}C NMR data for **1** and enokipodin A (**3**) strongly suggested that they had identical carbon skeletons.

Three protons were attributed: one (δ 4.35) to phenolic OH and two (δ 2.79 and 1.89) to alcoholic hydroxyl protons. The proton (δ 2.79) assigned to a hemiketal OH, 10-C-OH, similarly resonated in the ^{1}H NMR spectrum of **3** (δ 2.74).[9] The proton at δ 1.89 was located on carbon C-8 (δ 77.2) because HMBC experiments revealed a clear correlation of C-8 (δ 77.2) with a methyl group C-14 (δ 1.29), via three atomic bonds. The presence of a hydroxyl group at C-8 was further supported by the fact that C-10-OH was correlated with the only methylene carbon attributable to the cyclopentane ring C-9 (δ 46.5). Full assignments of ^{1}H and ^{13}C data for **1** were done on the basis of 2D NMR experiments, and NOE correlations revealed the relative stereostructure of **1** depicted in Figure 1, Supporting Information.

The molecular formula of enokipodin D (**2**) was established as $C_{15}H_{18}O_4$ by HREIMS. IR spectroscopy revealed the presence of a hydroxyl group (3446 cm^{-1}) and carbonyl groups (1733 and 1690 cm^{-1}) that were attributed to cyclopentanone and benzoquinone moieties in **2**. The ^{13}C NMR spectrum revealed the presence of 15 carbons comprised of four methyls, one methylene, three methines, and seven quaternary carbons. The quaternary carbons included an isolated ketonic carbonyl (δ 216.0), two benzoquinone carbonyls (δ 188.7 and 187.5), four sp^2 carbons, and a methine carbon bearing a hydroxyl group (δ 69.2). Enokipodin D (**2**) appeared to be an oxocyclopentylbenzo-

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10.1021/np000593r CCC: $20.00
Published on Web 07/07/2001

Table 1. Antibacterial Activities of Enokipodins A−D (**1**−**4**)

	C (**1**)		D (**2**)		A (**3**)		B (**4**)		PCP	
species/strain	25 μg[a]	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	12.5 μg	25 μg
Gram positive										
Bacillus subtilis LMA0011	20[b]	26	11	16	28	32.6	17	21	26.6	31
Staphylococcus aureus AHU1142	18	21.2	0	11.6	19.2	25.2	0	14	nt[c]	nt
Gram negative										
Escherichia coli AHU1714	0	0	0	0	0	0	0	0	nt	nt
Pseudomonas fluorescens AHU1719	0	0	nt	nt	0	0	nt	nt	nt	nt

[a] Loaded on 8 mm ϕ paper disk. [b] Diameter of inhibitory zone in mm (average from three replicates). [c] nt = Not tested.

quinone-type derivative, like enokipodin B (**4**),[9] with an additional hydroxyl group on C-8. Enokipodin A (**3**) and C (**1**) were autoxidized to enokipodin B (**4**) and D (**2**) on thin-layer plates, via the keto-type tautomer illustrated as **6**[9] and **5**, respectively, which has not yet been detected. Therefore, the structure of enokipodin D (**2**) was concluded to be that illustrated in Scheme 1. Full assignment of the ^{1}H and ^{13}C spectroscopic data for **2** was accomplished in a manner similar to that with **1**.

The absolute configuration of enokipodin C (**1**) was determined by the 2-(2′-methoxy-1′-naphthyl)-3,5-dichlorobenzoic acid (MNCB) method.[10] First, enokipodin C (**1**) was methylated with dimethyl sulfate, in the presence of K_2CO_3, 18-crown-6, and CH_3CN, to give 1,4-di-*O*-methylenokipodin C (**7**). Then, the secondary alcohol (C-8) in **7** was esterified with (a*S*)- and (a*R*)-MNBC to give **8** and **9**, respectively. In the NOESY spectra for **9**, NOE was observed between the methyl protons (H-14) of the alcohol moiety and methoxy protons of the reagent moiety (Figure 2, Supporting Information). The chemical shift differences of the proton signals, $\Delta\delta$ = **8**(a*S*) − **9**(a*R*), on the right side of the CB plane have positive values and those on the left side of the plane have negative values. The value of proton signals under the border plane were not considered.[10] Therefore, C-8 of **7** was confirmed to be *R* and C-7 and C-10 of **1** were *R*.

Total synthesis of α-cuparenones has been accomplished in a variety of ways,[11−15] despite the challenge due to steric congestion created by two contiguous quaternary centers around the cyclopentane ring.[16] However, synthesis or isolation of α-cuparenone-type sesquiterpenes with high oxidation levels, such as enokipodins A−D (**1**−**4**), has not yet been reported.

Compounds **1**−**4** exhibited antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, but were inactive against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescens*. For *B. subtilis*, the inhibition zones observed with 50 μg of enokipodins C (**1**) and A (**3**) were equivalent to those produced by 12.5 and 25 μg of pentachlorophenol (Table 1).

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on JEOL EX270 and Bruker AMX500 spectrometers, respectively. 2D NMR (^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY) was performed on a Bruker AMX500. The chemical shifts are relative to TMS (^{1}H) and the solvent peak (δ = 77.0 ppm; ^{13}C). EIMS and HREIMS 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 spectrophotometers, respectively. 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 digital polarimeter.

Fungus and Cultivation. The strain of *Flammulina velutipes* Fv-4 used in this work is kept in the culture collection of the Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University, and maintained on potato dextrose agar. The mycelia were cultured in 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 in diameter) of mycelia freshly grown on malt agar plates and cultured for 45 days at 25 °C under stationary conditions.

Antimicrobial Assay. An antifungal assay was carried out against *Cladosporium herbarum* AHU9262 (Hyphomycetes). The culture medium was filtered, and the filtrate was partitioned between EtOAc and H_2O. EtOAc extract equivalent to 0.25 mL of the culture medium was charged on thin layer plates and developed in $CHCl_3$−MeOH = 25:1. A spore suspension of *C. herbarum* was sprayed over the developed TLC plates, which were incubated at 25 °C under humid conditions for 3 days.[17] The observed inhibitory zones were correlated with the spots seen on the TLC plates under UV 254 nm light. The Gram-positive bacteria *Bacillus subtilis* LMA0011 and *Staphylococcus aureus* AHU1142 and the Gram-negative bacteria *Escherichia coli* AHU1714 and *Pseudomonas fluorescens* AHU1719 were used in the antibacterial assays. A 25 or 50 μg portion of **1**, **2**, **3**, or **4** in acetone (20 μL) was applied onto a paper disk of ϕ 8 mm, and the paper disks were air-dried. Then, the disks were placed on agar plates seeded with respective organisms. The Petri dishes were allowed to stand overnight at 4 °C, so that the metabolites could diffuse into the 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. Pentachlorophenol (PCP) (12.5 and 25 μg) was used as positive control antimicrobial compound. All experiments were done in triplicate.

Extraction and Isolation. After incubation, 1400 mL of culture medium was separated from the mycelia by filtration. The culture filtrate was extracted with EtOAc (750 mL × 3). The combined extracts were washed with a saturated solution of NaCl (1000 mL × 2), dried ($MgSO_4$), and evaporated to give 937 mg of an oily residue. Part of the crude extract (200 mg) was charged on PTLC (Silica Gel 60 F_{254} plates, 0.25 mm thick, Merck) and developed in $CHCl_3$−MeOH = 25:1. The bands at R_f 0.68 (1.4 mg), 0.35 (38.8 mg), and 0.07 (56.2 mg) were collected. The constituents were rechromatographed on TLC plates in toluene−acetone = 4:1, along with authentic **3** and **4**. The eluate from the top band (R_f 0.68) yielded a single product (1.4 mg), indistinguishable from **4**. The central band yielded 35.6 mg of **3** and a small amount of **2**. The bottom band gave 45.7 mg of **1** and 2.4 mg of **2**.

Enokipodin C (1): colorless oil; $[\alpha]^{24}_D$ − 9.4° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 209 (4.24) and 299 (3.63) nm; IR ν_{max} (NaCl cell) 3384, 2947, 2362, 1507, 1457, 1417, 1308, 1174, and 1066 cm^{-1}; ^{1}H NMR ($CDCl_3$, 270 MHz) δ 6.55 (1H, s, H-5), 6.51 (1H, s, H-2), 4.35 (1H, s, 4-OH), 3.89 (1H, d, J = 8.1 Hz, H-8), 2.79 (1H, s, 10-OH), 2.26 (1H, dd, J = 8.1, 15.3 Hz, H-9b), 2.22 (1H, dd, J = 2.9, 15.3 Hz, H-9a), 2.17 (3H, s, H-15), 1.89 (1H, br, J = 2.9 Hz, 8-OH), 1.29 (3H, s, H-14), 1.25 (3H, s, H-12), 0.74 (3H, s, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 147.8 (s, C-4), 145.8 (s, C-1), 128.5 (s, C-6), 123.4 (s, C-3), 117.2 (s, C-2), 111.1 (s, C-5), 108.7 (s, C-10), 77.2 (s, C-8), 51.7 (s, C-7), 46.5 (s, C-9), 42.6 (s, C-11), 19.7 (s, C-12), 16.7 (s, C-13), 15.5

(s, C-15), 11.4 (s, C-14); EIMS *m/z* 264 $[M]^+$ (70), 246 $[M - H_2O]^+$ (100), 231 (67), 205 (63), 203 (24), 177 (20), 176 (38), 175 (50), 161 (20), 151 (43); FDMS *m/z* 265 $[M + 1]^+$ (17), 264 $[M]^+$ (100); HREIMS *m/z* 264.1403 (calcd for $C_{15}H_{20}O_4$, 264.1356).

Enokipodin D (2): yellow powder; mp 116.0−117.0 °C; $[\alpha]^{24}_D$ +130° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 254 (3.83) nm; IR ν_{max} (NaCl cell) 3446, 2970, 2340, 1733, 1690, 1653, 1457, 1375, and 1250 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ 6.86 (1H, s, H-5), 6.59 (1H, d, J = 1.6 Hz, H-2), 5.04 (1H, dd, J = 8.6, 9.6 Hz, H-8), 2.83 (1H, dd, J = 8.6, 19.0 Hz, H-9a), 2.43 (1H, dd, J = 9.6, 19.0 Hz, H-9b), 2.05 (3H, d, J = 1.6 Hz, H-15), 1.28 (3H, s, H-14), 1.24 (3H, s, H-12), 0.83 (3H, s, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 216.0 (s, C-10), 188.7 (s, C-1), 187.5 (s, C-4), 150.6 (s, C-6), 144.5 (s, C-3), 135.8 (s, C-5), 135.2 (s, C-2), 69.2 (s, C-8), 55.4 (s, C-11), 53.3 (s, C-7), 41.7 (s, C-9), 22.8 (s, C-13), 19.8 (s, C-12), 17.0 (s, C-14), 15.0 (s, C-15); EIMS *m/z*: 262 $[M]^+$ (6.6), 244 $[M - H_2O]^+$ (20), 202 (20), 192 (78), 191(92), 190 (36), 178 (33), 177 (60), 176 (22), 175 (100); FDMS *m/z* 264 $[M + 2]^+$ (63), 263 $[M + 1]^+$ (60), 262 $[M]^+$ (100); HREIMS *m/z* 262.1223 (calcd for $C_{15}H_{18}O_4$, 262.1200).

1,4-Di-*O*-methylenokipodin C (7). The alcohol **1** (12.3 mg, 47.7 μmol), anhydrous K_2CO_3 (40.8 mg, 286.2 μmol), 18-crown-6 (1.3 mg, 4.9 μmol), and dimethyl sulfate (18.1 μL, 190.8 μmol) in CH_3CN (0.5 mL) were stirred at room temperature for 4 h.[18] The reaction mixture was directly applied to PTLC ($CHCl_3$−MeOH = 20:1) to give 1,4-di-*O*-methylenokipodin C (**7**) (R_f 0.53, 5.2 mg, 42% yield); EIMS *m/z* 292 $[M]^+$ (35), 206 (30), 179 (100); HREIMS *m/z* 292.1667 (calcd for $C_{17}H_{24}O_4$, 292.1668).

(a*S*)- and (a*R*)-MNCB Ester of 1,4-Di-*O*-methylenokipodin C (8 and 9). To a solution of **7** (2.6 mg, 13.5 μmol), (a*S*)-MNCB (4.9 mg, 14.1 μmol), and 4-pyrrolidinopyridine (0.13 mg, 0.8 μmol) in $CHCl_3$ (0.5 mL) was added dicyclohexylcarbodiimide (DCC) (2.4 mg, 11.6 μmol), and the solution was stirred at room temperature for 12 h.[19] The reaction mixture was directly applied to PTLC (hexanes−EtOAc = 3:1) to give **8** (R_f 0.29, 1.7 mg, 65% yield). Under essentially the same conditions, compound **7** was esterified with (a*R*)-MNCB to give **9** (R_f 0.40, 2.0 mg, 91% yield). EIMS *m/z*: **8**, 622 $[M+2]^+$ (8), 620 $[M]^+$ (11), 348 (46), 346 $[M - C_{17}H_{22}O_3]^+$ (70), 274 (85), 259 (100); **9**. 622 $[M + 2]^+$ (24), 620 $[M]^+$ (33), 348 (12), 346 $[M - C_{17}H_{22}O_3]^+$ (19), 329 (20), 275 (22), 274 (100), 259 (50).

Acknowledgment. The authors are grateful to Dr. Kiyoshi Miura, Dr. Takashi Yajima, and Dr. Masato Shibuya for encouragement during the course of this work. Thanks are also due to Mr. Kenji Watanabe and Dr. Eri Fukushi (GC-MS & NMR Laboratory) and Mr. Hiroshi Ishimoto and Mr. Hiroki Fukui (Laboratory of Ecological Chemistry), in our Graduate School, for recording the EI- and FDMS and 2D NMR spectra. Financial support (to S.T. and Y.F.) from CREST, Japan Science and Technology Corporation, and a Scholarship to N.K.I. from the Ministry of Education, Science, Sports and Culture of Japan, are gratefully acknowledged.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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NP000593R