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Illudalane sesquiterpenoids, echinolactones A and B, from a mycelial culture of *Echinodontium japonicum*

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Abstract

Illudalane sesquiterpenoids, echinolactones A and B, were isolated from the culture broth of the fungus *Echinodontium japonicum*, and their structures spectroscopically determined.

Keywords: *Echinodontium japonicum*; Echinolactones A and B; Illudalane

1. Introduction

As a part of our research program that involves studying naturally occurring bioactive metabolites, we isolated and clarified the structure of four protoilludane-derived sesquiterpenoids, echinocidins A, B, C, and D, from the whole culture broth of the Basidiomycete fungus *Echinodontium tsugicola* that was grown under agitation (Shiono et al., 2004, 2005). Echinocidins B, C, and D induced radicle elongation in lettuce seedling assay. In earlier studies conducted by other groups, lanostane triterpenoids, such as echinodone, diacetylechinodone, 3-epiechinodol, and deacetyl-3-echinodol, were isolated from the dried fruiting bodies of *E. tsugicola* (Kanematsu and Natori, 1972), while Arnone et al. (1995, 1998, 1999) reported the isolation of several protoilludane sesquiterpenes, such as tsugicolines A–I, from a liquid culture of this fungus. Tsugicoline A had allelopathic activity against *Lepidium sativum*, and tsugicolines F–I showed antimicrobial activity. As a continuation of our previous studies on the genus *Echinodontium*, we examined the chemical constituents of *Echinodontium japonicum* Imazeki (*kouyakuman-nenharitake* in Japanese). This fungus is found in southern Japan, where it produces fruiting bodies on dead Japanese blue oak trees (*Quercus glauca*) (Imazeki, 1935). To the best of our knowledge, the secondary metabolites of *E. japonicum* have not been previously studied. In this study, we isolated and clarified the structures of two novel illudalane sesquiterpenoid derivatives, echinolactones A (**1**) and B (**2**), along with a known protoilludane-type compound, 3-*epi*-illudol (**3**), from a liquid culture of *E. japonicum*.

2. Results and discussion

The strain *E. japonicum* was grown in a malt extract liquid culture. After 1 week of incubation, the culture filtrate was extracted using EtOAc. The organic phase of this extract was concentrated in a vacuum to yield a residue (1.0 g), which was subjected to silica gel CC and separated into fractions 1–13. Fraction 8 was purified further by silica gel CC to yield echinolactone A (**1**).

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Further column chromatographic separation of fractions 9 and 10 resulted in isolation of echinolactone B (**2**) and 3-*epi*-illudol (**3**).

The structure of 3-*epi*-illudol (**3**) was determined by comparing the obtained spectroscopic data with that reported in the literature (Arnone et al., 1989).

Compound **1** has the molecular formula $C_{15}H_{16}O_3$, which was established by analysis of the HRFABMS data. The UV spectrum of **1** showed a maximum absorption at 233 nm. Its IR spectrum indicated absorption bands at 1722, 1709, and 1606 cm^{-1}, which are characteristic of ester, carbonyl, and aromatic groups, respectively. In the ^{13}C NMR and DEPT spectra, two carbonyl carbon signals for a lactone [δ 165.0 (*s*)] and an α,β-unsaturated ketone group [δ 209.8 (*s*)], one aromatic methine [δ 125.1 (*d*)], five aromatic quaternary carbons [δ 125.5 (*s*), 133.1 (*s*), 134.2 (*s*), 143.7 (*s*), and 155.8 (*s*)], one sp^3 quaternary carbon [δ 45.2 (*s*)], three methylene signals [δ 25.3 (*t*), 42.6 (*t*), and 66.2 (*t*)], and three methyl groups [δ 14.8 (*q*) and 25.1 (*q*)] were observed. The eight unsaturation equivalents required by the molecular formula indicated that **1** has three rings. The ^1H NMR spectrum of **1** (Table 1) showed one aromatic methine signal at δ 8.42 (1H, *s*, H-9), two singlet methyl signals at δ 1.25 (6H, *s*, H_3-14 and 15), an arylic methyl signal at δ 2.30 (3H, *s*, H_3-13), and three methylene signals at δ 2.96 (2H, *s*, H_2-1), δ 3.08 (2H, t, $J = 5.9$ Hz, H_2-5), and δ 4.52 (2H, *t*, $J = 5.9$ Hz, H_2-6). A detailed analysis of the ^1H-^1H COSY spectrum in conjunction with an HMQC experiment established the presence of a four-proton spin system [$–CH_2(5)–CH_2(6)–$] and an isolated methylene [$–CH_2(1)–$]. For the connectivity of partial structures, we conducted HMBC experiments (Table 1). HMBC correlations from H_3-14 to C-12 and C-15 and from H_3-15 to C-12 and C-14, indicated that H_3-14 and H_3-15 were *geminal* methyls at C-12. The H_3-13 and H-9 signals correlated with C-2 and C-4; and that of H-1 correlated with C-10, C-11, C-14, and C-15. This data suggested the presence of a 2,2-dimethyl-4-methyl-indan-1-one. The chemical shift of C-6 indicated that the carbon was attached to a lactonic oxygen atom. HMBC correlations between H-9 and C-7 and between H_2-6 and C-7 were also observed. This data suggested the presence of a δ lactone ring composed of C-4, C-5, C-6, C-7, and C-8; therefore, the gross structure of echinolactone A was determined to be **1**.

Table 1
^1H and ^{13}C NMR spectroscopic data of echinolactone A (**1**)

No.	δ_H	δ_C	HMBC (H to C)
1	42.6 *t*	2.96 (2H, *s*)	2, 10, 11, 12, 14, 15
2	155.8 *s*		
3	133.1 *s*		
4	143.7 *s*		
5	25.3 *t*	3.08 (2H, *t*, 5.9)	3, 4, 6, 8
6	66.2 *t*	4.52 (2H, *t*, 5.9)	4, 5, 7
7	165.0 *s*		
8	125.5 *s*		
9	125.1 *d*	8.42 (1H, s)	2, 4, 7
10	134.2 *s*		
11	209.8 *s*		
12	45.2 *s*		
13	14.8 *q*	2.30 (3H, *s*)	2, 3, 4
14	25.1 *q*	1.25 (3H, *s*)	1, 11, 12, 15
15	25.1 *q*	1.25 (3H, *s*)	1, 11, 12, 14

Measured in $CDCl_3$, values in parentheses are coupling constants in Hz.

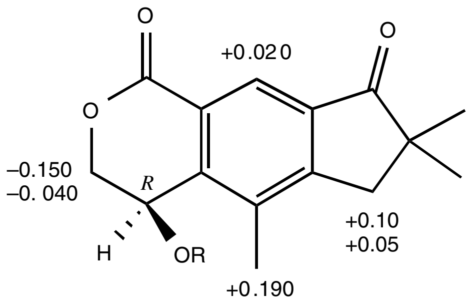
The molecular formula of **2** was determined to be $C_{15}H_{16}O_4$ by HRFABMS, suggesting the presence of an additional oxygen atom, in comparison with **1**. The ^1H and ^{13}C NMR spectrum (Table 2) of **2** was very similar to that of **1**; however, it lacked a methylene signal at C-5 which had been observed in **1**, and it was accompanied by the appearance of a new oxymethine signal [δ_H 5.00 (1H, *br. s*)]. HMBC experiments (Table 2) were conducted on **2** in order to determine the connectivity of the hydroxyl group. HMBC correlation from H-5 to C-3 and C-8 indicated that the hydroxyl group was placed at C-5. The ^1H and ^{13}C NMR shifts of **2** were fully assigned by 2D spectra, including HMBC, and are summarized in Table 2. The gross structure of echinolactone B was therefore determined as **2**. The absolute configuration of **2** at C-5 was confirmed by the modified Mosher's method (Ohtani et al., 1991). Treatment of **2** with α-methoxy(trifluoromethyl)phenylacetic acid (MTPA), dimethylaminopyridine (DMAP), and dicyclohexylcarbodiimide (DCC) yielded (−)-MTPA monoester **2a** and (+)-MTPA monoester **2b**. On comparing the ^1H NMR spectroscopic data for the (−)- and (+)-MTPA ester derivatives (Fig. 1), the *R* configuration was assigned

Table 2
^1H and ^{13}C NMR spectroscopic data of echinolactone B (**2**)

No.	δ_H	δ_C	HMBC (H to C)
1	42.5 *t*	2.95 (1H, *d*, 17.6)	2, 3, 11, 14, 15
		3.02 (1H, *d*, 17.6)	2, 3, 11, 14, 15
2	156.8 *s*		
3	134.5 *s*		
4	142.4 *s*		
5	61.8 *d*	5.00 (1H, *br. s*)	3, 4, 6, 8
6	72.2 *t*	4.53 (1H, *d*, 12.7)	4, 5, 7
		4.76 (1H, *d*, 12.7)	4, 5, 7
7	164.1 *s*		
8	124.4 *s*		
9	124.9 *d*	8.31 (1H, *s*)	2, 4, 7, 11
10	135.6 *s*		
11	210.0 *s*		
12	45.7 *s*		
13	13.5 *q*	2.46 (3H, *s*)	2, 3, 4
14	25.1 *q*[a]	1.23 (3H, *s*)[a]	1, 11, 12, 15
15	25.3 *q*[a]	1.26 (3H, *s*)[a]	1, 11, 12, 14

Measured in $CDCl_3$, and values in parentheses are coupling constants in Hz.

[a] Interchangeable.

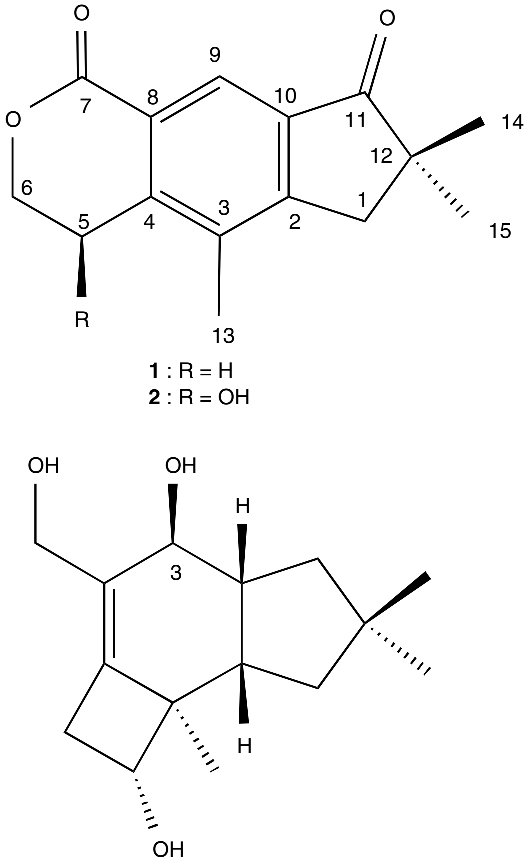


2a : R :(*S*)–(−)–MTPA
2b : R :(*R*)–(+)–MTPA

Fig. 1. Chemical shift differences for the (*S*)-(−)-MTPA Esters (**2a**) and (*R*)-(+)-MTPA Esters (**2b**) in ppm at 400 MHz.

to the C-5 chirality, thus establishing the absolute structure of echinolactone B as **2**.

Although some illudalane class of sesquiterpenoids have been isolated (Bardouille et al., 1978 and Murakami et al., 1980), compounds containing a lactone ring like **1** and **2** are scarcely known except for radulactone (Fabian et al., 1998) as fungal metabolites. Radulactone, a reductive derivative of **1** with a hydroxyl group attached to C-11, was reported to show weak antimicrobial activity.



1 : R = H
2 : R = OH

3

Chart 1.

We studied the biological activity of **1** and **2** using the antimicrobial activity assay and the lettuce bioassay. At a concentration of 200 μg/disk, compounds **1** and **2** were inactive against *Candida albicans* ATCC 2019, *Staphylococcus aureus* NBRC 13276, and *Pseudomonas aeruginosa* ATCC 15442. Further, in the lettuce bioassay, compounds **1** and **2** at a concentration of 100 ppm induced radicle elongation of 160% and 190%, respectively, in the controls.

It has been postulated that the illudalane skeleton is biosynthetically derived from the protoilludane skeleton, which is formed by the cyclization of farnesylpyrophosphate via a humulene intermediate (Ayer et al., 1981). Accordingly, the two echinolactones A (**1**) and B (**2**) would be biosynthesized from 3-*epi*-illudol (**3**) (see Chart 1).

3. Experimental

3.1. 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. CC 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 vanillin–sulphuric acid reagent followed by heating or by UV irradiation.

3.2. Fungus and cultivation

The producing strain *E. japonicum* Imazeki (NBRC 30308) was purchased from biological resource center, National Institute of Technology and Evaluation, Chiba, Japan. For fermentation, the fungal strain was grown in three 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 l of water. The inoculated flask was incubated at 25 °C for 1 weeks on a rotary shaker.

3.3. Extraction and isolation of echinolactones A and B

After the incubation period, 300 ml of culture broth was separated from the mycelia by filtration. The filtrate was extracted using EtOAc, with the organic solubles concentrated in vacuo to give an oily residue (1.0 g).

The latter was subjected to silica gel CC using a gradient of *n*-hexane–EtOAc (100:0–0:100) and then a gradient of EtOAc–MeOH (100:0–50:50) as eluting solvent systems to give fractions 1 through 13 (fr. 1–13). The purification of the eluates was monitored by the characteristic coloration with vanillin–sulfuric acid reagent or by UV irradiation. Fraction 8 (34.0 mg, *n*-hexane–EtOAc, 30:70) was further subjected to silica gel CC using a gradient of $CHCl_3$–EtOAc (100:0–0:100) to afford 40–50% EtOAc eluates. These fractions (99.0 mg) were combined and rechromatographed on ODS using a gradient of H_2O–MeOH (100:0–0:100) to yield echinolactone A (**1**, 4.3 mg). Fractions 9 and 10 (188.0 mg, *n*-hexane–EtOAc, 20:80 and 10:90) were combined and purified further by silica gel CC using a gradient of $CHCl_3$–EtOAc (100:0–0:100), and then a gradient of EtOAc–MeOH (100:0–50:50), as eluting solvent systems to afford fractions 1 through 13 (fr. 2–1–2–13). 3-*epi*-illudol (**3**, 61.0 mg) was isolated from fr. 2–11 ($CHCl_3$–EtOAc, 0:100). Fr. 2–13 (157.0 mg, EtOAc–MeOH, 50:50) was further purified by ODS CC with mixtures of H_2O–MeOH to yield echinolactone B (**2**, 5.3 mg).

3.4. Echinolactone A (*1*)

White powder; m.p. 223–226 °C; UV λ_{max} (MeOH) nm ($\log\varepsilon$): 233 (4.6); IR (KBr) ν_{max} cm^{-1}; 1722, 1709, 1606, 1211, 1171, 769. HRFABMS *m/z* $[M+H^+]$: 245.1178, calcd. for $C_{15}H_{17}O_3$, 245.1180. FABMS *m/z* 245 $[M+H^+]$. For ^1H and ^{13}C NMR spectra, see Table 1.

3.5. Echinolactone B (*2*)

Oil. $[\alpha]_D^{25}$ −24° (*c* 0.3, $CHCl_3$); UV λ_{max} (MeOH) nm ($\log\varepsilon$): 231 (4.4); IR (KBr) ν_{max} cm^{-1}; 3419, 1717, 1699, 1606, 1214, 1171, 754. HRFABMS *m/z* 261.1127 $[M+H^+]$, calcd. for $C_{15}H_{17}O_4$, 261.1127. FABMS *m/z* 261 $[M+H^+]$. For ^1H and ^{13}C NMR spectra, see Table 2.

3.6. Preparation of (S)- and (R)-MTPA esters (2a and 2b) from echinolactones B (2)

To **2** (1.3 mg) in CH_2Cl_2 (1.0 ml) were added, (*S*)-(−)-α-methoxy(trifluoromethyl)phenylacetic acid (MTPA, 1.5 mg), dicyclohexylcarbodiimide (2.0 mg) and 4-(dimethylamino)pyridine (5.0 mg), and the mixture was stirred at room temperature for 6 h. EtOAc was added to the reaction mixture, before the resulting solution was washed with a saturated solution of aqueous $NaHCO_3$ and brine, and concentrated in vacuo. Purification by CC on silica gel (*n*-hexane–EtOAc) gave the (*S*)-(−)-MTPA ester (**2a**, 1.0 mg) of **2**. Compound **2** (1.3 mg) was treated with (*R*)-(+)-MTPA (1.5 mg) in the same manner to afford the (*R*)-(+)-MTPA ester (**2b**, 1.0 mg).

(*S*)-(−)-MTPA ester **2a**: oil; FABMS *m/z* 477 $[M+H^+]$. ^1H NMR δ_H (400 MHz, $CDCl_3$): 1.26 (3H, *s*, CH_3-14 or 15), 1.29 (3H, *s*, CH_3-14 or 15), 2.36 (3H, *s*, CH_3-13), 2.97 (1H, *s*, $j = 17.6$ Hz, CH_2-1), 3.02 (1H, *s*, $J = 17.6$ Hz, CH_2-1), 3.39 (3H, *s*, MTPA-OCH_3), 4.60 (1H, *d*, $J = 13.7$ Hz, CH_2-6), 4.80 (1H, *d*, $j = 13.7$ Hz, CH_2-6), 6.37 (1H, *s*, CH-5), 8.46 (1H, *s*, CH-9), 7.42 (3H, *m*, MTPA-ArH), 7.50 (2H, *m*, MTPA-ArH).

(*R*)-(+)-MTPA ester **2b**: oil; FABMS *m/z* 477 $[M+H^+]$. ^1H NMR δ_H (400 Hz, $CDCl_3$): 1.25 (3H, *s*, CH_3-14 or 15), 1.31 (3H, *s*, CH_3-14 or 15), 2.17 (3H, *s*, CH_3-13), 2.92 (2H, *s*, CH_2-1), 3.49 (3H, *s*, MTPA-OCH_3), 4.64 (1H, *d*, $j = 13.7$ Hz, CH_2-6), 4.95 (1H, *d*, $j = 13.7$ Hz, CH_2-6), 6.26 (1H, *s*, CH-5), 8.44 (1H, *s*, CH-9), 7.40 (3H, *m*, MTPA-ArH), 7.52 (2H, m, MTPA-ArH).

3.7. Lettuce seedling assay

Lettuce seeds (*Lactuca sativa* L.) were used for bioassay. Fifteen seeds were sown in filter paper containing a definite concentration of test compound in Petri dish (5 cm i.d.). Distilled water (1 ml, containing 100 ppm (w/v) Tween 80) was added to Petri dish and incubation was carried out at 25 °C under continuous light for 7 days. The control experiments were conducted in the distilled water. The elongation of roots and shoots were measured compared to those of the control.

3.8. Assay for antimicrobial activity

Antimicrobial activities were determined using the agar diffusion test using paper disks (8 mm in diameter, thin, ADVANTEC) against *S. aureus*, *P. aeruginosa*, *C. albicans*, and *Aspergillus clavatus*. An antibiotic paper disk was loaded with a sample solution and then dried for 2 h in vacuo to the remove the solvent. Each test sample-loaded disk was placed on the agar plates inoculated with tester strains, which were incubated 25 °C. Antimicrobial activities were estimated measuring diameter of inhibition zone formed on the agar.

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