New Monoterpentriols from the Fruiting Body of Flammulina velutipes

Yuichi Hirai, Michimasa Ikeda*, Tetsuya Murayama, and Toshiharu Ohata

Department of Bioproduction, Faculty of Agriculture, Yamagata University, I-23 Wakabamati, Tsuruoka, Yamagata 997-0037, Japan

Received January 14, 1998

Two monoterpentriols were isolated from the stipe segment without either the pileus or apical growth zone of the harvested fruiting body of F. velutipes. The structures of these monoterpentriols were elucidated as (1R, 2R, 4R, 8S)-(-)-p-menthane-2,8,9-triol (1) and its 8-epimer (2) based on a spectroscopic analysis and stereoselective chemical transformation from an authentic monoterpenic alcohol. These monoterpentriols showed growth-promoting activities on the excised stipe with the pileus segment, which had been excised from a fruiting body just under the growth zone before the middle stage of the rapid growth period, at concentrations below 10 ppm, while the growth of the segment was inhibited at concentrations of 100 ppm and above.

Key words: monoterpentriol; p-menthane-2,8,9-triol; stipe elongation; Flammulina velutipes; fruiting body constituent

Since Borriss first suggested in 1934 that the stipe elongation of a fruiting body is controlled by a hormonal mechanism, subsequent studies by many workers have demonstrated the existence of this active substance in the lamellae of some mushrooms. Although there are reports about several substances which induced fruiting on the mycelium from fungi, the existence of a hormone-like substance influencing the stipe elongation of a fruiting body remains unknown. We have investigated the active substance and related constituents in the fruiting body of Flammulina velutipes based on the hypotheses that a rapid elongation of the stipe would only be induced in the presence of an adequate amount of an active substance, and would stop with a large excess, or that there is no growth-promoting substance in a fruiting body but rather a growth inhibitor that accumulates in the appropriately elongated stipe cell. A preliminary analysis by silica gel TLC revealed that three substances (A-1, A-2 and A-3) were present in the stipe segment without either the pileus or apical growth zone, and that these substances could not be detected by TLC of either the pileus or apical growth zone of the stipe. In this paper, we describe the isolation, structural elucidation, and biological activity of the two major substances, A-1 and A-2, of these three.

Materials and Methods

Fungi and cultivation. F. velutipes (3–6 strain) was kindly supplied by Dr. H. E. Gruen and cultured by the usual method for ENOKITAKE production with rice bran-sawdust medium.

Chemicals. (−)-dihydrocarveol, AD-mix-α, AD-mix-β, and Ni(acac)2 were purchased from Aldrich Chemical Company. All other chemicals were from Kanto Chemical Co.

Instrumental analyses. Optical rotation was measured with a JASCO DIP-4 digital polarimeter in a 10-cm cuvette. CD spectra were recorded by a JASCO J-20A spectropolarimeter equipped with a DP-501 data processor, while IR spectra were recorded with a JASCO A-202 spectrophotometer. HPLC was performed with a Shimazu LC-6A HPLC system equipped with an SPD-6A UV detector model. NMR spectra were recorded with a JEOL GX-400 spectrometer, and mass spectra were run on a JEOL JMX-D 300 spectrometer, using 70 eV as the ionizing energy.

Preparation of the fruiting bodies of F. velutipes. The dikaryotic mycelium from a slant culture of F. velutipes (3–6 strain) was inoculated into the rice bran-sawdust medium (bran, 40 g; beech sawdust, 160 g; glucose, 4.0 g; distilled water, 200 ml) in a bottle (800 ml) with a wide spout and cultivated at 25°C for a month. After scratching the face of the medium and spreading the mycelium on the bottle bottom, it was again cultivated at 13°C under light and high humidity for 7 to 10 days to induce a number of primordia and then under the same conditions for a further 8 days to induce growth of the fruiting bodies. The final growth of the fruiting body was 14 cm in height on average.

Stipe segment growth test. A fruiting body at nearly the end of the rapid-growth period was excised at 2.4 cm under the pileus. Five such segments were individually vertically planted on a 5% glucose-3% agar medium containing A-1 or A-2 at a concentration of 2.5, 10, 50, 100 or 500 ppm in a Petri dish (4 cm in diameter) and incubated at 13°C for 4 days. Four Petri dishes for each test solution were used in one assay experiment, and the assay was run in tandem. The growth of the control segment in length was 6.0 mm on average (number of segments per treatment was 20 x 2). The results are shown in Fig. 4.

* To whom correspondence should be addressed. Michimasa Ikeda, Department of Bioproduction, Yamagata University, 1-23, Wakabamati, Tsuruoka, Yamagata 997-0037, Japan. (Fax: +81-235-28-2884, E-mail: mikeda@tdsl.tr.yamagata-u.ac.jp)
Detection of A-1, A-2 and A-3 by TLC. A TLC analysis of each EtOAc-MeOH-soluble fraction from the pileus, apical growth zone of the stipe and residual under part of the stipe was conducted, using a silica gel plate (Merk, silica gel 60 F253, Art. 1.05554) with CHCl₃:MeOH (9:1) as the developer and detecting by spraying 5% vanillin-H₂SO₄ with subsequent heating at 120°C for a few minutes. A-1, A-2 and A-3 were clearly detectable in the fraction from the residual part of the stipe as clear red spots at Rₛ 0.17, 0.19 and 0.30, respectively.

Isolation method for A-1, A-2 and A-3. The acetone extract obtained from fresh fruiting bodies (8 kg) was partitioned between water and EtOAc. The EtOAc-soluble fraction (3.3 g) was chromatographed in a silica gel column (64 × 22 cm; Kanton Chemical, less than 150 µm), eluting with CHCl₃:MeOH (96: 4,600 ml; 94: 6,300 ml; 90:10, 300 ml). The eluate from 90:10 (a brown syrup, 80.1 mg) was dissolved in a small amount of MeOH and loaded into an LH-20 column (φ1.8 × 85 cm). This column was eluted with MeOH, and the fraction containing A-1 and A-2 (retention volume of 140–160 ml) was collected and concentrated. This fraction (27.0 mg) was further chromatographed in a low-pressure silica gel column (φ2.2 × 30 cm; Kusano Chemical, 50 µm), eluting with CHCl₃:MeOH (94:6) to yield A-1 (retention volume, 230 ml; 14.2 mg) and A-2 (retention volume, 330 ml; 5.0 mg) both as a colorless oil, and A-3 (retention volume, 220 ml; 25.8 mg) as colorless needles.

A-1 (1), [α]D₂₀ = 17° (c 1.0, MeOH). IR ν max (CHCl₃) cm⁻¹: 3590, 3430 (OH), 2930, 1450, 1380, 1040. HR-EIMS m/z: 157.1225 (calcd. for C₈H₁₀O₂, 157.1227; C₈H₁₀O₂·CH₂OH, 157.1227). EIMS m/z: 157, 139, 121, 95, 81 (base peak). CD (0.2 M i-PrOH/CCl₄, 1 × 10⁻³ M Ni(acac)₂) δ₆₅₁₅ +115.2, δ₆₃₀ 0.0, δ₆₂₉₂ −48.5 (c 1 × 10⁻³ M, 0.1-cm cell and 0.1° full scale). ¹H- and ¹³C-NMR data are presented in Table 1.

A-2 (2), [α]D₂₀ = −5.0° (c 2.0, MeOH). HR-EIMS m/z: 157.1222 (calcd. for C₈H₁₀O₂, 157.1227; C₈H₁₀O₂·CH₂OH, 157.1222). EIMS m/z: 157, 139, 121, 95, 81 (base peak). IR and EIMS data agree very closely with those of A-1. CD (0.2 M i-PrOH/CCl₄, 1 × 10⁻³ M Ni(acac)₂) δ₆₅₁₅ −92.0, δ₆₃₀ 0.0, δ₆₂₉₂ +60.6 (c 1 × 10⁻³ M, 0.1-cm cell and 0.1° full scale). ¹H- and ¹³C-NMR data are presented in Table 1.

Purification of commercially available (−)-dihydrocarveol. The purchased dihydrocarveol ([α]D₂₀ = −7.8° (c 1.0, MeOH)) was chromatographed in MPLC (Kusano Chemical, pre-packed C.I.G. Si-10 column; φ2.2 × 10 cm), eluting with n-hexane:EtOAc =4:1, at a flow rate 2 ml/min. The fractions of retention volume 88–102 ml were collected and concentrated under reduced pressure. The purified (−)-dihydrocarveol showed [α]D₂₀ = −27° (c 2.0, MeOH); ([α]D₂₀ = −34.15° (neat) in the literature).

Dihydroxylation of (−)-dihydrocarveol by OsO₄/H₂O₂. To a solution of purified (−)-dihydrocarveol (500 mg, 3.25 mmol) in benzene (1 ml), OsO₄ (20 mg, 0.08 mmol) in benzene (1 ml) and 30% H₂O₂ (1 ml) were added, and the mixture was stirred at room temp. for 5 h. The reaction was quenched by adding a 10% NaHSO₃ solution (10 ml) and then stirred at room temp. for 1 h. The reaction mixture was extracted with n-BuOH. The combined organic layers were washed with brine and concentrated by way of azeotropic mixtures under reduced pressure. Purification of the residue by silica gel chromatography (Kanto Chemical, φ2.5 × 30 cm, CHCl₃:MeOH =5:1, retention volume 190–320 ml) and subsequent MPLC (Kusano Chemical, pre-packed C.I.G. Si-10 column, φ2.2 × 10 cm, CHCl₃:MeOH = 94:6) gave SA-1 (114 mg) at the retention volume of 70–82 ml and SA-2 (128 mg) at 84–96 ml. The spectral data for SA-1 ([α]D₂₀ = −16° (c 1.9, MeOH)) and SA-2 ([α]D₂₀ = −4.0° (c 1.0, MeOH)) are in reasonable agreement with those of A-1 and A-2, respectively.

Stereoselective syntheses of A-1 and A-2. (1) Benzylolation of (−)-dihydrocarveol: To a solution of 60% NaH (108 mg, 2.7 mmol) in tetrahydrofuran (1 ml), (−)-dihydrocarveol (154 mg, 1 mmol) in tetrahydrofuran (0.2 ml) was added dropwise, and the mixture was stirred for 30 min. at 0°C under nitrogen. To the mixture, benzyl bromide (462 mg, 2.7 mmol) was added, and the mixture was stirred at 0°C for 1 h and then at room temp. for 16 h. The reaction was quenched by adding MeOH (0.2 ml) and then water (2.5 ml), and finally extracted with EtOAc. The combined extracts were washed with brine, dried over sodium sulfate, and concentrated under reduced pressure. Column chromatography of the residue on silica gel (φ2.6 × 24 cm, n-hexane:EtOAc = 50:1, retention volume 30–95 ml) gave the benzyl ether (167 mg, 68% yield). EIMS m/z: 244(M⁺), 187, 153, 138, 91 (base peak).

(2) Sharpless asymmetric dihydroxylation of the benzyl ether: A solution of 0.7 g of AD-mix-α for 8(S), 9-di-hydroxylation in i-PrOH (1 ml) and water (2.5 ml) was stirred until the aqueous layer changed to light yellow at room temp. To the cooled (0°C) mixture, the benzyl ether (122 mg, 0.5 mmol) in i-PrOH (1.5 ml) was added, and the mixture was stirred vigorously at 0°C for 18 h. The reaction was quenched by adding solid sodium sulfite (150 mg), before the reaction mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over sodium sulfate, and concentrated under reduced pressure. After removing the ligand by silica gel column chromatography (φ2.6 × 20 cm, n-hexane:EtOAc =4:1), the reaction product was successively subjected to standard hydrogenolysis (10% palladium/C in EtOH, H₂, room temperature) without further purification. The debenzylated reaction mixture was chromatographed by MPLC (Kusano Chemical, 50 µm, φ2.2 × 30 cm), eluting with CHCl₃:MeOH (94:6) to separate A-1 (retention volume 230 ml, 50.4 mg (53.6%) and A-2 (retention volume 330 ml, 12.5 mg (13.3%)). By a similar procedure to that just described, the reaction with AD-mix-β and the same benzyl ether gave 43.0 mg (45.7%) of A-2 and 8.5 mg (9.0%) of A-1. The spectral data for these compounds are in reasonable agreement with those for natural A-1 and A-2.
HPLC analysis of the diastereoisomeric excess of the reaction products from the Sharpless method, and a quantitative comparison of A-1 and A-2 for various parts of the fruiting body. (1) The debenzylated reaction mixture (see the above-mentioned experiment, 2.5 mg) was reacted with benzoyl chloride (10 mg) to give 2,8-dibenzooates (EIMS m/z: 261, 179, 152, 139, 134, 122, 119, 105 (base peak), 71, 37, 35 for A-1; 261, 256, 179, 152, 139, 134, 122, 119, 105, 77 (base peak) for A-2) in pyridine (0.3 ml) at room temp. for 12 h, and then treated in the usual manner. The resulting residue was analyzed by reversed-phase HPLC in a column of TOSOH TSK gel. The chromatographic conditions are as follows: column, ODS-80Ts (64.6 × 250 mm); eluent, acetonitrile-water (7:3); flow rate, 1 ml/min.; uv monitoring, 274 nm. The results are shown in Fig. 1.

(1) Each 100 g (fresh weight) of the pileus, growth zone part and residual part of the stipe was extracted with MeOH. The resulting aqueous concentrate was extracted with EtOAc. The EtOAc extract was chromatographed in a silica gel column (φ1 × 22 cm, Kusane Chemical, less than 150 μm), eluting with CHCl₃-MeOH (94:6, 40 ml; 90:10, 40 ml). The eluate from the 9:1 eluent was reacted with benzoyl chloride (100 mg) in pyridine (1 ml) at room temp for 12 h, and then treated in the usual manner. The resulting residue was analyzed by the above-mentioned method for (1), except that the eluent was acetonitrile-water (6:4). The results are shown in Fig. 3.

Results and Discussion

Both A-1 and A-2 were visualized as clear red spots on TLC by 0.5% vanillin-sulfuric acid. Monitoring these spots by TLC, repeated column chromatography of the oily syrup obtained from an acetone extract of the harvested fruiting bodies, and subsequent preparative TLC led to the isolation of A-1 (1) and A-2 (2) as colorless oils, and of A-3 as colorless needles. The molecular formulas of 1 and 2 were both established to be C₁₆H₂₇O₃ based on HR-EIMS data and ¹H- and ¹³C-NMR spectral data (Table 1), thus requiring one degree of unsaturation. Their spectral data showed general features similar to each other, except that the specific rotation values were −17° and −5.3°, respectively. These facts suggest that 1 and 2 were diastereomers of each other. The ¹³C-NMR and C-H COSY spectra of 1 and 2 each revealed the presence of one carbon of a secondary methyl (δc: 19.3 for 1, 19.2 for 2), a tertiary methyl (δc: 21.7 for 1, 21.5 for 2), a hydroxy methyl (δc: 68.9 for 1, 69.0 for 2), a hydroxy methylene (δc: 76.2 for 1, 76.3 for 2) and a hydroxy methine (δc: 73.9 for 1, 73.8 for 2), two carbons of a methine (δc: 41.0, 44.2; for 1, 41.0, 44.1 for 2), and three carbons of a methylene (δc: 26.7, 33.9, 38.1 for 1; 27.5, 34.0, 37.2 for 2). These findings strongly suggest 1 and 2 to have been a monocyclic monoterpenene having three hydroxyls. A detailed analysis of the ¹H-¹³C H and ¹H-NMR spectra (Table 1) and 2 suggested that their stereostructures were related to 8-epimers of 1R,2R,4R- or 1S,2S,4S-p-methane-2,8,9-triol. To confirm their structures, dihydroxylation of (−)-dihydrocarveol, (1R,2R,4R-p-methane-8-ene-2,ol), by osmium tetroxide-hydrogen peroxide in benzene was carried out. The two separated and purified main products having specific rotations of −16.2° and −4.0° were confirmed as 1 and 2, respectively, providing close agreement in their TLC data, and IR, ¹H- and ¹³C-NMR spectra with those of 1 and 2. The stereochemistry of the asymmetric C8 carbon was established according to the method for the stereoselective synthesis of (S)- and (R)-2-cyclohexylopropene-1,2-diolis from 2-cyclohexylopro-pane using asymmetric dihydroxylation reagent AD-mix-α for (S) and AD-mix-β for (R) by Sharpless et al., the 2-benzyloxy derivative of (−)-dihydrocarveol was treated with the AD-mix-α reagent for 8(5),9-dihydroxylation or with the AD-mix-β reagent for 8(R),9-dihydroxylation in t-BuOH-water, each reaction product then being hydrogenated on palladium-carbon under a hydrogen atmosphere. 1 and 2 were each obtained as the main product from the reaction with AD-mix-α and AD-mix-β, respectively. The diastereoisomeric excess (de) of each was determined by an HPLC analysis of their dibenzoate derivatives to be approxi-

<table>
<thead>
<tr>
<th>Position</th>
<th>δH</th>
<th>δC</th>
<th>δH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.57 (H, dddq, J=3.3, 9.9, 6, 12.1)</td>
<td>41.0</td>
<td>1.60 (H, dddq, J=3.3, 9.9, 6.2, 12.1)</td>
</tr>
<tr>
<td>2</td>
<td>3.45 (H, d, J=4.0, 9.9, 11.0)</td>
<td>76.2</td>
<td>3.49 (H, d, J=4.0, 9.9, 11.0)</td>
</tr>
<tr>
<td>3a</td>
<td>2.58 (H, ddd, J=2.6, 2.9, 4.0, 12.1)</td>
<td>38.1</td>
<td>2.87 (H, ddd, J=2.6, 2.9, 4.0, 12.1)</td>
</tr>
<tr>
<td>3b</td>
<td>1.64 (H, ddd, J=11.0, 12.1, 12.5)</td>
<td>1.76 (H, ddd, J=11.0, 12.1, 12.5)</td>
<td>44.1</td>
</tr>
<tr>
<td>4</td>
<td>2.09 (H, ddd, J=2.9, 3.3, 12.1, 12.5)</td>
<td>44.2</td>
<td>2.14 (H, ddd, J=2.9, 3.3, 12.1, 12.5)</td>
</tr>
<tr>
<td>5a</td>
<td>2.19 (H, dddd, J=2.6, 2.9, 3.3, 3.7, 12.5, 12.8)</td>
<td>26.7</td>
<td>1.96 (H, ddd, J=2.6, 2.9, 3.3, 3.7, 12.5, 12.8)</td>
</tr>
<tr>
<td>5b</td>
<td>1.42 (H, ddd, J=3.7, 12.5, 12.5, 12.8)</td>
<td>1.35 (H, ddd, J=3.7, 12.5, 12.5, 12.8)</td>
<td>34.0</td>
</tr>
<tr>
<td>6a</td>
<td>1.08 (H, ddd, J=3.7, 12.1, 12.8, 13.2)</td>
<td>33.9</td>
<td>1.14 (H, ddd, J=3.7, 12.1, 12.8, 13.2)</td>
</tr>
<tr>
<td>6b</td>
<td>1.80 (H, ddd, J=2.9, 3.3, 3.7, 13.2)</td>
<td>1.84 (H, ddd, J=2.9, 3.3, 3.7, 13.2)</td>
<td>19.2</td>
</tr>
<tr>
<td>7</td>
<td>1.24 (H, d, J=6.2)</td>
<td>19.3</td>
<td>1.30 (H, d, J=6.2)</td>
</tr>
<tr>
<td>8</td>
<td>73.8</td>
<td>73.8</td>
<td>68.9</td>
</tr>
<tr>
<td>9</td>
<td>3.96 (H, d, J=10.6)</td>
<td>3.96 (H, d, J=10.6)</td>
<td>3.98 (H, d, J=10.6)</td>
</tr>
<tr>
<td>10</td>
<td>1.43 (H, s)</td>
<td>21.7</td>
<td>1.46 (H, s)</td>
</tr>
</tbody>
</table>

* Taken in pyridine-D₂O at 400 MHz for ¹H and at 100 MHz for ¹³C; multiplicity and J values (Hz) are shown in parentheses.
Fig. 1. HPLC Profiles for the Diastereoisomeric Excess Analysis of the Reaction Products obtained from Benzylxy (−)-Dihydrocaryovel with AD-mix-α and β.

The sample used was the dibenzoate after standard hydrogenolysis of the reaction products.

Fig. 2. Structures of A-1 (1) and A-2 (2).

Fig. 3. HPLC Profiles for Various Parts of the Fruiting Body of F. velutipes.

Fig. 4. Effect of A-1 and A-2 on the Elongation of the Stipe Segment.

Activity ( %) = (length of test stipe with sample-24 mm) / (length of test stipe without sample-24 mm) × 100

Each bar shows the mean ± S.E. of duplicate assays.

segment growth test which observes the increase in length of the stipe segment that has been excised just under the growth zone on a 5% sucrose-agar medium. In this test, both A-1 and A-2 promoted growth of the stipe segments by about 20% compared with the control at a concentration of 2.5 ppm in the medium and clearly inhibited growth at 500 ppm (Fig. 4). These results suggest that these monoterpenoids would play an important role in stipe elongation. Further studies on the promoting or inhibitory activity of A-1 and A-2 toward the growth of the intact F. velutipes fruiting body and of other fungal fruiting bodies, for example, by the agaricus test, seem to be desirable. The structural elucidation of A-3 and its biological activity will be reported elsewhere.

Acknowledgment

The authors thank Dr. Masazumi Miyakoshi for recording the NMR spectra, and Professor Katsuhide Okada of the Faculty of Education, Yamagata university for the CD measurements.

References

2) For example: (a) Urayama, T., Das Wuchshormon des Fruchtkörpers von Agaricus campestris L. (Vorläufige Mitteilung). Bot. Mag. (Tokyo), 69, 298–299 (1956); (b) Higimoto, H. and Konishi, M., Studies on the growth of fruiting of fungi I. Existence of a hormone active to the growth of fruitbody in Agaricus


4) Gruen, H. E., Control of stipe elongation by the pileus and mycelium in fruitbodies of Flammulina velutipes and other Agaricales.


