# Periconicins, Two New Fusicoccane Diterpenes Produced by an Endophytic Fungus *Periconia* sp. with Antibacterial Activity

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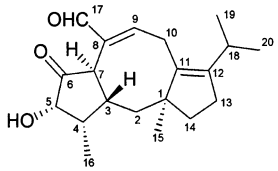
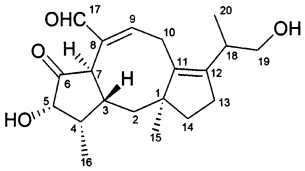
Two new fusicoccane diterpenes, named periconicins A (**1**) and B (**2**), with antibacterial activities have been isolated by bioassay-guided fractionation from an endophytic fungus *Periconia* sp., collected from small branches of *Taxus cuspidata*. The structures of the new compounds were determined by combined spectroscopic methods.

Endophytic fungi are widely recognized as prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents.[1−5] Since more than $1.5 \times 10^6$ endophytic fungi are thought to thrive inside the estimated 270 000 species of vascular plants, the prospects for additional discoveries of fungal metabolites are bright.[6]

In connection with our program on the isolation of new biologically active metabolites from microbes, we have characterized two new fusicoccane diterpene antibiotics, trivially named periconicins, from the culture broth of an endophytic fungus OBW-15. The strain (*Periconia* sp.)[7] was isolated from the inner bark of *Taxus cuspidata*. NMR data were used to characterize the structures of periconicins A and B (**1** and **2**).



**Periconicin A (1)**



**Periconicin B (2)**

The crude EtOAc extract of fungal culture broth was passed through an open column of silica gel, further purified by preparative TLC, and crystallized from EtOAc to obtain pure compounds (**1** and **2**). Periconicin A (**1**) was obtained as a white solid. The HREIMS assigned the molecular formula as $C_{20}H_{28}O_3$, indicating seven degrees of unsaturation. The IR spectrum exhibited absorption bands for hydroxyl ($\nu_{max}$ 3430 $cm^{-1}$) and carbonyl ($\nu_{max}$ 1630, 1750 $cm^{-1}$) groups. The $^1H$ NMR spectrum of **1** displayed four methyl resonances at $\delta_H$ 0.98 (s, 3H), 0.76 (d, $J$ = 7.2 Hz, 3H), 0.95 (d, $J$ = 6.7 Hz, 3H), and 0.96 (d, $J$ = 6.8 Hz, 3H). In addition, distinctive proton signals for an olefinic methine at $\delta_H$ 7.01 (dt, $J$ = 0.8, 8.8 Hz, 1H), an oxymethine at $\delta_H$ 4.80 (d, $J$ = 7.8 Hz, 1H), and an aldehyde at $\delta_H$ 9.16 (s, 1H) were observed. The $^{13}C$ NMR spectrum of **1** showed 20 carbon signals, which were classified into four methyl, four methylene, seven methine, and five

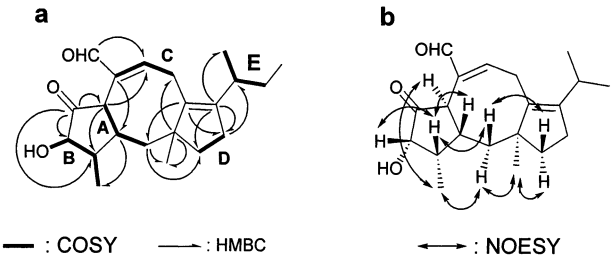



**Figure 1.** Selected HMBC and NOESY correlations of **1**.

quaternary carbons, including one quaternary $sp^3$, three olefinic, and one cabonyl carbon (Table 1).

The $^1H-^1H$ spin systems and one-bond $^1H-^{13}C$ connectivites were analyzed by $^1H-^1H$ COSY and HMQC data, respectively (Table 1). On the basis of the combined $^1H$ COSY and HMQC analyses, the following five fragments were assigned: >CH−CH($CH_2$−)− (**A**), −CH($CH_3$)−CH(OH)− (**B**), >C=CH−$CH_2$− (**C**), −$CH_2$−$CH_2$− (**D**), and −CH($CH_3$)$_2$ (**E**) (Figure 1a). Long-range $^1H-^{13}C$ couplings ($^2J_{C-H}$ and $^3J_{C-H}$) obtained from the HMBC experiment supported the connectivity of these spin systems (Table 1 and Figure 1a). Long-range $^1H-^{13}C$ couplings between $\delta_C$ 50.66 (C-1) and five $^1H$ resonances at $\delta_H$ 0.98 (H-15), 1.84 (H-2$\beta$), 1.70 (H-2$\alpha$), 2.00 (H-3), and 2.08 (H-13$\beta$) revealed the quaternary carbon (C-1) to be attached to the fragments **A** and **D** and one methyl group (C-15). Further analysis suggested that the methine carbon (C-3) in fragment **A** was connected with the methine carbon (C-4) in fragment **B**, while the methine carbon (C-7) in fragment **A** was connected with the olefinic quaternary carbon (C-8) in fragment **C**. Also, long-range $^1H-^{13}C$ couplings between $\delta_C$ 216.7 (C-6) and three $^1H$ resonances at $\delta_H$ 2.43 (H-4), 4.81 (H-5), and 2.65 (H-7) indicated the connections of C-4, C-5, and C-7 to the C-6 quaternary carbonyl carbon. The connectivites of the fragments **A** and **C** and one aldehyde carbon (C-17) were determined by the relationships between $\delta_C$ 48.0 (C-7) and a $^1H$ resonance at $\delta_H$ 7.01 (H-9), $\delta_C$ 141.3 (C-8), a $^1H$ resonance at $\delta_H$ 2.00 (H-3), $\delta_C$ 192.2 (C-17), and a $^1H$ resonance at $\delta_H$ 7.01 (H-9). Furthermore, the quaternary olefinic carbon (C-8) connected with the aldehyde carbon (C-17) and methine carbon (C-7) in fragment **A**. The connectivity of the remaining olefinic carbons (C-11 and C-12) with fragment **C** (C-10), fragment **E** (C-18), fragment **D** (C-13), and the quaternary carbon (C-1) was also determined by HMBC data analysis as shown in Table 1. Analysis of these data suggested that compound

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**Table 1.** NMR Spectral Data of Periconicin A (**1**) and B (**2**) in $CDCl_3$

| | periconicin A, **1** | | | | periconicin B, **2** | |
|---|---|---|---|---|---|---|
| | $^{13}C$ | $^{1}H$ | HMBC | NOESY | $^{13}C$ | $^{1}H$ |
| 1 | 50.6 (C) | | H-2, H-3, H-10$\beta$, H-13$\beta$, H-15 | | 50.9 (C) | |
| 2$\beta$ | 45.5 ($CH_2$) | 1.84 (d, 15.1) | H-3, H-7, H-14$\beta$, H-15 | H-4, H-15, H-16 | 45.3 ($CH_2$) | 1.86 (d, 15.0) |
| 2$\alpha$ | | 1.70 (dd, 9.9, 15.2) | | H-7, H-10$\alpha$, H-15, H-16 | | |
| 3 | 37.5 (CH) | 2.00 (ddd, 6.2, 9.5, 9.5) | H-2$\alpha$, H-7, H-16 | H-4, H-5, H-14$\beta$ | 37.6 (CH) | 1.98 (ddd, 6.3, 9.6, 9.6) |
| 4 | 39.0 (CH) | 2.43 (ddq, 6.9, 6.9, 7.1) | H-2$\alpha$, H-5, H-16 | H-2$\beta$, H-3, H-5 | 39.0 (CH) | 2.42 (ddq, 6.9, 6.9, 7.2) |
| 5 | 79.3 (CH) | 4.81 (d, 7.8) | H-4, H-16 | H-3, H-4 | 79.3 (CH) | 4.78 (d, 7.8) |
| 6 | 216.7 (C) | | H-4, H-5, H-7 | | 216.7 (C) | |
| 7 | 48.0 (CH) | 2.65 (d, 9.3) | H-2, H-4, H-9, H-17 | H-2$\alpha$, H-16 | 48.0 (CH) | 2.63 (d, 9.3) |
| 8 | 141.3 (C) | | H-3, H-10, H-17 | | 141.1 (C) | |
| 9 | 156.3 (CH) | 7.01 (dt, 0.8, 8.8) | H-10 | H-17, H-19, H-20 | 156.6 ($CH_2$) | 7.14 (dt, 0.9, 8.7) |
| 10$\beta$ | 24.6 ($CH_2$) | 2.66 (dd, 9.2, 13.0) | | H-19, H-20 | 24.4 ($CH_2$) | |
| 10$\alpha$ | | 3.05 (dd, 8.4, 12.5) | | H-2$\alpha$ | | 3.07 (dd, 8.4, 12.3) |
| 11 | 137.8 (C) | | H-2$\alpha$, H-10, H-13, H-14$\alpha$, H-15 | | 139.5 (C) | |
| 12 | 144.2 (C) | | H-13, H-14$\alpha$, H-18, H-19, H-20 | | 142.9 (C) | |
| 13$\beta$ | 26.3 ($CH_2$) | 2.08 (m) | H-14, H-18 | H-14$\beta$, H-19, H-20 | 26.6 ($CH_2$) | 2.04 (m) |
| 13$\alpha$ | | 2.19 (m) | | H-19, H-20 | | |
| 14$\alpha$ | 36.2 ($CH_2$) | 1.52 (m) | H-2$\beta$, H-15 | H-15 | 36.0 ($CH_2$) | 1.53 (m) |
| 14$\beta$ | | 1.58 (m) | | H-3, H-13$\beta$ | | |
| 15 | 26.5 ($CH_3$) | 0.98 (s) | H-2, H-14 | H-2, H-14$\alpha$ | 26.4 ($CH_3$) | 1.00 (s) |
| 16 | 7.3 ($CH_3$) | 0.76 (d, 7.2) | H-5 | H-2, H-7 | 7.3 ($CH_3$) | 0.75 (d, 7.2) |
| 17 | 192.2 (CH) | 9.16 (s) | H-7, H-9 | H-9 | 192.6 (CH) | 9.15 (s) |
| 18 | 28.4 (CH) | 2.61 (heptet, 6.8) | H-19, H-20 | | 36.9 (CH) | 2.69 (m) |
| 19 | 21.9 ($CH_3$) | 0.96 (d, 6.8) | H-18, H-20 | H-9, H-10$\beta$, H-13 | 65.8 ($CH_2$) | 3.50 (m) |
| 20 | 20.9 ($CH_3$) | 0.95 (d, 6.7) | H-18, H-19 | H-9, H-10$\beta$, H-13 | 15.2 ($CH_3$) | 0.94 (d, 6.7) |

**1** has a 5−8−5 ring system and is related to the fusicoccane diterpene class.[8−11] Interpretation of the NOESY data (Table 1 and Figure 1b) allowed us to assign the relative stereochemistry of **1**. The signal for $H_3$-16 showed NOESY correlations with H-7 and H-2$\alpha$, while the signal for H-4 exhibited correlations with H-5, H-3, and H-2$\beta$, which further coupled to H-14$\beta$. These data suggested the relative configuration of ring A as shown in Figure 1. NOESY correlations of $H_3$-15 with H-2$\alpha$ and H-14$\alpha$, combined with those of H-2$\beta$ with H-4 and H-14$\beta$, supported the $\alpha$-orientation of the C-15 methyl group.

Periconicin B (**2**) had a molecular formula $C_{20}H_{28}O_4$ as determined by HREIMS. The $^1H$ NMR data of **2** were highly compatible with those of **1**, implying that **2** could be a congener of **1** with one additional oxygen atom. The $^1H$ NMR spectrum of **2** displayed a new two-proton oxymethylene signal at $\delta_H$ 3.50 (H-19), and a methylene carbon signal appeared at $\delta_C$ 65.81 in the $^{13}C$ NMR spectrum (Table 1). The new methylene was assigned to C-19 on the basis of $^1H-^1H$ COSY data, in which correlation was found between H-18 and H-19.

Periconicins A and B (**1** and **2**) have the same carbon skeleton as the fusicoccins, which are a group of plant growth regulators isolated from the cultures of fungus *Fusicoccum amydali*.[8,9] The cotylenins possess the same carbon skeleton, which are glycosidal leaf growth substances of fungal origin.[12] The ophiobolins also have the same carbon skeleton.[10] It is intriguing to note that periconicins have the trans relative stereochemistry between the C-1 methyl group and C-3 hydrogen, while all other fusicoccanes have the cis relationship.

Periconicin A (**1**) exhibited significant antibacterial activity against Gram-positive and Gram-negative bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* with minimum inhibitory concentration (MIC) in the range of 3.12−12.5 $\mu$g/mL, as shown in comparison to gentamicin (Table 2). Periconicin B (**2**) exhibited modest antibacterial activity against the same strains of bacteria with MIC in the range of 25−50 $\mu$g/mL. Therefore, these compounds could be suggested as lead compounds for the development of antibacterial agents for many bacterial strains.

**Table 2.** Antibacterial Activity (MIC $\mu$g/mL) of Periconicins A (**1**) and B (**2**)

| | MIC ($\mu$g/mL) | | |
|---|---|---|---|
| microorganism | **1** | **2** | gentamicin |
| *Bacillus subtilis* ATCC 6633 | 3.12 | 25 | 3.12 |
| *Staphylococcus aureus* ATCC 6538p | 12.5 | 50 | 3.12 |
| *Staphylococcus epidermis* ATCC 12228 | 12.5 | 100 | 3.12 |
| *Micrococcus leuteus* IFO 12708 | 25 | >100 | 6.25 |
| *Klebsiella pneumoniae* IFO 13541 | 3.12 | 25 | 12.5 |
| *Proteus vulgaris* ATCC 3851 | 6.25 | 50 | 6.25 |
| *Salmonella typhimurium* ATCC 14028 | 6.25 | 50 | 3.12 |
| *Escherichia coli* ATCC 25922 | 100 | >100 | 1.56 |

## Experimental Section

**General Experimental Procedures.** The $^1H$ NMR (300 MHz) and $^{13}C$ NMR (75 MHz) spectra were taken at the Central Instrumental Facility of Natural Products Research Institute on a Bruker DPX300 spectrometer, with TMS ($\delta_H$ 0 ppm) and $CDCl_3$ ($\delta_C$ 77.0 ppm) as internal references, respectively. Melting points were determined using an Electrothermal melting point apparatus and were uncorrected. Optical rotations were measured with a JASCO J-810 spectropolarimeter. IR spectra were recorded on an FT-IR system 2000 (Perkin-Elmer) spectrometer. Silica gel 60 and precoated reversed-phase preparative thin-layer chromatography plates, RP-18 F254S, were purchased from Merck & Co., Inc. Mass spectra were recorded on a JEOL JMS-AX505WA mass spectrometer.

**Fungal Isolation.** The periconicin-producing fungus was one of many endophytic fungi isolated from small branches of *T. cuspidata* collected from the Kangwon region of Korea. The stems were washed in running $H_2O$ and surface sterilized by successive soaking in 70% EtOH for 1 min and then rinsed twice with water. Aseptically, the stem rods were cut open, and 1 × 1 $cm^2$ pieces of the inner bark were excised and placed on water agar in Petri plates. After incubation at 25 °C for several days, individual hyphal tips of the emerging fungi were removed and placed on potato dextrose agar (PDA). During the first two weeks of incubation, the cultures were periodically checked for purity and successively subcultured by hyphal tipping method until pure cultures were obtained. A fungal isolate, OBW-15, grew on PDA as a pale brown filamentous fungus colony and produced conidia upon maturation. The taxonomic identification of OBW-15 as *Periconia* sp. was

performed based on fungal morphology.[7,13] The fungus typically possesses conidiophores up to 550 $\mu$m long, 8−17 $\mu$m thick at the base, and 5−10 $\mu$m immediately below the head. The conidia are spherical, straw-colored to pale brown, verruculose, and mostly 4−6 $\mu$m in diameter.

**Bioassay Procedures.** The following eight microorganisms, obtained from the stock culture collection at the American Type Culture Collection (Maryland) and the Institute for Fermentation (Osaka, Japan), were used in this study: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* IFO 13541, *Proteus vulgaris* ATCC 3851, *Salmonella typhimurium* ATCC 14028, *Bacillus subtilis* ATCC 6633, *Micrococcus leuteus* IFO 12708, *Staphylococcus aureus* ATCC 6538p, *Staphylococcus epidermis* ATCC 12228. The antibacterial activity was determined by the 2-fold microtiter broth dilution method.[14] Dilutions of test compound dissolved in dimethyl sulfoxide (DMSO) were added to each well of a 96-well microtiter plate containing a fixed volume of standard methods broth (SM broth, Difco) (final 0.5% DMSO). Each well was inoculated with an overnight culture of bacteria ($10^5$ CFU/mL) and incubated at 37 °C for 24 h. The MIC was taken as the concentration at which no growth was observed.

**Extraction and Isolation of Periconicins.** *Periconia* sp. OBW-15 was grown on a PDA plate as an inoculum for 7 days at 25 °C. Ten pieces of 5 × 5 mm blocks of the well-grown culture were inoculated into 500 mL of liquid S-7 medium[15] in a 3 L narrow-mouth prescription bottle. The fungus was grown by still culture at 25 °C for 21 days. The EtOAc extracts, which were active in antibacterial assays, of the broth (total 15 L; thirty 3 L bottles, each containing 500 mL of S-7 medium) were prepared according to the modified Kupchan scheme[16] and subjected to an open column of silica gel with solvent A (7:6:1 $CHCl_3$−hexane−MeOH). Chromatographic fractions were pooled depending on their antimicrobial activities to yield two different fractions, each containing **1** and **2**, respectively. The active fractions were then subjected to PTLC on a Merck (RP-18 F254S, 20 × 20 cm) 1 mm ODS silica gel plate developed in solvent B (2:1:1 $CH_3CN$−$H_2O$−MeOH). The PTLC plate was cut into small strips longitudinally (1 × 6 cm). Each strip was placed on the SM agar plate (agar 1.5%), inoculated with test bacteria, and incubated at 37 °C overnight to detect the zone of inhibition corresponding to the antibacterial compound. After the separation in solvent B, the area of the plate containing active compound was carefully removed by scraping off the silica at the appropriate $R_f$ (1, 0.32; 2, 0.67; solvent B), and it was exhaustively extracted with acetonitrile. After solvent evaporation, white solid residues were crystallized from EtOAc to afford **1** (25 mg) and **2** (18 mg).

**Periconicin A (1):** white solid; mp 81.5 °C; $[\alpha]_D$ +63.1 (*c* 0.45, $CHCl_3$); UV (MeCN) $\lambda_{max}$ (log $\epsilon$) 276 (0.623), 226 (1.636), 198 (1.868) nm; IR $\nu_{max}$ (KBr) 3430, 1750,1630 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, see Table 1; HREIMS *m*/*z* 316.2046 $[M + H]^+$ (calcd for $C_{20}H_{28}O_3$, 316.2038).

**Periconicin B (2):** white solid; mp 71.4 °C; $[\alpha]_D$ +93.3 (*c* 0.120, $CHCl_3$); UV (MeCN) $\lambda_{max}$ (log $\epsilon$) 274 (0.089), 224 (0.260), 200 (0.354) nm; IR $\nu_{max}$ (KBr) 3433, 1749, 1628 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, see Table 1; HREIMS *m*/*z* 332.2008 $[M + H]^+$ (calcd for $C_{20}H_{28}O_4$, 332.1988).

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**Supporting Information Available:** $^1H$ and $^{13}C$ NMR spectra for periconicins A and B (**1** and **2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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