Protoilludane Sequiterpenoids, Echinocidins C and D Produced by a Decay Causing Fungal Strain *Echinodontium tsugicola*

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New protoilludane sequiterpenoids, echinocidins C (**3**) and D (**4**) have been isolated from liquid culture of *Echinodontium tsugicola*. Their structures have been established on the basis of spectral analysis. The biological activities of **3** and **4** were examined by bioassay with lettuce seedlings.

Key words: Echinodontium tsugicola, Echinocidins C and D

Introduction

Echinodontium tsugicola (Japanese name; mannenharitake) belonging to the Echinodontiaceae family is a wood-decay fungus saprotrophtic to *Tsuga diversifolia* MAST (Pinaceae), where it produces fruiting bodies called conks on the underside of branches and branch stubs. The conks are hard, woody, shelf-like or hoof-shaped, and toothed on the underside. Previously, some lanostane triperpenoids, echinodone, diacetylechinodone, 3-epiechinodol, and deacetyl-3-echinodol were isolated from the dried conks of *E. tsugicola* [1], while Arnone *et al*. have reported the isolation of several novel protoilludane sesquiterpenes named tsugicolines A-I from the culture filtrates of this fungus [2 – 4]. Tsugicoline A exhibited the growth of the water cress *Lepidium sativum*. We also reported that chemical investigation of culture broth of *E. tsugicola* in agitation condition, yielded two novel protoilludane-type derivative, echinocidins A (**1**) and B (**2**) possessing primary root growth activities toward lettuce seedlings [5]. In continuing our investigation for the metabolites of this fungal strain, we now isolated two additional new echinocidins C (**3**) and D (**4**). This paper describes fermentation, isolation, structure elucidation, and biological activities of these substances.

Result and Discussion

The producing microorganism was cultured at 25 °C for 4 weeks. The cultured filtrate was extracted with EtOAc. Concentration of the extract yielded 11.4 g of crude extract which was fractionated by silica column chromatography, followed by further repeated column chromatography on silica gel and ODS, resulting in the isolation of two new echinocidin derivatives, echinocidin C (**3**) and D (**4**).

C	**3**[a]	**3a**[b]	**4**[a]
1	43.4 t	44.0 t	41.8 t
2	46.1 d	46.7 d	45.0 d
3	44.2 s	45.4 s	44.0 s
4	71.0 d	70.7 d	35.9 t
5	35.2 t	34.6 t	73.2 d
6	33.3 d	33.0 d	72.7 s
7	49.4 d	44.1 d	134.7 s
8	75.5 d	71.7 d	134.0 d
9	46.6 d	44.1 d	38.7 d
10	42.4 t	42.5 t	47.7 t
11	36.4 s	36.3 s	37.9 s
12	18.2 q	19.3 q	22.3 q
13	66.5 t	64.6 t	65.3 t
14	31.5 q	31.8 q	31.9 q
15	32.2 q	32.6 q	32.0 q
16		98.9 s	
17		30.5 q	
18		19.4 q	

Table 1. ^{13}C NMR spectral data of **3**, **3a** and **4** (100 MHz).

[a] Measured in $CDCl_3$; [b] measured in C_5D_5N.

The HRFABMS data indicated that molecular formula of **3** was $C_{15}H_{26}O_3$ with three rings or unsaturations. The IR spectrum of **3** suggested the presence of hydroxyl group from the absorption bands at 3376 cm^{-1}. The ^{13}C NMR (Table 1) and DEPT spectra of **3** indicated 15 carbons comprised of the following; three methyls, four sp^3 methylenes, six sp^3 methines, and two quaternary carbons. The ^{1}H NMR spectrum

Table 2. ^{1}H NMR spectral data of **3**, **3a** and **4** (400 MHz).

H	**3**[b]	**3a**[c]	**4**[b]
1 α	1.41 (1H, dd, 13.6, 6.8)	α 1.38 (1H, m)	1.35 – 1.42[d]
β	1.26 (1H, d, 13.6)	β 1.31 (1H, m)	
2	2.26 (1H, m)	2.33 (1H, m)	1.99 (1H, m)
4	4.22 (1H, t, 7.8)	4.54 (1H, t, 8.3)	α 1.35 – 1.42[d]
			β 1.83 (1H, dd, 13.7, 6.4)
5	2.03 (2H, m)	α 2.22 (1H, dd, 10.7, 8.3)	3.94 (1H,d, 6.4)
		β 1.95 (1H, m)	
6	1.30 (1H, m)	1.28 (1H, m)	
7	1.49 (1H, m)	1.66 (1H, qd, 10.7, 4.9)	
8	3.31 (1H, t, 10.7)	3.40 (1H, t, 10.7)	5.60 (1H, s)
9	2.00 (1H, m)	2.13 (1H, m)	2.60 (1H, m)
10 α	1.59 (1H, dd, 14.2, 7.3)	α 1.91 (1H, m)	α 1.49 (1H, dd, 13.2, 2.0)
β	1.69 (1H, d, 14.2)	β 1.50 (1H, m)	β 1.81 (1H, dd, 13.2, 7.8)
12	1.02 (3H, s)	1.26 (3H, s)	1.25 (3H, s)
13	3.53 (1H, dd, 10.7, 8.8)	α 3.51 (1H, t, 11.2)	4.06 (1H, d, 12.2)
	3.66 (1H, d, 10.7)	β 3.83 (1H, dd, 11.2, 4.9)	4.32 (1H, d, 12.2)
14	1.00 (3H, s)	0.89 (3H, s)	0.98 (3H, s)
15	1.11 (3H, s)	1.09 (3H, s)	1.03 (3H, s)
17		1.53 (3H, s)	
18		1.48 (3H, s)	

[a] Values in parentheses are coupling constants in Hz; [b] measured in $CDCl_3$; [c] measured in C_5D_5N; [d] multiplicity patterns were unclear due to signals overlapping.

Table 3. HMBC correlations for **3** and **4** (400 MHz, in $CDCl_3$).

H		**3**		**4**
1	α	C-9, 10, 11, 14, 15		C-2, 9, 10, 11, 14, 15
	β	C-2, 3, 11, 14, 15		C-2, 9, 10, 11, 14, 15
2		C-1, 3, 4, 6, 9, 10		C-1, 6, 8, 9
4		C-2, 5, 12	β	C-3, 5, 6, 12
			α	C-2, 3, 5, 12
5		C-3, 4, 6		C-3, 7
6		C-2, 3, 4, 8		
7		C-8, 9, 13		
8		C-10, 13		C-2, 6, 7, 9, 10, 13
9		C-1, 3, 7		C-1, 2, 7, 8, 10, 11
10	α	C-1, 9, 11, 14, 15		C-1, 2, 8, 9, 11, 14, 15
	β	C-2, 9, 11, 14, 15		C-1, 8, 9, 11, 14, 15
12		C-2, 3, 4, 6		C-2, 3, 4, 6
13		C-8		C-7, 8
14		C-1, 10, 11, 15		C-1, 10, 11, 15
15		C-1, 10, 11, 14		C-1, 10, 11, 14

(Table 2) of **3** showed signals due to three singlet methyls [$\delta = 1.00$ (s, 3H), 1.02 (s, 3H), 1.11 (s, 3H)], four methylene groups, one of which was oxygenated [$\delta = 1.26$ (d, 1H, $J = 13.6$ Hz), 1.41 (dd, 1H, $J = 13.6$, 6.8 Hz), 1.59 (dd, 1H, $J = 14.2$, 7.3 Hz), 1.69 (d, 1H, $J = 14.2$ Hz), 2.03 (m, 2H), 3.53 (dd, 1H, $J = 10.7$, 8.8 Hz), 3.66 (d, 1H, $J = 10.7$ Hz)], six sp^3 methines, two of which were linked to oxygen [$\delta = 1.30$ (m, 1H), 1.49 (m, 1H), 2.00 (m, 1H), 2.26 (m, 1H), 3.31 (t, 1H, $J = 10.7$ Hz), 4.22 (t, 1H, $J = 7.8$ Hz)]. Based on these data, compound **3** was assumed to be tricyclic sesquiterpenoid. The ^{13}C and ^{1}H NMR spectra of **3** were similar to those of echinocidins A (**1**) and B (**2**). In the ^{1}H-^{1}H COSY spectrum, correlations between 1-H$_2$ and 2-H, 4-H and 5-H$_2$, 5-H$_2$ and 6-H, 6-H and 7-H, 7-H and 8-H, 7-H and 13-H$_2$, 8-H and 9-H, and 9-H and 10-H$_2$ were observed. The HMBC (Table 3) correlations from 12-H$_3$ to C-3, C-4 and C-6, 5-H$_2$ to C-3, and 6-H to C-4 supported the structure of the 1-hydroxy-2-methylcyclobutane ring moiety. The HMBC correlations from 14-H$_3$ to C-1, C-10, C-11 and C-15, 15-H$_3$ to C-1, C-10, C-11 and C-14, 9-H to C-1 and 2-H to C-10, were indicative of the *geminal*-dimethylcyclopentane ring moiety. Furthermore, the ^{1}H-^{1}H COSY spectrum combined with the HMBC correlations from 2-H to C-6, 12-H$_3$ to C-6, 8-H to C-10 and 9-H to C-7 indicated for **3** a tricyclic structure consisting of 4,6,5-membered rings as shown in Fig. 1. The stereostructure of **3** was determined as follow. Compound **3** was treated with 2,2-dimethoxypropane and a catalytic amount of *p*-TsOH in DMSO to afford

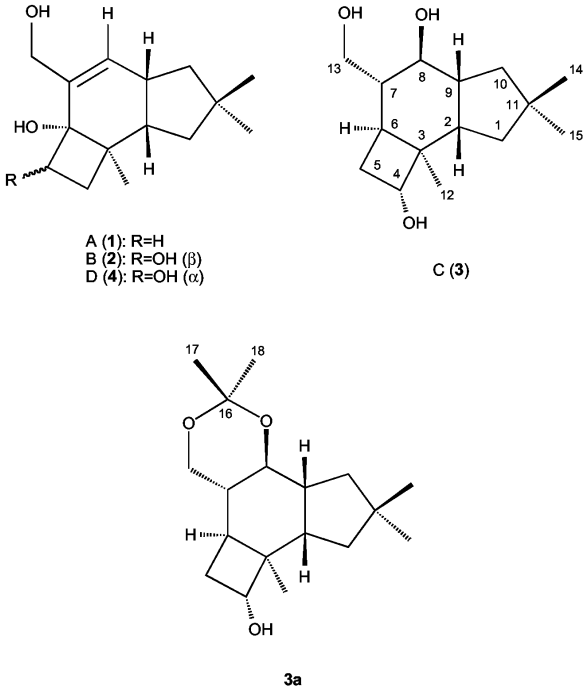

Fig. 1. The structures of echinocidins A (**1**), B (**2**), C (**3**), D (**4**) and acetonide derivative **3a**.

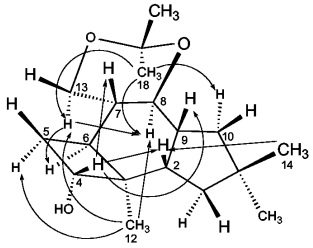

Fig. 2. Selected NOE correlations for acetonide derivative **3a**.

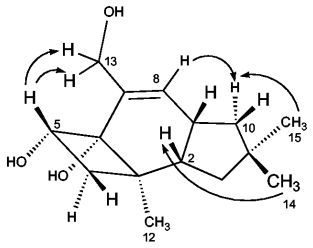

Fig. 3. Selected NOE correlations for echinocidin D (**4**).

acetonide **3a**. The *cis*-junction of the cycylohexane and cyclopentane rings was inferred from NOEs between 14-H_3 and 2-H, 4-H and 2-H, 4-H and 7-H, and 4-H and 9-H (Fig. 2). These NOEs data also required a *cis*-juncture between the cycylohexane and cyclobutane rings having an α-methyl group at C-3 and an α-hydrogen at C-6. The value of $J_{7-H/8-H}$ (10.7 Hz) in **3** implied that 7-H is arranged axially and *trans* to 8-H (Table 2). Furthermore, NOE correlations from 13-H α to 6-H, 12-H_3 to 13-H α, 18-H_3 to 13-H α, 18-H_3 to 8-H, indicated that these protons were all α-oriented. These findings allowed the assignment of the relative stereostructure of **3**. To determine the absolute configuration at C-4 of **3**, **3a** was converted into its (*S*)- and (*R*)-MTPA esters. However, the ^{1}H chemical-shift differences due to the MTPA esters did not allow us to suggested any absolute configuration at C-4.

The molecular formula of **4** was established to be $C_{15}H_{24}O_3$ by HRFABMS, indicating that **4** had the same molecular formula as **2**. The IR, ^{1}H and ^{13}C NMR spectra (Table 1 and 2) of **4** resembled those of **2**. It was possible to hypothesize that **4** was a diastereomer of **2**. A detailed comparison of the chemical shifts and coupling constants in the ^{1}H NMR spectra of **2** and **4** revealed only differences in the signal of the methine proton at C-5, while the other signals remained unchanged. This difference suggested that **4** was the epimer at C-5 of **2**. This was confirmed by NOEs experiments (Fig. 3), in which correlations between 5-H and 13-H_2 were observed, however, a NOE between 5-H and 12-H_3 was not visible, indicating that the structure of **4** was the C-5 epimer of echinocidin B (**2**). The unambiguous assignments of the signals in the ^{1}H and ^{13}C NMR spectra were based on HMBC experiments (Table 3). Therefore, the structure of echinocidin D (**4**) was concluded to be that shown in Fig. 1.

The absolute configuration for echinocidin C (**3**) and D (**4**) has not been established independently, but is assumed to be the same as in tsugicoline A (3-*epi*-illudole-5-one) isolated from still liquid culture of *E. tsugicola* [2].

These protoilludane-derived sesquiterpenoids have been isolated from natural sources and characterized [6–8]. The 4,6,5-tricyclic framework of protoilludane sesquiterpene should be derived biosynthetically from humulene by intramolecular cyclization *via* a protoilludyl cation. The formation of echinocidins A (**1**)-D (**4**) is thought to arise *via* oxidation from the protoilludyl cation or two possible precursors, Δ^6-protoilludene and Δ^7-protoilluden-6-ol [9].

We examined the biological activities of **3** and **4** against the lettuce seedlings. Compounds **3** and **4** accelerated the radical growth activity to 190% and 179% of control at concentration of 100 ppm, respectively.

Experimental Section

General experimental procedures

Melting points (mp) 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 producing strain *E. tsugicola* was grown on slant of potato dextrose agar. A loopful of the culture was transferred into a 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. The inoculated flask was incubated at 25 °C for 4 weeks on a rotary shaker.

Extraction and isolation of echinocidins C and D

6.5 Liters of culture broth was separated from the mycelia by filtration. The filtrate was extracted with EtOAc. The or-

ganic layer was concentrated *in vacuo* to give a oily residue (11.4 g). The residue was subjected to silica gel column chromatography with mixtures of *n*-hexane–EtOAc, and mixtures of EtOAc-MeOH to give fractions 1 through 13 (Fr. 1 – 13). Fr. 8 (70% MeOH eluate, 710 mg) was further chromatographed on silica gel by eluting with $CHCl_3$ and increasing volume of EtOAc to afford 40 – 50% EtOAc eluates (99 mg). These fractions combined and rechromatographed on ODS with H_2O-MeOH (10% stepwise gradient) to yield crude echinocidin D, which was finally purified by silica gel column chromatography with *n*-hexane–EtOAc (1:1, v/v) to obtain 6.9 mg of echinocidin D (**4**). Fr. 12 (50% MeOH eluate, 1.0 g) was further chromatographed on silica gel with mixtures of $CHCl_3$–EtOAc. The 20 – 30% EtOAc eluates (107 mg) was further purified by ODS column chromatography with mixtures of H_2O-MeOH (10% stepwise gradient), followed by silica gel column chromatography with mixtures of $CHCl_3$–EtOAc to obtain 2.9 mg of echinocidin C (**3**).

*Echinocidin C (**3**)*

White powder; m.p. 151 – 153 °C. – $[\alpha]_D^{20} - 29^\circ$ (*c* 0.09, $CHCl_3$). – IR (KBr): $\nu = 3376$ (OH), 2950, 2865, 1115 and 1070 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Tables 1 and 2. – HRMS (positive mode, FAB): $m/z = 255.1961$ [M+H^+] (calcd. for $C_{15}H_{27}O_3$: 255.1960). – MS (positive mode, FAB) $m/z =$ 255 [M+H^+].

*Echinocidin D (**4**)*

White powder; m. p. 135 – 137 °C. – $[\alpha]_D^{20} + 8.2^\circ$ (*c* 0.15, $CHCl_3$). – IR (KBr): $\nu = 3372$ (OH), 2950, 2865, 1135 and 1058 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Tables 1 and 2. – HRMS (positive mode, FAB): $m/z = 253.1805$ [M+H^+] (calcd. for $C_{15}H_{25}O_3$: 253.1804). – MS (positive mode, FAB): $m/z =$ 253 [M+H^+].

*Acetonide of echinocidin C (**3**)*

To echinocidin C (**3**, 0.7 mg) in 2,2-dimethoxylpropane (0.3 ml) and DMSO (0.3 ml), was added catalytic amounts of *p*-toluensulfonic acid, with the mixture left to stir at room temperature for 4 h. After evaporation residue was subjected to a silica gel column chromatography ($CHCl_3$:MeOH=20:1, v/v) to yield 8,13-*O*-isopropylidene-echinocidin C (**3a**, 0.3 mg) as an amorphous powder.

8,13-*O*-Isopropylidene-echinocidin C (**3a**): – ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Tables 1 and 2. – MS (EI) *m/z* (%) = 296 (10) [M^+], 279 (100) [H^+-OH], 251 (34), 235 (65), 219 (70).

Lettuce seedling assay

This assay was performed as reported [10].

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[1] A. Kanematsu, S. Natori, Chem. Pharm. Bull. **20**, 1993 (1972).
[2] A. Arnone, U. Brambilla, G. Nasini, O. V. Pava, Tetrahedron **51**, 13357 (1995).
[3] A. Arnone, C. D. Gregorio, U. Brambilla, G. Nasini, O. V. Pava, Tetrahedron. **54**, 10199 (1998).
[4] A. Arnone, C. D. Gregorio, S. V. Meille, G. Nasini, G. Sidoti, J. Nat. Prod. **62**, 51 (1999).
[5] Y. Shiono, T. Seto, M. Kamata, C. Takita, S. Suzuki, T. Murayama, M. Ikeda, Z. Naturforsch. **59b**, 925 (2004).
[6] D. M. X. Donnelly, T. Konishi, O. Dunne, P. Cremin, Phytochemistry. **44**, 1473 (1997).
[7] F. Rasser, T. Anke, O. Sterner, Phytochemistry. **55**, 511 (2000).
[8] M. Hirota, Y. Shimizu, T. Kamo, H. Makabe, H. Shibata, Biosci. Biotechnol. Biochem. **67**, 1597 (2003).
[9] W. A. Ayer, L. M. Browne, Tetrahedron **37**, 2199 (1981).
[10] Y. Goto, Y. Kojima, T. Nakayama, M. Terasawa, Phytochemistry. **57**, 109 (2001).