Protoilludane-Type Sesquiterpenes, Echinocidins A and B, from a Mycelial Culture of Echinodontium tsugicola

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Two novel protoilludane sesquiterpenoids, echinocidins A (3) and B (4), have been isolated from an artificial liquid culture of the basidiomycetous fungus Echinodontium tsugicola and their structures clarified spectroscopically. Echinocidin A (3) accelerated primary root growth to 117% and echinocidin B to 180% of controls at a concentration of 100 ppm.

Key words: Echinodontium tsugicola, Echinocidins A and B

Results and Discussion

The producing strain was cultured at 25 °C on a rotary shaker for 4 weeks in a medium (6.5 liters) containing 4% malt extract, 4% glucose, and 0.1% peptone in distilled water. The culture filtrate was extracted with EtOAc. The EtOAc extract was column-chromatographed on silica gel, yielding 7 fractions. From a second fraction, echinocidin A (3) was isolated by column chromatography and finally by preparative TLC. Echinocidin B (4) was obtained from the sixth fraction by Sephadex LH-20 column chromatography.

The molecular formula of echinocidin A (3) was determined to be C_{15}H_{24}O_{2}, by HRFABMS, implying four degrees of unsaturation or cycles. The IR spectrum of 3 had absorption for hydroxyl groups (3305 cm\(^{-1}\)). \(^{13}\)C NMR spectra (Table 1) of 3 showed the presence of one carbon-carbon double bond at δ = 135.6 (C-7) and 132.6 (C-8), suggesting that 3 is tricyclic. The \(^{13}\)C NMR spectrum of 3 also showed the presence of three methyls at δ = 31.5 (C-15), 31.9 (C-14) and 20.7 (C-12), one hydroxymethyl at δ = 65.2 (C-13), four methylenes at δ = 24.6 (C-4), 34.9 (C-5), 41.9 (C-1) and 47.5 (C-10), two sp\(^{3}\) me-
ternary carbons, one of which was linked to oxygen.

Detailed study of the 1H-1H-COSY and HMQC spectra (Table 1) of thiones at δ = 38.6 (C-9) and 44.5 (C-2) and three quaternary carbons, one of which was linked to oxygen at δ = 38.0 (C-11), 45.1 (C-3), and 73.3 (C-6). The 1H NMR spectrum (Table 1) of 3 showed three singlet methyls at δ = 0.97 (s, 3H, 14-H), 1.01 (s, 3H, 15-H) and 1.21 (s, 3H, 12-H) and two protons of hydroxymethyl at δ = 4.03 (d, J = 11.7 Hz, 1H, 13-H) and 4.40 (d, J = 11.7 Hz, 1H, 13-H), and a proton of a trisubstituted double bond at δ = 5.55 (s, 1H, 8-H). Detailed study of the 1H-1H-COSY and HMQC spectra showed the presence of -C(4)H2-C(5)H2- [δ = 1.28 (td, J = 10.3, 2.4 Hz, 1H, 4-β-H), 1.40 (m, 1H, 4-α-H), 2.06 (td, J = 10.3, 2.4 Hz, 1H, 5-β-H)] and 2.17 (q, J = 10.3 Hz, 1H, 5-α-H) and -CH2(CH2)-CH(9) in place of one methylene signal, 2.97 (m, 1H, 1-α-H), 3.37 (m, 1H, 1-β-H), 2.00 (m, 1H, 2-β-H), 2.69 (m, 1H, 9-H), 1.43 (dd, J = 13.2, 2.5 Hz, 1H, 10-α-H) and 1.80 (1H, dd, J = 13.2, 8.8 Hz, 10-β-H) moieties. Based on these findings, 3 was deduced to be a protoilludane sesquipiperine. In the comparison with spectral data for a sesquipiperine isolated from Laurilia tsugicola (E. tsugicola), its 13C NMR spectral data for C-1, C-2, C-3, C-10, C-11, C-12, C-13, C-14, and C-15 resembled that of tsugicoline A [1]. In the HMBC spectrum of 3 (Table 1), HMBC correlations of 13-H2 to C-6 and C-8, and 8-H to C-13 indicated that a double bond can be placed between C-7 and C-8. Signals of 4-H2 correlated with C-2, C-6, and C-12; signals of 5-H2 with C-3 and C-7; and signals of 12-H3 with C-2, C-3, C-4, and C-6, suggesting a planar structure for 3 as shown in Fig. 1. The relative stereochemistry of 3 was established by a nuclear Overhauser effect (NOE) difference experiment (Fig. 2) in which NOEs from 14-H3 to 1-β-H, 14-H3 to 10-β-H, 14-H3 to 2-H, 15-H3 to 1α-H, 15-H3 to 10α-H, 15-H3 to 8-H, and 9-H to 2-H. These results indicated a cis-junction between cyclopentane and cyclohexene rings. The configuration at C-3 was elucidated to be α-CH3 based on NOEs from 12-H3 to 1-H2, 12-H3 to 4α-H and 12-H3 to 5α-H. In NOE experiments on monoacetate 3a, irradiation of 12-H3 caused NOEs on 5α-H and 6-OH, thus establishing the cyclobutane/cyclohexene rings junction as cis, i.e., the relative structure of echinocidin A was 3. The stereostructure of 3 was similar to those of tsugicolines A (1) and B (2), whose absolute structures have been determined as a (2S,3R,9R)-configuration by application of the Horeau chirality rule [1]. The absolute configurations of hydrogen at C-2, C-9 and of a methyl at C-3 were therefore presumed to be the same as those of 1 from a biosynthetic pathway.

Echinocidin B (4) showed spectral characteristics similar to those of 3. The molecular formula of 4, C15H20O3, was determined by HRFABMS, corresponding to one oxygen atom more than that of 3. The IR spectrum showed a hydroxyl group at 3322 cm⁻¹. The 1H and 13C NMR spectra (Table 2) of 4 correspond well to those of 3, except for the presence of an oxymethylene signal [δH = 4.29 (t, J = 8.8, 1H, 5-H), δC = 75.9 (C-5)] in place of one methane signal, indicating 4 was probably an oxygenated metabolite of 3. The location of a hydroxyl group at C-5 was established by HMBC (Table 2) in which methine (5-H) correlated with C-4, C-6, and C-7. Upon acetylation, 4
from 9-H to 10 and neoilludol from Clitocybe illudens [1]. The biological activity of 3 and 4 was examined via antimicrobial activity and lettuce bioassay. Compounds 3 and 4 were inactive against Candida albicans ATCC 2019, Staphylococcus aureus NBRC 13376, and Pseudomonas aeruginosa ATCC 15442 at a concentration of 100 µg / disk. With the lettuce seedling assay, 3 showed radicle elongation activity of 117% and 4 180% of controls at a concentration of 100 ppm.

**Experimental Section**

**General experimental procedures**

Melting points (m. p.) data are uncorrected. Optical rotation was measured with a Horiba model SEPA-300 polarimeter, IR spectra were recorded with a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were recorded with a JEOL JMS-700 instrument, and 1H and 13C NMR spectra were obtained with a JEOL EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was conducted on Sephadex LH-20 (Pharmacia) and silica gel 60 (Kanto Chemical Co., Inc.). TLC was done on a precoated silica gel plate (Merck), and spots were detected by spraying 10% vanillin in H2SO4 followed by heating.

**Fungus and cultivation**

The strain of E. tsugicola used in this work was kindly supplied by Mori & Company, Ltd., Gunma, Japan. The mycelia were cultured in sixty five 500 ml-Sakaguchi flasks containing 100 ml of a medium consisting of 40 g of malt extract, 40 g of glucose, and 1.0 g peptone per 1 liter of water. Each flask was inoculated with some disks (5 mm i. d.) of the mycelia freshly grown on malt agar plates, and cultured at 25 °C for 4 weeks on a rotary shaker (120 rpm).

**Extraction and isolation of echinocidins A and B**

After the incubation period, 6.5 liters of culture broth was separated from the mycelia by filtration. The culture filtrate was extracted with EtOAc. The organic layer was dried over anhydrous sodium sulfate, the solvent was evaporated to afford a brown residue (3.3 g). This crude extract was subjected to silica gel column chromatography with CHCl3-MeOH (98:2 and 95:5, v/v) to give seven fractions (fr. 1.1 – 1.7). These fractions were examined by preparative TLC with CHCl3-MeOH (94:6, v/v) to afford echinocidin A (3, 64.9 mg). Fr. 1.6 was separated by Sephadex LH-20 with MeOH to yield echinocidin B (4, 20.4 mg).

**Echinocidin A (3)**

White needles; m.p. 77 – 78 °C. - [α]D20 = -35 (c 1.5, CHCl3). - IR (KBr): ν = 3305 (OH), 2950, 2861, 1234, 772, 737 cm⁻¹. MS (FAB): m/z = 329 [M + Na]+. 1H NMR: δ (ppm, J): 0.96 (3H, s) at C-3, 1.15 (3H, s) at C-12, 1.24 (3H, s) at C-11, 1.32 (3H, s) at C-14, 1.35 (1H, m) at C-13, 1.37 (1H, m) at C-15, 1.78 (1H, m) at C-5, 2.40 (2H, d, 12.2) at C-6, 2.68 (1H, d, 12.2) at C-1, 2.76 (1H, d, 12.2) at C-10, 3.61 (1H, d, 14.2) at C-14, 3.74 (1H, d, 14.2) at C-15, 4.20 (1H, d, 12.2) at C-6, 4.39 (1H, d, 12.2) at C-8, 4.91 (1H, m) at C-1, 5.40 (1H, d, 12.2) at C-10, 5.77 (1H, d, 12.2) at C-13, 5.84 (1H, d, 12.2) at C-15, 7.02 (1H, d, 12.2) at C-1. 13C NMR: δ (ppm): 20.0 (C-13), 21.9 (C-2), 25.2 (C-14), 26.6 (C-11), 30.3 (C-12), 37.1 (C-10), 37.4 (C-1), 39.3 (C-15), 39.9 (C-3), 40.3 (C-5), 42.3 (C-8), 44.5 (C-1), 44.8 (C-9), 45.6 (C-14), 46.9 (C-6), 49.8 (C-15), 51.7 (C-11), 52.0 (C-14), 53.2 (C-10), 70.3 (C-12), 76.2 (C-10), 78.1 (C-8), 78.5 (C-6), 87.0 (C-11), 129.3 (C-12), 130.0 (C-11), 132.2 (C-10), 132.3 (C-8), 149.1 (C-10), 149.2 (C-8), 154.9 (C-10), 155.2 (C-8), 164.2 (C-11), 164.3 (C-14), 165.1 (C-15). Table 2. 13C (100 MHz) and 1H (400 MHz) NMR data for 4.

<table>
<thead>
<tr>
<th>No.</th>
<th>δC (Mult.)</th>
<th>δH (Mult. J, Hz)</th>
<th>HMBC (1H to 13C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.6 t</td>
<td>β 1.26 (1H, t, 12.7)</td>
<td>3, 9, 10, 11, 14, 15</td>
</tr>
<tr>
<td>2</td>
<td>44.4 d</td>
<td>α 1.35 (1H, m)</td>
<td>3, 9, 10, 11, 14, 15</td>
</tr>
<tr>
<td>3</td>
<td>37.1 s</td>
<td>2.08 (1H, m)</td>
<td>1, 6, 8, 9, 11</td>
</tr>
<tr>
<td>4</td>
<td>35.6 t</td>
<td>β 1.34 (1H, m)</td>
<td>2, 5, 12</td>
</tr>
<tr>
<td>5</td>
<td>75.9 d</td>
<td>α 1.84 (1H, dd, 10.7, 8.8)</td>
<td>5, 12</td>
</tr>
<tr>
<td>6</td>
<td>77.8 s</td>
<td>2.49 (1H, t, 8.8)</td>
<td>4, 6, 7</td>
</tr>
<tr>
<td>7</td>
<td>133.1 s</td>
<td>2.66 (1H, s)</td>
<td>2, 6, 10, 13</td>
</tr>
<tr>
<td>9</td>
<td>27.0 s</td>
<td>2.70 (1H, m)</td>
<td>1, 7, 10, 11</td>
</tr>
<tr>
<td>10</td>
<td>47.5 t</td>
<td>α 1.39 (1H, dd, 13.7, 2.0)</td>
<td>1, 8, 11, 14, 15</td>
</tr>
<tr>
<td>11</td>
<td>38.0 s</td>
<td>β 1.20 (1H, dd, 13.7, 8.3)</td>
<td>1, 8, 11, 14, 15</td>
</tr>
<tr>
<td>12</td>
<td>21.9 q</td>
<td>0.97 (3H, s)*</td>
<td>1, 10, 11, 15</td>
</tr>
<tr>
<td>13</td>
<td>31.9 q</td>
<td>0.96 (3H, s)*</td>
<td>1, 10, 11, 14</td>
</tr>
</tbody>
</table>

* Values in parentheses are coupling constants in Hz. * These values might be interchangeable.

Fig. 3. Selected NOE correlations for diacetylchelinocidin B (4a).
1126 and 1078 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) and ¹³C {¹H} NMR (100 MHz, CDCl₃) data see Table 1. – HRMS (negative mode, FAB): m/z = 235.1702 [M-H⁻] (calcd. for C₁₅H₂₃O₃: 251.1641). – MS (negative mode, FAB): m/z = 235 [M-H⁻]. – MS (EI): m/z (%) = 236 (2) [M⁺], 208 (38) [M⁺ -CH₂CH₂ -H₂O], 190 (35) [M⁺ -CH₂CH₂ -H₂O], 151 (13), 134 (48), 122 (100), 91 (30).

**Echinocidin B (4)**

White powder. M. p. 85 – 87 °C. – [α]D +5.5 (c 0.69, CHCl₃). – IR (KBr); ν = 3322 (OH), 2965, 2867, 1205, 1118 and 1074 cm⁻¹. – ¹H NMR (400 MHz, CDCl₃) and ¹³C {¹H} NMR (100 MHz, CDCl₃) data see Table 2. – HRMS (negative mode, FAB): m/z = 251.1647 [M-H⁻] (calcd. for C₁₅H₂₃O₃: 251.1641). – MS (negative mode, FAB): m/z = 251 [M-H⁻]. MS (EI): m/z (%) = 252 (2) [M⁺], 234 (4) [M⁺ -H₂O], 208 (100) [M⁺ -CH₂CH₂OH], 190 (85) [M⁺ -CH₂O], 175 (57), 134 (82), 121 (35), 94 (44).

**Acetylation of echinocidin A (3)**

Echinocidin A (3, 7.0 mg) in pyridine (0.5 ml) was acetylated with acetic anhydride (0.5 ml) at room temperature overnight. The reaction mixture was poured into water and extracted with EtOAc (5 ml × 3). The organic layer was dried with NaCl and dried over Na₂SO₄ and concentrated in vacuo to give a residue which was purified by silica gel chromatography to yield the monoacetate (3a, 5.5 mg) as an amorphous powder.

Monoacetate 3a: ¹H NMR (400 MHz, CDCl₃): δ = 0.97 (s, 3H, 14-H₃ and 15-H₃), 1.21 (s, 3H, 12-H₃), 1.27 (td, J = 10.3, 1.9 Hz, 1H, 1-H), 1.39 (m, 2H, 1-H), 1.42 (m, 1H, 4-α-H), 1.46 (m, 1H, 10-α-H), 1.80 (dd, J = 13.2, 8.8 Hz, 1H, 10-β-H), 1.90 (br. s, 3H, 6-OH), 1.99 (m, 1H, 2-H), 2.04 (m, 1H, 5-β-H), 2.07 (s, 3H, CH₃CO), 2.13 (q, J = 10.3 Hz, 1H, 5-H), 2.71 (q, J = 8.8 Hz, 1H, 9-β-H), 4.66 (d, J = 12.2 Hz, 1H, 13-H), 4.71 (d, J = 12.2 Hz, 1H, 13-H), 5.63 (s, 1H, 8-H). – ¹³C {¹H} NMR (100 MHz, CDCl₃): δ = 21.0 (q, C'H₂CO), 21.2 (q, C'H₂), 25.0 (t, C-4), 31.5 (q, C-15), 31.8 (q, C-14), 34.3 (t, C-5), 37.8 (s, C-11), 38.7 (s, C-9), 41.9 (t, C-1), 44.5 (t, C-2), 45.0 (s, C-3), 47.5 (t, C-10), 65.0 (t, C-13), 71.7 (s, C-6), 133.0 (d, C-8), 133.6 (s, C-7), 171.1 (s, CH₃CO). – MS (EI): m/z (%) = 278 (12) [M⁺], 261 (100) [M⁺ -OH].

**Acetylation of echinocidin B (4)**

Echinocidin B (4, 2.0 mg) in pyridine (0.5 ml) was acetylated with acetic anhydride (0.5 ml) at room temperature overnight. The reaction mixture was treated in the same manner as described above to furnish the diacetate (4a, 1.3 mg) as an amorphous powder.

Diacetate 4a: ¹H NMR (400 MHz, CDCl₃): δ = 0.94 (s, 3H, 14-H₃), 0.98 (s, 3H, 15-H₃), 1.21 (s, 3H, 12-H₃), 1.26 (m, 1H, 1-β-H), 1.38 (dd, J = 11.2, 8.8 Hz, 1H, 4-β-H), 1.41 (m, 1H, 1-α-H), 1.44 (m, 1H, 10-α-H), 1.82 (dd, J = 13.2, 8.3 Hz, 1H, 10-β-H), 1.92 (dd, J = 11.2, 8.8 Hz, 1H, 4-α-H), 2.01 (s, 3H, CH₃CO), 2.07 (s, 3H, CH₃CO), 2.13 (1H, m, 2-H), 2.69 (br. t, J = 8.3 Hz, 1H, 9-β-H), 4.56 (d, J = 12.7 Hz, 1H, 13-H), 4.75 (d, J = 12.7 Hz, 1H, 13-H), 5.09 (t, J = 8.8 Hz, 1H, 5-α-H), 5.82 (s, 1H, 8-H). – ¹³C {¹H} NMR (100 MHz, CDCl₃): δ = 20.6 (q, C'H₂CO), 21.2 (q, C'H₂CO), 21.9 (q, C-12), 32.2 (q, C-14), 32.4 (q, C-15), 33.2 (t, C-13), 38.0 (s, C-3), 39.1 (s, C-11), 39.3 (d, C-9), 41.7 (t, C-1), 44.1 (d, C-2), 51.0 (t, C-10), 64.8 (t, C-13), 76.4 (d, C-5), 77.8 (s, C-6), 131.2 (s, C-7), 136.1 (d, C-8), 169.6 (s, CH₃C=O), 170.7 (s, CH₃C=O). MS (EI): m/z (%) = 336 (5) [M⁺], 276 (18) [M⁺ -CH₂COOH], 251 (54), 234 (45), 216 (95), 189 (88), 162 (93), 133 (95), 105 (100), 95 (65), 79 (62).

**Lettuce seedling assay**

This assay was performed as reported [7].

**Antimicrobial activity**

Testing for antimicrobial activity was performed using agar diffusion method [8].

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