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Brefeldin A, a cytotoxin produced by *Paecilomyces* sp. and *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis*

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Abstract

Paecilomyces sp. and *Aspergillus clavatus*, which were isolated from *Taxus mairei* and *Torreya grandis* from southeast China, produced toxic metabolites when grown in liquid culture. Nuclear magnetic resonance techniques, infrared spectrometry, electrospray ionization mass spectroscopy and X-ray analysis identified brefeldin A, a bioactive metabolite produced by a number of fungal species belonging to the genera *Alternaria*, *Ascochyta*, *Penicillium*, *Curvularia*, *Cercospora* and *Phyllosticta*. This is the first report of the isolation of the cytotoxin from *Paecilomyces* sp. and *A. clavatus*. The relevance of brefeldin A to the association between these fungi and their host plants is discussed.

Keywords: Brefeldin A; Endophytic fungi; *Paecilomyces* sp.; *Aspergillus clavatus*; *Taxus mairei*; *Torreya grandis*

1. Introduction

Fungi are fundamental to the health and prosperity of every terrestrial ecosystem and are essential to the sustainability of biodiversity. Endophytic fungi, which live inside plants as an inconspicuous embroidery of thread-like filaments, provide yet another dimension to the fungal support system. The role of endophytic fungi in plant–herbivore interactions and their production of extremely beneficial compounds (such as taxol) have received particular attention in recent years [1–4].

We have isolated hundreds of endophytic fungi from *Taxus mairei*, *Cephalataxus fortunei* and *Torreya grandis*, which are traditional Chinese pharmaceutical plants [5]. 14.5 and 52.3% of endophytic fungi fermentation broths showed antitumor and antifungal activity, respectively, demonstrating that endophytic fungi are to be a promising source of beneficial bioactive products [5].

Paecilomyces and *Aspergillus*, belonging to fungi imperfecti, were commonly found as endophytic fungi in our investigation. However, the former fungus was encountered more frequently than the latter [5]. The genus *Paecilomyces* includes several species that are able to produce a wide array of bioactive secondary metabolites of different chemical classes and with different biological activities, such as antimicrobial activity, cytotoxic activity and immunostimulating activity [6]. *Aspergillus clavatus* has usually been found as a saprophytic fungus, producing mycotoxins such as patulin and cytochalasin E [7]. Further investigation of *Paecilomyces* sp. and *A. clavatus* will not only be a way of finding useful bioactive compounds, but also provide us with much more beneficial ecological information.

The present paper describes the isolation and chemical identification of a metabolite from three endophytic fungi of *T. mairei* and *T. grandis*. The systematic identification of these endophytic fungi and the relevance of the cytotoxin to their association with their host plants are also discussed.

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2. Materials and methods

2.1. Identification of endophytic fungi

Endophytic fungi strains W-001, H-036 and H-037 were studied in this work. They were isolated from bark samples of *T. mairei* and *T. grandis*, which were gathered at Wuyi Mountain nature conservation area, Fujian province, southeast China, at an altitude of about 900 m. Fungal identification was based on the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores [8–10].

2.2. Scanning electron microscopy

For scanning electron microscopy, the materials were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h and then step dehydrated (15 min each) with 50, 70, 90, 100% (v/v) ethanol, isoamyl acetate/ethanol (1:1 v/v) and finally 100% isoamyl acetate. Then the samples were critical-point dried, gold coated with a sputter coater and observed and photographed with a Hitachi S520 scanning electron microscope.

2.3. Fermentation and compound isolation

Each fungus was cultured in potato–dextrose liquid (PDA) medium for 7 days at 25°C, 120 rpm. Crude fermentation broth was blended thoroughly and centrifuged at 4000 rpm for 5 min. The supernatant was passed through a filtration membrane (0.22 μm, Minipore) before its bioactivity was assayed. 1 l of homogenized broth was extracted three times with ethyl acetate. The combined organic extract was evaporated under reduced pressure yielding a crude semi-solid. The crude extract was separated by silica gel column chromatography applying gradient elution from petroleum ether to ethyl acetate to methanol to yield 50 fractions, which were analyzed by thin-layer chromatography (TLC) and combined to eight fractions. The spots on silica plates were visualized by exposure to UV radiation and/or by spraying with 1% vanillin in H_2SO_4–methanol (4:1, v/v), followed by heating at 110°C for 10 min. These eight fractions were screened for their ability to inhibit the growth of KB cells (see Section 2.4). Fraction 5 was found to be most active and was further purified by re-crystallization to yield 'compound 1' as colorless prism crystals. The compound was homogeneous by TLC in solvent (A) benzene/ethyl acetate/diethylamine 7:2:1 (v/v) (R_f 0.23), solvent (B) chloroform/methanol 7:1 (v/v) (R_f 0.51) and solvent (C) chloroform/methanol 7:3 (v/v) (R_f 0.68).

2.4. Cell culture and cytotoxicity study

Human tumor cell lines HL60, KB, Hela, SPC-A-1 and MCF-7 were obtained from the Cell Line Bank of the Chinese Academy of Sciences. All the cell lines were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin, 80 U ml^{-1} kanamycin and 100 U ml^{-1} streptomycin. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The cytotoxic effect of the fermentation broth was tested using a protocol (the MTT assay) adapted from that described by Mosmann [11]. The optical density of the wells was measured with a microplate reader (M-3550, Bio-Rad) at 595 nm with 655 nm as reference. Growth inhibition rate was calculated by the following equation:

$$\text{Inhibition rate} = \frac{OD_{\text{control well}} - OD_{\text{treated well}}}{OD_{\text{control well}}} = \times 100\%$$

ID_{50} and IC_{50} were respectively defined as the dilution of fermentation broths and concentration of compounds that resulted in (at least) a 50% inhibition of growth rate.

2.5. Spectroscopic measurements

X-ray crystallographic data were collected on an Enraf-Nonius CAD-4 diffractometer equipped with a graphite monochromator Mo$K\alpha$ radiation ($\lambda = 0.7013$). A total of 1742 independent reflections were collected in the range of $1 < 2\theta < 26°$ by the ω–2θ scan technique at room temperature, and the structure was solved by direct methods that yielded the positions of all non-hydrogen atoms, and which were refined with anisotropic thermal parameters. All hydrogen atoms were generated assuming idealized geometry (C–H bond lengths fixed at 0.96 Å), assigned appropriate isotropic thermal parameters, and allowed to ride on their parent atoms. The computation was performed on a PIII-600 compatible PC with the SHELXL97 for full-matrix least-square refinement.

Nuclear magnetic resonance spectroscopy was undertaken on a Brucker 500 instrument using deuterated ethyl ester. The mass spectra were taken on a Brucker Esquire 3000 Plus mass spectrometer. The infrared ray spectrum was taken on a Nicolet Avatar FT-IR360 IR spectrometer.

Table 1
Cytotoxicity of the endophytic fungi and brefeldin A

	HL-60	KB	Hela	MCF-7	Spc-A-1
	ID_{50}				
W-001	1:300	1:1000	1:5000	1:4000	1:1000
H-036	1:1500	1:3000	1:3000	1:2000	1:3000
H-037	1:200	1:200	1:1000	1:200	1:4000
	IC_{50} (ng ml^{-1})				
Taxol	1.2	0.16	1.8	5.0	0.8
Brefeldin A	10.0	9.0	1.8	2.0	1.0

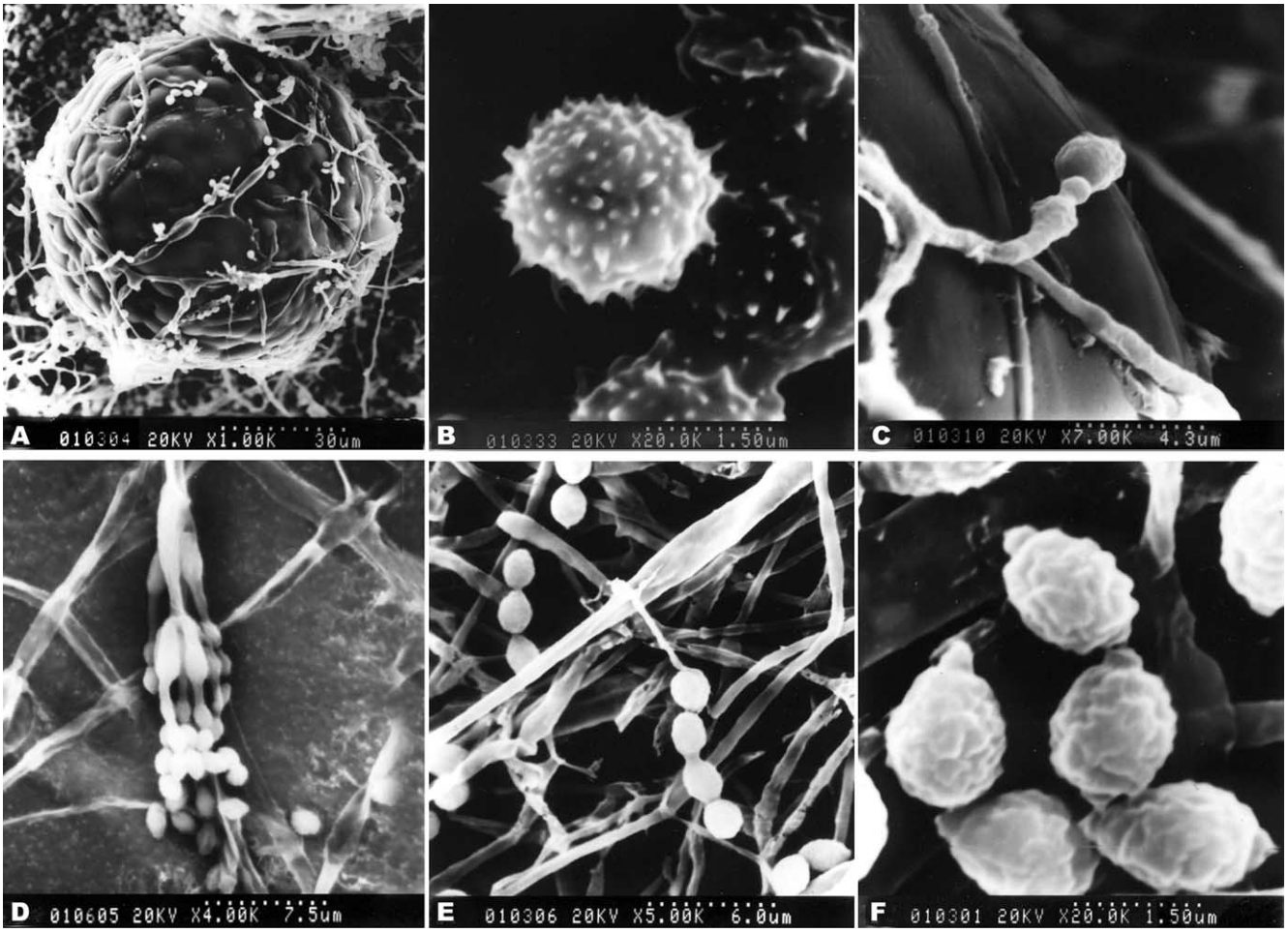

Fig. 1. The morphological observation of *Paecilomyces* sp. strain H-036. A: eleistothecia; B: ascospore inside the eleistothecium; C: conidiophore in single branch; D: conidiophore in three level branches; E: conidio-chain; F: conidia.

3. Results

3.1. Identification of endophytic fungi

Three endophytic fungi were studied in this work. Strains W-001 from *T. mairei* and H-036 and H-037 from *T. grandis* were chosen for study because they possessed excellent antitumor activity (Table 1).

Stain H-036 grew rapidly in PDA medium at 25°C. The mycelium surface was white when young and turned green when mature. The aerial hyphae were hyaline and branched. After 7 days of culture, the fungus produced numerous sexual fruiting bodies, which indicated that it was an eleistothecia. Each eleistothecium was about 50–200 μm in diameter with a thick wall and smooth wavy surface (Fig. 1A). The eleistothecia were engorged with ascospores, which were approximately 2.5 μm in diameter, hyaline, cylindrical and with spinous protuberances on the surface (Fig. 1B). Several kinds of conidiophores grew in the colony with a single branch or two to four levels of branching (Fig. 1C,D). Generally, dozens of conidiospores formed a conidio-chain (Fig. 1E). The conidia were smaller than the ascospores. They were hyaline, and cylindrical to pyliform with verruciform protuberances on

Table 2
Bond lengths [Å] for brefeldin A

Bond	Å	Bond	Å	Bond	Å
O(1)–C(1)	1.331(7)	C(3)–C(4)	1.495(8)	C(9)–C(10)	1.494(7)
O(1)–C(15)	1.446(6)	C(4)–C(5)	1.541(7)	C(10)–C(11)	1.314(7)
O(2)–C(1)	1.202(7)	C(5)–C(6)	1.528(7)	C(11)–C(12)	1.477(8)
O(3)–C(4)	1.416(6)	C(5)–C(9)	1.563(7)	C(12)–C(13)	1.526(8)
O(4)–C(7)	1.434(8)	C(6)–C(7)	1.523(9)	C(13)–C(14)	1.533(9)
C(1)–C(2)	1.477(8)	C(7)–C(8)	1.500(8)	C(14)–C(15)	1.518(8)
C(2)–C(3)	1.304(7)	C(8)–C(9)	1.541(8)	C(15)–C(16)	1.514(9)

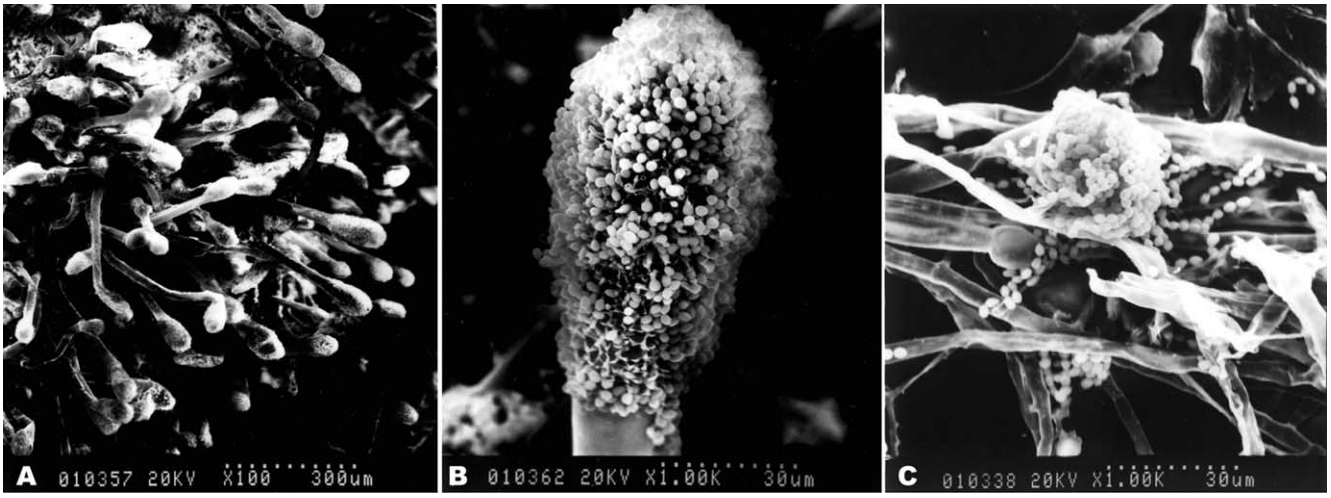

Fig. 2. The morphological observation of *A. clavatus* strain H-037. A: conidiophores; B: head of conidiophore; C: conidia in chain.

the surface (Fig. 1F). According to the characteristics described above, strain H-036 is a species of *Paecilomyces* [8–10].

Strain W-001 displayed similar characteristics to H-036 and was likewise identified as a *Paecilomyces* (data not shown).

Strain H-037 showed excellent growth on PDA medium. The color of the mycelium surface changed from laurel-green to ashen when it matured. This fungus produced numerous asexual fruiting bodies – baseball stick-shaped conidiophores of 200–250 μm in length and 9–12 μm in diameter (Fig. 2A). The head of the conidiophore had a single radiating conidia layer (Fig. 2B). The conidia were hyaline, pyliform and 1.8–2.4×2.3–2.8 μm in size and occurred in chains (Fig. 2C). The fungus was accordingly identified as *A. clavatus* [8–10].

3.2. Structure elucidation of cytotoxic constituent

The organic extracts of the cultures of W-001, H-036 and H-037 all showed high cytotoxicity in the MTT assay. The crude extract of H-036 was fractionated and purified by column chromatography and re-crystallization to give the toxic 'compound 1' as crystalline colorless prisms having R_f 0.23 on silica gel TLC (solvent A) and giving a blue spot when visualized with vanillin–H_2SO_4–MeOH at 110°C. The same cytotoxic compound was subsequently isolated from both W-001 and H-037. Compound 1 isolated from the three fungi had the same crystal parameters, TLC R_f and ions peaks (281.2$[M+H]^+$, 303.2$[M+Na]^+$, 561.4$[[2M+H]^+$, 583.3$[2M+Na]^+$) in electrospray ionization mass spectroscopy spectra. The yield of compound 1 from W-001, H-036 and H-037 was approximately 50, 150 and 100 mg l^{-1}, respectively.

The structure of compound 1 was elucidated by X-ray crystallography. The cell parameters were: $\alpha = 7.3779(2)$ Å; $\beta = 10.994(2)$ Å; $\gamma = 18.829(4)$ Å; $V = 1527.3(5)$ Å. The space system was orthorhombic and the space group $P2_12_12_1$, with four molecules per asymmetric unit ($Z = 4$). The structure was solved by direct methods and refined by least-squares to a final R_f of 5.4% for 1742 independent reflections. Tables 2 and 3 show the bond lengths and bond angles, respectively. The structure of compound 1 (Fig. 3) was determined to be the macrolide, brefeldin A, (4H-cyclopent [f] oxacyclotridecin-4-one-1,6,7,8,9,11a,12, 13,14,12a-decahydro-1, 13-dihydroxy-6-methyl). Furthermore, all other spectroscopic data were in agreement with this structural assignment.

Table 3
Bond angles [°] for brefeldin A

Angle	°	Angle	°	Angle	°
C(1)–O(1)–C(15)	118.5(4)	C(6)–C(5)–C(9)	105.0(4)	C(8)–C(9)–C(5)	103.5(4)
O(2)–C(1)–O(1)	124.2(5)	C(4)–C(5)–C(9)	113.8(4)	C(11)–C(10)–C(9)	127.4(5)
O(2)–C(1)–C(2)	123.8(5)	C(7)–C(6)–C(5)	102.7(4)	C(10)–C(11)–C(12)	126.5(5)
O(1)–C(1)–C(2)	112.0(5)	O(4)–C(7)–C(8)	108.7(5)	C(11)–C(12)–C(13)	114.2(5)
C(3)–C(2)–C(1)	124.0(5)	O(4)–C(7)–C(6)	110.7(5)	C(12)–C(13)–C(14)	115.5(5)
C(2)–C(3)–C(4)	127.1(5)	C(8)–C(7)–C(6)	102.2(5)	C(15)–C(14)–C(13)	113.8(5)
O(3)–C(4)–C(3)	112.5(5)	C(7)–C(8)–C(9)	107.7(5)	O(1)–C(15)–C(16)	110.4(5)
O(3)–C(4)–C(5)	108.4(4)	C(10)–C(9)–C(8)	111.9(5)	O(1)–C(15)–C(14)	104.2(5)
C(3)–C(4)–C(5)	109.4(4)	C(10)–C(9)–C(5)	114.1(4)	C(16)–C(15)–C(14)	113.9(5)
C(6)–C(5)–C(4)	113.0(4)				

Symmetry transformations used to generate equivalent atoms.

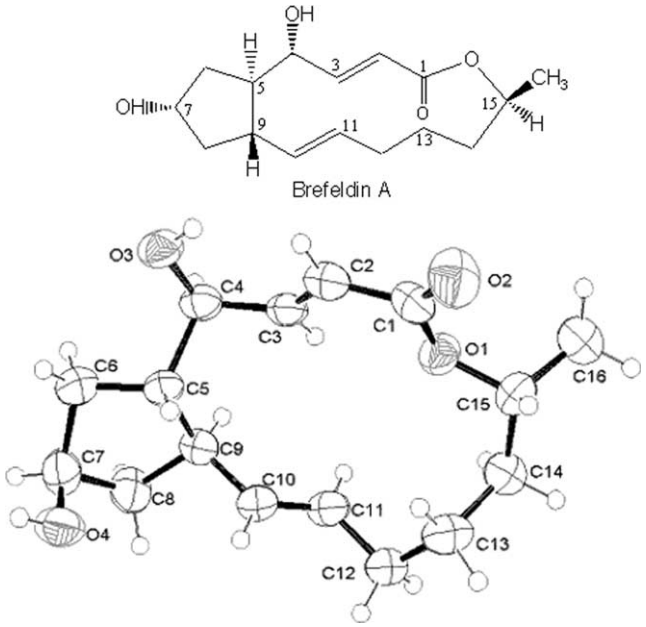

Fig. 3. Chemical structure of brefeldin A.

3.3. Cytotoxic activities of the endophytic fungi and brefeldin A

Cytotoxic activities against the human tumor cell lines HL-60, KB, Hela, MCF-7 and Spc-A-1, were compared with those of taxol, which is used clinically for cancer patients. The degree of cell growth inhibition was measured by the MTT assay after 3 days of exposure.

All three fungi showed high cytotoxicity against the tumor cell lines, and their values of 50% inhibitory dilution ranged from 1:200 to 1:5000. HL-60 was the cell line least sensitive to the fermentation broth of these three fungi and to brefeldin A (Table 1).

The cell line most sensitive to taxol was KB, while the most sensitive to brefeldin A was Spc-A-1. Values for the IC_{50} of taxol and brefeldin A are given for each cell line in Table 1.

4. Discussion

Our previous work showed that endophytic fungi were abundant in *T. mairei*, *T. grandis* and *C. fortunei* with great diversity [4–5]. This study focused on the taxonomic position and cytotoxic metabolites of three endophytic fungi (H-036, W-001 and H-037) with high antitumor activity.

H-036 and W-001, isolated from *T. grandis* and *T. mairei*, respectively, had almost identical morphological characteristics and were found to belong to the genus *Paecilomyces*. H-037 was identified as *A. clavatus*. This is the first time that *A. clavatus* has been isolated from *T. grandis* as an endophytic fungus.

Up to now, a number of bioactive compounds from *Paecilomyces* sp. have been reported. These include: leucinostatins A, D, H and K as peptide antibiotics [12–14]; polygalactosamine with antitumor activity [15]; saintopin and UCE1022 as antitumor antibiotics with topoisomerase-dependent DNA cleavage activity [16–17]; sphingofungins E and F as novel serinepalmitoyl transferase inhibitors [18]; paeciloquinones A, B, C, D, E and F, protein tyrosine kinases inhibitors [19]; kurasoins A and B, inhibitors of protein farnesyltransgerase [20]; lipohexin, an inhibitor of prolyl endopeptidase [21]; asetoxyscirpenediol and ergosterol peroxide with cytotoxic activities [6]; beauvericin and beauvericin A, two antimycobacterial and antiplasmodial cyclodepsipeptides [22]. Recently, apoptotic antitumor activity [23], antioxidant and immunostimulating activities of *Paecilomyces japonica* were reported [24]. *A. clavatus* produces patulin, clavatol, c-sarcin, cytochalasin E and tremorgenic mycotoxins [7,25,26]. These reports indicate that *Paecilomyces* sp. and *A. clavatus* are promising sources of natural bioactive agents.

Strains H-036, W-001 and H-037 can produce brefeldin A with high yield (50–150 mg l^{-1}) when grown in liquid culture. Brefeldin A, also named decumbin, cyanein, ascotoxin and sinergisidin [27], is a 16-membered macrolide antibiotic which had previously been isolated from a number of fungal genera: *Alternaria*, *Ascochyta*, *Penicillium*, *Curvularia*, *Cercospora*, and *Phyllosticta* [28]. We report in this work for the first time brefeldin A isolation as a metabolite of *Paecilomyces* sp. and *A. clavatus*.

Brefeldin A has several important bioactive activities, including antifungal, antivitral and anticancer activity [25–27]. It can even be used in weed management [28]. Recently, brefeldin A was found to induce a wide variety of human cancer cells to differentiate and apoptose, and now it is in development as an anticancer agent [29]. We also found it to show high cytotoxicity against HL-60, KB, Hela, MCF-7 and Spc-A-1 cell lines (IC_{50} = 1.0–10.0 ng ml^{-1}). Cytotoxicities against Hela, MCF-7 and Spc-A-1 cell lines were close to that of taxol. Brefeldin A is therefore a very promising compound in the area of cancer therapy.

Moreover, we found the three fungi studied in this work to have different anticancer activities against the different cancer cell lines (Table 1), which suggested that there were some other cytotoxic metabolites besides brefeldin A in the fungal cultures. Hence, the metabolites of the three fungi are worth further study.

The fungi we isolated from *T. grandis* and *T. mairei* were traditionally obtained from the phloem/cambial region after thorough treatment of the bark with 70% ethanol, and the bark samples were collected from the plant without obvious symptoms. We suggest that some kind of mutualism has developed between H-036, H-037, W-001 and their hosts. The fungi primarily live in the intercellular spaces of the hosts tissue acquiring support, protection and food from the nutrient-rich phloem, while producing one or more antibiotics that provide protection from bac-

terial infection to the tree hosts [30]. When the host is stressed, however, endophytic fungi may become pathogenic. This delicate equilibrium between endophytic fungi and their host seems to be controlled partially by chemical factors. In this study, it is conceivable that brefeldin A may play an important role in regulating the relationship between fungi (*Paecilomyces* H-036, W-001 and *A. clavatus* H-037) and plant hosts (*T. grandis* and *T. mairei*). It can affect the normal function of the secretory system of plant cells by inhibiting vesicle formation at the Golgi apparatus [31], which could enable the fungi to obtain food from their hosts more easily. On the other hand, brefeldin A may primarily function to protect the plant host from attacks by animals, insects or microbes.

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