Pestalotiopsins A and B: New Caryophyllenes from an Endophytic Fungus of *Taxus brevifolia*

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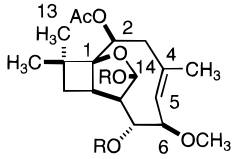
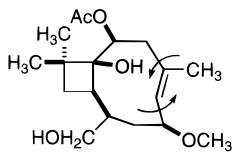
Two new caryophyllene sesquiterpenes have been isolated from *Pestalotiopsis* sp., an endophytic fungus associated with the bark and leaves of *Taxus brevifolia*. Pestalotiopsin A (**1**) has a novel oxatricyclic ring system while pestalotiopsin B (**3**) exists as two slowly equilibrating atropisomers (6:5 ratio of $\beta\beta$:$\beta\alpha$) in chloroform solution at room temperature. Structures of **1** and **3** were determined by spectroscopic analyses and single-crystal X-ray diffraction.

The Pacific yew, *Taxus brevifolia*, produces the well-known diterpene taxol,[1] which was recently approved by the FDA for use against breast and ovarian cancers.[2] The intense interest in taxol has prompted a thorough examination of the Pacific yew's secondary metabolites, and this examination has grown to embrace some microorganisms associated with the tree. In a provocative report, Stierle et al. isolated an endophytic fungus, *Taxomyces andreanae*, that produced taxol in pure cultures.[3] As a consequence of that report, we began examining other endophytic fungi associated with the genus *Taxus* as an approach to discovering new biologically active substances. In this paper we report the isolation and structure determination of two new caryophyllene-type sesquiterpenes from *Pestalotiopsis* sp., an endophytic fungus from *Taxus brevifolia*. Caryophyllene sesquiterpenes are not common fungal metabolites although the punctaporonins B[4] and G,[5] which were isolated a few years ago from the dung fungus *Poronia punctata*, are also highly functionalized caryophyllenes. The oxatricyclic structure of pestalotiopsin A (**1**) is unprecedented among natural products.[6]

At least two different strains of *Pestalotiopsis* sp. have been isolated from a single *T. brevifolia* tree, and as other authors have noted,[4] the production of secondary metabolites by this genus is variable. The closely related compounds reported here have been given the trivial names pestalotiopsin A (**1**) and B (**3**). The producing strain was grown on a modified S7 medium in still culture for 21 days at room temperature. The filtrate (6 L) was extracted at neutral pH with dichloromethane (0.5 L × 6), and evaporation of the solvent yielded 159 mg of a yellow waxy material. A portion (59 mg) was purified by silica gel chromatography using 95:5 chloroform−methanol. Pestalotiopsin A (**1**) was crystallized from



1 R = H
2 R = Ac



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dichloromethane to give 7.1 mg of pure **1** (3.2 mg/L of filtrate) with a mp of 212−213 °C. A molecular formula of $C_{18}H_{28}O_6$ was determined by HRFABMS (314.1964 calculated for $C_{18}H_{29}O_6$, found 341.1924), and this formula was consistent with the ^{1}H- and ^{13}C-NMR spectra. Acetylation of **1** afforded a triacetate (**2**) (mp 158−159 °C), and the downfield shift of two different protons in the ^{1}H-NMR spectrum upon acetylation suggested the presence of two 1° or 2° hydroxyl groups. The ^{1}H-NMR spectrum of **1** showed 26 nonexchangeable protons including an olefinic proton, a methoxyl group, and four methyl groups. Analyses of the ^{13}C-NMR and the PFG-HMQC[7] spectra of **1** allowed the further identification of two quaternary sp^3-hybridized carbons, a carbonyl carbon, an sp^2-hybridized quaternary carbon, two methylene carbons, and seven methine carbons—three of which had an oxygen substituent.

The skeleton of **1** was constructed through analysis of the PFG-DQFCOSY[8] and PFG-HMBC[9] spectra. An important aspect of the analysis was the observed HMBC correlations of the signal at δ 5.83 (H-14) with C-1, C-7, and C-9, suggesting a hemiacetal moiety at C-14 (δ 108.7) with C-1 contributing the hydroxyl group. The gross stereochemistry of **1** was deduced from NOE difference spectroscopy experiments. For example, the enhancement of the signal for H-3α that arose from H-5 irradiation coupled with the failure to observe an H-5 enhancement upon H-15 irradiation clearly indicated an *E*-geometry for the C-4 to C-5 double bond. Irradiation of H-2 resulted in enhancements for H-13, H-3β, and H-15; irradiation of H-7 enhanced the signals for H-8 and H-14; and irradiation of H-14 enhanced the signals for H-7 and H-5, but not H-8.

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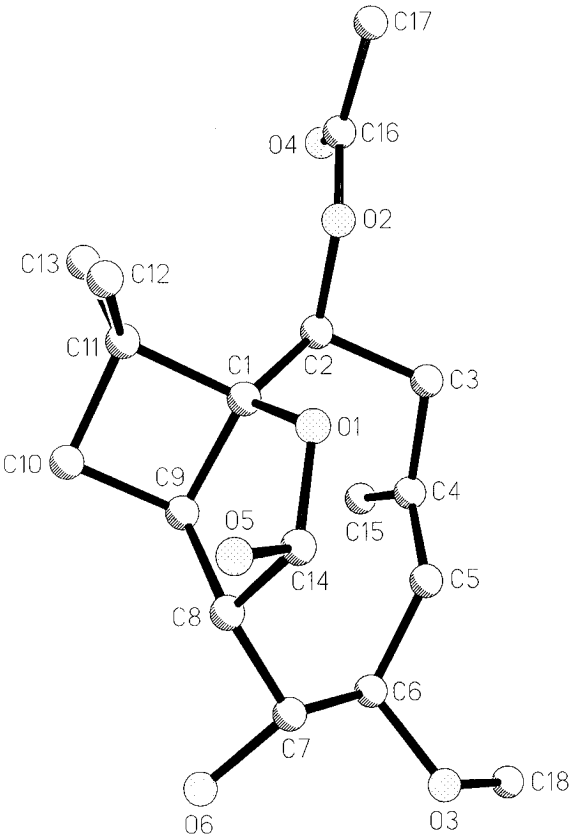

Figure 1. Computer-generated perspective drawing of pestalotiopsin A (**1**). Hydrogens are omitted for clarity, and no absolute configuration is implied.

In order to clarify both the stereochemistry and the conformation of **1**, we selected a suitable crystal for X-ray diffraction analysis, and the results, which gave only the relative stereochemistry, are shown in Figure 1. Compound **1** crystallized in the monoclinic space group $P2_1$ ($Z = 2$) with $a = 8.181(3)$, $b = 6.117(2)$, and $c = 18.120(6)$ Å and $\beta = 97.97(3)°$. The structure was solved by direct methods, and full matrix least-squares refinements with 1228 observed ($|F_o| \geq 4\sigma F_o$) reflections (97%), anisotropic non-hydrogen atoms, and isotropic riding hydrogens have converged to a final *R*-factor of 4.7%. The torsional angle about the double bond involving C-3, C-4, C-5, and C-6 is 152°.

Pestalotiopsin B (**3**) was recrystallized from ethyl acetate (11.8 mg, 5.3 mg/L, mp 156 °C), and HRFABMS indicated a molecular formula of $C_{18}H_{28}O_5$ (325.2015 calculated for $C_{18}H_{29}O_5$, found 325.2014). Although compound **3** appeared to be a single entity in HPLC and HPTLC analyses, the ^{1}H-NMR spectrum recorded at 600 MHz revealed the presence of two closely related compounds. In addition 36 resonances could be detected in the ^{13}C-NMR spectrum of **3**. Despite these complicating features, the overall patterns of the spectra of **3** strongly resembled those for **1** and suggested that **3** was also a functionalized caryophyllene. PFG-HMQC, PFG-DQ-COSY, and PFG-HMBC experiments allowed all of the signals to be assigned and suggested the structure shown in **3**. The spectra also indicated that two slowly equilibrating conformers, in a 6:5 ratio, exist in $CDCl_3$ at room temperature. In DMSO solutions at 150 °C, the exchange between the two conformers became rapid on the ^{1}H-NMR time scale. The existence of two conformational isomers for caryophyllenes has been reported in the literature[10] and has been attributed to atropisomers generated by coupled hindered rotations about the C-3 to C-4 and C-5 to C-6 bonds as shown in **3**. In the case of **3**, NOE enhancements between 15-CH_3 and 2-H, 3-Hα, 6-H, and 9-H for the minor component clearly indicate that in the minor conformer the olefinic methyl group is oriented on the α face of the molecule.[11]

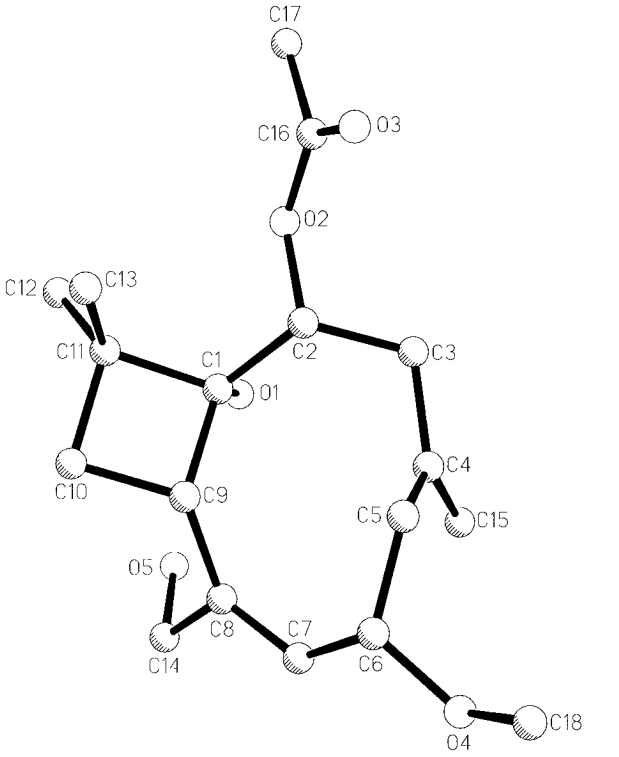

Figure 2. Computer-generated perspective drawing of pestalotiopsin B (**3**). Hydrogens are omitted for clarity, and no absolute configuration is implied.

The structural and conformational complexities of **3** were further elucidated by a single-crystal X-ray diffraction analysis, and the results, which gave only the relative stereochemistry, are shown in Figure 2. Compound **3** crystallized in the monoclinic space group $P2_1$ ($Z = 2$) with $a = 10.188(5)$, $b = 8.164(4)$, and $c = 11.471(6)$ Å and $\beta = 98.034(4)°$. The structure was solved by direct methods, and full matrix least-squares refinements with 930 observed ($|F_o| \geq 4\sigma F_o$) reflections (72%), anisotropic non-hydrogen atoms, and isotropic riding hydrogens have converged to a final *R*-factor of 5.5%. The conformer that crystallized is the major conformer, and the C-3, C-4, C-5, and C-6 double bond has a torsional angle of 152°.

Compounds **1** and **2** both show immunosuppressive activity in the mixed lymphocyte reaction with IC_{50}s of 3−4 μg/mL and cytotoxicity with IC_{50}s at roughly the same level.

Experimental Section

General Procedures. All reagents and fermentation media components were from the Aldrich Chemical Co. or Wako Chemical Industries and were used without further

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purification. TLC plates were Merck (Darmstadt) silica gel $60F_{254}$, with layer thickness of 0.25 mm, and the determination of R_f values was done by using plates of 20 cm length. $CHCl_3$−MeOH (96:4 v/v) was used as the solvent system, and after developing, the compounds were visualized by spraying with a mixture of anisaldehyde−sulfuric acid (5% w/v) in MeOH and subsequent heating at 110 °C for 2−3 min. The stationary phase for column chromatography was Merck silica gel (70−230 mesh). Melting points were measured on a Yanaco micro melting point apparatus and are uncorrected.

Spectral Procedures. NMR measurments were performed on a JEOL JNM-A 600 or JNM-A400 spectrometer with pulsed field gradient (PFG) unit.

1D, ^{1}H-, and ^{13}C-NMR spectra were recorded in $CDCl_3$ or mixtures of $CDCl_3$ and CD_3OD solutions at 600 at 150 MHz, and the chemical shifts are given relative to the TMS and $CDCl_3$ solvent peaks at 0.00 and 77.0 ppm, respectively. NOE difference spectra were recorded using a standard pulse sequence with a relaxation delay of 3 s 2D: All spectra were recorded using standard pulse sequence with *z*-axis PFG. The PFG DQFCOSY spectra were obtained using a data set of 1K × 1K points with 512 increments with 8 transients at magnitude mode. Sine bell shaped gradient pulses were used with a 2:1:4 ratio and 1 ms duration, and maximum strength was 25.6 G/cm. The PFG-HMQC spectra were recorded using a 512 × 1K data set with 16 scans per increment, using a maximum gradient strength of 25.6 G/cm. The PFG-HMBC experiments were performed using the same data set and PFG strength of PFG-HMQC experiments, with 64 scans per increment. For long range C−H coupling, a 60 ms delay time was used.

FAB (fast atom bombardment) mass spectra were run using a glycerol matrix. These spectra, as well as HR (high resolution)-FAB mass spectra, were taken on a JEOL JMS-AX505WA mass spectrometer.

The IR absorption spectra was measured using a Nicolet Impact 400 instrument on KBr pressed disk samples, while the specific rotations were recorded on JASCO DIP-370 digital polarimeter.

Collection. Leaves of *Taxus brevifolia* were collected in Bozeman, MT. The strain was selected by culturing several small leaf pieces onto water agar, and subsequently transferring the mycelial tips several times. The strain was identified as *Pestalotiopsis* sp. Small agar plugs were then transfered into a 2 L Erlenmayer flask containing 1 L of a culture broth consisting of (per L of water) 1 mg of biotine, 1 mg of thiamine, 1 mg of calcium, pantothenate, 1 mg of pyrodoxine, 3.6 mg of $MgSO_4$, 6.5 mg of $Ca(NO_3)_2$, 1 mg of $Cu(NO_3)_2$, 2.5 mg of $ZnSO_4$, 5 mg of $MnCl_2$, 2 mg of $FeCl_3$, 5 mg of phenylalanine, 100 mg of Na^+ benzoate, 1 g of glucose, 3 g of fructose, 6 g of sucrose, 1 g of sodium acetate, 1 g (Bacto) of Soytone, and 1 mL of 1 M of KH_2PO_4. The fungus was grown in the dark in still culture at 25 °C for 21 days.

Extraction and Isolation. The culture was filtered through cheesecloth to remove the mycelia, and the fluid (6 L) was extracted with CH_2Cl_2 (3 L). The organic extract was concentrated under vacuum and yielded 159 mg of crude extract. A portion (59 mg) was applied to silica gel (2 × 13 cm), the column was developed in the solvent system $CHCl_3$−CH_3OH (95:5), and the eluant was collected in 1.5 mL fractions. Tubes 39−52, on evaporation, yielded 12.5 mg of a solid material which was crystallized from ethyl acetate, giving 11.8 mg of a pure compound (pestalotiopsin B (**3**)). Tubes 57−68, on evaporation, yielded 7.8 mg of a compound, further purified by crystallization from dichloromethane (7.2 mg) (pestalotiopsin A (**1**)).

Acetylation of Pestalotiopsin A. Pestalotiopsin A (**1**) (5 mg) was dissolved in a 2 mL mixture of acetic anhydride and pyridine (3:1) and stirred overnight. The solution was then poured into water and extracted with ethyl acetate (3 × 3 mL). The organic layer was evaporated under vacuum, yielding a solid material, which was crystallized from dichloromethane (5.1 mg) (pestalotiopsin A 7,14-diacetate (**2**)).

Pestalotiopsin A: solid; $[\alpha]_D$ (22 °C) +76.8° (*c* 1, MeOH); mp 212−213 °C; R_f ($CHCl_3$−MeOH 98:2) = 0.11; FAB-MS *m*/*z* (relative intensity) 341.2 (M^+ + H, 2.5), 323.2 (8), 263.2 (12), 227 (4.5), 213 (4.5), 147 (3); HR-MS calcd for $C_{18}H_{29}O_6$ 341.1964, found 341.1924; IR λ_{max} (KBr) 3600−3200, 2935, 1704, 1269 cm^{-1}; ^{1}H NMR (600 MHz in $CDCl_3$, δ ppm from TMS) δ 1.01 (s, 3H), 1.13 (s, 3H), 1.64 (dd, 1H, *J* = 12.2, 6.4 Hz), 1.90 (s, 3H), 1.98 (dd, 1H, *J* = 12.2, 9.8 Hz), 2.04 (s, 3H), 2.41 (dd, 1H, *J* = 10.7, 10.7 Hz), 2.43 (br, s, 1H), 2.53 (dd, 1H, *J* = 10.7, 5.1 Hz), 2.60 (dd, 1H, *J* = 9.8, 6.4 Hz), 3.28 (s, 3H), 3.83 (dd, 1H, *J* = 11.7, 6.4 Hz), 3.94 (d, 1H, *J* = 6.4 Hz), 5.05 (d, 1H, *J* = 11.7 Hz), 5.23, (dd, 1H, *J* = 10.7, 5.1 Hz), 5.83 (d, 1H, *J* = 2.4 Hz); ^{13}C NMR (600 MHz in $CDCl_3$) δ 17.6 (q), 21.4 (q), 23.9 (q), 27.2 (q), 38.4 (d), 39.5 (s), 41.1 (t), 42.4 (t), 56.1 (q), 65.1 (d, C-8), 73.7 (d), 77.23 (d), 83.0 (d), 98.1 (s), 108.7 (d), 124.0 (d), 137.7 (s), 170.4 (s).

Pestalotiopsin A 7,14-diacetate: solid; $[\alpha]_D$ (22 °C) +35.7° (*c* 0.8, MeOH); mp 158−159 °C; R_f ($CHCl_3$−MeOH 98:2) = 0.60; FAB-MS *m*/*z* (relative intensity) 425 (M^+ + H, 3), 365 (100), 305 (59), 263, (30), 245 (56), 231 (39), 213 (100), 185 (90); HR-MS calcd for $C_{22}H_{33}O_8$ 425.2175, found 425.2180; IR λ_{max}(KBr) 2938, 1738, 1231 cm^{-1}; ^{1}H NMR (600 MHz in $CDCl_3$, δ ppm from TMS) δ 1.05 (s, 3H), 1.09 (s, 3H), 1.56 (dd, 1H, *J* = 12.3, 6.3 Hz), 1.93 (s, 3H), 2.01 (dd, 1H, *J* = 12.3, 9.5 Hz), 2.05 (s, 3H), 2.06 (s, 3H), 2.12 (s, 3H), 2.47 (br s, 1H), 2.52 (dd, 1H, *J* = 10.7, 5.4 Hz), 2.55 (dd, 1H, *J* = 10.7, 10.7 Hz), 2.68 (dd, 1H, *J* = 9.5, 6.3 Hz, 3.25 (s, 3H), 4.04 (dd, 1H, *J* = 11.2, 6.1 Hz), 5.22 (dd, 1H, *J* = 6.1, 2.5 Hz), 5.23 (d, 1H, *J* = 11.2 Hz), 5.24 (dd, 1H, *J* = 10.7, 5.9 Hz), 6.69 (d, 1H, *J* = 2.0 Hz); ^{13}C NMR (600 MHz in $CDCl_3$) δ 17.6 (q), 21.3 (q), 21.3 (q), 21.4 (q,), 23.6 (q), 27.4 (q), 38.1 (d), 39.8 (s), 40.9 (t), 42.1 (t), 56.0 (q), 61.3 (d), 73.0 (d), 78.2 (d), 79.7 (d), 99.4 (s), 106.3 (d), 124.2 (d), 137.8 (s), 169.8 (s), 169.9 (s), 170.4 (s).

Pestalotiopsin B: $[\alpha]_D$ (22 °C) −240.5 (*c* 0.49, MeOH); mp 156 °C; R_f ($CHCl_3$−MeOH 98:2) = 0.23; FAB-MS *m*/*z* (relative intensity) 325 (M + H, 16), 307 (12), 275 (12), 265 (11), 247 (38), 233 (51), 215 (89), 187 (63), 159 (74), 145 (100), 131 (100); HR-MS calcd for $C_{18}H_{29}O_5$ 325.2015, found 325.2014; IR λ_{max} (KBr) 3350, 3212, 2978, 2947, 1727, 1250 cm^{-1}; ^{1}H NMR (600 MHz in $CDCl_3$, δ ppm from TMS) major δ 1.05 (s, 3H), 1.13 (s, 3H), 1.68 (dd, 1H, *J* = 12.2, 10.7 Hz), 1.96 (s, 3H), 2.06 (s, 3H), 2.07 (dd, 1H, *J* = 12.2, 8.3 Hz), 2.39 (dd, 1H, *J* = 10.7, 3.9 Hz), 2.62 (dd, 1H, *J* = 11.2, 10.7 Hz), 3.14 (dd, 1H, *J* = 10.7, 8.3 Hz), 3.31 (s, 3H), 3.92 (d, 1H, *J* = 11.2 Hz), 4.31 (d, 1H, *J* = 11.2 Hz), 4.48 (br d, 1H, *J* = 9.8 Hz), 5.23 (d, 1H, *J* = 9.8 Hz), 5.27 (dd, 1H, *J* = 11.2, 3.9 Hz), 5.97 (s, 1H); minor δ 1.06 (s, 3H), 1.12 (s, 3H), 1.61 (dd, 1H, *J* = 10.8, 8.8 Hz), 1.79 (s, 3H), 1.81 (dd, 1H, *J* = 14.2, 10.7 Hz), 2.06 (s, 3H), 2.18 (dd, 1H, *J* = 10.8, 10.8 Hz), 3.02 (dd, 1H, *J* = 14.2, 7.8 Hz), 3.26 (dd, 1H, *J* = 10.8, 8.8 Hz), 3.31 (s, 3H), 3.93 (d, 1H, *J* = 10.7 Hz), 4.18 (d, 1H, *J* = 10.7 Hz), 4.31 (br d, 1H, *J* = 6.8 Hz), 5.25 (dd, 1H, *J* = 10.7, 7.8 Hz), 5.74 (d, 1H, *J* = 6.8 Hz), 6.00 (s, 1H); ^{13}C NMR (600 MHz in $CDCl_3$) major δ 17.55 (q), 21.53* (q), 24.28 (q), 27.07 (q), 33.81 (t), 40.34 (t), 40.86 (d), 41.59 (s), 55.61 (q), 66.63 (t), 76.00 (d), 76.19 (d), 81.59 (s), 130.02 (d), 132.82 (s), 136.79 (s), 140.03 (d), 170.09 (s); minor δ 21.41* (q), 23.60 (q), 24.54 (q), 25.23 (q), 45.41 (t), 36.03 (t), 40.34 (s), 45.81 (d), 55.97 (q), 67.27 (t), 75.85 (d), 79.29 (d), 83.38 (s), 126.37 (d), 133.54 (s), 140.49 (d), 141.81 (s), 170.15 (s) (* indicates tentative assignments).

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