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A diterpene quinone from the bark of *Cryptomeria japonica*

Hisayoshi Kofujita[a,*], Michikazu Ota[a], Kouetsu Takahashi[b], Yasushi Kawai[c], Yoshiki Hayashi[d]

[a]*Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka 020-8550, Japan*
[b]*Faculty of Agriculture, Yamagata University, Tsuruoka 997-8555, Japan*
[c]*Institute for Chemical Research, Kyoto University, Uji 611-0011, Japan*
[d]*Tamanoi Research Center, Tamanoi Vinegar Co., Kohriyama 639-1038, Japan*

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Abstract

A diterpene, cryptoquinone, was isolated from the bark of *Cryptomeria japonica*, the structure, 7,11,14-trioxoabieta-8,12-diene, was established by spectral analyses and X-ray crystallography. This diterpene quinone showed moderate antifungal activities against *Pyricularia orizae* and *Alternaria alternata*, and cytotoxic activity against mouse lymphoid neoplasm (P388) cells with an IC_{50} of 0.26 μg/ml.

Keywords: Cryptomeria japonica; Abietane; Diterpene; Quinone; Antifungal activity; Cytotoxic activity

1. Introduction

Cryptomeria japoinica D. Don (Sugi) is a widely distributed conifer in Japan. The wood is used as a raw material for production of furniture and other wooden structures. Because of its industrial importance, its constituents including terpenoids have been investigated by many researchers (Kondo et al., 1960; Ito et al., 1969; Matsui et al., 1989; Nagahama et al., 2000). However, there have been few investigations concerning the constituents of its bark, even though it is a major by-product formed during wood processing. On the other hand, the bark extracts have been found to exhibit antimicrobial activities against some plant pathogenic fungi (Mori et al., 1995; Kofujita et al., 2001). Recently, the bark of *C. japonica* was developed as a substrate for horticultural crops in soil-less culture, and it has also been reported that replacement of rockwool with processed bark reduces losses caused by soil-borne plant pathogens (Yu and Komada, 1995; Yu et al., 1997). As the major extractable substance, several abietane-type diterpenes have been found in bark of *C. japonica* (Sogo, 1971). Some abietane diterpenoids are known to be bioactive compounds, having antimicrobial and/or cytotoxic activities (Fukui et al., 1978; Dellar et al., 1996; Ohtsu et al., 1999; Chang et al., 1999; Kinouchi et al., 2000). In this paper, the isolation and characterization of a novel abietane-type diterpene quinone from the bark of *C. japonica* and its antifungal and cytotoxic activities is reported.

2. Results and discussion

The *n*-hexane extract of the bark of *C. japonica* was subjected to silica gel column chromatography using solvent systems of *n*-hexane–Et_2O. The components in the *n*-hexane: $Et_2O = 7:3$ eluate were separated further by HPLC (Shimadzu Shim-pack PREP-H, *n*-hexane:$Et_2O = 8:2$ as the solvent) to afford compound **1** (68 mg).

Compound **1** gave a molecular ion at *m*/*z* 314.1846, corresponding to a molecular formula $C_{20}H_{26}O_3$. The ^1H NMR spectrum showed the presence of an olefinic proton at δ_H 6.43 (1H, *s*, H-12), whereas an isopropyl group was observed at δ_H 3.03 (1H, *sept*, $J = 6.8$ Hz, H-15) together with methyl signals at 1.12 and 1.19 (each 3H, *d*, $J = 6.8$ Hz, Me-16 and Me-17). Three

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* Corresponding author. Tel.: +81-19-621-6171; fax: +81-19-621-6171.

E-mail address: kofujita@iwate-u.ac.jp (H. Kofujita).

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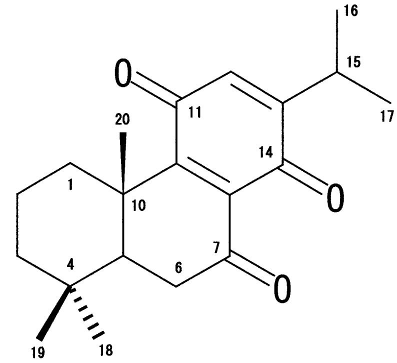

(1)

other methyl groups attached to quaternary carbons were observed at δ_H 0.91 (3H, *s*, Me-18), 0.95 (3H, *s*, Me-19) and 1.38 (3H, *s*, Me-20). The relation between H-5 and H_2-6 observed at δ_H 1.78 (1H, *dd*, $J = 14.4$, 3.9 Hz, H-5), 2.51 (1H, *dd*, $J = 18.1$, 14.4 Hz, H-6α) and 2.66 (1H, *dd*, $J = 18.1$, 3.9 Hz, H-6β) as well as among H_2-1 (δ_H 1.22, 2.77), H_2-2 (δ_H 1.59, 1.75) and H_2-3 (δ_H 1.25, 1.49) were deduced by using the DQF-COSY method. The ^{13}C NMR (DEPT) spectrum showed the presence of five methyl, four methylene, three methine and eight quaternary carbon signals for 20 carbon atoms. The three quaternary carbon signals at δ_C 184.9, 188.9 and 197.3 (C-14, C-11 and C-7, respectively) indicated the presence of three carbonyl groups including a quinone moiety, which was supported by the UV maximum (264 and 512 nm) and the IR absorbance (1705 and 1654 cm^{-1}). Four other downfield carbon signals at δ_C 131.4 (C-12), 132.0 (C-8), 154.3 (C-13) and 159.9 (C-9) were assignable to olefinic carbon atoms as members of the quinone ring. All these spectral data showed that the structure of compound **1** was an abietane-type diterpene quinone. The proton bearing carbons were unambiguously assigned by an HMQC experiment, and complete assignments of carbon signals were performed by an HMBC experiment. Consequently, the original diterpene quinone should be 7,11,14-trioxoabieta-8,12-diene (**1**) and is named cryptoquinone **1**. The relative stereo structure was confirmed by X-ray crystallographic analysis as shown in Fig. 1.

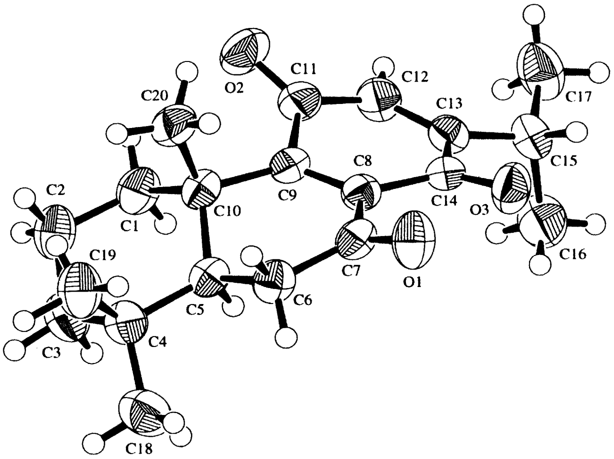

Fig. 1. ORTEP drawing of compound **1**.

Table 1
Antifungal activity of 1 and authentic compounds

Fungus	Dose[a] (μg/culture)	Inhibition rate (%)[b]		
		1	Ferruginol	Hinokitiol
A. alternata	400	80	61	100
	100	77	52	100
	25	68	Inhibition	28
P. oryzae	400	72	68	100
	100	66	53	100
	25	57	43	54

[a] Samples dissolved in acetone were spread on PDA media.

[b] Inhibition rate (%) = 100 × (the area of hyphal growth on the control medium −the area of hyphal growth on the test medium)/the area of hyphal growth on the control medium.

The antifungal activity of compound **1** against two phytopathogenic fungi was investigated, together with two authentic compounds, ferruginol and hinokitiol, as positive controls (Muhammad et al., 1992; Inamori et al., 1999). As shown in Table 1, the order of inhibition rate of these compounds for both fungi was hinokitiol > compound **1** > ferruginol at 400 μg/culture, whereas it was compound **1** > hinokitiol > ferruginol at 25 μg/culture. These results suggest that compound **1** has a moderate inhibitory effect at a wide dose range. Compound **1** was also cytotoxic against P388 cells with an IC_{50} of 0.26 μg/ml. Many abietane-type diterpene quinones have been isolated from *Salvia* species (Sabri and El-Lakany, 1990) and some of them have been reported to show cytotoxic activities (Moujir et al., 1996). More detailed analyses of the mechanisms of the cytotoxic action against target tumor cells are in progress.

3. Experimental

3.1. General experimental procedure

The melting point was determined with Yanagimoto micro melting point apparatus and was uncorrected. The optical rotation was measured on a JASCO DIP-1000 polarimeter in $CHCl_3$ and the UV spectrum was measured with a Shimadzu UV-mini in $CHCl_3$. NMR spectra (^1H: 400 MHz, ^{13}C: 100 MHz) were recorded using a JEOL AL-400 spectrometer using $CDCl_3$ as a solvent and Me_4Si as an internal standard. Electron impact mass spectra (EI-MS) were taken on a JEOL

JMS-SX102A mass spectrometer using a direct inlet system (ionization voltage; 70 eV).

3.2. Extraction and isolation

The bark of *Cryptomeria japonica* D. Don (Sugi) collected at Kuzumaki, Iwate prefecture, Japan was air-dried, milled and screened. Powdered samples under 40 mesh size (1.2 kg) were extracted with 4.8 l of *n*-hexane at ambient temperature for 24 h. The solution was evaporated in vacuo to give an *n*-hexane extract. The extract (33.0 g) was subjected to silica gel CC using solvent systems of *n*-hexane–Et_2O (9:1, 8:2, 7:3, 6:4, 1:1) and Et_2O. The *n*-hexane:Et_2O = 7:3 eluate was further separated by HPLC (Shimadzu Shim-pack PREP-H, 20 mm i.d. × 250 mm, *n*-hexane:Et_2O = 8:2) to yield compound **1** (68 mg).

3.3. Cryptoquinone (*1*)

Red needles, mp 105–106 °C, $[\alpha]_D^{25}$ $-680°$($CHCl_3$; *c* 0.1). EIMS *m*/*z* (rel. int.): 314 ([M^+], 100), 299 (61), 271 (24), 245 (63), 231 (41), 205 (54), 177 (12), 115 (14), 91 (11), 77 (9), 69 (8), 55 (8). HREIMS $C_{20}H_{26}O_3$ (*m*/*z*: 314.1846; calc., 314.1882). $[\lambda]_{max}^{CHCl_3}$ nm (logϵ): 216 (3.93), 264 (4.07), 512 (1.54). IR $[\upsilon]_{max}^{KBr}$ cm^{-1}: 2925, 1705, 1654, 1250. ^1H NMR ($CDCl_3$):δ0.91 (3H, *s*, Me-18), 0.95 (3H, *s*, Me-19), 1.12, 1.19 (each 3H, *d*, *J* = 6.8 Hz, Me-16 and Me-17), 1.22 (1H, *m*, H-1α or β), 1.25 (1H, *m*, H-3α or β), 1.38 (3H, *s*, Me-20), 1.49 (1H, *m*, H-3 βor α), 1.59 (1H, *m*, H-2 α or β), 1.75 (1H, *m*, H-2β or α), 1.78 (1H, *dd*, *J* = 14.4, 3.9 Hz, H-5), 2.51 (1H, *dd*, *J* = 18.1, 14.4 Hz, H-6α), 2.66 (1H, *dd*, *J* = 18.1, 3.9 Hz, H-6β), 2.77 (1H, *m*, H-1β or α), 3.03 (1H, *sept*, *J* = 6.8 Hz, H-15), 6.43 (1H, *s*, H-12). ^{13}C NMR ($CDCl_3$):δ18.1 (C-20), 18.6 (C-2), 21.3 (C-19), 21.4 (C-16, 17), 26.4 (C-15), 32.8 (C-18), 33.4 (C-4), 35.7 (C-1), 36.8 (C-6), 40.1 (C-10), 40.8 (C-3), 49.2 (C-5), 131.4 (C-12), 132.0 (C-8), 154.3 (C-13), 159.9 (C-9), 184.9 (C-14), 188.9 (C-11), 197.3 (C-7).

3.4. X-Ray crystallographic analysis

A red prismatic crystal of compound 1, having approximate dimensions of 0.20 × 0.02 × 0.93 mm was used for data collection. Crystallographic parameters measured on a Rigaku AFC7R diffractometer, with filtered CuKα radiation and a rotating anode generator, were as follows: space group, $P2_1$ (No.4); lattice parameters, a = 11.064(1)Å, b = 6.167(1)Å, c = 12.961(2)Å, β = 104.16(1)°, V = 857.7(2)Å^3; Z = 2; D_c = 1.218 g/cm^3; radiation, $CuK_\alpha(\lambda = 1.54178$Å); $\mu(Cu_{K\alpha}) = 6.37$ cm^{-1}; $2\theta_{max} = 136.2°$. The structure was solved by direct methods, and the non-hydrogen atoms were refined anisotropically. The final cycle of full-matrix least-squares refinement was based on 2889 observed reflections to give R = 0.054 and R_w = 0.073. All calculations were performed using the TEXSAN crystallographic software package developed by Molecular Structure Corp. (1985, 1992).

3.5. Bioassays

The fungi tested for antifungal activities were *Pyricularia orizae* P-2, provided by the National Institute of Agro-Environmental Sciences in Japan, and *Alternaria alternata* Am 21–9/5–2, provided by the Iwate Biotechnology Research Center in Japan. Each strain was pre-incubated on potato-dextrose agar (PDA) in a petri dish at 25°C until the fungus covered most of the surface of the plate. A portion (5 mm diameter) of each culture was transferred onto a testing PDA plate (30 mm diameter). Before inoculation of the fungus, the sample dissolved in 0.05 ml of acetone was spread on the agar medium. An equal amount of acetone was spread on the control medium, without a dissolved sample. The antifungal activity was evaluated as inhibition rate. Inhibition rate (%) = 100 × (the area of hyphal growth on the control medium—the area of hyphal growth on the test medium)/the area of hyphal growth on the control medium.

Cytotoxic activity against the P388 cell line (mouse lymphoid neoplasm derived from ATCC) was assayed by the cell count method (Hayashi et al., 1996). Each sample was initially dissolved in DMSO and diluted 2^n-fold with an experimental growth medium. The evaluation is expressed as an average of duplicated assays, the value for IC_{50} being determined as the concentration required to inhibit 50% of cell growth of the control.

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