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, 1H and 13C, and 2D NMR). The absolute configuration of enokipodin C was determined from the observed 1H NMR chemical shifts and NOEs in NOESY experiments after conversion into the corresponding esters with the chiral reagent 2-(2-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.

Results and Discussion

The molecular formula of enokipodin C (1) was established as C15H20O4 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 carbonyl carbon appeared at δ 77.2, indicating that 1 was a secondary alcohol. UV absorption (λmax at 206 and 299 nm) and signals in the 13C NMR for six sp² carbons (4×C–I+2×CH) suggested the presence of a tetrastubstituted benzene ring. The remaining nine carbons were classified as four methyls, one methylene, one methine, and three quaternary carbons. Comparison of 13C 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 1H NMR spectrum of 3 (δ 2.74). The proton at δ 1.89 was located on carbon C-8 (δ 77.2) because HMB experiments revealed a close 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 1H and 13C 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 C15H18O4 by HREIMS. IR spectroscopy revealed the presence of a hydroxyl group (3446 cm⁻¹) and carbonyl groups (1882 and 1786 cm⁻¹), which were attributed to cyclopentanone and benzoquinone moieties in 2. The 13C 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 sp² carbons, and a methine carbon bearing a hydroxyl group (δ 69.2). Enokipodin D (2) appeared to be an oxocyclopentylbenzo-
Table 1. Antibacterial Activities of Enokipodins A–D (1–4)

<table>
<thead>
<tr>
<th>species</th>
<th>strain</th>
<th>C (1)</th>
<th>D (2)</th>
<th>A (3)</th>
<th>B (4)</th>
<th>PCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive</td>
<td></td>
<td>25 µg</td>
<td>50 µg</td>
<td>25 µg</td>
<td>50 µg</td>
<td>25 µg</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> LMA0011</td>
<td></td>
<td>20</td>
<td>26</td>
<td>11</td>
<td>16</td>
<td>28</td>
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<tr>
<td><em>Staphylococcus aureus</em> AHU1142</td>
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<td>18</td>
<td>21.2</td>
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<td>11.6</td>
<td>19.2</td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> AHU1719</td>
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<td>0</td>
<td>0</td>
<td>nt</td>
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</tr>
</tbody>
</table>

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

**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 H2O. EtOAc extract equivalent to 0.25 mL of the culture medium was charged on thin layer plates and developed in CHCl3–MeOH = 25:1. A spore suspension of *C. herbarum* was sprayed 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 Ø 8 mm, and the paper disks were air-dried. 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 a positive control antifungal 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 (MgSO4), and evaporated to give 937 mg of an oily residue. Part of the crude extract (200 mg) was charged on PTLC (Silica Gel 60 F254 plates, 0.25 mm thick, Merck) and developed in CHCl3–MeOH = 25:1. The bands at Rf 0.68 (1.4 mg), 0.35 (38.8 mg), and 0.07 (56.2 mg) were collected. The constituents were chromatographed on TLC plates in taneune-acetone = 4:1, along with authentic 3 and 4. The eluate from the top band (Rf 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; [α]D25 −9.4° (c 1.0, MeOH); UV (MeOH): λmax (log ε) 209 (4.24) and 299 (3.63) nm; IR (KBr) νmax (NaCl cell) 3384, 2947, 2362, 1507, 1457, 1417, 1308, 1174, and 1066 cm−1; 1H NMR (CDCl3, 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), 2.27 (1H, dd, J = 2.9, 15.3 Hz, H-9a), 1.27 (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); 13C NMR (CDCl3, 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-14), 14.8 (s, C-11), 13.08 (s, C-10), 1174 (s, C-8), and 1066 (s, C-1).

**Experimental Section**

**General Experimental Procedures.** 1H and 13C NMR spectra were recorded on a J EOL EX270 and Bruker AMX500 spectrometers, respectively. 2D NMR spectra were recorded on a Bruker AMX500. The chemical shifts are relative to TMS (1H) and CDCl3 (13C). EIMS (70 eV) and HREIMS spectra were recorded on Hitachi model QMS-1000 spectrometers, respectively. Melting points were determined on a Yanaco MP-30 micro melting point apparatus and are uncorrected. Optical rotations were recorded on Jasco DIP-370 digital polarimeter.

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Encyclopedia of Natural Products, 2001, Vol. 64, No. 7

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