# Echinolactones C and D: Two Illudalane Sesquiterpenoids Isolated from the Cultured Mycelia of the Fungus *Echinodontium japonicum*

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Echinolactones C and D, two compounds with an illudalane sesquiterpenoid skeleton, were isolated from the cultured mycelia of the basidiomycetous fungus *Echinodontium japonicum* (Echinodontiaceae). The structures of echinolactones C and D were determined by 1D and 2D NMR spectroscopy. Their biological activities were determined using antimicrobial activity and lettuce seedling assays.

*Key words: Echinodontium japonicum* Imazeki, Echinolactones C and D

## Introduction

In the course of a screening program undertaken to identify new natural products produced by microorganisms, we previously reported on the isolation and characterization of four protoilludane sesquiterpenoids, namely echinocidins A, B (**1**), C, and D, from a liquid culture of the basidiomycete fungus *Echinodontium tsugicola* (Japanese name: Mannen-haritake) [1, 2]. *E. tsugicola* is a very rare Japanese indigenous decay fungus in which the sporophores are produced on the boles of living conifers (*Tsuga diversifolia* Mast.) [3, 4]. Wood decay initiated by the fungus progresses from the heartwood to the sapwood and eventually causes tree death. In earlier papers, Kanematsu *et al.* described the isolation of the lanostane triterpenoids, echinodone, diacetylechinodone, 3-epiechinodol, and deacetyl-3-echinodol from the dried fruiting bodies of *E. tsugicola* [4]. From a liquid culture of *E. tsugicola*, Arnone *et al.* reported the structural elucidation of tsugicolines A – I, L, and M [5 – 8]. Tsugicoline A showed allelopathic activity. As a part of our chemosystematic study of the *Echinodontiaceae* family, we isolated two new illudalane sesquiterpenoids, echinolactones A (**2**) and B (**3**) possessing a lactone moiety in the molecule. In addition, we isolated a known protoilludane-type sesquiterpenoid, illudol, from a liquid culture of *Echinodontium japonicum* Imazeki (Japanese name: Kouyaku–mannen–haritake). *E. japonicum* is also a rare Japanese indigenous fungus in which the fruiting bodies, called conks, are found on the boles of dead Japanese blue oak trees (*Quercus glauca*) [9]. Using a lettuce seedling assay, **2** and **3** were found to stimulate radicle elongation. In our ongoing search for the chemical constituents of *E. japonicum*, we isolated two new echinolactones C (**4**) and D (**5**) together with a known compound, neoilludol (**6**). Here, we report on their isolation and structural elucidation.

*E. japonicum* Imazeki was cultured at 25 °C for 4 weeks. The culture filtrate was extracted with EtOAc. Concentration of the extract yielded 8.8 g of crude extract that was fractionated by silica gel column chromatography, followed by repeated column chromatography on silica gel and ODS. These procedures resulted in the isolation of two new echinolactone derivatives echinolactones C (**4**) and D (**5**) and neoilludol (**6**).

The molecular formula of echinolactones C (**4**) was determined to be $C_{15}H_{16}O_4$ by HR–FABMS. Compound **4** had one more oxygen than **2**. The ultraviolet (UV) spectrum of **4** revealed the presence of aromatic rings [$\lambda_{max}$ = 234]. The infrared (IR) spectrum showed the presence of the hydroxyl (3412 $cm^{-1}$) and carbonyl (1725 and 1696 $cm^{-1}$) groups, which corresponded with the presence of ester and ketone functions. The $^1H$ and $^{13}C$ NMR data (Table 1) of **4** indicated the presence of a 4-methyl-indan-1-one moiety [$\delta_H = 2.32$ (s, 3H, 13-$H_3$), 2.85 (d, $J = 17.6$ Hz, 1-$H_2$), 3.30 (d, $J = 17.6$ Hz, 1-$H_2$), 8.31 (s, 9-H); $\delta_C = 14.3$, 51.3, 37.6, 124.6, 125.3, 133.1, 134.8, 144.2, 156.9, and 209.8] and a lactone moiety [$\delta_H = 3.07$ (t, $J = 6.4$ Hz, 2H, 5-H), 4.53 (t, $J = 6.4$ Hz, 2H, 6-H); $\delta_C = 25.7$,

Table 1. $^{1}$H and $^{13}$C NMR data of echinolactone C (**4**).

| No. | $\delta_C$ (Mult) | $\delta_H$ (Mult. *J* Hz) | HMBC ($^{1}$H to $^{13}$C) |
|---|---|---|---|
| 1 | 37.6 t | 2.85 (1H, d, 17.6) | 2, 10, 11, 12, 14, 15 |
| | | 3.30 (1H, d, 17.6) | 2, 10, 11, 12, 14, 15 |
| 2 | 156.9 s | | |
| 3 | 133.1 s | | |
| 4 | 144.2 s | | |
| 5 | 25.7 t | 3.07 (2H, t, 6.4) | 3, 4, 6, 8 |
| 6 | 66.2 t | 4.53 (2H, t, 6.4) | 4, 5, 7 |
| 7 | 164.8 s | | |
| 8 | 125.3 s | | |
| 9 | 124.6 d | 8.31 (1H, s) | 2, 4, 7, 11 |
| 10 | 134.8 s | | |
| 11 | 209.8 s | | |
| 12 | 51.3 s | | |
| 13 | 14.3 q | 2.32 (3H, s) | 2, 3, 4 |
| 14 | 67.6 t | 3.61 (1H, d, 10.7) | 1, 11, 12, 15 |
| | | 3.89 (1H, d, 10.7) | 1, 11, 12, 15 |
| 15 | 20.6 q | 1.21 (3H, s) | 1, 12, 14 |

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

Table 2. $^{1}$H and $^{13}$C NMR data of echinolactone D (**5**).

| No. | $\delta_C$ (Mult) | $\delta_H$ (Mult. *J* Hz) | HMBC ($^{1}$H to $^{13}$C) |
|---|---|---|---|
| 1 | 42.2 t | 2.64 (1H, d, 16.6) | 2, 10, 12, 14, 15 |
| | | 2.96 (2H, d, 16.6)* | 2, 10, 12, 14, 15 |
| 2 | 148.4 s | | |
| 3 | 130.9 s | | |
| 4 | 136.3 s | | |
| 5 | 25.1 t | 2.94 (2H, t, 5.9) | 3, 4, 6, 8 |
| 6 | 66.7 t | 4.48 (2H, t, 5.9) | 4, 5, 7 |
| 7 | 166.1 s | | |
| 8 | 123.7 s | | |
| 9 | 124.2 d | 7.80 (1H, s) | 2, 4, 7, 11 |
| 10 | 141.5 s | | |
| 11 | 42.6 t | 2.71 (1H, d, 16.6) | 2, 10, 12, 14, 15 |
| | | 2.96 (2H, d, 16.6)* | 2, 10, 12, 14, 15 |
| 12 | 44.4 s | | |
| 13 | 15.2 q | 2.19 (3H, s) | 2, 3, 4 |
| 14 | 70.3 t | 3.53 (2H, s) | 1, 11, 12, 15 |
| 15 | 24.1 q | 1.18 (3H, s) | 1, 11, 12, 14 |

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

66.2, and 164.8]. An isolated oxymethylene group was newly observed at $\delta_H = 3.61$ (d, $J = 10.7$ Hz, 1H), and 3.89 (d, $J = 10.7$ Hz, 1H); $\delta_C = 67.6$, in place of the methyl group observed in **2**. In the HMBC spectrum of **4**, $^{13}$C-$^{1}$H long-range correlations were observed between the signals at $\delta_H = 3.61$ and 3.89 and the carbon signals at $\delta_C = 20.6$ (C-15), 37.6 (C-1), 51.3 (C-12), and 209.8 (C-11), suggesting that an oxygenated methylene group was located at C-12. These observations indicated that **4** is a 12-$CH_2OH$ derivative of **2**. Compound **4** was optically active $[[\alpha]_D -2.5^\circ$, (*c* 0.67, MeOH)]. The configuration of the hydroxymethyl group at C-12 of **4** was considered to be the same as that of tsugicoline L, because the specific rotation value of **4** was similar to that of tsugicoline L [lit.[8]; $[\alpha]_D -1.6^\circ$, (*c* 0.1, MeOH)].

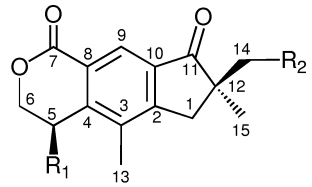
Table 3. $^{1}$H and $^{13}$C NMR data of neoilludol (**6**).

| No. | $\delta_C$ (Mult) | $\delta_H$ (Mult. *J* Hz) | HMBC ($^{1}$H to $^{13}$C) |
|---|---|---|---|
| 1 | 43.1 t | $\alpha$ 1.30 (1H, dd, 12.5, 7.2) | 2, 9, 10, 11, 14, 15 |
| | | $\beta$ 1.42 (1H, t, 12.5) | 2, 3, 9, 11, 14, 15 |
| 2 | 44.7 d | 2.16 (1H, m) | 1, 3, 6, 8 |
| 3 | 52.4 s | | |
| 4 | 65.7 d | 3.62 (1H, t, 8.6) | 2, 5, 12 |
| 5 | 45.8 t | $\alpha$ 2.06 (1H, dd, 11.0, 8.6) | 4, 6, 7 |
| | | $\beta$ 2.35 (1H, dd, 11.0, 8.6) | 3, 4, 6, 7 |
| 6 | 68.5 s | | |
| 7 | 140.0 s | | |
| 8 | 129.5 d | 5.40 (1H, br. s) | 2, 6, 10, 13 |
| 9 | 39.4 d | 2.62 (1H, m) | 1, 7, 8, 10, 11 |
| 10 | 49.0 t | $\alpha$ 1.37 (1H, dd, 13.2, 2.3) | 2, 8, 11, 14, 15 |
| | | $\beta$ 1.76 (1H, dd, 13.2, 8.3) | 8, 9, 11, 14, 15 |
| 11 | 38.5 s | | |
| 12 | 15.2 q | 1.08 (3H, s) | 2, 3, 4, 6 |
| 13 | 64.2 t | 3.99 (1H, dt, 13.1, 1.1) | 6, 7, 8 |
| | | 4.24 (1H, ddd, 13.1, 3.5, 1,1) | 6, 7, 8 |
| 14 | 32.6 q[a] | 0.93 (3H, s)[a] | 1, 10, 11, 15 |
| 15 | 32.3 q[a] | 0.94 (3H, s)[a] | 1, 10, 11, 14 |

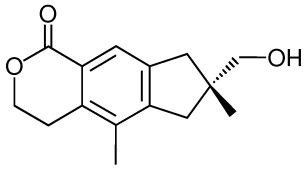
Measured in acetone-$d_6$, and values in parentheses are coupling constants in Hz. [a] Interchangeable.

The molecular formula of echinolactones D (**5**) was determined to be $C_{15}H_{18}O_3$ by HR–FABMS. The IR spectrum of **5** revealed the presence of a lactone moiety (1717 $cm^{-1}$) and the absence of the ketone carbonyl that was observed in **4**. No signal due to the carbonyl carbon at C-11 in **4** was apparent in **5**, however, new signals of a benzylic methylene group [$\delta_C = 42.6$; $\delta_H = 2.71$ (d, $J = 16.6$, 1H); 2.96 (d, $J = 16.6$, 1H)] were seen in the $^{1}$H and $^{13}$C NMR spectra (Table 2), indicating the lack of carbonyl functions. In the HMBC spectrum (Table 2) of **5**, correlations between 14-$H_3$, 15-$H_3$ and 9-H and C-11 indicated that **5** was an 11-deoxo-derivative of **4**. On the basis of its optical rotations {**5**, ($[\alpha]_D + 4.6^\circ$, *c* 0.45, MeOH)}, the absolute configuration at C-12 of **5** was opposite to that of **4**.

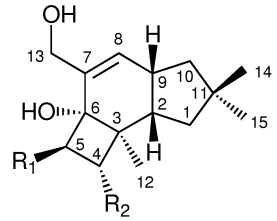
The results of the HR–EIMS and $^{13}$C and $^{1}$H NMR spectroscopy of **6** (Table 3) revealed a molecular formula of $C_{15}H_{24}O_3$, indicating that **6** had the same molecular formula as **1**. The $^{1}$H and $^{13}$C NMR spectra of **6** correspond well to those of **1**, with the exception of the presence of an oxymethine signal [$\delta_H = 3.62$ (t, $J = 8.6$ Hz, 1H); $\delta_C = 65.7$] and chemical shift differences in some proton and carbon signals. The location of a hydroxyl group at C-4 in the cyclobutane ring, in which methine (4-H) correlated with C-2, C-5, and C-12, was established by HMBC (Table 3). The planar



**2**: $R_1$=H, $R_2$=H
**3**: $R_1$=OH, $R_2$=H
**4**: $R_1$=H, $R_2$=OH



**5**



**1**: $R_1$=OH, $R_2$=H
**6**: $R_1$=H, $R_2$=OH

Fig. 1. The structures of echinocidin B (**1**), echinolactones A (**2**), B (**3**), C (**4**), D (**5**), and neoilludol (**6**).

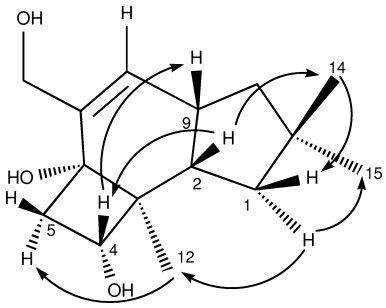


Fig. 2. Selected NOE correlations for **6**.

structure of **6** was elucidated as shown in Fig. 1. This planar structure was identical to that of the neoilludol isolated from *Clitocybe illudens* [10]. However, the relative stereochemistry of neoilludol remains to be clarified, and no biological activity has yet been reported. The *cis*-junction of the cycylohexene and cyclopentane rings was inferred from the nuclear Overhauser effects (NOEs) between 14-$H_3$ and 2-H, 4-H and 2-H, 4-H and 9-H, and 1$\alpha$-H and 12-$H_3$, and 5$\alpha$-H and 12-$H_3$ (Fig. 2). These NOEs also required a *cis*-junction between the cyclohexene and cyclobutane rings having an $\alpha$-methyl group at C-3 and an $\beta$-hydrogen at C-4. These findings allowed the assignment of a relative stereostructure to **6**.

We studied the biological activities of **4**, **5**, and **6** by using an antimicrobial activity assay and a lettuce seedling bioassay. At a concentration of 100 $\mu$g/disk, compounds **4**, **5**, and **6** were inactive against *Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442. Further, in the lettuce bioassay, compound **5** stimulated weak radicle growth at a concentration of 100 ppm. Both **1** and echinocidin D (the epimer of **1** at C-5) have previously been shown to stimulate 180% greater radicle elongation compared to controls [2], whereas **6** showed no activity. These results indicated that the hydroxyl group at C-5 in **1** and echinocidin D is important for the radicle elongation activity.

## Experimental Section

*General experimental procedures*

Melting points (mp) 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 $\delta$ (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 vanillin-sulphuric acid reagent followed by heating or by UV irradiation.

*Fungus and cultivation*

The producing strain *E. japonicum* Imazeki (NBRC 30308) was purchased from the biological resource center, National Institute of Technology and Evaluation, Chiba, Japan. For fermentation, the fungal strain was grown in twenty two 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 4 weeks on a rotary shaker.

*Extraction and isolation of echinolactones A and B and neoilludol*

After the incubation period, 2200 ml of culture broth were separated from the mycelia by filtration. The filtrate was extracted with EtOAc. The organic layer was concentrated *in vacuo* to give an oily residue (8.8 g). The residue was

subjected to silica gel column chromatography 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. 1-1 ∼ 1-13). The purification of the eluates was monitored by the characteristic coloration with vanillin-sulphuric acid reagent or by UV irradiation. Fractions 1-6 ∼ 1-8 (700 mg, *n*-hexane–EtOAc, 50 : 50-30 : 70) were combined and further chromatographed on silica gel using a gradient of $CHCl_3$ –EtOAc (100 : 0-0 : 100) to afford 20% EtOAc eluates. This fraction (198 mg) was rechromatographed on ODS using a gradient of $H_2O$-MeOH (100 : 0-0 : 100) to yield echinolactone D (**5**, 5.8 mg). Fraction 1-10 (2.1 g, *n*-hexane–EtOAc, 10 : 90) was chromatographed on silica gel 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). Fr. 2-6 (212 mg, $CHCl_3$-EtOAc, 50 : 50) was further purified by ODS column chromatography with mixtures of $H_2O$-MeOH to yield echinolactone C (**4**, 15.3 mg). Fr. 2-10 and 2-11 (2.1 g, $CHCl_3$-EtOAc, 10 : 90 and 0 : 100) was subject to ODS column chromatography with mixtures of $H_2O$-MeOH to yield neoilludol (**6**, 7.0 mg).

*Echinolactone C (**4**)*

White powder. m. p. 192 – 194 °C. – $[\alpha]_D^{20}$ −2.5° (*c* 0.67, MeOH). – UV (MeOH) $\lambda_{max}$ $(\lg \varepsilon) = 234$ nm (4.4). – IR (KBr): $\nu = 3412$ (OH), 1725, 1696, 1606, 1203 and 1164 $cm^{-1}$. – $^1H$ NMR (400 MHz, $CDCl_3$) and $^{13}C$ $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Table 1. – HRMS (positive mode, FAB): $m/z = 261.1127$ [M+H$^+$] (calcd. for $C_{15}H_{17}O_4$: 261.1127). – MS (positive mode, FAB): $m/z =$ 261 [M+H$^+$].

*Echinolactone D (**5**)*

Oil. – $[\alpha]_D^{20} + 4.6°$ (*c* 0.45, MeOH). – UV (MeOH) $\lambda_{max}$ $(\lg \varepsilon) = 254$ nm (4.2). – IR (KBr): $\nu = 3412$ (OH), 1717, 1609, 1202 and 1171 $cm^{-1}$. – $^1H$ NMR (400 MHz, $CDCl_3$) and $^{13}C$ $\{^1H\}$ NMR (100 MHz, $CDCl_3$) data see Table 2. – HRMS (positive mode, FAB): $m/z = 247.1337$ [M+H$^+$] (calcd. for $C_{15}H_{19}O_3$: 247.1334). – MS (positive mode, FAB): $m/z = 247$ [M+H$^+$].

*Neoilludol (**6**)*

White powder. m. p. 149 – 152 °C. – $[\alpha]_D^{20} - 45°$ (*c* 0.27, MeOH). – IR (KBr): $\nu = 3326$ (OH), 2948, 2861, 1455, 1220, 1033 $cm^{-1}$. – $^1H$ NMR (400 MHz, acetone-$d_6$) and $^{13}C$ $\{^1H\}$ NMR (100 MHz, acetone-$d_6$) data see Table 3. – HRMS (positive mode, FAB): $m/z = 253.1805$ [M+H$^+$] (calcd for $C_{15}H_{25}O_3$: 253.1804). – MS (positive mode, FAB): $m/z = 291$ [M+K$^+$].

*Lettuce seedling assay*

This assay was performed as reported [9].

*Assay for antimicrobial activity assay*

This assay was performed as reported [9].

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