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ANTIFUNGAL COMPOUND AGAINST AZOLE-RESISTANT *CANDIDA ALBICANS* FROM A MARINE-DERIVED FUNGUS, *PARABOEREMIA SELAGINELLAE*

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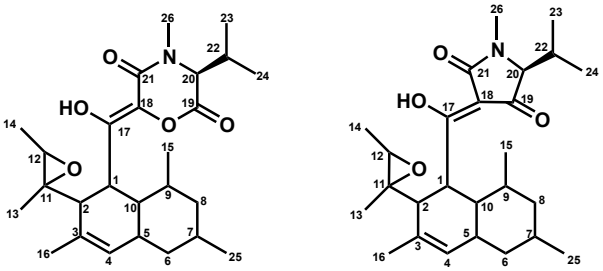
Abstract – A new compound (**1**) and AB4063-A (**2**), whose stereochemistry was unstated, were isolated from the marine-derived fungus *Paraboeremia selaginellae.* The structure of **1** was elucidated from spectroscopic data (NMR, MS, IR), and the absolute configuration of the *N*-methylvaline moiety was determined. Compound **2** showed antifungal activity against azole-resistant *Candida albicans.*

Clinically used antifungal drugs are very limited compared to antibacterial and antiviral drugs. Azole antifungals are the most widely used to treat fungal infections. However, the number of azole-resistant fungal strains is increasing, which causes clinical problems. Thus, novel antifungal drugs are needed.[1] We investigated the antifungal activities of marine-derived fungal metabolites. Marine-derived fungi have great potential as a source of drugs because they produce a diversity of natural products.[2] We previously reported several novel compounds from marine-derived fungi including phomactins,[3-5] didymellamides A-D,[6] and myrocin D.[7] In this paper, we report a new compound **1** and a compound **2** for which the planar structure is known. Isolated compounds were evaluated for their antifungal activities. Compound **2** showed antifungal activity against azole-resistant *Candida albicans* (minimum inhibitory concentration (MIC) was 2.0 μg/mL).



1 **2**

Paraboeremia selaginellae was grown on an artificial seawater-barley medium, then extracted with $CHCl_3$. The $CHCl_3$ extract showed antifungal activity against azole-resistant *C. albicans*. The $CHCl_3$ extract was subjected to silica gel column chromatography and ODS column chromatography by activity-guided separation and compounds **1** and **2** were obtained.

The molecular formula of compound **1** was determined to be $C_{26}H_{39}NO_5$ by HRFABMS and NMR data, which indicated 8 degrees of unsaturation. The ^1H and ^{13}C NMR (Table 1), DEPT, and HMQC spectra revealed the presence of eight methyls, two methylenes, ten methines including one olefinic methine, two oxygenated quaternary carbons, two carbonyls, and three other sp^2 quaternary carbons, which accounted for four of the 8 degrees of unsaturation. Thus, compound **1** was determined to have four rings in the structure. COSY correlations between δ_H 2.22 and δ_H 3.56, δ_H 3.56 and δ_H 2.09, and δ_H 2.09 and δ_H 2.02 were observed and the connections from H-2, H-1, and H-10 were clarified (Figure 1). The connectivities from H-4 to H-8, from H-25 to H-7, and from H-15 to H-9 were determined by the COSY correlations as shown in Figure 1. The HMBC correlations from the methine proton at δ_H 3.56 (H-1) to the methine carbon at 40.0 (C-5), from the olefinic methine proton at δ_H 5.57 (H-4) to the methine carbon at δ_C 52.6 (C-2), from methyl protons at δ_H 0.99 (H_3-15) to the methylene carbon at δ_C 38.0 (C-8), from the methyl protons at δ_H 1.58 (H_3-16) to the olefinic quaternary carbon at δ_C 130.9 (C-3), from the methine proton at δ_H 2.22 (H-2) to the methyl carbon at δ_C 21.6 (C-16), and from the methylene protons at δ_H 1.63 (H-6) to the methine carbon at δ_C 39.6 (C-10) were observed. These COSY and HMBC correlations allowed us to elucidate the structure of the decalin derivative moiety. The presence of an epoxide was indicated by the chemical shifts of δ_C 61.8 (C-11) and δ_C 59.5 (C-12) having an HMQC correlation with δ_H 2.80. Moreover, the methyl protons at δ_H 1.25 (H_3-14) had a COSY correlation with the methine proton at δ_H 2.80 (C-12), and the HMBC correlation from the methyl protons at δ_H 1.58 (H_3-13) and δ_H 1.25 (H_3-14) to the quaternary carbon at δ_C 61.8 (C-11) confirmed the epoxide moiety. The HMBC correlation from the methine proton at δ_H 3.56 (H-1) to the quaternary carbon at δ_C 61.8 showed that the epoxide moiety was bonded to C-2. The position of the last ring was suggested by the HMBC correlations from the proton of the hydroxy group at δ_H 12.5 to the

methine carbon at δ_C 38.0 (C-1) and olefinic quaternary carbon at δ_C 122.1 (C-18), and the methine protons at δ_H 3.56 (H-1) and δ_H 2.09 (H-10) to the olefinic quaternary carbon at δ_C 164.7 (C-17). The last ring was linked via this quaternary carbon at δ_C 164.7 (C-17).

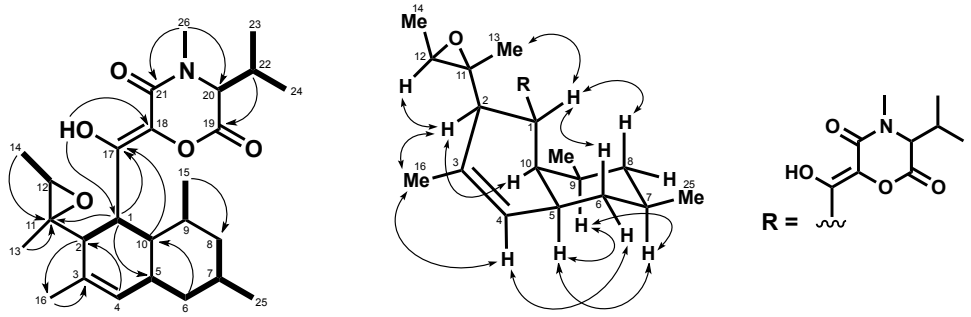


Figure 1. Key DQF COSY (bold), HMBC (arrows), and NOESY (double arrows) correlations for **1**

The last ring was elucidated by subtracting the previous structure from the molecular formula of compound **1** and the presence of three methyls including an *N*-methyl, two carbonyl carbons, a quaternary carbon, and one oxygen was considered. The presence of an isopropyl was deduced from COSY correlations shown in Figure 1 and the presence of an *N*-methyl was indicated by the chemical shifts of δ_C 32.8 and δ_H 3.01. The IR spectrum exhibited absorption at 1759 cm^{-1} (carbonyl) and 1643 cm^{-1} (amido). HMBC correlations from the *N*-methyl protons δ_H 3.01 to the carbonyl carbon at δ_C 162.5 (C-21) and methine carbon at δ_C 66.9 (C-20), and from the methine proton of the isopropyl moiety at δ_H 2.25 to the carbonyl carbon at δ_C 161.2 (C-19) were observed. The chemical shift value of the carbonyl carbon at δ_C 161.2 suggested the presence of an ester. These data indicated the structure of 3-isopropyl-4-methylmorpholine-2,5-dione. The absolute configuration of the *N*-methylvaline moiety in the structure of 3-isopropyl-4-methylmorpholine-2,5-dione was determined to be *N*-methyl-L-valine by Marfey's analysis.[10] The relative stereochemistry of **1** was deduced by NOESY correlations between H-5 and H-7, H-5 and H-9, and H-7 and H-9. These correlations suggested that H-5, H-7, and H-9 were in the 1,3-diaxial orientation (Figure 1). The *cis* orientation of H-5 and H-10 was suggested by NOESY correlations between H-1 and H-6β, H-1 and H-8β, H-2 and H-10, H-4 and H-6α, and H-4 and H-16. Moreover, NOESY correlations between the methyl protons at δ_H 1.19 (H_3-13) and the methine proton at δ_H 3.56 (H-1), and the methine proton at δ_H 2.80 (H-12) and the methine proton at δ_H 2.22 (H-2) were observed, indicating that the methyl group of the epoxy moiety was in the *cis* orientation. Hence, the structure of **1** including the relative configuration of the decalin derived moiety and epoxy part, and absolute configuration of the *N*-methyl-L-valine, were determined as shown in Figure 1. **1** is a new compound and named selaginemine A.

The molecular formula of compound **2** was determined to be $C_{26}H_{39}NO_4$ by HRFABMS and NMR data, which indicated 8 degrees of unsaturation. The IR spectrum exhibited absorptions at 1700 cm^{-1} (carbonyl) and 1642 cm^{-1} (amido). The ^1H and ^{13}C NMR (Table 1), DEPT, and HMQC spectra revealed the presence of eight methyls, two methylenes, ten methines including one olefinic methine, one oxygenated quaternary carbon, two carbonyls, and two other sp^2 quaternary carbons, which accounted for four of the 8 degrees of unsaturation. These data (Table 1) indicated that the structure of **2** was similar to compound **1** except for the ring including nitrogen. DQF-COSY correlations were observed between H-1 and H-2, H-1 and H-10, H-6 and H-7, and H-7 and H-8 (Figure 2). The HMBC correlations from H-4 to C-5, H-10 to C-5, H-15 to C-8 and C-10, H-16 to C-2, and H-25 to C-7 suggested the presence of a decalin derivative moiety (Figure 2). The presence of an epoxide was confirmed by the chemical shifts of C-11 (δ_C 62.2) and C-12 (δ_C 60.5, δ_H 2.80). The HMBC correlation from H-14 to C-11 and C-12 suggested the presence of methyl group at epoxy moiety. The HMBC correlation from H-1 to C-11 suggested the linkage between C-2 and C-11. The presence of an *N*-methyl was indicated by the chemical shift of C-26 (δ_C 27.2, δ_H 2.91). The HMBC correlations from H-20 to C-19, C-21, C-22, C-23 and C-24, H-23 to C-22 and C-24, H-24 to C-22 and C-23, and H-26 to C-20 and C-21 were obtained. The molecular formula of compound **2** ($C_{26}H_{39}NO_4$) has one less oxygen than compound **1** ($C_{26}H_{39}NO_5$). The chemical shift value of one carbonyl carbon appeared at δ_C 194.0 (C-19) instead of δ_C 161.2 (C-19) for compound **1**. Thus, the structure of 5-isopropyl-1-methylpyrrolidine-2,4-dione was considered. The planar structure of compound **2** had been reported as AB4063-A in a Japanese patent,[8] and the NMR and MS data for compound **2** were in good agreement with those for AB4063-A.

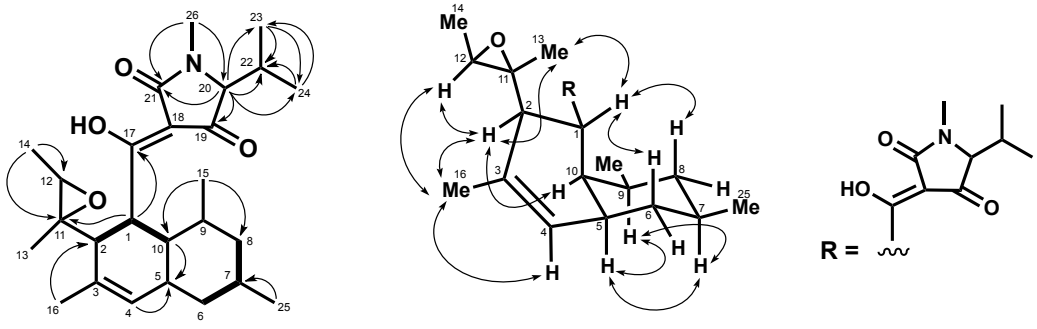
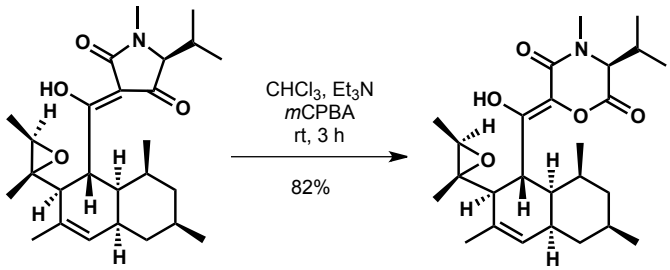


Figure 2. Key DQF COSY (bold), HMBC (arrows), and NOESY (double arrows) correlations of **2**

However, the ^1H and ^{13}C NMR chemical shift values in the patent were not assigned. Hence, the structure of **2** was identified as AB4063-A, and all proton and carbon chemical shifts were assigned for the first time. Compound **1** was derived by Baeyer-Villiger oxidation using compound **2**, supporting its

assigned structure. The relative configuration of compound **2** was considered to be the same as that of compound **1**.



2
38.6 mg

1
32.5 mg

Scheme 1. Baeyer-Villiger oxidation of **2**

Compounds **1** and **2** were evaluated for antifungal activities against azole-resistant *C. albicans* J2-36. Compound **2** inhibited growth of azole-resistant *C. albicans* with a MIC of 2 μg/mL, whereas compound **1** did not exhibit any antifungal activity at a concentration of 10 μg/mL. Although the antifungal activity of compound **2**[8,11] was known, its activity against azole-resistant *C. albicans* J2-36 is reported here for the first time.

Table 1. ^{1}H and ^{13}C NMR Data for **1** and **2** in $CDCl_3$

	1			**2**		
position	δ_C	type	δ_H (mult., *J* in Hz)	δ_C	type	δ_H (mult., *J* in Hz)
1	31.9	CH	3.56 (t, 10.7)	35.6	CH	4.35 (t, 10.7)
2	52.6	CH	2.22 (d, 10.7)	54.0	CH	2.27 (m)
3	130.9	C		130.6	C	
4	130.7	CH	5.57 (d, 5.8)	130.7	CH	5.59 (d, 5.5)
5	40.0	CH	2.02 (m)	40.1	CH	2.02 (m)
6	37.9	CH_2	α 0.91 (m)	37.7	CH_2	α 1.64 (m)
			β 1.63 (m)			β 1.05 (m)
7	33.2	CH	1.46 (m)	33.2	CH	1.44 (m)
8	37.9	CH_2	α 1.28 (m)	37.7	CH_2	α 1.03 (m)
			β 0.97 (m)			β 1.26 (m)
9	38.0	CH	1.72 (m)	37.9	CH	1.64 (1H, m)
10	39.6	CH	2.09 (m)	41.9	CH	2.15 (dt, 3.2, 10.7)
11	61.8	C		62.2	C	
12	59.5	CH	2.80 (q, 5.4)	60.5	CH	2.80 (q, 5.5)
13	11.6	Me	1.19 (s)	11.7	Me	1.15 (s)
14	13.6	Me	1.25 (d, 5.4)	13.6	Me	1.23 (d, 5.5)
15	20.1	Me	0.99 (d, 7.2)	20.3	Me	0.88 (d, 7.5)
16	21.6	Me	1.58 (s)	21.4	Me	1.57 (s)
17	164.7	C		190.0	C	
18	122.2	C		103.9	C	
19	161.2	C		194.0	C	
20	66.9	CH	3.93 (d, 4.3)	71.1	CH	3.59 (d, 3.1)
21	162.5	C		173.8	C	
22	31.7	CH	2.25 (m)	29.1	CH	2.21 (m)
23	17.8	Me	1.00 (d, 6.9)	17.2	Me	0.91 (d, 7.1)
24	19.4	Me	1.14 (d, 6.9)	17.5	Me	1.13 (d, 7.1)
25	22.4	Me	0.93 (d, 6.7)	22.3	Me	0.96 (d, 6.4)
26	32.8	Me	3.01 (s)	27.2	Me	2.91 (s)
17-OH			12.50 (brs)			

EXPERIMENTAL

General Experimental Procedures

Optical rotation was measured with a Horiba SEPA-300 polarimeter. UV spectra were recorded with a Thermo Genesys 10S UV-vis spectrophotometer. IR spectra were recorded with a Thermo FT-IR Nicolet iS5 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with JNM-ECA500 spectrometers using tetramethylsilane as the internal standard. Low- and high-resolution FABMS spectra were measured with a JEOL JMS-700 spectrometer. Column chromatography was performed using silica gel 60N (Kanto Chemical) and ODS (YMC). HPLC was performed using a JASCO PU2089 PLUS pump equipped with a JASCO PU2075 PLUS detector at 340 nm, with an ODS (SHISEIDO CAPCELLPAK C_{18} MG II S5 4.6 mm × 250 mm) column.

Fungal Material

The fungus was isolated from the surface of an unidentified marine alga collected off the Senbonhama coast of Numazu-shi, Shizuoka Prefecture, Japan, in July 2006. The isolate was identified by DNA sequence analyses of the genes encoding β-tubulin and the rRNA internal transcribed spacer (ITS) region. The DNA sequences of b-tubuline (tub2) and ITS rDNA of the fungus were identical to those of the type strain of *Paraboeremia selaginellae* (GenBank accession numbers: GU237656 for tub2 and NR135980 for ITS). A voucher specimen *Paraboeremia selaginellae* (MPUC229) was deposited at the Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University.

Fermentation

Paraboeremia selaginellae was inoculated into 1000 mL Roux flasks (18 flasks) containing wheat (150 g per flask) and artificial seawater (Instant Ocean, 50 mL per flask). The flasks were incubated at 26 °C in the dark for 20 days.

Extraction and Isolation

The fermented wheat substrate was extracted with $CHCl_3$. The $CHCl_3$ extract (140.44 g) was fractionated by silica gel column chromatography (7 × 17 cm, *n*-hexane−acetone−MeOH, step gradient elution from 1:0:0, 10:1:0, 5:1:0, 3:1:0, 1:1:0, 0:0:1) to obtain seven fractions, p1−p7. Fraction p5 (2.35 g) was subjected to ODS column chromatography (3 × 10 cm, MeCN−H_2O−$CHCl_3$, step gradient elution from 8.5:1.5:0, 9:1:0, 9.5:0.5:0, 1:0:0, 0:0:1) to obtain four fractions, p5-1−p5-4. p5-2 (277.7 mg) was chromatographed on an ODS column (2 × 17 cm, MeCN−H_2O−$CHCl_3$, step gradient elution from 8.5:1.5:0, 9:1:0, 9.5:0.5:0, 0:0:1) to obtain six fractions, p52-a−p52-f. p52-b (63.3 mg) was fractionated by silica gel column chromatography (2 × 18 cm, *n*-hexane−acetone−MeOH, step gradient elution from 5:1:0, 4:1:0, 3:1:0, 2:1:0, 0:1:0, 0:0:1) to obtain six fractions, p52b-1−p52b-6, and afforded compound **1** from fraction p52b-2 (11.1 mg). p52-c (103.8 mg) was subjected to silica gel column chromatography (2 × 16 cm, *n*-hexane−acetone−MeOH, step gradient elution from 5:1:0, 4:1:0, 3:1:0,

2:1:0, 1:1:0, 0:1:0, 0:0:1) to obtain five fractions, p52c-1−p52c-5, and afforded compound **2** (26.0 mg) from fraction p52c-3.

Compound **1**: pale yellow oil; $[\alpha]^{27}_{D}$ +6.8 (*c* 1.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 245 (4.26), 275 (4.21) nm; IR (ATR) ν_{max} 2961, 1759, 1262 cm^{-1}; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$), shown in Table 1; FABMS *m/z*: 446 $[M+H]^+$, 428, 374; HR-FABMS *m/z*: 446.2907 $[M + H]^+$ (calcd for 446.2906, $C_{26}H_{40}NO_5$).

Compound **2**: pale yellow oil; UV (MeOH) λ_{max} (log ε) 203 (3.84), 292 (3.99) nm; IR (ATR) ν_{max} 2960, 1604, 1452 cm^{-1}; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$), shown in Table 1; FABMS *m/z*: 430 $[M+H]^+$, 412, 358; HR-FABMS *m/z*: 430.2960 $[M+H]^+$ (calcd for 430.2955, $C_{26}H_{40}NO_4$).

Baeyer-Villiger Oxidation

To a solution of compound **2** (38.6 mg, 93.41 μmol) in $CHCl_3$ (3.0 mL) were added a solution of *m*CPBA (77.2 mg, 81.83 μmol) in $CHCl_3$ (900 μL) and triethylamine (390 μL, 2.798 mmol) at room temperature. The mixture was stirred for 3 h. The reaction was quenched by the addition of saturated sodium thiosulfate (4.0 mL). The mixture was extracted three times with $CHCl_3$. The combined organic layers were washed with water, dried over magnesium sulfate and filtered. The filtrate was concentrated under reduced pressure to give compound **1** (32.5 mg, 73.03 μmol) in 82% yield.

Marfey's Analysis

To a solution of compound **1** (0.5 mg, 1.21 μmol) in MeOH (500 μL) was added 12 M HCl (500 μL). The mixture was heated at 100 °C for 17 h. The mixture was cooled to room temperature and neutralized with 12 M NaOH solution (500 μL). The resulting aqueous amino acid containing solution was treated by addition of 100 μL of a 1% (w/v) solution of N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in acetone, 1 M $NaHCO_3$ (50 μL) and H_2O (100 μL). The mixture was heated at 40 °C for 1 h, then cooled to room temperature, and neutralized with 1 M HCl (50 μL) to give the sample for the analysis. Aliquots (10 μL) of the sample were then analyzed by HPLC with an ODS column using an elution system consisting of solvent A (0.1% TFA/10% MeCN in H_2O) and solvent B (0.1% TFA/90% MeCN in H_2O). The sample was eluted using a linear gradient of 0% to 10% over the course of 10 min, then 10% to 50% over the course of 20 min, and finally 50% to 100% over the course of 15 min. The flow rate was kept constant at 1 mL/min, and UV detection was employed at 340 nm. Amino acid standards (*N*-methyl-L-valine and *N*-methyl-DL-valine) were prepared by dissolving 50 μg of amino acids in H_2O (50 μL) followed by treatment as above using a 1% solution (50 μL) of FDAA in acetone, 1 M $NaHCO_3$ (25 μL) and 1 M HCl (25 μL) for neutralization. The mixture was then processed for HPLC in a fashion similar to that used for sample hydrolysate analyses. The retention times for FDAA derivatives of *N*-methyl-D-valine and *N*-methyl-L-valine were 32.5 and

31.1 min, respectively. The *N*-methylvaline fragment in compound **1** was assigned as *N*-methyl-L-Val (t_R 31.3 min).

Compound Susceptibility Testing

Azole-resistant *C. albicans* J2-36 (resistant to fluconazole, itraconazole, and voriconazole) was examined. The minimum inhibitory concentration (MIC) for growth was determined as the lowest antifungal concentration that caused ≥90% reduction in growth of the fungus. Susceptibility testing was performed in triplicate according to the CLSI M27-A2 microdilution method.[12]

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