Eremophilane-Type Sesquiterpenoids from the Fermentation Broth of Plant Endophytic Fungus *Pestalotiopsis photiniae* Isolated from the Chinese Podocarpaceae Plant *Podocarpus macrophyllus*

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Two new sesquiterpenoid esters, pestalotiopins B and C (**1** and **2**, resp.) related to the eremophilane class, together with one known compound, berkleasmin C (**3**), were isolated from the fermentation broth of the plant endophytic fungus *Pestalotiopsis photiniae*. Their structures and relative configurations were elucidated by extensive spectroscopic analysis including 1D- and 2D-NMR (HSQC, HMBC, and NOESY), and MS experiments. Compound **1** has an interesting lactam structure, and this type of sesquiterpenoid with a lactam structure is reported for the first time.

Introduction. – Endophytic fungi grow within normal tissues of host plants without causing apparent disease symptoms [1][2]. The widely distributed fungi of the genus *Pestalotiopsis* (Amphisphaeriaceae) are known as endophytes of tropical higher plants [2]. Since discovery of the anticancer agent taxol from an endophytic fungal strain of the genus *Pestalotiopsis* [3][4], interest in searching for bioactive metabolites from this fungal genus has increased considerably. Previous chemical studies of some species of this genus have afforded a variety of bioactive metabolites [5–17].

Our previous investigation of *Pestalotiopsis photiniae* grown in the fermentation broth has led to the isolation of several structurally diverse and biologically active known compounds, such as 10-norparvulenone, an anti-influenza virus antibiotic, reported from *Microsphaeropsis* sp. FO-5050 [18], and zinniol-related phytotoxins [19]. To search further for the minor active components, the fungus *P. photiniae* isolated from the branch of *Podocarpus macrophyllus* in Hainan, P. R. China, was refermented in a larger scale using the liquid fermentation culture. As a continuation of this work, two new eremophilane-type sesquiterpenoids, named pestalotiopins B and C (**1** and **2**, resp.), and one known compound, berkleasmin C (**3**) [20], were obtained (*Fig. 1*). Details of the isolation and structure elucidation of these compounds are reported herein.

Results and Discussion. – Pestalotiopin B (**1**) was obtained as an optically active white powder ($[\alpha]_D^{19} = -40$ ($c = 0.3$, MeOH) that gave a *quasi*-molecular-ion peak at m/z 544.3638 ($[M+H]^+$) in the HR-ESI-MS (positive-ion mode), consistent with a

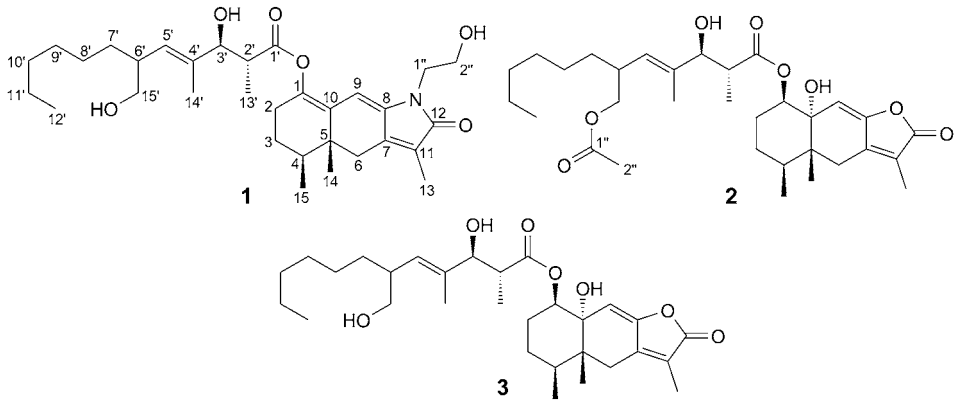


Fig. 1. *The structures of compounds* **1**–**3**

molecular formula of $C_{32}H_{49}NO_6$ (calc. for $C_{32}H_{50}NO_6^+$, 544.3633), requiring nine degrees of unsaturation. The IR spectrum revealed absorption bands of C=C (1678 cm^{-1}), OH (3318 cm^{-1}), and CO (1779 cm^{-1}) groups. The ^{13}C-NMR (DEPT; *Table 1*) exhibited 32 C-atom resonances, attributed to two CO groups, eight olefinic C-atoms, eleven CH_2 and four CH groups, one sp^3 quaternary C-atom, and six Me groups. Eight olefinic C-atom signals could be assigned to four olefins, which together with the two CO groups accounted for six of the nine required degrees of unsaturation. The remaining degrees of unsaturation had to be present as three rings. The ^{1}H-NMR spectrum (*Table 1*) exhibited six Me H-atom signals (δ(H) 1.85 (*s*), 1.66 (*s*), 1.04 (*d*, *J* = 7.2), 1.00 (*d*, *J* = 6.6), 0.97 (*s*), 0.86 (*t*, *J* = 6.9, 14.1)), two olefinic H-atom signals (δ(H) 6.45 (*s*), 5.17 (*d*, *J* = 10.0)), three CH_2 H-atom signals (δ(H) 3.31–3.35 (*m*, 1 H), 3.59–

Table 1. *^{1}H- and ^{13}C-NMR Data for* **1** (at 600 and 150 MHz, resp., in $CDCl_3$)

	δ(H)	δ(C)		δ(H)	δ(C)
C(1)		146.2	H–C(2′)	2.74–2.76 (*m*)	43.7
CH_2(2)	2.37–2.39 (*m*), 2.27–2.29 (*m*)	27.7	H–C(3′)	4.16 (*d*, *J* = 10.2)	81.3
CH_2(3)	1.69–1.71 (*m*), 1.64–1.66 (*m*)	26.4	C(4′)		137.2
H–C(4)	1.77–1.79 (*m*)	38.7	H–C(5′)	5.17 (*d*, *J* = 10.0)	133.1
C(5)		38.3	H–C(6′)	2.53–2.55 (*m*)	40.7
CH_2(6)	2.78 (*d*, *J* = 16.0, H_α),	34.8	CH_2(7′)	1.10, 1.27 (2m_c)	31.3
	2.35 (*d*, *J* = 16.0, H_β)		CH_2(8′)	1.24–1.26 (*m*)	27.2
C(7)		138.4	CH_2(9′)	1.24–1.26 (*m*)	29.3
C(8)		137.7	CH_2(10′)	1.24–1.26 (*m*)	31.8
H–C(9)	6.45 (*s*)	103.2	CH_2(11′)	1.24–1.26 (*m*)	22.6
C(10)		127.8	Me(12′)	0.86 (*t*, *J* = 6.9, 14.1)	14.1
C(11)		124.9	Me(13′)	1.04 (*d*, *J* = 7.2)	14.0
C(12)		171.9	Me(14′)	1.66 (*s*)	10.6
Me(13)	1.85 (*s*)	8.4	CH_2(15′)	3.31–3.35 (*m*), 3.59–3.62 (*m*)	66.6
Me(14)	0.97 (*s*)	19.5	CH_2(1″)	3.46–3.50 (*m*), 3.84–3.86 (*m*)	41.7
Me(15)	1.00 (*d*, *J* = 6.6)	15.4	CH_2(2″)	3.68–3.69 (*m*), 3.63–3.65 (*m*)	60.8
C(1′)		173.8			

3.62 (*m*, 1 H); 3.68 – 3.69 (*m*, 1 H), 3.63 – 3.65 (*m*, 1 H); 3.46 – 3.50 (*m*, 1 H), 3.84 – 3.86 (*m*, 1 H)), and one O-bearing CH H-atom signal (δ(H) 4.16 (*d*, $J = 10.2$)). By careful analysis of NMR data, we found that the NMR data of **1** were similar to those of berkleasmin C (*Table 1*) recently reported from the saprobic fungus *Berkleasmium nigroapicale* [20], and this suggested that **1** is composed of a tricyclic sesquiterpene core attached to a long-chain acid through an ester linkage. Further interpretation of HMBC spectrum showed the following long-range correlations (*Fig. 2*): from H–C(3′) to C(1′), C(2′), C(5′), C(13′), and C(14′), from H–C(5′) to C(3′), C(6′), C(7′), C(14′), and C(15′), and from H–C(6′) to C(4′), C(5′), and C(15′), and from H–C(15′) to C(5′), C(6′), and C(7′). These spectral evidences, along with the H-atom spin system: H–C(2′)/H–C(3′) and H–C(2′)/H–C(13′); H–C(5′)/H–C(6′)/H–C(7′)/H–C(8′)/H–C(9′)/H–C(10′)/H–C(11′)/H–C(12′), and H–C(6′)/H–C(15′) deduced from ^{1}H,^{1}H-COSY (*Fig. 2*) correlations, led to the establishment of a long-chain acid unit. In addition, the HMBC spectrum also showed the long-range couplings from H–C(2) to C(1), C(3), C(4), and C(10), from H–C(3) to C(2) and C(5), from H–C(6) to C(5), C(8), C(10), C(11), and C(14), from H–C(9) to C(1), C(5), and C(7), from H–C(13) to C(7), C(11), and C(12), from H–C(14) to C(4), C(5), C(6), and C(10), from H–C(15) to C(3), C(4), and C(5), and from H–C(1″) to C(2″), C(8), and C(12). These spectral data, coupled with another two H-atom systems, H–C(2)/H–C(3)/H–C(4)/H–C(15); H–C(1″)/H–C(2″), established by ^{1}H,^{1}H-COSY correlations (*Fig. 2*), gave

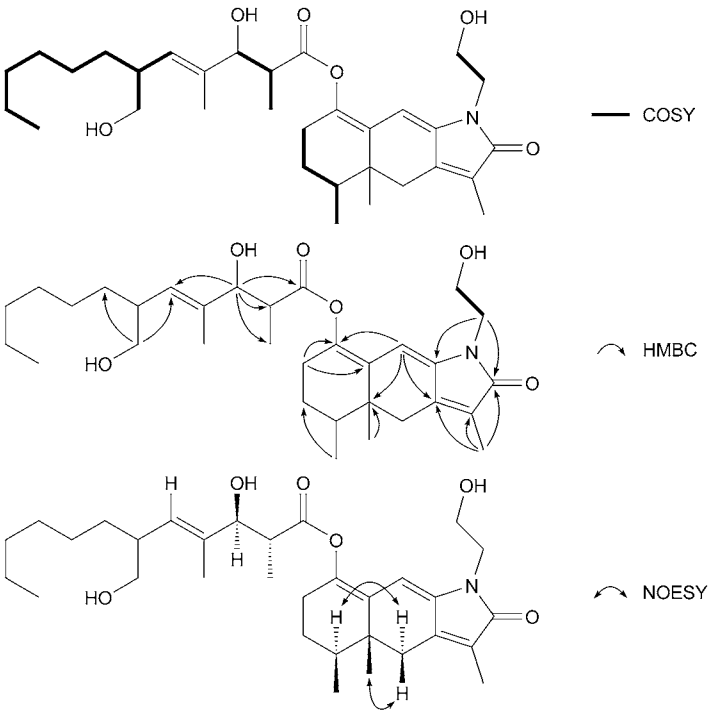
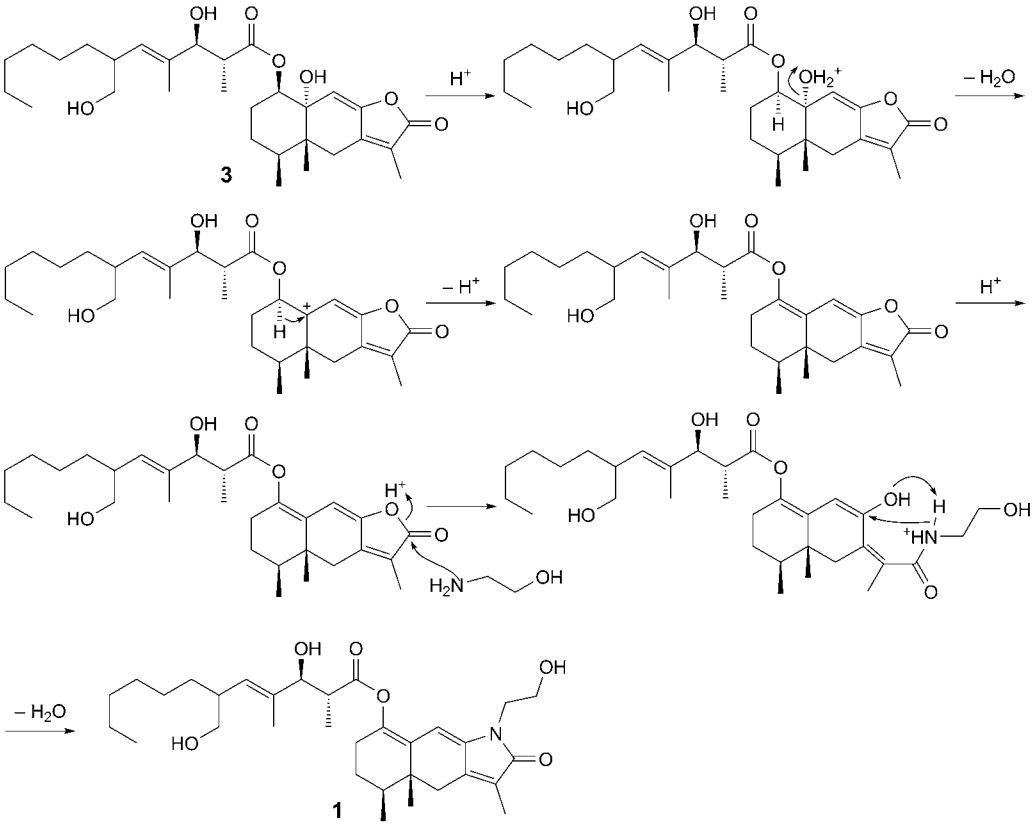


Fig. 2. *The ^{1}H,^{1}H-COSY, selected key HMBC, and NOESY correlations of* **1**

rise to a tricyclic sesquiterpene core partial structure. Accordingly, the structure of **1** was determined as shown in *Fig. 1*.

The relative configuration of **1** was elucidated by analysis of the partial NOESY data and comparison of chemical shifts with those of berkleasmins A – E and some related eremophilane-type sesquiterpenoids [20 – 22]. The same relative configuration at C(4), C(5), C(2′), C(3′), and C(4′) in **1** as in berkleasmins A – E were deduced from the very similar C- and H-atom chemical shifts. The NOE (*Fig. 2*) interaction observed between H_α–C(6) (δ(H) 2.78 (*d*, $J = 16.0$)) and H–C(4), along with no NOE interaction observed between H_α–C(6) and Me(15), indicated that the Me(15) is β-configurated. The NOE interaction observed between H_β–C(6) (δ(H) 2.35 (*d*, $J = 16.0$)) and Me(14) suggested that Me(14) should be β-configurated. Because of some significant signal overlap, we tried to crystallize **1** in different solvents and finally failed to obtain suitable crystals. Due to small quantity of sample, we could not further determine the relative configuration of **1** by chemical methods. Finally, the relative configurations of remaining stereogenic centers of **1** except for C(6′) were determined by comparison of the chemical shifts with those of berkleasmins A – E. The relative configuration of C(6′) remains unassigned. The proposed conversion route from **3** to **1** is depicted in the *Scheme*.

Scheme. *The Proposed Conversion Route from* **3** *to* **1**



Pestalotiopin C (**2**) showed an HR-ESI-MS *pseudo*-molecular-ion peak at *m/z* 583.3232 ($[M+Na]^+$), corresponding to the molecular formula $C_{32}H_{48}O_8$ (calc. for $C_{32}H_{48}NaO_8^+$, 583.3241). The IR spectrum revealed absorption bands of C=C (1667 cm^{-1}), OH (3472 cm^{-1}), and CO (1739 cm^{-1}) groups. Inspection of ^{1}H- and ^{13}C-NMR (DEPT) revealed 32 C-atom signals including those of seven Me, nine CH_2, seven CH (involving two olefinic and two O-bearing C-atoms), and nine quaternary C-atoms (including four olefinic, three CO, one O-bearing, and one sp^3 quaternary C-atoms). The NMR data of **2** (*Table 2*) very closely resembled to those of berkleasmin C except for one additional AcO group [20]. The AcO group was assigned to C(15′) through ester bond, since HMBCs from H–C(15′) to C(1″), and from H–C(2″) to C(1″) were observed. In addition, the resonances of CH_2 H-atoms at C(15′) (δ(H) 3.59 (*dd*, $J = 10.5, 4.6$, 1 H), 3.33 (*dd*, $J = 10.5, 9.2$, 1 H) and C-atom (δ(C) 66.6) in berkleasmin C were shifted downfield in **2** (δ(H) 3.94–3.96 (*m*, 2 H); δ(C) 67.5) owing to the OH group acetylated at C(15′). Therefore, the structure of **2** was determined as shown in *Fig. 1*.

Table 2. *^{1}H-* (600 MHz) *and ^{13}C-NMR* (150 MHz) *Data for* **2** *in $CDCl_3$*

	δ(H)	δ(C)		δ(H)	δ(C)
H–C(1)	5.06–5.07 (*m*)	74.9	C(1′)		175.1
CH_2(2)	2.17–2.20 (*m*), 1.69–1.72 (*m*)	25.8	H–C(2′)	2.58–2.60 (*m*)	43.7
CH_2(3)	1.52–1.55 (*m*), 1.39–1.42 (*m*)	25.6	H–C(3′)	4.06 (*d*, $J = 9.6$)	80.1
H–C(4)	2.17–2.20 (*m*)	33.8	C(4′)		136.6
C(5)		43.2	H–C(5′)	5.14 (*d*, $J = 9.0$)	130.7
CH_2(6)	2.70 (*d*, $J = 16.4$, H_α),	32.6	H–C(6′)	2.64–2.69 (*m*)	37.3
	2.47 (*d*, $J = 16.4$, H_β)		CH_2(7′)	1.41–1.46 (*m*), 1.29–1.18 (*m*)	31.7
C(7)		147.6	CH_2(8′)	1.29–1.18 (*m*)	27.1
C(8)		152.0	CH_2(9′)	1.29–1.18 (*m*)	29.2
H–C(9)	5.64 (*s*)	109.9	CH_2(10′)	1.29–1.18 (*m*)	31.7
C(10)		73.6	CH_2(11′)	1.29–1.18 (*m*)	22.6
C(11)		123.5	Me(12′)	0.86 (*t*, $J = 7.0, 14.1$)	14.0
C(12)		171.3	Me(13′)	1.00 (*d*, $J = 7.1$)	14.3
Me(13)	1.91 (br. *s*)	8.6	Me(14′)	1.61 (*s*)	11.1
Me(14)	1.02 (*s*)	15.4	CH_2(15′)	3.94–3.96 (*m*)	67.5
Me(15)	0.91 (*d*, $J = 6.9$)	15.3	C(1″)		170.7
			Me(2″)	2.03 (*s*)	21.0

Comparison of the physicochemical properties with reported data allowed identification of the compound **3** as berkleasmin C [20], recently reported from the saprobic fungus *B. nigroapicale* and shown to possess cytotoxicity against cancer cell lines (NCI-H187, MCF-7, and KB) and antimalarial activities.

Eremophilane-type sesquiterpenes, including those with similar skeletons as berkleasmins A–C, widely exist as constituents of various plants, while there have been several reports as fungal secondary metabolites mostly from family Xylariaceae. According to the literature, there is no report about eremophilane-type sesquiterpenes from the genus *Pestalotiopsis*. Compounds **1**–**3** were isolated from this genus for the

first time. Compound **2** has the same skeleton as berkleasmins A – C, while compound **1** has an interesting lactam structure instead of the typical lactone.

Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200 – 300 mesh; *Yantai Zhi Fu chemical Co., Ltd.*, P. R. China), *RP-18* (12 nm, *S-50* μm, *YMC Co., Ltd.*, Japan), TLC: silica gel GF_{254} plates (*Yantai Zhi Fu chemical Co., Ltd*, P. R. China), and *Sephadex LH-20* gel (25 – 100 μm, *GE Healthcare Co., Ltd.*, Sweden). Optical rotations: *Perkin-Elmer 341* spectropolarimeter. UV Spectra: *UV-210* spectrometer, λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer* 577 spectrometer; KBr pellets; in cm^{-1}. NMR Spectra: *Bruker AM-600* spectrometer; δ in ppm, J in Hz; Me_4Si as internal standard. FT-MS Spectra: *Bruker apex-ultra 7.0 T* spectrometer in m/z.

Fungal Material and Cultivation Conditions. Pestalotiopsis photiniae was isolated from the branch of *Podocarpus macrophyllus* in Hainan, P. R. China, in April, 2008, and identified by Prof. *Jing-Ze Zhang*, and assigned the accession No. L328. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 28° for 7 d, and then inoculated into 500-ml *Erlenmeyer* flask containing 100 ml of PDA medium (20.0 g of glucose, 200.0 g of potato (peeled), 3.0 g of KH_2PO_4, 1.5 g of $MgSO_4$, 0.1 g of citric acid, and 10.0 mg of thiamin hydrochloride, in 1 l of deionized H_2O). The final pH of the media was adjusted to 6.5 before sterilization. After 7 d of incubation at 28° on rotary shakers at 150 rpm, 25 ml of culture liquid were transferred as seed into each 1000-ml *Erlenmeyer* flask containing 250 ml of PDA medium, and the fermentation was carried out on a shaker for 30 d.

Extraction and Isolation. The culture broth (60 l) was extracted three times with AcOEt (60 l for each time). Evaporation of the solvent *in vacuo* gave a brown oily residue (18.0 g), which was subjected to CC (SiO_2; petroleum ether (PE)/acetone 100 : 0, 98 : 2, 95 : 5, 90 : 10, 80 : 20, 50 : 50 (*v*/*v*) to afford six fractions, *Frs. 1 – 6*. *Fr. 5* (3.0 g) eluted with PE/acetone 80 : 20 was further purified by repeated CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1 : 1) and prep. TLC (PE/AcOEt 1 : 6) to afford compounds **1** (3.0 mg) and **2** (2.5 mg). Compound **3** (3.5 mg) was obtained from *Fr. 6* after repeated CC (*Sephadex LH-20*; acetone) and prep. TLC ($CHCl_3$/MeOH 5 : 1).

Pestalotiopin B (= rel-*(4a*R,5S*)-2,4,4a,5,6,7-Hexahydro-1-(2-hydroxyethyl)-3,4a,5-trimethyl-2-oxo-1*H-*benzo[*f*]indol-8-yl* rel-*(2*R,*3*S,*4*E*)-3-Hydroxy-6-(hydroxymethyl)-2,4-dimethyldodec-4-enoate*; **1**). White powder. $[\alpha]_D^{19} = -40$ ($c = 0.3$, MeOH). IR (KBr): 3318 (OH), 1779 (CO), 1678 (C=C). UV ($CHCl_3$): 254 (2.60), 280 (2.86), 345 (3.42). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS (pos.): 544.3638 ($[M+H]^+$, $C_{32}H_{50}NO_6^+$; calc. 544.36326).

Pestalotiopin C (= rel-*(4a*R,5S,*8*R,*8aR)-2,4,4a,5,6,7,8,8a-Octahydro-8a-hydroxy-3,4a,5-trimethyl-2-oxonaphtho[2,3-*b*]furan-8-yl* rel-*(2*R,*3*S,*4*E*)-6-[(Acetyloxy)methyl]-3-hydroxy-2,4-dimethyldodec-4-enoate*; **2**). White powder. $[\alpha]_D^{19} = +50$ ($c = 0.15$, MeOH). IR (KBr): 3472 (OH), 1739 (CO), 1667 (C=C). UV ($CHCl_3$): 254 (4.10), 280 (3.99), 300 (3.36). ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS (pos.): 583.3232 ($[M+Na]^+$, $C_{32}H_{48}NaO_8^+$; calc. 583.3241).

This work was supported by the programs for *New Century Excellent Talents in University* (NCET-09-0112), the *Key Project of Chinese Ministry of Education* (209010) and the *Key Applied Basic Research Programs of Hebei Province* (0996030917D), and *National Natural Science Foundation of China* (31071701).

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Received January 11, 2011