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

The mushroom *Echinodontium tsugicola* belongs to the Echinodontiaceae family and is indigenous to Japan and saprophytic to *Tsuga diversifolia*. Phytochemical investigation of the dried fruiting bodies of *E. tsugicola* revealed four lanostane triterpenoids – echinodone, deacetylechinodone, 3-epiechinodol, and deacetyl-3-echinodol [1]. When grown in a malt extract liquid culture, *E. tsugicola* produces a complex mixture of sesquiterpene metabolites. Previously, from culture filtrates of this fungal source, Arnone *et al.* reported the isolation of several novel protoilludane sesquiterpenes, *e. g.* tsugicoline A (**1**) and B (**2**) [2 – 4]. We studied the chemical constituents of the liquid culture of *E. tsugicola* and isolated two compounds. Below, we detail the production, isolation, and structure of these two compounds.

Results and Discussion

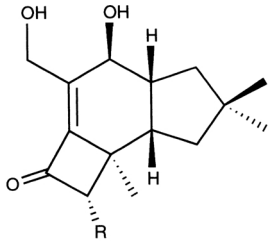
The producing strain was cultured at 25 °C on a rotary shaker for 4 weeks in a medium (6.5 liters) containing 4% malt extract, 4% glucose, and 0.1% peptone in distilled water. The culture filtrate was extracted with EtOAc. The EtOAc extract was column-chromatographed on silica gel, yielding 7 fractions. 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 $\delta = 135.6$ (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 $\delta = 31.5$ (C-15), 31.9 (C-14) and 20.7 (C-12), one hydroxymethyl at $\delta = 65.2$ (C-13), four methylenes at $\delta = 24.6$ (C-4), 34.9 (C-5), 41.9 (C-1) and 47.5 (C-10), two sp^3 me-

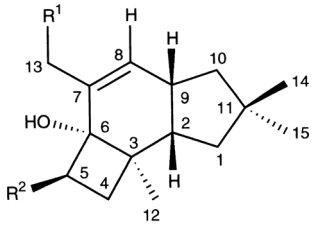
Table 1. ^{13}C (100 MHz) and ^1H (400 MHz) NMR data for **3**.

No.	δ_C (Mult.)	δ_H (Mult. *J* Hz)	HMBC (^1H to ^{13}C)
1	41.9 t	α 1.35 (1H, m)	9, 10, 14, 15
		β 1.37 (1H, m)	9, 10, 14, 15
2	44.5 d	2.00 (1H. m)	6, 8, 11
3	45.1 s		
4	24.6 t	β 1.28 (1H, td, 10.3, 2.4)	2, 6, 12
		α 1.40 (1H, m)	2, 6, 12
5	34.9 t	β 2.06 (1H, td, 10.3, 2.4)	3, 4, 6, 7
		α 2.17 (1H, q, 10.3)	3, 4, 6, 7
6	73.3 s		
7	135.6 s		
8	132.6 d	5.55 (1H, s)	2, 6, 9, 10, 13
9	38.6 d	2.69 (1H, m)	1, 7, 8, 11
10	47.5 t	α 1.43 (1H, dd, 13.2, 2.5)	1, 8, 9, 11, 14, 15
		β 1.80 (1H, dd, 13.2, 8.8)	1, 2, 8, 9, 11, 14, 15
11	38.0 s		
12	20.7 q	1.21 (3H, s)	2, 3, 4, 6
13	65.2 t	4.03 (1H, d, 11.7)	6, 7, 8
		4.40 (1H, d, 11.7)	6, 7, 8
14	31.9 q	0.97 (3H, s)	1, 10, 11, 15
15	31.5 q	1.01 (3H, s)	1, 10, 11, 14

Values in parentheses are coupling constants in Hz.



1 : R=H
2 : R=OH



3 : R^1=OH, R^2=H
3a: R^1=OAc, R^2=H
4 : R^1=OH, R^2=OH
4a: R^1=OAc, R^2=OAc

Fig. 1. The structures of tsugicolines A (**1**) and B (**2**), echinocidin A (**3**), monoacetylechinocidin A (**3a**), echinocidin B (**4**), and diacetylechinocidin B (**4a**).

thines at $\delta = 38.6$ (C-9) and 44.5 (C-2) and three quaternary carbons, one of which was linked to oxygen at $\delta = 38.0$ (C-11), 45.1 (C-3), and 73.3 (C-6). The ^{1}H NMR spectrum (Table 1) of **3** showed three singlet methyls at $\delta = 0.97$ (s, 3H, 14-H_3), 1.01 (s, 3H, 15-H_3) and 1.21 (s, 3H, 12-H_3) and two protons of hydroxymethyl at $\delta = 4.03$ (d, $J = 11.7$ Hz, 1H, 13-H_2) and 4.40 (d, $J = 11.7$ Hz, 1H, 13-H_2), and a proton of a trisubstituted double bond at $\delta = 5.55$ (s, 1H, 8-H). Detailed study of the ^{1}H-^{1}H-COSY and HMQC spectra showed the presence of -C(4)H_2-C(5)H_2- [$\delta = 1.28$ (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 -CH_2(1)-CH(2)-CH(9)[-CH_2(10)-]-CH(8)- [$\delta = 1.35$ (m, 1H, 1 α-H), 1.37 (m, 1H, 1 β-H), 2.00 (m, 1H, 2-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 sesquiterpene. In the comparison with spectral data for a sesquiterpene isolated from *Laurilia tsugicola* (*E. tsugicola*), its ^{13}C 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-H_2 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-H_2 correlated with C-2, C-6, and C-12; signals of 5-H_2 with C-3 and C-7; and signals of 12-H_3 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-H_3 to 1 β-H, 14-H_3 to 10β-H, 14-H_3 to 2-H, 15-H_3 to 1α-H, 15-H_3 to 10α-H, 15-H_3 to 8-H, and 9-H to 2-H. These results indicated a *cis*-juncture between cyclopentane and cyclohexene rings. The configuration at C-3 was elucidated to be α-CH_3 based on NOEs from 12-H_3 to 1-H_2, 12-H_3 to 4α-H and 12-H_3 to 5α-H. In NOE experiments on monoacetate **3a**, irradiation of 12-H_3 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 (2*S*, 3*R*, 9*R*)-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.

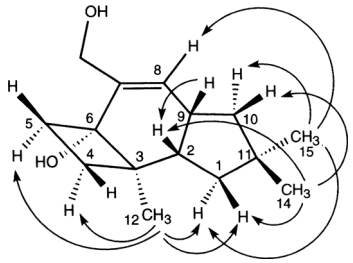


Fig. 2. Selected NOE correlations for echinocidin A (**3**).

Echinocidin B (**4**) showed spectral characteristics similar to those of **3**. The molecular formula of **4**, $C_{15}H_{24}O_3$, 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 ^{1}H and ^{13}C NMR spectra (Table 2) of **4** correspond well to those of **3**, except for the presence of an oxymethine signal [$\delta_H = 4.29$ (t, $J = 8.8$, 1H, 5-H), $\delta_C = 75.9$ (C-5)] in place of one methylene 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**

Table 2. ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR data for **4**.

No.	δ_C (Mult.)	δ_H (Mult. *J* Hz)	HMBC (^{1}H to ^{13}C)
1	41.6 t	β 1.26 (1H, t, 12.7)	3, 9, 10, 11, 14, 15
		α 1.35 (1H, m)	3, 9, 10, 11, 14, 15
2	44.4 d	2.08 (1H, m)	1, 6, 8, 9, 11
3	37.1 s		
4	35.6 t	β 1.34 (1H, m)	2, 5, 12
		α 1.84 (1H, dd, 10.7, 8.8)	5, 12
5	75.9 d	4.29 (1H, t, 8.8)	4, 6, 7
6	77.8 s		
7	133.1 s		
8	134.8 d	5.68 (1H, s)	2, 6, 10, 13
9	39.1 d	2.70 (1H, m)	1, 7, 10, 11
10	47.5 t	α 1.39 (1H, td, 13.7, 2.0)	1, 8, 11, 14, 15
		β 1.80 (1H, dd, 13.7, 8.3)	1, 8, 11, 14, 15
11	38.0 s		
12	21.9 q	1.15 (3H, s)	2, 3, 4, 6
13	64.9 t	4.20 (1H, d, 12.2)	6, 8
		4.41 (1H, d, 12.2)	6, 8
14	31.7 q	0.97 (3H, s)*	1, 10, 11, 15
15	31.9 q	0.96 (3H, s)*	1, 10, 11, 14

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

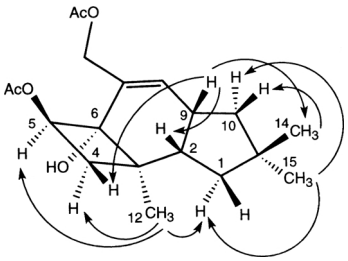


Fig. 3. Selected NOE correlations for diacetylechinocidin B (**4a**).

gave diacetate **4a**. In NOE experiments (Fig. 3), diacetate **4a** showed NOEs from 9-H to 2-H, 9-H to 14-H_3, 14-H_3 to 10β-H, 15-H_3 to 1α-H, and 15-H_3 to 10α-H, indicating that the cyclopentane-cyclohexene ring junction was *cis*. It also required a *cis* juncture between cyclobutane and cyclohexene rings with α-12-H_3 and an $\alpha-$hydroxyl group at C-6 from NOEs observed from 9-H to 4β-H, 12-H_3 to 1α-H and 12-H_3 to 4α-H. The methine proton at C-5 was α-oriented, which was supported by an NOE from 12-H_3 to 5-H. These data let us to formulate the structure of echinocidin B (**4**) to be that shown in Fig. 1.

Other protoilludane-derived sesquiterpenoids, *i. e.*, Δ^7-protoilluden-6-ol from *Fomitopsis insularis*, [5] and neoilludol from *Clitocybe illudens* [6] have also been isolated but their activity not reported. Tsugicoline A (**1**) had allelopathic activity against *Lepidium sativum* [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 13276, 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 ^{1}H and ^{13}C 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 H_2SO_4 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 flasks 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 $CHCl_3$-MeOH (98:2 and 95:5, v/v) to give seven fractions (fr. 1.1 – 1.7). Fr. 1.2 was chromatographed on silica gel column by eluting with hexane-EtOAc (2:1), followed by preparative TLC with $CHCl_3$-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. – $[\alpha]_D^{20}$ – 35 (*c* 1.5, $CHCl_3$). – IR (KBr): ν = 3305 (OH), 2950, 2861, 1234,

1126, and 1078 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C $\{^1H\}$

NMR (100 MHz, $CDCl_3$) data see Table 1. – HRMS (negative mode, FAB): $m/z = 235.1702$ [M-H$^-$] (calcd. for $C_{15}H_{23}O_2$: 235.1692). – MS (negative mode, FAB) $m/z =$ 235 [M-H$^-$]. – MS (EI): *m/z* (%) = 236 (2) [M$^+$], 208 (38) [M$^+$ -CH_2CH_2], 190 (35) [M$^+$ -CH_2CH_2 -H_2O], 151 (13), 134 (48), 122 (100), 91 (30).

*Echinocidin B (**4**)*

White powder. M. p. 85 – 87 °C. – $[\alpha]_D^{20} + 5.5$ (*c* 0.69, $CHCl_3$). – IR (KBr): $\nu = 3322$ (OH), 2965, 2867, 1205, 1118 and 1074 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Table 2. – HRMS (negative mode, FAB): $m/z = 251.1647$ [M-H$^-$] (calcd. for $C_{15}H_{23}O_3$: 251.1641). – MS (negative mode, FAB): $m/z =$ 251 [M-H$^-$]. MS (EI): *m/z* (%) = 252 (2) [M$^+$], 234 (4) [M$^+$ -H_2O], 208 (100) [M$^+$ -CH_2CHOH], 190 (85) [M$^+$ -H_2O -CH_2CHOH], 175 (57), 134 (82), 121 (35), 91 (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 washed with satd. NaCl and dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was purified by silica gel column chromatography to yield the monoacetate (**3a**, 5.5 mg) as an amorphous powder.

Monoacetate **3a**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.97$ (s, 6H, 14-H_3 and 15-H_3), 1.21 (s, 3H, 12-H_3), 1.27 (td, $J =$ 10.3, 1.9 Hz, 1H, 4 β-H), 1.39 (m, 2H, 1-H_2), 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, 1H, 6-OH), 1.99 (m, 1H, 2-H), 2.04 (m, 1H, 5 β-H), 2.07 (s, 3H, C*H*$_3$ CO), 2.13 (q, $J = 10.3$ Hz, 1H, 5 α-H), 2.71 (br. t, $J = 8.8$ Hz, 1H, 9-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). ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$): $\delta = 21.0$ (q, *C* H_3CO), 21.2 (q, C-12), 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 (d, C-9), 41.9 (t, C-1), 44.5 (d, 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, CH_3*C* O). – MS (EI): *m/z* (%) = 278 (12) [M$^+$], 261 (100) [M$^+$ -OH], 252 (25), 245 (22), 208 (38), 201 (90), 183 (75), 157 (75), 115 (70), 103 (71), 80 (74), 78 (73).

*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, $CDCl_3$): $\delta = 0.94$ (s, 3H, 14-H_3), 0.98 (s, 3H, 15-H_3), 1.21 (s, 3H, 12-H_3), 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, C*H*$_3$ CO), 2.07 (s, 3H, C*H*$_3$ CO), 2.13 (1H, m, 2-H), 2.69 (br. t, $J = 8.3$ Hz, 1H, 9-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). ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$): $\delta = 20.6$ (q, *C* H_3CO), 21.2 (q, *C* H_3CO), 21.9 (q, C-12), 32.2 (q, C-14), 32.4 (q, C-15), 33.2 (t, C-4), 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, CH_3*C* O), 170.7 (s, CH_3*C* O). MS (EI): *m/z* (%) = 336 (5) [M$^+$], 276 (18) [M$^+$ -CH_3COOH], 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].

Acknowledgements

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