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Azole-synergistic Anti-Candidal Activity of Altenusin, a Biphenyl Metabolite of the Endophytic Fungus *Alternaria alternata* Isolated from *Terminalia chebula* Retz.

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In this study, a tropical endophytic fungus, *Alternaria alternata* Tche-153 was isolated from a Thai medicinal plant *Terminalia chebula* Rezt. The ethyl acetate extract prepared from the fermentation broth exhibited significant ketoconazole-synergistic activity against *Candida albicans*. Bioassay-directed fractionation of the ethyl acetate extract led to the isolation of altenusin (1), isoochracinic acid (2), and altenuic acid (3) together with 2,5-dimethyl-7-hydroxychromone (4). Using the disc diffusion method and the microdilution chequerboard technique, only altenusin (1) in combination with each of three azole drugs, ketoconazole, fluconazole or itraconazole at their low sub-inhibitory concentrations exhibited potent synergistic activity against *C. albicans* with the fractional inhibitory concentration index range of 0.078 to 0.188. This first discovery of altenusin (1) as a new azole-synergistic prototype possessing a biphenyl structure is of significance for further development of new azole-synergists to treat invasive candidiasis.

***Keywords*:** endophytic fungus, *Alternaria alternata*, *Terminalia chebula*, altenusin, biphenyl, azole-synergism, anticandidal activity

Introduction

Candida species are opportunistic fungal pathogens that can cause diverse diseases ranging from mucocutaneous infections to severe invasive candidiasis. Oropharyngeal candidiasis (OPC) is commonly seen in patients with human immunodeficiency virus (HIV) infection, acquired immune deficiency syndrome (AIDS) (Greenspan, 1994) and cancer (Gligorov *et al*., 2011). Immunocompromized people, especially patients with hematopoietic cells and solid organ transplants, are particularly susceptible to invasive candidiasis (Kriengkauykiat *et al*., 2011). Candidemia is a life-threatening infection in critically ill patients and *C. albicans* is globally the most common species causing bloodstream infection (Pfaller *et al*., 2011). Azoles, including ketoconazole, fluconazole, itraconazole, and voriconazole, are a group of systemic antifungal agents available for treatment of candidiasis (Patton *et al*., 2001; Kriengkauykiat *et al*., 2011). Azole-resistant *Candida* infections have been reported in immunocompromized patients (Pfaller *et al*., 2011). Therefore, several approaches have been investigated to combat these infections, involving the search for new antifungal entities (Tempone *et al*., 2007), the innovation of new dosage formulations (Ricci *et al*., 2004), and the search for a new combination therapy to improve the antifungal efficacy. A number of studies on drug combinations with well-known azoles showing a synergistic effect against *C. albicans* have recently been reported, such as fluconazole and amiodarone (Guo *et al*., 2008), fluconazole and cyclosporine (Li *et al*., 2008), and fluconazole and micafungin (Nishi *et al*., 2009). In addition, several groups of natural products have been reported to exhibit synergistic effects with azoles such as fluconazole and berberine chloride (Quan *et al*., 2006), ketoconazole and xanthorrhizol from *Curcuma xanthorrhiza* (Rukayadi *et al*., 2009), azoles and curcumin from *Curcuma longa* (Sharma *et al*., 2010), ketoconazole and tetrandrine (Zhang *et al*., 2010), fluconazole and eugenol, fluconazole and methyleugenol (Ahmad *et al*., 2010), and fluconazole and curvularide B from the endophytic fungus *Curvularia geniculata* (Chomcheon *et al*., 2010).

Endophytic fungi live asymptomatically and inconspicuously in plant tissues (Strobel and Daisy, 2003). It has been hypothesized that a symbiotic interaction exists in which the fungi produce metabolites to promote the growth of the host plants which in turn provide nutritional benefit to the fungi (Tan and Zou, 2001). Interestingly, endophytic fungi have been recognized as a new source of diverse bioactive secondary metabolites with potential pharmacological and agrochemical properties (Strobel, 2003; Yu *et al*., 2010).

In the course of our screening program for azole-synergistic effects of endophytic fungi, we found that the crude ethyl acetate (EtOAc) extract from the endophytic fungus *Alternaria alternata* Tche-153 showed significant synergistic activity with ketoconazole against *C. albicans*. The endo-

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phytic fungus, *A. alternata* Tche-153 was isolated from the fresh leaves of a Thai medicinal plant *Terminalia chebula* Retz. Traditionally, the leaves of *T. chebula* have been used as a mild laxative, antipyretic, and carminative while the unripe fruits have been used as a mild laxative, antipyretic, astringent for dysentery and hemorrhoids, and as an expectorant for sore throat (Bunyapraphatsara and Chokechaijaroenporn, 2000). *Alternaria* species are plant pathogens and have also been isolated as endophytic fungi from various plants, for example, *Polygonum senegalense* (Aly *et al.*, 2008), *Trixis vauthieri* (Cota *et al.*, 2008), and *Ficus carica* (Chengliang and Yangmin, 2010). Several groups of secondary metabolites produced by *Alternaria* species have been reported to possess various bioactive properties, including cytotoxicity (Aly *et al.*, 2008; Phuwapraisirisan *et al.*, 2009; Huang *et al.*, 2011; Zheng *et al.*, 2012), antibacterial activity (Wang *et al.*, 2009), antifungal activity (Chengliang and Yangmin, 2010), and trypanothione reductase inhibitory activity (Cota *et al.*, 2008).

This paper reports on the isolation and identification of secondary metabolites responsible for the azole-synergistic property of the isolated endophytic fungus *A. alternata* Tche-153 against *C. albicans.*

Materials and Methods

General

^{1}H, ^{13}C and 2-D NMR measurements were acquired with Bruker Avance DPX-300 spectrometers (Bruker, Germany), Varian Mercury plus 400 (Varian, USA), JEOL JMN-A 500 (Jeol, Japan), and Bruker Biospin Avance AV-500 FTNMR (Bruker) spectrometers using solvent signals ($CDCl_3$, CD_3OD, or DMSO-d_6) as internal references. ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and ^{1}H-detected heteronuclear multiple bond correlation (HMBC) experiments were optimized for $^{1}J_{CH}$ = 145.0 Hz and $^{n}J_{CH}$ = 8.0 Hz, respectively. Electron impact mass spectra (EIMS) were recorded on a JEOL JMS 700 mass spectrometer (Jeol). Electrospray ionization time-of-flight mass spectra (ESI TOFMS) were recorded on a Micromass LCT mass spectrometer (Bruker). Chromatographic techniques were performed using Silica gel (Merck, Germany) for column chromatography and Sephadex LH-20 (GE Healthcare Bio-Sci, Sweden) for gel filtration chromatography. Analytical thin layer chromatography was performed on precoated Merck F254 silica gel plates. All chemicals used in the study were of analytical grade.

Isolation of endophytic fungi

Apparently healthy leaves of *T. chebula* (Family Combretaceae) were collected from Suanluang Rama IX Public Park, Bangkok, Thailand. The leaf samples were cleaned under running tap water and then air-dried. The cleaned leaves were surface-sterilized using sodium hypochlorite solution and subjected to isolation of endophytic fungi as previously described (Wiyakrutta *et al.*, 2004). A fungal isolate designated Tche-153 was selected for this investigation.

Identification of the endophytic fungus *A. alternata* Tche-153

Conventional method: The endophytic fungus Tche-153 was cultured on potato dextrose agar (PDA) plates and water agar plates with 5-6 small pieces of sterilized banana leaf at 25°C for 10 days. Its macroscopic and microscopic characteristics were then observed.

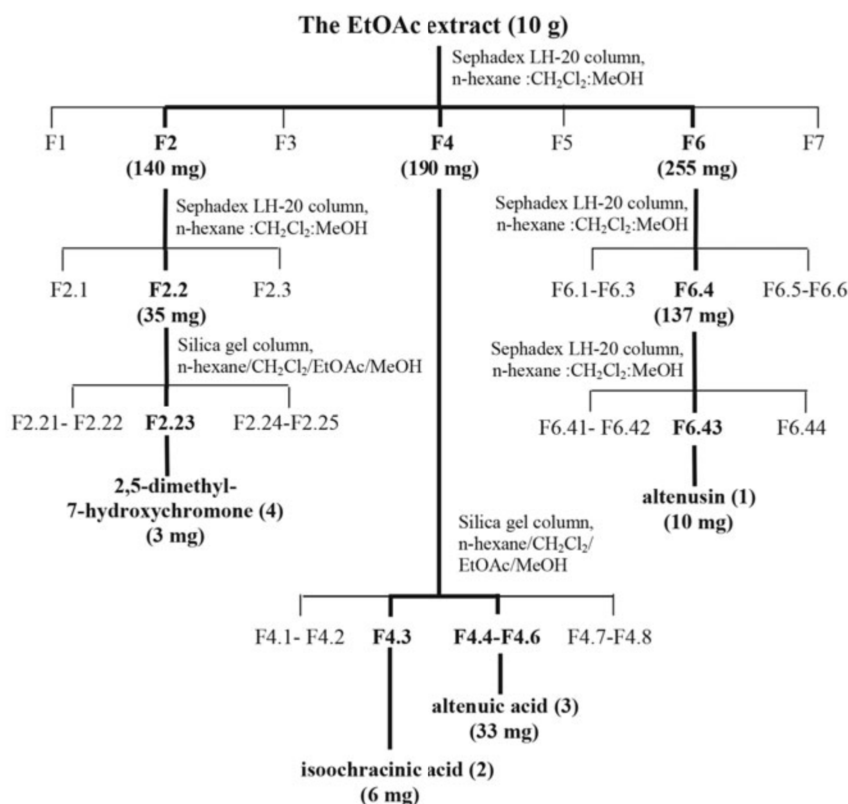
Molecular method: The Tche-153 fungus was grown in a potato dextrose broth for 4 days and total cellular DNA was extracted from the washed fungal mycelium using a FTA® Plant Kit (Whatman®, USA) according to the manufacturer's instructions. The ITS1-5.8S-ITS2 of the ribosomal RNA gene region was amplified from the fungal genomic DNA by PCR with TopTaq™ PCR Master Mix (QIAGEN, Germany) using the ITS5 (GGAAGTAAAAGTCGTAAC AAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.*, 1990) as previously described (Prachya *et al.*, 2007). The PCR products were gel-purified and directly subjected to DNA sequencing (Bioservice Unit, NSTDA, Thailand) in both directions and primed with either of the two primers used to originally amplify the fragment. The DNA sequence of the ITS1-5.8S-ITS2 rRNA gene obtained was used as a query sequence to search for similar sequences in GenBank using BLASTN 2.2.24+ (Zhang *et al.*, 2000). DNA sequences of reference and published strains of *Alternaria* were retrieved for phylogenetic analysis. The DNA sequences were aligned using the Clustal W multiple sequence alignment program in the CLC Main Workbench software package version 6.6.2 (CLC Bio, Denmark) with manual final adjustment. The phylogenetic relationship was estimated with the neighbor-joining method. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

Cultivation of the endophytic fungus *A. alternata* Tche-153

The endophytic fungus *A. alternata* Tche-153 was cultivated on PDA agar plates at 25°C for 7 days. Six pieces (@ 0.5 × 0.5 cm) of mycelial agar plugs were inoculated into a 1000-ml Erlenmeyer flask containing 200 ml of yeast extract sucrose (YES) broth (Paterson and Bridge, 1994) and incubated at 25°C under still conditions for 21 days. Twenty liters of the culture broth were collected for isolation and purification of fungal metabolites.

Extraction, fractionation, and isolation

Twenty liters of the endophytic fungus *A. alternata* Tche-153 culture broth were filtered and the combined filtrate was exhaustively partitioned with equal volumes of EtOAc. The EtOAc part was evaporated under reduced pressure to give a crude EtOAc extract as a dark brown mass (10 g). The crude EtOAc extract (10 g) was subjected to fractionation by column chromatography over a Sephadex LH-20 column (2.5× 150 cm) using n-hexane:dichloromethane (CH_2Cl_2):methanol (MeOH) (2:2:1) as the eluent to yield seven pooled-fractions (fractions 1–7). All fractions were tested for azole-synergistic activity against *C. albicans.* Only fraction 6 (255 mg) showed antifungal activity and was chosen for further fractionation by a Sephadex LH-20 column using n-hexane: CH_2Cl_2:MeOH (2:2:1) as the eluent to yield six fractions (fractions 6.1–6.6). The active fraction (fraction 6.4, 137 mg)



Scheme 1. The flow-chart of the isolation process for compounds 1-4 from *A. alternata* Tche-153.

was repeatedly purified using a Sephadex LH-20 column eluted with a mixture of n-hexane:CH_2Cl_2:MeOH (2:2:1) to yield four fractions (fractions 6.41–6.44). Fraction 6.43 was finally recrystallized with a mixture of n-hexane:CH_2Cl_2:MeOH to provide altenusin (**1**, 10 mg). Fractions 2 and 4 exhibited no antifungal activity but showed interesting silica TLC patterns. Therefore, the fractions were investigated for their chemical compositions. Fraction 4 (190 mg) was fractionated with vacuum liquid column chromatography over silica gel using gradient solvent systems of n-hexane:CH_2Cl_2:EtOAc:MeOH to yield eight fractions (fractions 4.1–4.8). Fraction 4.3 was recrystallized with a mixture of CH_2Cl_2:EtOAc to give isoochracinic acid (**2**, 6 mg). Fractions 4.4–4.6 were combined and recrystallized with CH_2Cl_2:EtOAc to give altenuic acid (**3**, 33 mg). Fraction 2 (140 mg) was repeatedly separated with a Sephadex LH-20 column using n-hexane:CH_2Cl_2:MeOH (2:2:1) as the eluent to yield three fractions (fractions 2.1–2.3). Fraction 2.2 (35 mg) was further separated by vacuum liquid column chromatography over silica gel using gradient mixtures of n-hexane:CH_2Cl_2:EtOAc:MeOH as the solvent system to yield five fractions (fractions 2.21–2.25). Fraction 2.23 was recrystallized with CH_2Cl_2 to give 2,5-dimethyl-7-hydroxychromone (**4**, 3 mg). The isolation flow-chart for compounds **1–4** is outlined in Scheme 1.

Altenusin (**1**): reddish prisms; $C_{15}H_{14}O_6$; MS: 290. ^{1}H-NMR (400 MHz, CD_3OD): δ 1.90 (3H, *s,* H_3-2′), 3.80 (3H, *s*, 5-OCH_3), 6.11 (1H, *d*, *J* = 2.7 Hz, H-6), 6.37 (1H, *d*, *J* = 2.7 Hz H-4), 6.43 (1H, *s*, H-6′), 6.52 (1H, *s*, H-3′). ^{13}C-NMR (100 MHz, DMSO-*d6*): δ 175.0 (C-7), 165.9 (C-3), 165.0 (C-5), 148.0 (C-1), 145.0 (C-4′), 143.0 (C-5′), 135.5 (C-1′), 127.5 (C-2′), 118.0 (C-3′), 117.0 (C-6′), 111.0 (C-6), 107.0 (C-2), 101.0 (C-4), 56.0 (5-OCH_3), 19.5 (2′-CH_3).

Isoochracinic acid (**2**): colorless amorphous powder; $C_{10}H_8O_5$; MS: 208. ^{1}H-NMR (500 MHz, CD_3OD): δ 2.75 (1H, *dd, J* = 16.7, 7.9 Hz, H-2′a), 3.00 (1H, *dd J* = 16.7, 4.9 Hz, H-2′b), 5.77 (1H, *dd*, *J* = 7.9, 4.9 Hz, H-1′), 6.85 (1H, *dd*, *J* = 8.1, 0.6 Hz, H-4), 7.02 (1H,*dd, J* = 7.7, 0.6 Hz, H-6), 7.52 (1H, *dd*, *J* = 8.1, 7.7 Hz, H-5). ^{13}C-NMR (125 MHz, CD_3OD): δ 172.9 (C-3′-COOH), 171.4 (C-7), 158.3 (C-3), 152.5 (C-1), 137.7 (C-5), 117.0 (C-4), 113.9 (C-6), 112.6 (C-2), 78.9 (C-1′), 40.2 (C-2′).

Altenuic acid (**3**): colorless needles; $C_{15}H_{14}O_8$; MS: 322. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 1.69 (3H, *s*, H_3-7′), 3.80 (3H, *s*, 5-OCH_3), 2.50 (1H, *d*, *J* =16.0 Hz, H-5′a), 2.76 (1H, *d*, *J* = 16.0 Hz, H-5′b), 2.95 (1H, *d*, *J* =18.0 Hz, H-2′a), 3.79 (1H, *d*, *J* =18.0 Hz, H-2′b), 6.45 (1H, *d* ,*J* = 2.0 Hz, H-4), 6.78 (1H, *d*, *J* = 2.0 Hz, H-6), 10.97 (3-OH). ^{13}C-NMR (125 MHz, DMSO-*d6*): δ 172.25 (C-3′), 169.9 (C-6′-COOH), 165.8 (C-5), 165.3 (C-7), 158.2 (C-3), 149.2 (C-1), 105.2 (C-23), 102.2 (C-4), 101.1 (C-6), 88.8 (C-1′), 88.1 (C-4′), 56.0 (5-OCH_3), 40.6 (C-2′), 39.0 (C-5′), 21.9 (C-7′).

2,5-Dimethyl-7-hydroxychromone (**4**): colorless needles; $C_{11}H_{10}O_3$; MS: 190. ^{1}H-NMR (500 MHz, CD_3OD): δ 2.32 (3H, *d*, *J* = 0.7 Hz, 2-CH_3), 2.72 (3H, *s*, 5-CH_3), 6.62 (1H, *dd*, *J* = 2.6,1.0 Hz, H-6), 6.64 (1H, *d*, *J* = 2.6 Hz, H-8). ^{13}C-NMR (125 MHz, CD_3OD): δ 182.1 (C-4), 166.6 (C-2), 163.1 (C-7), 161.5 (C-8a), 143.7 (C-5), 118.0 (C-6), 115.6 (C-4a), 111.4 (C-3), 101.7 (C-8), 23.1 (5-CH_3), 19.8 (C-2-CH_3).

Table 1. Fungal reference and published strains used in the phylogenetic analysis

Species	Strains	GenBank accession no. of ITS1-5.8S-ITS2 sequence
A. alternata	ATCC 13963	AY625056
A. alternata	ATCC 28329	AF229459
A. brassicae	BMP 21-61-02	AF229463
A. brassicicola	EEB 2232	AF229462
A. carotiincultae	EGS 26-010	AF229465
A. cheiranthi	EGS 41-188	AF229457
A. crassa	DGG Acr1	AF229464
A. dauci	ATCC 36613	AF229466
A. japonica	ATCC 13618	AF229474
A. longissima	ATCC 18552	AF229489
A. macrospora	DGG Ams1	AF229469
A. malorum var. *polymorpha*	STE-U 457	AY251080
A. petroselini	EGS 09-159	AF229454
A. porri	ATCC 58175	AF229470
A. radicina	ATCC 6503	AF307014
A. radicina	ATCC 58405	AF307015
A. radicina	ATCC 96831	AF229471
A. selini	EGS 25-198	AF229455
A. smyrnii	EGS 37-093	AF229456
A. solani	ATCC 58177	AF229475
A. tagetica	ATCC 58771	DQ100420
A. tenuissima	ATCC 16423	AF229476
Chalastospora gossypii[a]	CBS 148.66	FJ214870
C. gossypii[a]	CBS 540.75	FJ214896
C. gossypii[a]	CBS 900.87	FJ214860
C. gossypii[a]	CBS 114809	FJ214861
C. gossypii[a]	CBS 114810	FJ214894
Lewia infectoria[b]	BMP 21-11-15	AF229458
Bipolaris tetramera	CBS 371.72	AY004777
Exserohilum mcginnisii	ATCC 60408	AF081453

[a] *Chalastospora gossypii* is the current name of *Alternaria malorum*.
[b] *Lewia infectoria* is the teleomorph and the current name of *Alternaria infectoria*.

Determination of anti-*Candida albicans* activity

The activity of each isolated metabolite against *C. albicans* ATCC 90028 was preliminarily determined using the disc diffusion method as previously described (Chomcheon *et al*., 2010). Briefly, an overnight culture of *C. albicans* ATCC 90028 diluted with 0.85% NaCl to 50% T_{580nm} was added at 1% inoculum into molten SDA with and without ketoconazole at a sub-inhibitory concentration (0.125 µg/ml). The seeded medium (9 ml) was transferred into a 9-cm petri dish. Ten microliter of each isolated metabolite (256 µg) in DMSO and DMSO (control) were applied to paper discs (6 mm dia.) placed on the inoculated agar surface. After storage at room temperature for 1 h, the test plates were incubated at 37°C for 24 h. The clear zone of inhibition around the disc indicating the antimicrobial activity of the compound was measured. The experiment was done in duplicate.

Determination of minimum inhibitory concentration (MIC) of altenusin (1)

Altenusin (**1**) was tested over a final concentration range of 256 to 0.5 µg/ml, with serial two-fold dilutions, against *C. albicans* ATCC 90028 using the broth microdilution method as described in the NCCLS document M27-A2 (NCCLS, 2002). The azole drugs, ketoconazole, fluconazole and itraconazole, in a final concentration range of 64 to 0.125 µg/ml were used as positive controls. The experiment was done in triplicate. The MIC-0 of the drug is defined as the lowest concentration that gives rise to no visible growth. According to the fungistatic activity of the azole drug that results in a trailing effect in growth inhibition, the MIC-2 is applied for the azole drug and defined as the lowest concentration causing a prominent decrease in turbidity as compared with the control growth (NCCLS, 2002).

Determination of synergistic activity using the microdilution chequerboard technique

Synergistic activity of altenusin (**1**) and azole drugs against *C. albicans* was determined with the broth microdilution chequerboard technique (Sun *et al*., 2008). The final drug concentrations after addition of 100 µl of the inocula ranged from 256 to 4 µg/ml for altenusin (**1**) and 16 to 0.25 µg/ml for the azole drugs. Plates were incubated at 37°C for 48 h. The experiment was done in triplicate.

A fractional inhibitory concentration (FIC) index was used to indicate the effect of the combination. The FIC index is

the sum of the MIC-0 of each drug when used in combination divided by the MIC-0 of the drug when used alone. Synergism, indifference and antagonism were defined as FIC indices of <0.5, >0.5 - 4 and >4, respectively (Odds, 2003).

Results and Discussion

Identification of the endophytic fungus *A. alternata* Tche-153

Morphological characteristics: The Tche-153 fungus grew on PDA as a gray cottony colony with brown diffusible pigment around the colony and brown color on the reverse side. On water agar with sterilized banana-leaf pieces, the Tche-153 fungus produced brown catenate conidia on a brown conidiophore. Conidia were obclavate with a short cylindrical beak, verrucose, with up to 8 transverse septa and a few longitudinal septa, and overall 25–49 μm long and 8–12 μm wide in the broadest part. These characteristics suggest that the Tche-153 isolate is *Alternaria alternata* (Ellis, 1993).

DNA sequence based identification: The complete ITS1-5.8S-ITS2 DNA sequence of the endophytic fungus Tche-153 was perfectly matched (100% homology) with those of *A. alternata* (HM003680, GQ916545, GQ169766, DQ156341), *A. porri* (DQ156345), *A. tenuissima* (AF276656, DQ323692), and *A. longipes* (AF267137). However, none of the perfectly matched strains were reference strains. Therefore, the complete ITS1-5.8S-ITS2 sequences of *Alternaria* reference and published strains available in NCBI databases were retrieved using CLC Mainworkbench 6.6.2 (CLC Bio, Denmark), as shown in Table 1, and aligned with that of the Tche-153 fungus. It was found that the complete ITS sequence of Tche-153 showed the highest homology (99.79%) to that of *A. alternata* ATCC 28329 (AF229459) and 99.59% homology to those of *A. alternata* ATCC MYA-4642 (HQ263343), *A. alternata* ATCC 13963 (AY625056), and *A. tenuissima* ATCC 16423 (AF229476). The phylogenetic analysis using a neighbor-joining method placed Tche-153 in the clade of the alternata species-group with 100% bootstrap support, as shown in Fig. 1.

Based on the morphological characteristics, molecular phylogenetic analysis and current systematics of fungi in the genus *Alternaria* (Pryor and Gilbertson, 2000; Andrew *et al.*, 2009), this endophytic fungus was identified as *Alternaria alternata* Tche-153. A culture of *A. alternata* Tche-153 has been deposited at the Department of Microbiology, Faculty of Science, Mahidol University, Thailand. The DNA sequences of the ITS1-5.8S-ITS2 region of this fungus have been submitted to the GenBank database with the accession number JN210895.

Identification of the isolated compounds

The crude EtOAc extract (1 mg/disc) of the endophyte *A. alternata* Tche-153 primarily exhibited ketoconazole-synergistic activity against *C. albicans* with an inhibition zone diameter of 23.59 mm. The extract was further purified using bioassay-guided fractionation with chromatographic combinations of Sephadex LH-20 and silica gel columns to afford four compounds (**1–4**) as shown in Fig. 2. Following extensive analyses of NMR spectral data (^{1}H and ^{13}C; 2D H-H

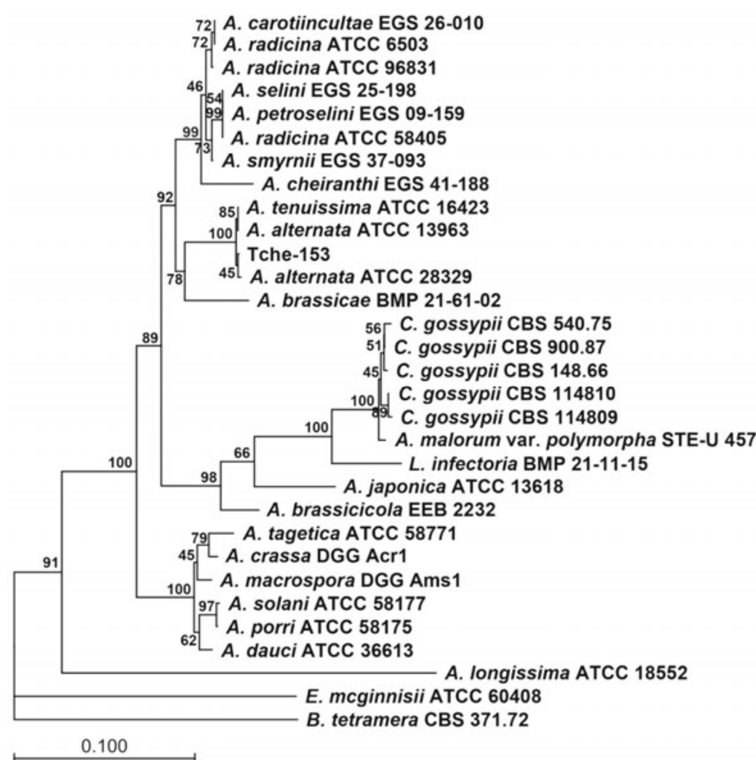


Fig. 1. Phylogenetic tree generated from neighbor-joining analysis of ITS1-5.8S-ITS2 sequences of Tche-153 with reference and published strains of *Alternaria* (see Table 1 for details). Bootstrap values (percentage from 1,000 replicates) are indicated at the nodes. The scale bar indicates the number of nucleotide change per site. *Bipolaris tetramera* and *Exserohilum mcginnisii* are used as outgroups.

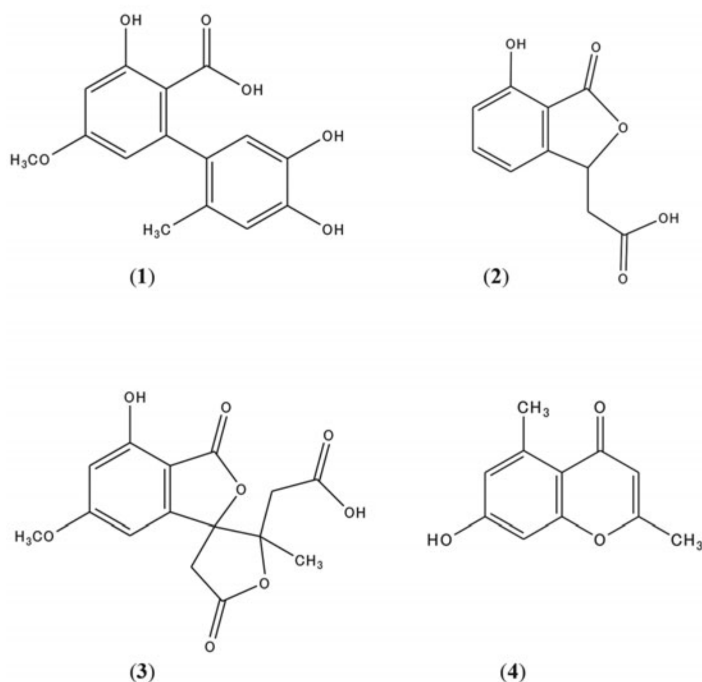


Fig. 2. Structures of the metabolites isolated from *A. alternata* Tche-153: altenusin (**1**), isoochracinic acid (**2**), altenuic acid (**3**), and 2,5-dimethyl-7-hydroxychromone (**4**).

Fig. 3. Disc diffusion assay determining synergistic activity with ketoconazole against *C. albicans* of the isolated secondary metabolites (256 µg/disc, each) from *A. alternaria* Tche-153: the left SDA plate without ketoconazole, the right SDA plate with a sub-inhibitory concentration of ketoconazole (0.125 µg/ml), A, altenuic acid (**3**); B, 2,5 dimethyl-7-hydroxychromone (**4**); C, altenusin (**1**) and D, dimethylsulfoxide (control).

COSY, HMQC, HMBC, and NOESY) and mass spectral data in combination with data comparison from the literatures, compounds **1–3** were respectively identified to have polyketide origins, including altenusin (Nakanishi *et al.*, 1995; Kamisuki *et al.*, 2004), isoochracinic acid (Stierle *et al.*, 1991) and altenuic acid (Rosett *et al.*, 1957; Williams and Thomas, 1973) and compound **4** as 2,5-dimethyl-7-hydroxychromone (Lee *et al.*, 2001).

The isolated compounds were previously reported from several natural sources as follows: altenusin (**1**) from microorganisms in different genera, including other *Alternaria* spp. (Aly *et al.*, 2008; Cota *et al.*, 2008; Johanna *et al.*, 2012), *Penicillium* and *Streptomyces* (Nakanishi *et al.*, 1995); isoochracinic acid (**2**) from *Mycosphaerella fijiensis* (Stierle *et al.*, 1991); altenuic acid (**3**) from *A. tenusis* (Rosett *et al.*, 1957); and 2,5-dimethyl-7-hydroxychromone (**4**) from *Cassia fistula* (Lee *et al.*, 2001) and *Alternaria* sp. (Kjer *et al.*, 2009).

Azole-synergistic activity of altenusin (1) against *C. albicans*

All isolated metabolites (**1–4**) were investigated for their anti-*C. albicans* activity with the disc diffusion assay. Altenusin (**1**) at the amount of 256 µg/disc exhibited weak activity against *C. albicans* with an unclear inhibition zone diameter of 8.3 mm. In the presence of a sub-inhibitory concentration of ketoconazole at 0.125 µg/ml, altenusin (**1**) produced a clear inhibition zone diameter of 19.2 mm while the other isolated metabolites (**2–4**) at the same concentration were completely inactive, as shown in Fig. 3. This suggests

Table 2. MICs (µg/ml) of altenusin (1) and selected azole drugs using the microdilution chequerboard technique indicating synergistic activity against *C. albicans*

Azole drugs	Azole drug alone		MIC-0 in combination		FIC index
	MIC-2[a]	MIC-0[b]	Azole drugs	Altenusin (**1**)[c]	
Ketoconazole	0.25	16	0.25	16	0.078
Fluconazole	2	>64	1	32	0.141
Itraconazole	2	>64	4	32	0.188

[a] MIC-2 is defined as the minimum inhibitory concentration that causes a prominent decrease in turbidity as compared with the growth control.
[b] MIC-0 is defined as the minimum inhibitory concentration that gives rise to no visible growth.
[c] MIC-0 of altenusin (**1**) alone was 256 µg/ml.

that altenusin (**1**) in combination with an azole drug might be synergistic against *C. albicans*. Therefore, the microdilution chequerboard technique was used to analyze the interaction of altenusin (**1**) with three representative azole drugs, including ketoconazole, fluconazole and itraconazole. As shown in Table 2, altenusin (**1**) showed strong synergistic activity against *C. albicans* with all three selected azole drugs with the FIC index range of 0.078–0.188. Among the three azole drugs, the combination of altenusin (**1**) with ketoconazole showed the highest synergistic activity with an FIC index of 0.078. The MIC-0s of ketoconazole and altenusin (**1**) were 16 and 256 μg/ml when used alone and dramatically reduced to 0.25 and 16 μg/ml when used in the combination, respectively. The synergistic activity determined by the microdilution chequerboard technique confirmed the results of the disc diffusion assay. The correspondence of results obtained from the two methods found in this study was in accordance with a previous study of curvularide B (Chomcheon *et al*., 2010). These results demonstrate that the simple disc diffusion assay using a culture medium containing a sub-inhibitory concentration of drug could be used as a preliminary method to screen a large number of test compounds for synergistic activity against *C. albicans*.

This is the first report of azole-synergistic activity of altenusin (**1**) against *C. albicans*. The biphenyl basic skeleton of altenusin (**1**) containing a salicylic moiety and a catechol moiety could be the important part for its interesting azole-synergistic activity. Previously, altenusin (**1**) was reported to possess broad antimicrobial activity against several multidrug-resistant bacterial and fungal strains (Kjer *et al*., 2009; Johann *et al*., 2012) and also exhibit cytotoxicity against the L5178Y mouse lymphoma cell line (Kjer *et al*., 2009).

Conclusion

In this report, we demonstrate that altenusin (**1**) exhibits significant synergistic activity, when combined with azole drugs, against *C. albicans* in low concentration while the related derivatives **2** and **3** are completely inactive. Because this is the first discovery of a biphenyl derivative exhibiting significant synergistic activity with azole drugs against *C. albicans*, altenusin (**1**) may serve as a potential lead candidate of the drug discovery program for more potent azole-synergistic drugs to effectively combat widespread candidiasis.

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