A Cleistanthane Diterpene From a Marine-derived *Fusarium* Species Under Submerged Fermentation

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A cleistanthane-type diterpene, compound **1**, was isolated from the marine-derived *Fusarium* sp. FH-146. The structure of **1** was determined by 1D and 2D NMR spectroscopy. Compound **1** showed moderate antimicrobial, cytotoxic, and phytotoxic activities.

Key words: Cleistanthane, Diterpenoid, Antimicrobial Activity, Cytotoxicity, *Fusarium* sp.

Introduction

Marine-derived fungi have become an important source of novel active metabolites with potential as agrochemicals and pharmaceutical agents [1]. During our previous screening for bioactive compounds from marine-derived fungi, three antimicrobial pyrone derivatives were isolated from unpolished rice fermented with *Fusarium* sp. FH-146, which was isolated from driftwood collected from a seashore in Akita, Japan [2]. In this study, we examined the effect of culturing *F.* sp. FH-146 in a sea water-based liquid fermentation by comparing the chemical composition of liquid fermentation broth and solid fermentation. A cleistanthane-type diterpenoid, **1**, was isolated from the liquid culture of *F.* sp. FH-146, which was not produced during the solid fermentation of this fungus. While three pyrone derivatives, neofusapyrone, fusapyrone, and deoxyfusapyrone, were identified in the initial investigation, they were not detected when this strain was cultured in sea water-based liquid medium. We describe in this paper the isolation and structural elucidation of compound **1**.

Results and Discussion

The purification of the metabolites from *F.* sp. FH-146 was guided by their characteristic intense blue coloration in vanillin-sulfuric acid solution on TLC plates. The culture broth supernatant (5 L) of the fungus *F.* sp. FH-146 was subjected to extraction with EtOAc, and the resulting EtOAc-soluble portions were chromatographed on a column of silica gel. Two of the obtained fractions were selected for further purification by ODS column chromatography to afford compound **1**.

As determined by HR-FABMS and NMR data (Table 1), compound **1** had a molecular formula of $C_{20}H_{30}O_4$, thus requiring six degrees of unsaturation. The IR spectrum of **1** showed the presence of a hydroxyl (3435 cm^{-1}) and a carbonyl group (1641 cm^{-1}). ^{13}C NMR and DEPT spectra showed 20 carbon signals, including characteristic signals due to two methyls [δ_C = 21.2 (C-18), 16.6 (C-20)], one carbonyl carbon [δ_C = 180.2 (C-19)], four sp^3 methine carbons [δ_C = 74.6 (C-3), 43.7 (C-8), 47.8 (C-9), 56.8 (C-14)], one sp^2 methine carbon [δ_C = 142.0 (C-15)], two sp^2 methylenes [δ_C = 117.3 (C-16), 107.2 (C-17)], six sp^3 methylenes [δ_C = 28.2 (C-7), 28.5 (C-2), 28.7 (C-11), 28.9 (C-6), 30.2 (C-1), 37.9 (C-12)], three quaternary carbon atoms [δ_C = 42.8 (C-10), 51.9 (C-4), 81.3 (C-5)] and one sp^2 quaternary carbon atom [δ_C = 153.4 (C-13)]. The ^1H NMR spectrum, which was analyzed using ^1H-^1H COSY and ^{13}C-^1H COSY data indicated the presence of two tertiary methyl groups [δ_H = 0.86 (3H, s, 20-Me), 1.37 (3H, s, 18-Me)], a monosubstituted double bond [δ_H = 5.64 (m, 1H, 15-H), 4.94 (dd, *J*=17.3, 2.2 Hz, 1H, 16-H), 5.11 (dd, *J* =10.2, 2.2 Hz, 1H, 16-H)], two *exo*-methylenic methylenes [δ_H = 4.51 (s, 1H, s, 17-H) and 4.61 (s, 1H, 17-H)], and one oxygenated methine group [δ_H = 4.09 (br.

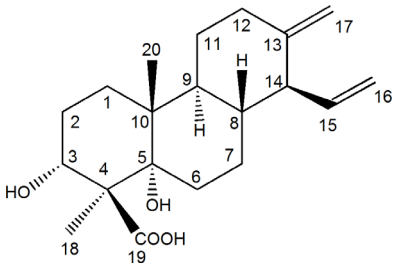

Fig. 1. Structure of compound **1**.

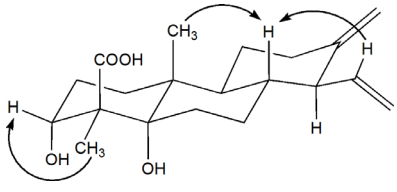

Fig. 2. NOEs for compound **1**.

s. 1H, 3-H)]. The ^{1}H and ^{13}C NMR spectra data showed that **1** had the characteristic pattern of a cleistanthane diterpene skeleton. The connectivities of the partial structures were determined by HMBC experiments (Table 1). Long-range correlations from 18-Me to C-3, C-4, C-5 and C-19, and from 3-H to C-18 and C-19 confirmed that the carboxyl and hydroxyl groups are connected to C-4 and C-3, respectively. Based on these results, the planar structure of **1** was deduced (Fig. 1), which is related to that of zythiostromic acid B (3α,5α-dihydroxycleistantha-13(17),15-dien-19-oic acid) isolated from *Zythiostroma* sp. [3].

The IR, ^{1}H, and ^{13}C NMR spectra of **1** closely resembled those of zythiostromic acid B, suggesting that the two compounds are diastereomers. A detailed comparison of the chemical shifts and coupling constants in the ^{1}H NMR spectra of **1** and zythiostromic acid B revealed differences in the signals due to the methines at C-14 and C-15, while other signals showed no significant differences, suggesting that **1** was the C-14 epimer of zythiostromic acid B. To confirm this speculation, NOE experiments were performed (Fig. 2). An observed NOE from 18-Me to 3-H implied that the hydroxyl at C-9 had an α-configuration, while the β-configuration of the mono-substituted double bond at C-14 was supported by the NOE from 20-Me and 15-H to 8-H. Therefore, the structure of **1** was concluded to be 14-*epi*-zythiostromic acid B (3α,5α-dihydroxy-14(β)-cleistantha-13(17),15-dien-19-oic acid).

In our initial investigation, pyrone derivatives were produced by solid-state fermentation of *F.* sp. FH-146. However, when this fungus was cultured under sea water-based liquid culture conditions, the profile of produced compounds was different; pyrone derivatives were not detected, and compound **1** appeared. The comparison of the spectral data of compound **1** with similar compounds reported in the literature revealed it to be closely related to cleistanthane-type diterpenes. Generally, cleistanthane-type diterpenes belong to a small group of diterpenenes that are mainly isolated from plant sources. However, the isolation of two cleistanthane sesquiterpenes, sonomolides A and B, were the first examples from fungal culture material [4].

Table 1. ^{1}H and ^{13}C NMR data of compound **1**[a].

No	δ_C	δ_H	HMBC
1	30.2 t	1.68 – 1.76[b]	
		2.23 – 2.33[b]	
2	28.5 t	1.30 (1H, m)	
		1.67 (1H, m)	
3	74.6 d	4.09 (1H, br. s)	1, 18, 19
4	51.9 s		
5	81.3 s		
6	28.9 t	1.68 – 1.76[b]	
		2.23 – 2.33[b]	
7	28.2 t	1.36 (1H, m)	
		1.61 (1H, m)	
8	43.7 d	1.59 (1H, m)	10, 15
9	47.8 d	1.82 (1H, m)	12, 14
10	42.8 s		
11	28.7 t	1.06 (1H, m)	
		1.68 – 1.76[b]	
12	37.9 t	2.03 (1H, t, 12.8 Hz)	
		2.38 (1H, m)	
13	153.4 s		
14	56.8 d	2.23 – 2.33[b]	
15	142.0 d	5.64 (1H, m)	8, 13, 14
16	117.3 t	4.94 (1H, dd, 17.3, 2.2)	14, 15
		5.11 (1H, dd, 10.2, 2.2)	14, 15
17	107.2 t	4.51 (1H, s)	12, 13, 14
		4.61 (1H, s)	12, 13, 14
18	21.2 q	1.37 (3H, s)	3, 4, 5, 19
19	180.2 s		
20	16.6 q	0.86 (3H, s)	1, 5, 9, 10

[a] Measured in CD_3OD, values in parentheses are coupling constants in Hz; [b] overlapped signals.

Compound **1** is structurally related to sonomolides A and B from a coprophilous fungus, and to zythiostromic acids A and B and zythiostromolide from *Zythiostroma sp.*. Sonomolides A and B, and zythiostromolide possess a lactone moiety. Sonomolides A and B were reported to show antimicrobial activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, while zythiostromic acids A and B and zythiostromolide exhibited no antimicrobial activity against *Ophiostroma crassivaginatum*. We therefore examined the biological activity of **1** using an antimicrobial activity assay. At a concentration of 100 μg per disk, **1** was active against *Staphy-*

lococcus aureus NBRC 13276 (15 mm) and *Pseudomonas aeruginosa* (13 mm). However, **1** did not exhibit antimicrobial activity against *C. albicans* or *Aspergillus clavatus* and only displayed weak inhibitory cell growth activity against the human breast cancer cell line YMB and human colon cancer cell line COLO 201 with observed IC_{50} values of 55 and 45 μM, respectively. The plant growth inhibitory activities of **1** were also examined using a seedling growth test of lettuce. At a concentration of 50 μg mL^{-1}, compound **1** inhibited root growth by 35 % compared to the untreated controls.

Experimental Section

General experimental procedures

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 obtained with a Shimadzu UV mini-1240 instrument. Mass spectra were recorded with a Jeol JMS-700 instrument, and ^{1}H NMR 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 ODS (Fuji Silysia, Japan) and silica gel 60 (Kanto Chemical Co., Inc.). TLC was performed on a precoated silica gel plate (Merck), and spots were detected by spraying 10 % vanillin in H_2SO_4 followed by heating.

Isolation and cultivation of the fungus

Endophytic fungus *Fusarium* sp. FH-146 was isolated from driftwood collected in May 2004 from the Oga peninsula, Akita, Japan [1].

*Isolation of compound **1***

For fermentation, the producing strain *Fusarium* sp. FH-146 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 of peptone per 1 L of artificial sea water. The inoculated flask was incubated at 25 °C for 4 weeks on a rotary shaker. 5.0 L of culture broth was separated from the mycelia by filtration. The filtrate was extracted with EtOAc. The organic layer was concentrated *in vacuo* to give an oily residue (10.3 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. 6 and 7 were combined (171 mg) and further chromatographed on ODS by eluting with mixtures of H_2O and MeOH to afford compound **1** (20.5 mg).

Compound **1**: colorless solid. – $[\alpha]_D^{20}$ = +24.4° (c = 0.26, MeOH). – IR (KBr) ν = 3435, 2106, 1641, 1398, 1261, 1099, 1014 cm^{-1}. – ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C {^{1}H} NMR (100 MHz, CD_3OD) data see Table 1. – MS ((+)-FAB): m/z = 335 [M+H]$^+$. – HRMS ((+)-FAB): m/z = 335.2245 (calcd. 335.2222 for $C_{20}H_{31}O_4$, [M+H]$^+$).

Assay for antimicrobial activity

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

Lettuce seedling assay

Lettuce seeds (*Lactuca sativa* L.) were used for the bioassay. 15 seeds were sown in filter paper containing a definite concentration of the test compound in a Petri dish (5 cm i. d.). Distilled water (1 mL, containing 100 ppm (w/v) Tween 80) was added to the Petri dish, and incubation was carried out at 25 °C under continuous light for 7 d. The control experiments were also conducted in distilled water. The elongation of roots and shoots was measured compared to those of the control.

Cell growth inhibitory activity

Human breast cancer YMB cells and human colon cancer COLO 201 cells were used for *in vitro* test. Both cell lines were cultivated in RPMI1640 medium (Nissui Co. Japan) containing 10 % fetal bovine serum (HyClone Inc. USA) and penicillin / streptomycin (Invitrogen Co., USA) at 37 °C in a humidified atmosphere of 5 % CO_2 in air. In brief, 5×10^3 cells of each line were inoculated in 96-well microtiter plates containing 270 μL of the medium. Compounds were dissolved in DMSO. The serially diluted solutions of the compounds were added to each well of the plates. At the end of the incubation, 100 μL of each cultured cell was transferred to the other 96-well plates, and 10 μL of WST-8 assay solution was added per well and incubated at 37 °C in humidified air supplemented with CO_2 for an additional 4 h. The amount of formazan formed was measured at an optical density of 450 nm using a microplate reader, and the cell viability was expressed as a percentage of the untreated control. The IC_{50} values were determined by the extrapolation of the dose-response viability curves. 5-Fluorouracil was used as positive control (IC_{50} = 1 μM).

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