Two Drimane-type Sesquiterpenes, Strobilactones A and B, from the Liquid Culture of the Edible Mushroom *Strobilurus ohshimae*

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Two novel drimane sesquiterpenoids, strobilactones A (**3**) and B (**4**), were isolated from the organic extract of a liquid culture of *Strobilurus ohshimae*. The structures of **3** and **4** were determined by spectroscopic methods. Compounds **3** and **4** exhibit cell growth inhibitory activities against cultured COLO 201 cells. Compound **4** also shows antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Key words: Strobilurus ohshimae, Drimane, Strobilactones A and B, Edible Mushroom

Introduction

In recent years, mushrooms have received significant attention from researchers due to their diverse nutritional, medicinal, and pharmacological characteristics [1]. In particular, edible mushrooms are important as functional foods and as rich sources of bioactive compounds [2, 3]. We are interested in evaluating the natural compounds present in wild edible Japanese mushrooms. Recently, during our investigations for bioactive compounds from edible mushrooms, we investigated field-collected samples of the mushroom *Strobilurus ohshimae* (Sugiedatake in Japanese). *S. ohshimae* is a wild edible mushroom that belongs to the order Agaricales, class Basidiomycetes, and family Tricolomataceae. In Japan, it grows wild on the fallen dead branches of the Japanese cedar at the end of fall. Although research regarding the analysis of the chemical composition and bioactive compounds of *S. ohshimae* is limited, in our preliminary chemical investigation of its fruiting bodies, we isolated and elucidated the structure of 4 new sesquiterpenoids – strobilols A (**1**), B, C (**2**), and D [4]. In addition, compound **1** demonstrated moderate activity against the brine shrimp *Artemia salina* (LC_{50} value of 100 μM). In a continuation of our study, we attempted to establish a liquid culture of *S. ohshimae* to investigate whether the mycelia produce strobilols or other bioactive compounds. The ethyl acetate (EtOAc) extract of the culture liquid of *S. ohshimae* showed antimicrobial activity against *Staphylococcus aureus*.

From the EtOAc extract, we isolated 2 new drimane-type sesquiterpenoids – strobilactones A (**3**) and B (**4**) – whose structures were elucidated by spectroscopic methods. Strobilactone B (**4**) demonstrated antimicrobial activity against *S. aureus* and *Pseudomonas aeruginosa*. This paper describes the isolation, structure elucidation, and antimicrobial activity of the 2 novel sesquiterpenoids **3** and **4**.

Results and Discussion

The microorganism-producing *S. ohshimae* NBRC 30370 was cultured at 25 °C for 4 weeks. The cultured filtrate was extracted with EtOAc. Concentration of the EtOAc extract yielded 10.5 g of crude extract that was purified by a combination of silica gel and ODS column chromatographies to afford strobilactones A (**3**) and B (**4**) together with strobilols A (**1**) and C (**2**) (Fig. 1).

The molecular formula of strobilactone A (**3**) was $C_{15}H_{22}O_4$, as determined by HREIMS. The IR spectrum exhibited the presence of hydroxyl (3355 cm^{-1}) and carbonyl (1770 cm^{-1}) moieties. The ^{13}C NMR

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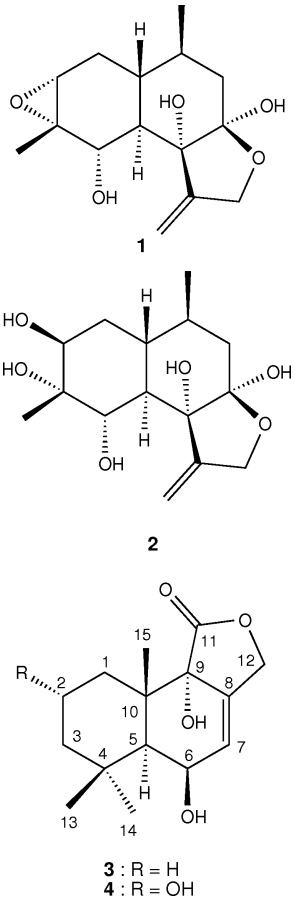

Fig. 1. Structures of strobilols A (**1**) and C (**2**), and strobilactones A (**3**) and B (**4**).

Table. 1. ^{1}H and ^{13}C NMR data of strobilactone A (**3**)[a].

No.	δ_C	δ_H	HMBC
1	30.5 t	α 1.67 – 1.72[b]	
		β 2.03 (1H, m)	2
2	17.8 t	α 1.58 (1H, m)	4, 10
		β 1.67 – 1.72[b]	
3	44.7 t	β 1.25 (1H, m)	2, 4, 5
		α 1.41 (1H, br d. 13.6)	2, 4, 5
4	34.1 s		
5	45.6 d	1.78 (1H, d, 4.8)	3, 7, 9, 13, 14, 15
6	65.4 d	4.54 (1H, br s)	4, 8
7	127.8 d	5.95 (1H, br s)	5, 12
8	132.8 s		
9	74.9 s		
10	37.6 s		
11	175.3 s		
12	69.1 t	β 4.74 (1H, d, 12.1)	7, 8, 9, 11
		α 4.95 (1H, dd, 12.1, 2.1)	7, 8, 9, 11
13	32.6 q	1.31 (3H, s)	3, 4, 5, 14
14	25.0 q	1.10 (3H, s)	3, 4, 5, 13
15	18.9 q	1.13 (3H, s)	1, 5, 9, 10

[a] In $CDCl_3$, values in parentheses are coupling constants in Hz; [b] overlapping signals.

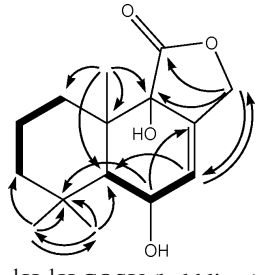

Fig. 2. Important ^{1}H-^{1}H COSY (bold lines) and HMBC correlations observed for **3**.

spectrum showed a total of 15 carbon atoms, and their multiplicity assignments using DEPT established the presence of 3 methyls, 4 sp^3 methylenes (one of them bearing an oxygen atom), 2 sp^3 methines, 1 sp^2 methine, 3 sp^3 quaternary carbons, 1 sp^2 quaternary carbon, and 1 ester.

The ^{1}H NMR spectral data of **3** revealed the presence of 3 tertiary methyls [δ_H = 1.10 (s, 3H), 1.13 (s, 3H) and 1.31 (s, 3H)], 1 olefinic methine [δ_H = 5.95 (br. s, 1H)], 1 oxygenated methine [δ_H = 4.54 (br. s, 1H)], and 1 methylene attached to an oxygen atom [δ_H = 4.74 (d, J = 12.1 Hz, 1H), 4.95 (dd, J = 12.1, 2.1 Hz, 1H)]. A detailed analysis of the ^{1}H-^{1}H COSY spectrum in conjunction with an HMQC experiment established the presence of 2 partial structures (Fig. 2). These data and detailed ^{13}C and ^{1}H NMR studies with the aid of HMQC let us to conclude that **3** may be a drimane-type sesquiterpene with a lactone ring and hydroxyl groups. In the HMBC spectrum of **3** (Fig. 2), HMBC correlations between 12-H_2 and C-7, between 12-H_2 and C-9, and between 12-H_2 and C-11 suggested the presence of a γ-lactone ring comprising C-8, C-9, C-11, and C-12. The position of the double bond between C-7 and C-8 was assigned on the basis of the HMBC correlations from 7-H to C-5 and C-12 (Table 1). HMBC correlations of the signal at δ_H = 4.54 with C-4 and C-8 were detected, implying that the oxygenated methine group was located at C-6. Therefore, the gross structure of strobilactone A was determined to be **3** (Fig. 1). The relative stereochemistry of **3** was determined on the basis of the NOEs observed in difference NOE experiments (Fig. 3). NOEs were observed from 13-Me to 15-Me, indicating that 13-Me had a β-configuration. NOEs from 14-Me to 5-H and from 14-Me to 6-H implied that 14-Me and 6-H had α-configurations. The α-configuration of the hydroxyl at C-9 was supported by the NOE from 15-Me to one

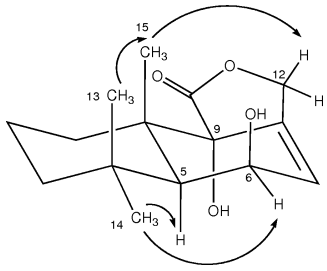

Fig. 3. Selected NOE Correlations for **3**.

of the methylene protons, namely, 12β-H. The relative structure was deduced as 6β,9α-dihydroxy-drim-7-ene-11,12-olide. The absolute stereochemistry of **3** was confirmed by the specific rotation that had the same negative sign ($[\alpha]_D = -110°$) as that of the analog (–)-drimenin ($[\alpha]_D = -58°$) [5].

The molecular formula of strobilactone B (**4**) was deduced as $C_{15}H_{22}O_5$ by the HREIMS data and indicated the presence of 1 more oxygen atom as compared to that present in **3**. The IR spectrum of **4** showed the presence of hydroxyl (3352 cm^{-1}) and carbonyl (1770 cm^{-1}) moieties. In general, the ^1H and ^{13}C NMR spectra of **4** closely resembled those of **3**, except for the presence of an oxymethine signal [δ_H = 3.95 (m, 1H, 2-H) and δ_C = 66.0 (C-2)] in place of a methylene signal, indicating that **4** was probably an oxygenated metabolite of **3**. The presence of a hydroxyl group at C-2 was established by HMBC correlations from 2-H to C-4 and C-10. The ^1H and ^{13}C NMR shifts in **4** were also fully assigned by 2D NMR spectra, including HMBC (Table 2). The stereochemistry of the hydroxyl at C-2 was determined as having an α-orientation based on the ^1H-^1H coupling constants ($J_{1,2}$ = 12.5 and 2.1 Hz) and the NOE from 2-H to 15-Me in the difference NOE experiments.

Strobilactones A (**3**) and B (**4**) were evaluated by the agar diffusion method against gram-positive and gram-negative bacteria, yeast, and fungus strains. Against *S. aureus* NBRC 13276 and *P. aeruginosa* ATCC 15442, **4** showed moderate activities with zones of inhibition of 13 mm and 12 mm in diameter, respectively, at a concentration of 100 μg/disk. At this concentration, **3** was inactive against *S. aureus* NBRC 13276 and *P. aeruginosa* ATCC 15442. These results indicated that a compound's polarity appears to play an important role in its antibacterial activity. Neither of the tested compounds **3** or **4** showed activity against *Aspergillus clavatus* F 318a and *Candida albicans* ATCC 2019 (> 100 μg/disk). Furthermore, we studied the anti-tumor effects of **3** and **4** on the human colon carcinoma cell line COLO 201. Compounds **3** and **4** weakly inhibited the cell growth activity of COLO 201 with IC_{50} values of 40 μg/mL and 35 μg/mL, respectively.

Table. 2. ^1H and ^{13}C NMR data of strobilactone B (**4**)[a].

No.	δ_C	δ_H	HMBC
1	47.4 t	α 1.87 (1H, t, 12.5)	2, 15
		β 2.24 (1H, ddd, 12.5, 2.5, 2.1)	
2	66.0 d	3.95 (1H, m)	4, 10
3	55.2 t	α 1.24 (1H, t, 11.5)	1, 2, 4, 14
		β 1.65 (1H, td, 11.5, 2.5)	
4	37.2 s		
5	50.6 d	1.72 (1H, d, 4.6)	1, 4, 6, 9, 14, 15
6	66.2 d	4.48 (1H, m)	4, 8
7	129.5 d	5.90 (1H, br s)	5, 12
8	135.1 s		
9	76.2 s		
10	40.9 s		
11	177.8 s		
12	70.9 t	4.74 (1H, br d, 12.1)	7, 8, 9, 11
		4.93 (1H, br d, 12.1)	7, 8, 9, 11
13	33.8 q	1.34 (3H, s)	3, 4, 5, 14
14	26.8 q	1.14 (3H, s)	3, 4, 5, 13
15	20.8 q	1.10 (3H, s)	1, 5, 9, 10

[a] In $CDCl_3$, values in parentheses are coupling constants in Hz.

Since the drimane 1,4-dialdehydes, warburganal and polygodial, are reported to be insect antifeedant agents [6], we investigated whether the isolated compounds **3** and **4** also exhibit antifeedant activity against *Spodoptera litura*. Compounds **3** and **4** did not show significant antifeedant activity at 100 ppm in a feeding bioassay. Previous studies performed by Kubo *et al.* revealed that the dialdehyde groups were responsible for the antifeedant activity of warburganal and polygodial [7]. Furthermore, the polygodial conversion products of sesquiterpene lactones did not show antifeedant activity [7]. The present result is in agreement with previous reports.

This study is a part of our efforts to evaluate the biological activity of this fungus grown under liquid culture conditions. Compounds **3** and **4** were isolated from the liquid fermented culture from *S. ohshimae*; these compounds are not produced in the fresh fruiting bodies of this fungus. A similar sesquiterpene core was recently found in the dendocarbins A–N obtained from the Japanese nudibranch *Dendrodoris carbunculosa* [8]. Furthermore, **3** was the sesquiterpene portion of the drimane ester with unsaturated fatty acids isolated from *Aspergillus ustus var. pseudodeflectus* by Hayes *et al.* [9].

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. 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 ODS (Fuji Silysia, Japan) and silica gel 60 (Kanto Chemical Co., Inc., Japan). TLC was done on a precoated silica gel plate (Merck), and spots were detected by spraying with 10 % vanillin in H_2SO_4 followed by heating.

Fungus and cultivation

The producing strain *Strobilurus ohshimae* NBRC 30370 was purchased from the biological resource center, National Institute of Technology and Evaluation, Chiba, Japan. For fermentation, the fungus strain *S. ohshimae* was grown on slant of potato dextrose agar. A loopful of the culture was transferred into fifty 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 strobilactones A and B, and strobilols A and B

The culture broth (5.0 L) 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 (10.5 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 % EtOAc 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 were combined and rechromatographed on silica gel with $CHCl_3$/MeOH (20 : 1) to yield strobilactone A (**3**, 14 mg), strobilol A (**1**, 33.0 mg) and strobilol C (**2**, 22.0 mg). Fr. 11 (100 % MeOH eluate, 650 mg) was chromatographed on silica gel by eluting with mixtures of $CHCl_3$ and EtOAc to afford 90 – 100 % EtOAc eluates (57 mg). These fractions were combined and further chromatographed on ODS by eluting with mixtures of H_2O and MeOH to afford strobilactone B (**4**, 10.2 mg).

*Strobilactone A (**3**) (6R,9S-dihydroxy-drim-7-ene-11,12-olide)*

Colorless oil. – $[\alpha]_D^{20} = -110°$ (c = 0.70, $CHCl_3$). – IR (KBr): ν = 3355 (OH), 2865, and 1770 (CO) cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): data see Table 1. – HRMS (positive mode, EI): m/z = 266.1519 (calcd. 266.1518 for $C_{15}H_{22}O_4$, $[M]^+$). – MS (positive mode, EI): m/z = 266 $[M]^+$.

*Strobilactone B (**4**) (2S,6R,9S-trihydroxy-drim-7-ene-11,12-olide)*

Colorless oil. – $[\alpha]_D^{20} = -41°$ (c = 0.073, $CHCl_3$). – IR (KBr): ν = 3352 (OH), 2950 and 1770 (CO) cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 2. – HRMS (positive mode, EI): m/z = 282.1463 (calcd. 282.1463 for $C_{15}H_{22}O_5$, $[M]^+$). – MS (positive mode, EI): m/z = 282 $[M]^+$.

Antimicrobial activity

Test organisms were *Staphylococcus aureus* NBRC 13276, *Pseudomonas aeruginosa* ATCC 15442, *Aspergillus clavatus* F 318a and *Candida albicans* ATCC 2019. Antimicrobial assays were carried out by the paper disk diffusion method using a published protocol [10].

Cell growth inhibitory activity

The cell growth assay was examined using human cancer cells COLO 201 as described earlier [11]. 5-Fluorouracil was used as positive control (IC_{50} values of 1.0 μM).

Insect antifeedant bioassay

The antifeedant bioassay was carried out with the larvae (n = 10) of the polyphagous pest insect *Spodoptera litura*. The larvae were cultured on an artificial diet purchased from Nippon Nosan Kogyo Co., Ltd. To one gram of the diet, 100 μL of a certain amount of a MeOH solution of the test compound was added in a petri dish. After removing the solvent, ten larvae of the third instar were placed in each petri dish. After five days (moist chamber, 25 °C), the percentage area eaten on each artificial diet was determined. The negative control insects were given diets treated with MeOH solution not containing the test compounds. After the end of the period, the amount of the diet eaten was determined gravimetrically. All diets were weighted before and after the experiments, and results were recorded as weight of the diet eaten.

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