Chiang Mai J. Sci. 2016; 43(3) : 476-482
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Ophiobolides, Polyketides Isolated from *Ophiobolus* sp. KTC 2293

Wilanfranco C. Tayone [α], Kazuaki Tanaka [b] and Noboru Takada*[b]

[α] Mathematics and Natural Sciences Department, Davao Oriental State College of Science and Technology, Mati City 8200, Davao Oriental, Philippines

[b] Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki 036-8561, Japan

*Author for correspondence; e-mail: takada@hirosaki-u.ac.jp

Received: 6 January 2015
Accepted: 27 April 2015

ABSTRACT

Three new polyketides, ophiobolides A, B**,** and C, were isolated from the culture broth of *Ophiobolus* sp. KTC 2293 along with structurally related achaetolide. The structures of these polyketides were elucidated on the basis of NMR and MS analyses. Ophiobolide B showed a weak antifungal activity against *Cochliobolus miyabeanus*.

Keywords: ophiobolide, polyketides, *Ophiobolus*, structural elucidation, antifungal activity

1. INTRODUCTION

Polyketides are a large family of structurally diverse natural products possessing a broad range of biological activities, including antibiotic and pharmacological properties [1-2]. In our continuing search for fungal metabolites with attractive chemical structures and/or biological activities [3-4], achaetolide [5] was isolated from a culture broth of *Ophiobolus* sp. KTC 2293. Our earlier investigation led us to report an interesting flexible conformational property of the 10-membered lactone ring through conformational analysis [6]. In order to further investigate the biosynthesis and flexible conformation of achaetolide, the related compounds were explored from the fungal extract. A recent study resulted in the isolation and structure elucidation of three polyketides, named ophiobolides A, B, and C, respectively. Among them, ophiobolide B showed a weak antifungal activity against *Cochliobolus miyabeanus*. In this paper, we report the details.

2. MATERIALS AND METHODS

2.1 General Experimental Procedure

The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL JNM-ECA500 spectrometer. In $CDCl_3$, the signal due to 7.24 ppm was used as the standard, while 3.30 ppm was employed for CD_3OD. Electrospray ionization (ESI) MS spectra were obtained from a HITACHI NanoFrontier LD spectrometer. Measurements of IR spectra were performed with a HORIBA FT-720 spectrometer on KBr cell. The optical rotation values were measured on a HORIBA SEP-700 spectrometer. Chemicals used in these experiments were obtained from Wako Pure Chemical Industries Ltd. and Nacalai Tesque Inc.

2.2 Fungal Material

Ophiobolus sp. KTC 2293 was collected from dead stems of *Achillea alpina* subsp. *pulchra* (Asteraceae) in Rishiri Island, Hokkaido,

Japan. The fungal isolate was deposited in the Japan Collection of Microorganisms (JCM) as JCM 17828.

2.3 Fermentation and Isolation

The culture broth of *Ophiobolus* sp. KTC 2293 in 200 mL potato-sucrose medium [prepared from a potato extract (40 g potato), 4 g of sucrose, and H_2O] in 500 mL Erlenmeyer flasks (×5) at 25 °C for 90 days on a rotary shaker (100 rpm). The combined extract (284 mg) was then extracted with *c.a.* 1.0 L of MeOH and a filtrate was concentrated *in vacuo*. The extract was then partitioned with EtOAc (1.0 L×3) followed by silica gel column chromatography (7.5 g, 12 mmID×150 mm) using $CHCl_3$/MeOH (1:0, 100:1, 30:1, 9:1, 4:1) solvent system to give fraction A (52.3 mg), fraction B (41.0 mg), achaetolide (48.0 mg) and ophiobolide A (54.8 mg). Further purification of fraction A (52.3 mg) yielded fractions A-1 (9.3 mg), A-2 (9.6 mg), A-3 (4.1 mg), and A-4 (9.8 mg) by silica gel column chromatography (2 g, 10 mmID×100 mm) employing hexane/EtOAc (15:1, 9:1, 5:1, 1:1, 1:5). Fraction A-4 (9.8 mg) was further purified by silica gel column chromatography (0.5 g, 5 mmID×50 mm) using hexane/EtOAc (9:1, 7:1, 5:1, 3:1, 1:1) to afford ophiobolide B (3.3 mg) and ophiobolide C (2.2 mg), respectively.

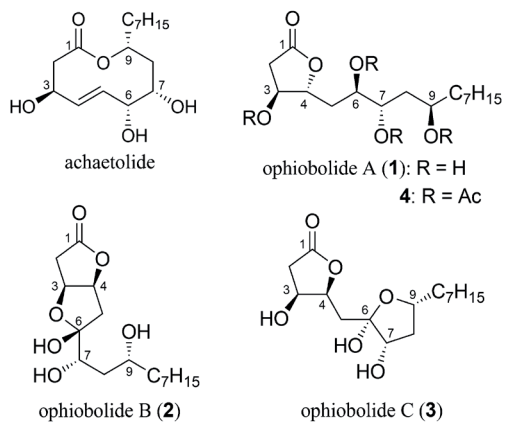

Figure 1. Structures of achaetolide and ophiobolides A-C.

Ophiobolide A (1): Colorless oil; $[\alpha]^{20}_{D}$ +28° (*c* 0.12, $CHCl_3$); IR (film) ν_{max} = 3533 (O-H), 3367 (O-H), 2954 (C-H), 2920 (C-H), 2854 (C-H), 1778 (O-C=O) cm^{-1}; HRESIMS *m/z* 341.1941 (calcd for $C_{16}H_{30}NaO_6$ 341.1940).

Ophiobolide B (2): Colorless oil; $[\alpha]^{20}_{D}$ +36° (*c* 0.11, $CHCl_3$); IR (film) ν_{max} = 3456 (O-H), 2927 (C-H), 2854 (C-H), 1770 (O-C=O) cm^{-1}; HRESIMS *m/z* 317.1954 (calcd for $C_{16}H_{29}O_6$ 317.1953).

Ophiobolide C (3): Colorless oil; $[\alpha]^{20}_{D}$ -22° (*c* 0.09, $CHCl_3$); IR (film) ν_{max} = 3448 (O-H), 2927 (C-H), 2854 (C-H), 1778 (O-C=O) cm^{-1}; HRESIMS *m/z* 339.1786 (calcd for $C_{16}H_{28}NaO_6$ 339.1780).

2.4 Acetylation of Ophiobolide A (1)

Compound **1** (4.2 mg, 13 mmol) was treated with acetic anhydride (0.2 mL) in pyridine (0.5 mL) and stirred overnight at room temperature. The mixture was diluted with EtOAc (20 mL) and washed successively with H_2O (20 mL) and 1.0 M aqueous $NaHCO_3$ (20 mL). After the organic layer was dried over $MgSO_4$, the filtrate was concentrated *in vacuo* to give the crude product, which was purified by silica gel column chromatography (0.5 g, 5 mmID×50 mm, hexane/$CHCl_3$ = 1:1) to give acetate **4** (3.4 mg, 52%) as a colorless oil.

Acetate 4: Colorless oil; ^{1}H NMR ($CDCl_3$) δ 5.15 (1H, m, H-7), 5.13 (1H, ddd, *J* = 7.1, 4.4, 2.6 Hz, H-3), 5.08 (1H, ddd, *J* = 10.7, 3.0, 2.7 Hz, H-6), 4.86 (1H, m, H-9), 4.48 (1H, ddd, *J* = 9.4, 4.4, 2.1 Hz, H-4), 2.95 (1H, dd, *J* = 18.6, 7.1 Hz, H-2a), 2.57 (1H, dd, *J* = 18.6, 2.6 Hz, H-2b), 2.08 (3H, s, C*H*$_3$COO), 2.05 (1H, m, H-5a), 2.03 (3H, s, C*H*$_3$COO), 2.02 (3H, s, C*H*$_3$COO), 2.00 (3H, s, C*H*$_3$COO), 1.79 (1H, ddd, *J* = 14.7, 9.4, 2.7 Hz, H-5b), 1.71 (2H, m, H-8), 1.51 (2H, m, H-10), 1.30 - 1.20 (10H, m, C-11, H-12, H-13, H-14, H-15), 0.86 (3H, t, *J* = 6.7 Hz, H-16); ^{13}C NMR ($CDCl_3$)

δ 173.2 (C, C-1), 170.8 (C, CH_3COO), 170.28 (C, CH_3COO), 170.25 (C, CH_3COO), 170.2 (C, CH_3COO), 81.4 (CH, C-4), 73.0 (CH, C-3), 70.7 (CH, C-6), 69.5 (CH, C-7), 69.4 (CH, C-9), 34.7 (CH_2, C-10), 34.1 (CH_2, C-2), 33.9 (CH_2, C-8), 32.4 (CH_2, C-5), 31.7 (CH_2, C-11, C-12, C-13, C-14, or C-15), 29.4 (CH_2, C-11, C-12, C-13, C-14, or C-15), 29.1 (CH_2, C-11, C-12, C-13, C-14, or C-15), 25.1 (CH_2, C-11, C-12, C-13, C-14, or C-15), 22.6 (CH_2, C-11, C-12, C-13, C-14, or C-15), 21.0 (CH_3, CH_3COO), 20.9 (CH_3, CH_3COO), 20.84 (CH_3, CH_3COO), 20.8 (CH_3, CH_3COO), 14.1 (CH_3, C-16); HRESIMS *m/z* 509.5436 (calcd for $C_{24}H_{38}NaO_{10}$ 509.5423).

2.5 Acetonidation of Ophiobolide A (1)

To a stirred solution of **1** (41.4 mg, 0.13 mmol) in acetone (1 mL) was added perchloric acid (1 drop). The reaction mixture was stirred at room temperature for 20 min. After neutralization with sat. NH_4Cl_{aq} (10 mL), the reaction mixture was extracted with EtOAc (10 mL×3). The combined EtOAc layer was washed with brine (5 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (0.5 g, 5 mmID×50 mm, $CHCl_3$/MeOH = 100/1) to give **5** (19.9 mg, 43%) and **6** (1.2 mg, 3%).

Acetonide 5: Colorless oil; ^{1}H NMR (CD_3OD) δ 4.51 (1H, ddd, *J* = 10.2, 3.4, 3.3 Hz, H-4), 4.40 (1H, ddd, *J* = 10.0, 5.9, 3.1 Hz, H-7), 4.28 (1H, ddd, *J* = 6.4, 3.4, 3.3 Hz, H-3), 4.25 (1H, ddd, *J* = 11.1, 5.9, 2.5 Hz, H-6), 3.72 (1H, m, H-9), 2.90 (1H, dd, *J* = 17.9, 6.4 Hz, H-2a), 2.39 (1H, dd, *J* = 17.9, 3.4 Hz, H-2b), 1.79 (1H, ddd, *J* = 14.2, 11.1, 3.4 Hz, H-5a), 1.64 (1H, ddd, *J* = 14.2, 10.2, 2.5 Hz, H-5b), 1.59 (1H, ddd, *J* = 14.0, 10.0, 2.6 Hz, H-8a), 1.42 (3H, s, acetonide CH_3), 1.41 (1H, ddd, *J* = 14.0, 10.0, 3.1 Hz, H-8b), 1.34 (3H, s, acetonide CH_3), 1.48-1.28 (12H, m, H10, H-11, H-12, H-13, H-14, H-15), 0.90 (3H, t, *J* = 6.9 Hz, H-16); ^{13}C NMR ($CDCl_3$) δ 177.9 (C, C-1), 109.1 (C, O-C-O), 87.0 (CH, C-4), 76.0 (CH, C-7), 75.5 (CH, C-6), 72.5 (CH, C-3), 69.4 (CH, C-9), 39.4 (CH_2, C-10), 38.3 (CH_2, C-8), 37.9 (CH_2, C-2), 34.8 (CH_2, C-5), 33.0 (CH_2, C-14 or C-15), 30.7 (CH_2, *C*-11, C-12, or C-13), 30.5 (CH_2, *C*-11, C-12, or C-13), 28.9 (CH_3, acetonide CH_3), 26.8 (CH_2, *C*-11, C-12, or C-13), 26.1 (CH_3, acetonide CH_3), 23.7 (CH_2, C-14 or C-15), 14.4 (CH_3, C-16); HRESIMS *m/z* 381.4580 (calcd for $C_{19}H_{34}NaO_6$ 381.4594).

Acetonide 6: Colorless oil; ^{1}H NMR (C_6D_6) δ 4.34 (1H, ddd, *J* = 10.7, 10.7, 2.9 Hz, H-4), 3.74 (1H, ddd, *J* = 10.1, 9.0, 1.9 Hz, H-6), 3.57 (1H, m, H-9), 3.40 (1H, m, H-3), 3.16 (1H, m, H-7), 2.15 (1H, dd, *J* = 17.7, 6.5 Hz, H-2b), 1.91 (1H, m, H-5b), 1.89 (1H, dd, *J* = 17.7, 3.6 Hz, H-2a), 1.87 (1H, m, H-5a), 1.65 (1H, m, H-8b), 1.48-1.30 (12H, m, H10, H-11, H-12, H-13, H-14, H-15), 1.39 (3H, s, acetonide CH_3), 1.25 (3H, s, acetonide CH_3), 1.16 (1H, dt, *J* = 12.8, 10.4, 10.4 Hz, H-8a), 0.93 (3H, t, *J* = 6.7 Hz, H-16); HRESIMS *m/z* 381.4585 (calcd for $C_{19}H_{34}NaO_6$ 381.4594).

2.6 Antifungal Test

Solutions of *Cochliobolus miyabeanus* spores provided by Mitsubishi Chemical Corporation were prepared containing 500, 100, 50, 10, 5.0 and 1.0 mg/mL for each compound with 2% sucrose in DMSO. After 36 h at 25°C, germination and the shapes of the spores were observed under a microscope. The IC_{50} values were determined by the concentration which showed 50% inhibition of germination.

3. Results and Discussion

A methanolic extract of culture broth of *Ophiobolus* sp. KTC 2293 was partitioned by ethyl acetate. The concentrated organic fraction was then subjected to a series of silica gel column chromatography to give three new compounds, ophiobolides A (**1**), B (**2**), and C (**3**) as colorless oils along with achaetolide.

The molecular formula of ophiobolide A (**1**) was established to be $C_{16}H_{30}O_6$ by HRESIMS at m/z 341.1941 $[M+Na]^+$. The ^1H, ^{13}C, and HMQC (Heteromolecular Multiple Quantum Coherence) NMR spectra indicated the presence of one ester carbonyl [δ_C 178.1], five oxygenated methines [δ_H 3.59/δ_C 72.4, 3.69/72.9, 3.79/69.2, 4.26/72.7, and 4.59/87.0], nine methylenes, and one methyl group (δ_H 0.89/δ_C 14.4) as shown in Table 1. The connectivity from C-2 to C-16 was addressed by COSY (Correlated Spectroscopy), HMQC, and HMBC (Heteromolecular Multiple Bond Correlation) spectra (Figure 2). HMBC correlations (H-2/C-1, H-3/C-1 and H-4/C-1) suggested the presence of g-butyrolactone which was also corroborated by its characteristic IR absorption at 1778 cm^{-1}. The positions of four hydroxyl groups were confirmed by downfield shifts of oxymethine protons [δ_{H3} 4.26→5.13, δ_{H6} 3.59→5.08, δ_{H7} 4.26→5.15, δ_{H9} 3.79→5.13] in acetate **4** prepared by acetylation (52%) of **1** using acetic anhydride in pyridine. Thus, the

Table 1. NMR spectroscopic data for ophiobolides A (**1**), B (**2**), and C (**3**).

	ophiobolide A (1)[a,c]		ophiobolide B (2)[b,c]		ophiobolide C (3)[b,c]	
No.	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**
1	178.1		175.3		175.0	
2	38.0	2.89, dd (17.9, 6.2) 2.40, dd (17.9, 3.3)	37.4	2.74, dd (18.4, 7.1) 2.61, d (18.4)	35.7	2.77, dd (18.4, 5.5) 2.71, d (18.4)
3	72.7	4.26, ddd (8.3, 6.2, 3.3)	78.1	4.91, dd (7.1, 6.0)	76.9	4.85, dd (5.5, 4.9)
4	87.0	4.59, ddd (10.3, 8.3, 3.4)	82.4	5.15, dd (6.0, 5.9)	83.4	5.10, ddd (6.8, 4.9, 3.0)
5	37.0	1.81, ddd (14.5, 10.3, 2.1) 1.68, ddd (14.5, 10.5, 3.4)	38.5	2.44, d (14.2) 2.28, dd (14.2, 5.9)	39.6	2.46, dd (15.2, 6.8) 2.42, dd (15.2, 3.0)
6	72.4	3.59, ddd (10.5, 5.5, 2.1)	116.7		112.7	
7	72.9	3.69, ddd (9.8, 5.5, 2.5)	76.7	4.14, dd (7.0, 3.0)	73.8	4.06, dd (10.8, 7.5)
8	40.9	1.58, ddd (14.2, 9.7, 2.5) 1.49, ddd (14.2, 9.8, 2.6)	39.9	2.46, dd (13.5, 7.0) 1.45, ddd (13.5, 6.7, 3.0)	38.1	2.38, ddd (13.5, 7.5, 6.5) 1.55, ddd (13.5, 10.8, 9.5)
9	69.2	3.79, m	77.4	3.96, m	76.9	3.96, m
10	39.3	1.45, m, 2H	36.3	1.60, 1.50, m	37.6	1.60, 1.42, m
11	26.8	1.45, 1.31, m	25.9	1.25, m, 2H	25.6	1.25, m, 2H
12	30.5	1.31, m, 2H	29.3	1.25, m, 2H	29.2	1.25, m, 2H
13	30.8	1.31, m, 2H	29.6	1.25, m, 2H	29.5	1.25, m, 2H
14	33.0	1.31, m, 2H	31.8	1.25, m, 2H	31.8	1.25, m, 2H
15	23.7	1.31, m, 2H	22.6	1.25, m, 2H	22.6	1.25, m, 2H
16	14.4	0.89, t (6.9), 3H	14.1	0.86, t (6.8), 3H	14.1	0.86, t (6.9), 3H

[a]in CD_3OD, [b]in $CDCl_3$, [c]Hydrogens of OH were not observed.

planar structure of **1** was determined as shown in Figure 1.

The molecular formula of ophiobolide B (**2**) was established to be $C_{16}H_{28}O_6$ by HRESIMS at m/z 317.1954 $[M+H]^+$. Initial perusal of its ^1H and ^{13}C NMR spectra reveals that compound **2** consists of 16 carbons similar with **1** and has an identical straight alkyl chain, suggesting the presence of similar carbon framework for **2** and **1**. The molecular formula of **2** is less two hydrogens than that of **1**. Compound **2** has a ketal carbon [δ_C 116.7 (C-6)], not an additional sp^2 carbon(s). These findings implied that **2** should be a bicyclic compound produced by oxidation and subsequent cyclic ketal formation of **1**. The structure of **2** was elucidated on the basis of the 2D NMR spectroscopic data (Figure 2, Table 1). The COSY and HMQC correlations indicated the connectivity of C-2 to C-5 and C-7 to C-16. The presence of γ-butyrolactone was confirmed by HMBC correlations such as H-2/C-1, H-3/C-1 and H-4/C-1 as well as IR adsorption at 1770 cm^{-1}. The linkage to C-6 hemiketal carbon was connected by the HMBC correlations such as H-3/C-6, H-5/C-6, H-5/C-7, and H-7/C-6. Thus, the planar structure of **2** was determined as shown in Figure 1.

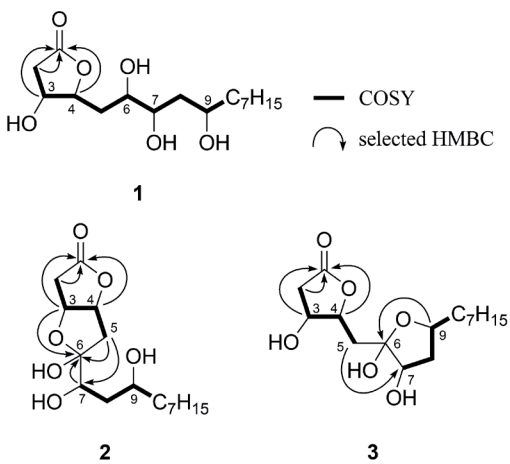

Figure 2. COSY and HMBC correlations of ophiobolides A-C.

The molecular formula of ophiobolide C (**3**) was identical ($C_{16}H_{28}O_6$) with that of **2** by HRESIMS at m/z 339.1786 $[M+Na]^+$. Furthermore, compound **2** easily transformed to **3** (and *vise versa*) to become a 2:3 mixture in NMR tube upon standing for 2-3 days, indicating that compound **3** is an interconvertible isomer of **2**. An HMBC correlation (H-9/C-6) indicated a location of 5-membered hemiketal ring in **3** to be different from that of **2** as shown in Figure 1. This structure could account for all the spectroscopic data of **3** without contradiction.

NOE (Nuclear Overhauser Effect) experiments of ophiobolide A (**1**) and its derivatives led us to disclose its relative configuration. Remarkable NOEs [H-3 to H-2a (5.3%), H-5a (2.4%) and H-5b (4.2%); H-5a to H-2a (3.4%) and H-3 (3.0%)] in **1** were observed to reveal a *trans*-relationship (3S^*,4R^*) in g-butyrolactone (Figure 3A), although the coupling constant between H-3 and H-4 (8.3 Hz) did not provide

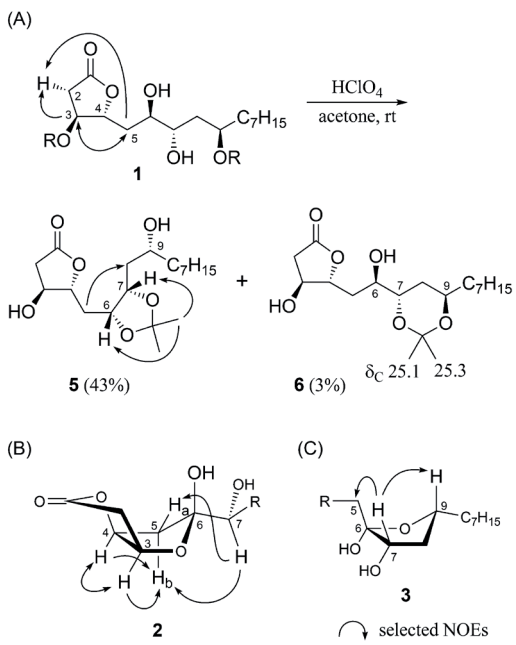

Figure 3. Relative configurations of ophiobolides A-C; (A) NOE experiments of **1** and preparations of acetonides **5** and **6**, (B) NOE experiments of **2**, (C) NOE experiments of **3**.

definitive information. On the other hand, in order to determine the relative configuration of right half in **1**, 6-*O*-7-*O*-isopropylidene **5** (43%) and 7-*O*-9-*O*-isopropylidene **6** (3%) were prepared by treating **1** with $HClO_4$ in acetone (Figure 3A). In NOE experiments of **5**, crutial NOEs [H-5a to H-8a (3.1%) and H-8b (0.9%); an acetal methyl to H-6 (3.4%) and H-7 (2.5%)] suggested *cis*-relationship on the dioxolane ring. The configuration of C7/C-9 moiety was determined using the empirical rule proposed by Rychnovsky and co-workers [7]. The HMQC spectrum showed the chemical shifts of two acetal methyls in **6** to be 25.1 and 25.3 ppm, suggesting a 1,3-*trans* relationship of C-7/C-9. These results clarified the configuration of C-6/C-9 moiety in **1** to be 6*R**,7*S**,9*R**.

The relative configurations of ophiobolide B (**2**) and C (**3**) were also elucidated using their NOE experiments. As described above, compounds **2** and **3** are readily convertible to each other. The corresponding configurations, excepting C-6 ketal carbon, must be same. The relative configuration of C-3/C-4 in **2** must be *cis* due to structural requirement of its 1,4-dioxabicycle[3.3.0]octa-2-one moiety (Figure 3B). Remarkable NOEs in **2** [H-3 to H-4 (3.6%) and H-5b (0.5%); H4 to H-3 (2.7%) and H-5b (3.2%); H7 to H-5a (5.1%) and H-5b (0.6%)] suggested C-3/C-6 in **2** to be 3*S**,4*S**,6*S**, suggesting C-3/C-4 in **3** should be also 3*S**,4*R**. Crucial NOEs in **3** [H-7 to H-5a (2.2%) and H-9 (1.3%); H-9 to H-7 (1.0%)] were observed to reveal C-6/C-9 on the hemiketal ring to be 6*R**,7*S**,9*R** (Figure 3C), indicating C-7/C-9 in **2** to be 7*S**,9*R**. Thus, thorough NOE experiments allowed us to establish the relative configuration in **2** and **3** as shown in Figure 1.

Although the genetic basis for the productions of both achaetolide and ophiobolides is not yet identified, their structural similarity indicates that compound **7** is a plausible common precursor for these compounds (Figure 4). Lactonization (C-1/C-9) leads compound **7** to achaetolide, while a conjugated addition at C-4 to ophiobolides. In latter case, the attack to C-4 re face followed by a reduction of C-6 carbonyl provides ophiobolide A. On the other hand, compound **8** obtained by the si face attack successively suffers a hemiacetal formation with neighboring C-3 or C-9 hydroxyls to give ophiobolides B and C, respectively. Thus, it is probable that the absolute configurations of ophiobolides are same with that of achaetolide as shown in Figure 1 due to the biosynthetic consideration. In order to elucidate the absolute configuration of ophiobolides, it was tried to prepare the corresponding bis-(*S*)- and bis-(*R*)-MTPA (methoxy-trifluoromethyl-phenyacetyl) esters of **5** for the modified Mosher's method. Unfortunately, the reaction yields were miserable and sufficient amounts of these MTPA esters have not been obtained to date. In order to identify the process, further exploration of plausible precursors such as **7** and **8** are actively pursued in our laboratory.

Finally, ophiobolides and achaetolide were subjected to antifungal test against *Cochliobolus miyabeanus*. Among them, compound **2** showed a weak activity with an IC_{50} value of 0.5 mg/mL. Further biological properties of these compounds are now under investigation in our laboratory.

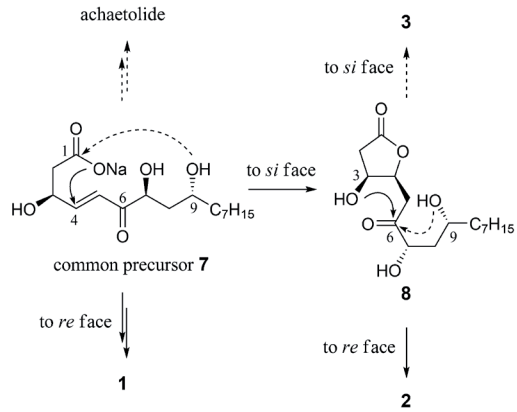

Figure 4. Plausible biosynthesis processes for ophiobolides and achaetolide.

4. Conclussion

We succeeded in isolations of three novel achaetolide analogs from the culture broth of *Ophiobolus* sp. KTC 2293. The structures were elucidated on the basis of spectral analyses. Ophiobolide B showed a weak antifungal activity against *Cochliobolus miyabeanus*.

Acknowledgements

Part of this work was supported by a Grant for Priority Research designated by the President of Hirosaki University. We thank Mr. Takaharu Okada and Ms. Eri Kogawa for their technical assistance.

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