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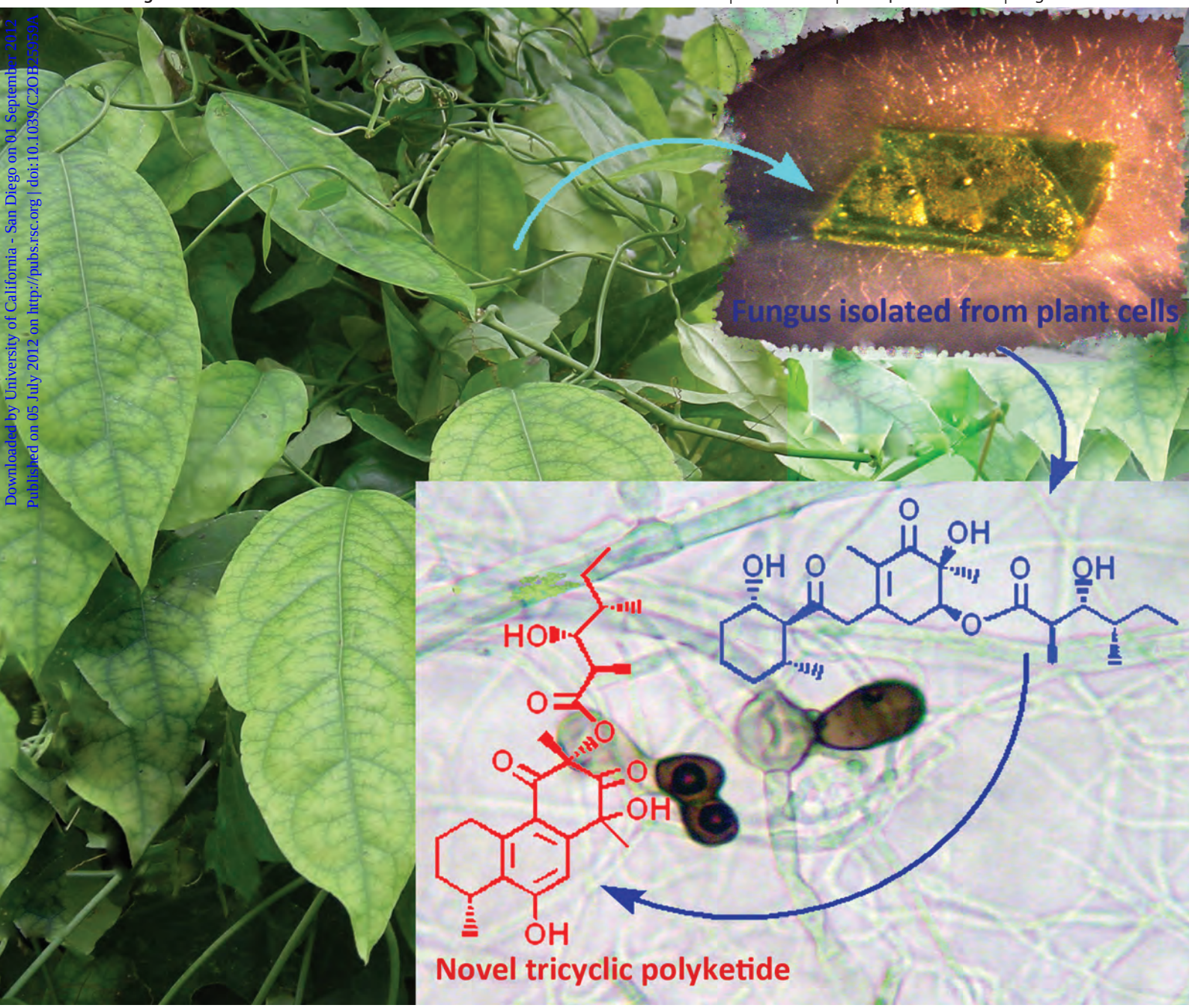

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PAPER

A novel tricyclic polyketide and its biosynthetic precursor azaphilone derivatives from the endophytic fungus *Dothideomycete* sp†

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Azaphilone derivatives **1** and **2** and a novel tricyclic polyketide **3**, together with a known azaphilone, austdiol (**4**), were isolated from the endophytic fungus *Dothideomycete* sp., which was isolated from a Thai medicinal plant, *Tiliacora triandra*. Compound **3** is the first polyketide having a tricyclic 6,6,6 ring system, which is similar to that of a terpenoid skeleton. The absolute configurations of stereogenic centers in **1–3** were addressed by Mosher's method and biosynthetic analogy with a known azaphilone isolated from the same fungus. Cytotoxic and antimicrobial activities of the isolated compounds were evaluated.

Introduction

Endophytic fungi are rich sources of bioactive compounds with pharmaceutical interest, and some metabolites from the endophytic fungi are in clinical trials.[1–4] As part of our on-going research on bioactive metabolites from endophytic fungi isolated from Thai medicinal plants,[5,6] we report herein the isolation, characterization, and biological activities of the metabolites **1–4**, which were isolated from the endophytic fungus *Dothideomycete* sp. (isolated from a Thai medicinal plant, *Tiliacora triandra*). Compound **3** was a novel terpene-like tricyclic polyketide, which was co-isolated with its biosynthetic congeners **1** and **2**, as well as a known azaphilone, austdiol (**4**). A skeleton of a tricyclic **3** is similar to that of a terpenoid, however, careful analysis reveals that it is biosynthetically related to azaphilone derivatives **1** and **2**, which are derived from polyketide biosynthetic pathway. Normally, skeletons of tricyclic polyketides are a linear 6,6,6 ring system, while other systems, such as 5,6,5 and 6,5,6, are exceptionally rare in nature (Fig. 1). Examples of a linear 6,6,6 ring system are mithramycin and chromomycin, which are biosynthesized by type II polyketide synthases.[7] Rare 5,6,5 and 6,5,6 ring systems are found in spinosyns[8,9] and hirsutellones[10,11] (Fig. 1). A terpene-like 6,6,6 ring system in **3** is the first tricyclic polyketide skeleton (Fig. 1), which shares a great similarity with a terpenoid skeleton. The present work reveals for the first time that a tricyclic 6,6,6 ring system, which is normally found in a terpenoid skeleton, is derived from a polyketide biosynthetic pathway.

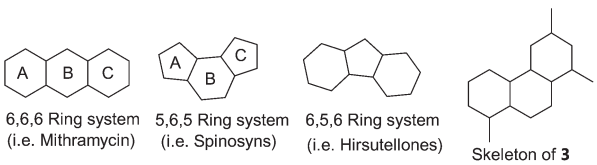
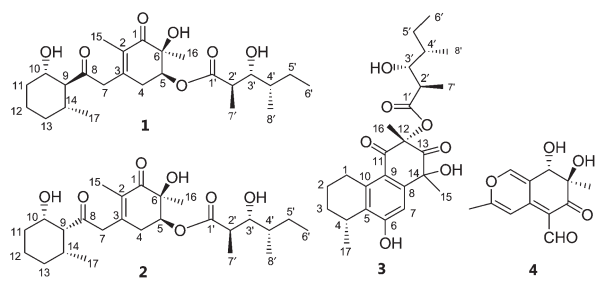


Fig. 1 Ring system of tricyclic polyketides.



Results and discussion

The extracts of fermentation broth and cells of the endophytic fungus *Dothideomycete* sp. CRI7 were purified by Sephadex LH-20 and silica gel column chromatography, as well as by reversed-phase C_{18} HPLC, to yield new azaphilone derivatives **1** and **2**, a novel compound **3**, and a known azaphilone **4**.

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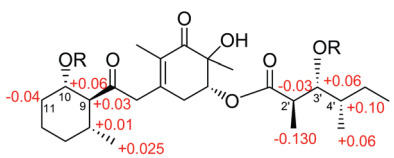
Spectroscopic data of a known azaphilone, austdiol (**4**), were identical to those reported in the literature.[12,13]

Compound **1**, named dothideomycetone A, had a molecular formula $C_{25}H_{40}O_7$ (by HRESI-MS). The IR spectrum of **1** showed absorption bands for carbonyl peaks at 1712, 1676 and 1640 cm^{-1}, while ^{13}C NMR demonstrated signals for two ketones (δ_C 200.5 and 209.9) and a carbonyl ester (δ_C 175.3). ^{13}C NMR and DEPT experiments revealed 25 signals ascribable to 6 methyl, 6 methylene, 7 sp^3 methine, and 6 non-protonated carbons. ^1H–^1H COSY spectrum of **1** established a partial structure from H-9 to H-14, and showed correlations of H-14/H_3-17, and H_2-4/H-5. The ^1H–^1H COSY spectrum established a structure of 3-hydroxy-2,4-dimethylhexanoic acid unit in **1**, showing correlations from H-2′ along the chain to H_3-6′, and correlations of H-2′/H_3-7′, and H-4′/H_3-8′. Allylic couplings between H_3-15 and H_2-4 and between H_3-15 and H_2-7 were evident from the ^1H–^1H COSY spectrum. HMBC spectrum was very useful for structural assembling in dothideomycetone A (**1**), particularly the HMBC correlations of protons to non-protonated carbons; key HMBC correlations in **1** were as follows: H-9 to C-7, C-8, and C-17; H-10 to C-8; H_2-7 to C-2, C-3, C-4, C-8, and C-9; H_2-4 to C-2, C-3, C-6, and C-7; H_3-15 to C-1, C-2, and C-3; H_3-16 to C-1, C-5, and C-6; H-5 to C-1, C-3, C-6, C-16, and C-1′; and H-2′, H-3′, and H_3-7′ to C-1′. Upon these spectroscopic data, a gross structure of **1** was established. Relative configuration of **1** was established by analysis of coupling constants and NOESY spectrum. Large coupling constants ($J_{H\text{-}9,H\text{-}10}$ and $J_{H\text{-}9,H\text{-}14}$ = 10.4 Hz) of H-9 indicated *trans* orientation between H-9 and H-10 and between H-9 and H-14, while the NOESY correlation between H-5 and H_3-16 revealed *cis* relationship between these two protons. It is known that the vicinal coupling constants (*i.e.*, $J_{H\text{-}2',H\text{-}3'}$ for 3-hydroxy-2,4-dimethylhexanoic acid unit) for *anti* diastereoisomer (7.0–9.0 Hz) are larger than those for the *syn* one (5.9–6.3 Hz).[14,15] The $J_{H\text{-}2',H\text{-}3'}$ of 9.6 Hz for 3-hydroxy-2,4-dimethylhexanoic acid unit in **1** indicated *anti* orientation of H-2′ and H-3′. Again the $J_{H\text{-}3',H\text{-}4'}$ for 3-hydroxy-2,4-dimethylhexanoic acid derivatives of *anti* diastereoisomer (8.5 Hz) are also larger than those of *syn* isomer (5.7 Hz).[16] Therefore, the $J_{H\text{-}3',H\text{-}4'}$ of 4.2 Hz in **1** suggested *syn* relationship between H-3′ and H-4′. On the basis of these data, the relative configurations of **1** were secured. The absolute configuration of **1** was addressed by Mosher's method. Accordingly, Mosher's (MTPA) esters **1a** and **1b** were prepared, and the $\Delta\delta$ values ($\delta_{(S)} - \delta_{(R)}$) indicated that the absolute configurations at C-3′ and C-10 in **1** were *R* and *S*, respectively (Fig. 2).

Therefore, the absolute configurations at C-2′, C-4′, C-9, and C-14 were assigned as *R*, *S*, *S*, and *R*, respectively. The absolute configuration at C-6 in dothideomycetone A (**1**) was addressed



1a; R = (*S*)-MTPA
1b; R = (*R*)-MTPA

Fig. 2 $\Delta\delta$ values for the MTPA esters **1a** and **1b**.

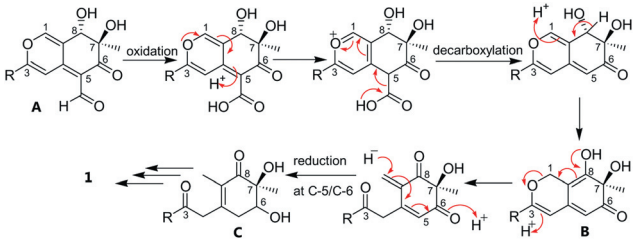


Fig. 3 Proposed biosynthetic pathway of **1** from austdiol.

by biosynthetic analogy with austdiol (**4**), a known azaphilone isolated from the same fungus. It is more likely that the cyclohexenone, dothideomycetone A (**1**), is biosynthetically derived from azaphilones (*i.e.*, **4**). Previously azaphilones were co-isolated with their corresponding cyclohexenone congeners.[17] As shown in Fig. 3, austdiol derivative **A** was oxidized, decarboxylated, followed by allylic shift to give an intermediate **B**; ring opening of **B** followed by reduction at C-5/C-6 furnish an intermediate **C**. Esterification of **C** gives rise to dothideomycetone A (**1**). Previously, azaphilones functionalized with a methylcyclohexane unit were isolated from the fungus *Hypoxylon multiforme*.[18] Upon the biosynthetic pathway in Fig. 3, the C-6 absolute configuration of **1** (C-7 of **C**, Fig. 3) was tentatively assigned to be *S*; accordingly, C-5 of **1** was *S*. Assignments of ^1H and ^{13}C resonances for **1** are in Table 1.

Dothideomycetone B (**2**) had the same molecular formula, $C_{25}H_{40}O_7$ (by HRESI-MS), as that of **1**. ^1H and ^{13}C NMR spectra of **2** and **1** were nearly superimposed, suggesting that **2** was a derivative of **1**. Detailed analysis of NMR spectra revealed that dothideomycetone B (**2**) was a diastereomer of **1**; small coupling constants ($J_{H\text{-}9,H\text{-}10}$ and $J_{H\text{-}9,H\text{-}14}$ = 0.5 Hz) of H-9 suggested *cis* relationships between H-9 and H-10 and between H-9 and H-14. ^1H–^1H COSY and HMBC correlations of **2** were similar to those of **1**. On the basis of these data, the structure of **2** was established as shown. Protons and carbons in **2** are assigned by analysis of 2D NMR data (Table 1).

Dothideomycetide A (**3**) exhibited a molecular formula $C_{25}H_{34}O_7$ as determined by HRESI-MS. ^1H NMR spectrum of **3** showed signals of a singlet aromatic proton (δ_H 7.08), six methyls, and a number of methine and methylene protons. ^{13}C and DEPTs NMR data revealed the presence of 25 carbons in **3**, which were classified as 6 methyl carbons, 4 methylene carbons, 5 methine (4 sp^3 and 1sp^2) carbons, and 10 quaternary carbons. Detailed analysis of ^1H and ^{13}C NMR data revealed that **3** had the same 3-hydroxy-2,4-dimethylhexanoic acid unit as that in **1** and **2**, and the structure elucidation of this moiety was carried out in the same fashion as that for **1** and **2**. Chemical shifts of the methyl groups at δ_H 1.67 (s, H_3-16) and 1.46 (s, H_3-15) suggested that they were attached to oxygenated sp^3 quaternary carbons (C-12 and C-14). ^1H–^1H COSY spectrum of **3** established a partial structure of H_2-1/H_2-2/H_2-3/H-4/H_3-17. The HMBC correlations from H_2-1 to C-5, C-9, and C-10; H-4 to C-5, C-6, and C-10; and H_3-17 to C-5 suggested the attachment of the substructure H_2-1/H_2-2/H_2-3/H-4/H_3-17 to C-5/C10 of an aromatic ring. The HMBC correlations from H-7 to C-5, C-6, C-8, C-9, and C-14 and from H_3-15 to C-8, C-13, and C-14 placed the singlet aromatic proton at C-7 and established both the linkage at C-8/C-14 and the position of C-13 ketone. The

Table 1 NMR data ($CDCl_3$, 600 MHz) of compounds **1–3**

	1		**2**		**3**	
Carbon no.	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)
1	200.5		200.4		27.4	2.48, ddd (16.6, 9.4, 9.3); 3.63, ddd (16.6, 4.6, 4.4)
2	130.1		130.4		29.3	1.62, m; 1.83, m
3	145.7		145.7		18.2	1.65, m; 1.75, m
4	35.3	2.88, br d (17.5); 2.55, br d (17.5)	35.7	2.85, d (19.5); 2.48, d (19.5)	27.2	3.14, m
5	74.8	5.29, dd (2.2, 2.3)	74.8	5.31, dd (2.2, 2.2)	130.7	
6	74.3		74.1		159.4	
7	51.5	3.46, d (17.0); 3.56, d (17.0)	52.3	3.33, d (17.5); 3.66, d (17.5)	109.5	7.08, s
8	209.9		209.4		143.9	
9	65.3	2.20, t (10.4)	56.8	3.14, br t (0.5)	119.6	
10	73.2	3.70, ddd (10.4, 10.4, 4.1)	71.4	3.88, br dd (11.6, 4.7)	142.4	
11	35.5	1.95, m; 1.29, m	30.4	1.60, m; 1.83, m	191.8	
12	23.4	1.30, m	23.7	1.30, m	83.3	
13	33.6	0.95, m	29.0	1.28, m; 1.40, m	206.2	
14	34.2	1.70, m	33.3	1.80, m	76.2	
15	11.6	1.83, s	11.6	1.80, s	34.7	1.67, s
16	23.6	1.42, s	23.7	1.42, s	23.4	1.46, s
17	19.9	0.88, d, (6.1)	18.9	1.00, d (7.0)	20.5	1.22, d (6.9)
1′	175.3		175.2		174.4	
2′	43.7	2.58, quin (7.0)	43.7	2.57, quin (7.1)	43.5	2.85, quin (7.1)
3′	75.6	3.57, dd (9.0, 3.0)	75.6	3.56, br d (7.7)	76.1	3.78, dd (8.5, 2.3)
4′	36.3	1.42, m	36.3	1.45, m	36.1	1.56, m
5′	26.7	1.27, m; 1.43, m	26.7	1.27, m; 1.40, m	26.8	1.37, m; 1.54, m
6′	11.7	0.88, t (7.0)	11.7	0.89, t (7.1)	12.2	0.93, t (7.4)
7′	14.3	1.07, d (7.1)	14.2	1.07, d (7.2)	13.8	1.25, d (7.0)
8′	12.3	0.82, d (6.5)	12.2	0.84, d (6.8)	11.8	0.90, d (6.5)

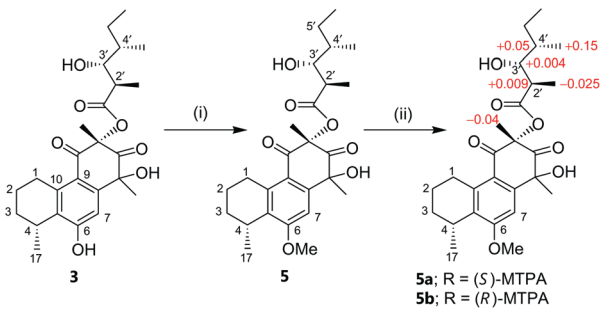


Fig. 4 $\Delta\delta$ values for the MTPA esters **5a** and **5b**. (i) MeI, K_2CO_3, acetone, 81%; (ii) a. (*R*)- or (*S*)-MTPA, DMAP, $CDCl_3$, 0 °C; b. DCC, 31%.

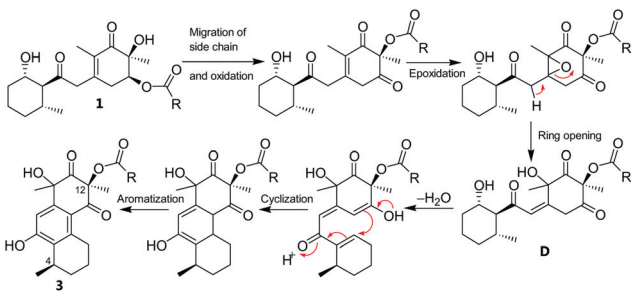


Fig. 5 Proposed biosynthetic pathway of dothideomycetide A (**3**).

HMBC correlations from H_3-16 to C-11, C-12, and C-13 suggested that the C-16 methyl group was flanked by C-11 and C-13 ketones. Chemical shift implied that C-11 (δ_C 191.8) was a conjugated ketone, which attached to an aromatic ring. On the basis of these data, a core structure of **3** was established. A downfield shift (δ_C 83.3) of C-12 suggested that 3-hydroxy-2,4-dimethylhexanoic acid unit was esterified at the C-12 hydroxyl group. Mosher's method was used to determine the absolute configuration of 3′-OH in **3**. A phenolic 6-OH of **3** was first protected by methylation to give **5**, which was esterified with Mosher's reagents, furnishing MTPA esters **5a** and **5b** (Fig. 4). As expected, the $\Delta\delta$ values indicated the *R* configuration of C-3′ in **3** (Fig. 4), which was the same as that in **1**. Dothideomycetide A (**3**) was possibly biosynthetically derived from the azaphilone derivative **1**, and its configurations at C-4 and C-12 were inferred from biosynthetic analogy with **1**. The acyl side chain in dothideomycetone A (**1**) is migrated from C-5 to C-6 with concomitant oxidation of C-5 alcohol to ketone (Fig. 5). Epoxidation of a double bond, followed by epoxide ring opening, gives rise to an intermediate **D**. Loss of H_2O from the intermediate **D**, intramolecular Michael addition, and aromatization lead to the formation of dothideomycetide A (**3**) (Fig. 5). The proposed biosynthetic pathway establishes the absolute configurations at C-4 and C-12 in **3** as *R* and *S*, respectively. However, the C-14 absolute configuration in **3** could not be established based on available spectral data. Upon these spectroscopic data, the structure of **3** was secured.

Dothideomycetone A (**1**) exhibited cytotoxic activity against MOLT-3 (acute lymphoblastic leukemia) cancer cell line with IC_{50} value of 24 μg mL^{-1}, but it was not active (at 50 μg mL^{-1}) toward HuCCA-1 (human cholangiocarcinoma), A549 (human lung carcinoma), and HepG2 (human hepatocellular liver carcinoma) cancer cell lines. While dothideomycetone B (**2**) did not exhibit cytotoxicity, dothideomycetide A (**3**) showed cytotoxic

activity against HuCCA-1, A549, HepG2, and MOLT-3 cell lines with IC_{50} values of 33, 36, 35, and 15 μg mL^{-1}, respectively. Dothideomycetide A (**3**) showed antibacterial activity against *Staphylococcus aureus* ATCC 25923 and ATCC 33591 (methicillin resistant strain) with respective MIC values of 128 and 256 μg mL^{-1}, but dothideomycetones A (**1**) and B (**2**) were inactive.

Experimental section

General

Optical rotations were measured with sodium D line (590 nm) on JASCO DIP-370 digital polarimeter. UV-Vis spectra were obtained using a Shimadzu UV-1700 PharmaSpec spectrophotometer. FT-IR data were recorded on a universal attenuated total reflectance (UATR) attachment on a Perkin-Elmer Spectrum One spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were acquired on a Bruker AVANCE 600 spectrometer (operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C). HRESI-MS spectra were recorded on a Bruker MicroTOF-LC spectrometer.

Fungal material and identification of the fungus

Roots of *Tiliacora triandra* were collected from Nakhonsawan Province, Thailand, and they were cleaned under running tap water. The cleaned roots of *T. triandra* were surface-sterilized, and the isolation of endophytic fungi was carried out according to the method previously described by Schulz and co-workers.[19]

The CRI7 fungus was cultured on potato dextrose agar and banana leaf agar at 25 °C in 12 h light–12 h light cycle and in darkness. The fungus grew as sterile mycelium without any reproductive structure or characteristic morphology formed during cultivation for up to 60 days. The CRI7 fungus was subjected to DNA sequence-based identification. The CRI7 fungus was cultivated in potato dextrose broth for 7 days, the mycelium was collected and washed with sterile water. Total cellular DNA was extracted from the washed fungal mycelium using 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 (GoTaq® Colorless Master Mix, Promega) using the ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers.[20] The thermal cycle program was as follows: 5 min at 95 °C followed by 30 cycles of 50 s at 95 °C, 40 s at 45 °C and 40 s at 72 °C, with a final extension period of 10 min at 72 °C (GeneAmp® PCR System 9700, Applied Biosystems). The amplified DNA fragment was purified and subjected to DNA sequencing on both strands using primers ITS5 and ITS4.

The NS1 (GTAGTCATATGCTTGTCTC) and NS8 (TCCGCAGGTTCACCTACGGA) primers were used to amplify the 18S rRNA gene from total DNA extracts using the PCR reagents and the thermal cycler as mentioned above.[20] The thermal cycle program was as follows: 5 min at 95 °C followed by 30 cycles of 50 s at 95 °C, 40 s at 42 °C, and 2 min at 72 °C, with a final extension period of 10 min at 72 °C. The amplified DNA was purified and subjected to DNA sequencing on both strands using the NS1, NS2, NS3, NS4, NS5, NS6, NS7 and NS8 primers.[20]

The DNA sequences of the ITS1-5.8S-ITS2 (549 nucleotides) and that of the 18S rRNA gene (1755 nucleotides) obtained were submitted to BLASTN 2.2.26+ program[21] to search for similar sequences in GenBank. 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. Phylogenetic relationship was estimated using the neighbor-joining method. Bootstrap analysis was performed with 1000 replications to determine the support for each clade.

The ITS1-5.8S-ITS2 DNA sequence of the CRI7 was shown to be similar to those of some *Mycoleptodiscus indicus* strains (GenBank accession number GU220382, GU980696, JF736515) with 94% sequence identity. No other sequence of higher per cent identity could be found in the GenBank. Comparison of 18S rRNA gene revealed that the CRI7 fungus was related to fungi in the class Dothideomycetes with 94–96% DNA sequence identity. A phylogenetic tree constructed from 18S rRNA sequence alignments showed that the CRI7 fungus formed a cluster with *Acrospermum compressum* UME 31704 and *A. gramineum* UME 31190 (GenBank accession number AF242258 and AF242259, respectively). However, the sequence identity of 96% was too low to reliably place the CRI7 fungus in this genus. Based on the above data and the current Ascomycota systematics,[22] the strain CRI7 was classified as a mycelia sterilia fungus *Dothideomycete* sp., belonging to the class Dothideomycetes, subphylum Pezizomycotina. The DNA sequence of 18S-ITS1-5.8S-ITS2rRNA gene region of the CRI7 fungus has been submitted to GenBank with an accession number of JQ867364. Culture of the CRI7 has been deposited at Chulabhorn Research Institute.

Extraction and isolation of the metabolites 1–4 from the endophytic fungus *Dothideomycete* sp. CRI7

The fungus *Dothideomycete* sp. CRI7 grown in PDB was filtered to obtain fermentation broth (15 L) and mycelia. Fermentation broth of the fungus was extracted with an equal volume of EtOAc, to obtain a crude broth extract (3.5 g). This crude extract was divided into two fractions, methanol soluble and methanol insoluble materials. Methanol insoluble fraction was found to be austdiol (**4**, 540 mg). Methanol soluble part was fractionated (approximately 25 mL for each fraction collected) on Sephadex LH-20 column chromatography (4 × 58 cm), eluted with MeOH, to yield 14 fractions (F1–F14). Each fraction was monitored by TLC and ^{1}H NMR spectrum. Fractions with same TLC patterns and ^{1}H NMR spectrum were pooled together to obtain six main fractions (A/I–A/VI). Fraction A/II (478 mg) was further fractionated using Sephadex LH-20 column chromatography (2 cm × 125 cm) to obtain 14 fractions (15 mL each), and fractions with similar TLC patterns were combined to yield five main fractions (B/I–B/V). Fraction B/II (74 mg) was purified by preparative TLC, developed with a solvent system of 50% EtOAc in hexane, yielding compound **1** (25 mg). Fraction B/III (230 mg) was further purified by silica gel column chromatography (3 cm × 35 cm) using a gradient system of EtOAc and hexane (150 mL)

(3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2, 9 : 1 and 100 : 0), giving three fractions (C/I–C/III). The fraction C/II (120 mg) was further separated by reversed-phase (C_{18}) HPLC, eluted with the solvent system of 70% MeOH in water, to yield compound **1** (40 mg) and compound **2** (5 mg). Fraction A/IV (740 mg) was further fractionated using Sephadex LH-20 column chromatography (75 cm × 3 cm), to obtain 18 fractions (15 mL each). These fractions were combined on the basis of TLC pattern, to yield six main fractions (D/I–D/VI). Fraction D/IV (300 mg) was further divided into hexane soluble (170 mg) and hexane insoluble (130 mg) materials. The hexane soluble part was further purified using silica column chromatography, eluted with a mixture of hexane and EtOAc with an increasing polarity (7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9 and finally with 100% EtOAc), to yield compound **3** (39 mg).

Fungal mycelia were extracted sequentially with MeOH (500 mL × 2) and CH_2Cl_2 (500 mL × 2). The MeOH and CH_2Cl_2 extracts were combined and extracted with EtOAc (500 mL × 3), yielding a crude cell extract (9 g). The cell extract was divided into two parts, hexane soluble and hexane insoluble materials. The hexane soluble material was further fractionated using Sephadex LH-20 column chromatography (4 cm × 58 cm), eluted with MeOH, to obtain 21 fractions (25 mL each). After monitoring TLC patterns of each fraction, fractions 7 and 8 were combined, and this material (320 mg) was further purified by silica gel column chromatography (35 cm × 3 cm), eluted with a mixture of hexane and EtOAc with an increasing polarity (7 : 3, 3 : 2, 5 : 5, 2 : 3, 3 : 7, 1 : 4, 1 : 9, and finally with 100% EtOAc), to yield 64 fractions (10 mL each). Fractions 48–55 were combined based on TLC profile, and this combined fraction contained compound **3** (90 mg).

Dothideomycetone A (1). Pale yellow amorphous solid; $[\alpha]_D^{27}$ +56.2 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 248 (3.9) nm; FT-IR ν_{max} 3464, 2953, 2931, 2873, 1712, 1676, 1640, 1458, 1379, 1173, 1042 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESI-MS *m/z* 453.2847 $[M + H]^+$ (calcd for $C_{25}H_{41}O_7$, 453.2852).

Dothideomycetone B (2). Pale yellow amorphous solid; $[\alpha]_D^{27}$ +33.0 (*c* 0.43, MeOH); UV (MeOH) λ_{max} (log ε) 249 (4.0) nm; FT-IR ν_{max} 3446, 2953, 2932, 2866, 1726, 1712, 1675, 1458, 1379, 1242, 1171, 1097, 1041 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESI-MS *m/z* 453.2852 $[M + H]^+$ (calcd for $C_{25}H_{41}O_7$, 453.2852).

Dothideomycetide A (3). Pale yellow amorphous solid; $[\alpha]_D^{27}$ +47.0 (*c* 1.8, MeOH); UV (MeOH) λ_{max} (log ε) 293 (3.8) nm; FT-IR ν_{max} 3372, 2960, 2934, 2873, 1732, 1688, 1579, 1455, 1421.65, 1371, 1340, 1295, 1273, 1243, 1169, 1136, 1097, 1053, 1024, 962, 890 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESI-MS *m/z* 447.2369 $[M + H]^+$ (calcd for $C_{25}H_{41}O_7$, 447.2383).

Austdiol (4). $[\alpha]_D^{27}$ +106.7 (*c* 1.0, pyridine), Lit.[12] $[\alpha]_D^{26}$ +160.3 (*c* 1.25, pyridine); UV (MeOH) λ_{max} (log ε) 256 (3.87), 380.5 (4.1) nm; FT-IR ν_{max} 3473, 3377, 1604, 1470, 1412, 1287, 1259, 1198, 1174, 1095, 1069, 1044, 932, 879, 852 cm^{-1}; ^1H and ^{13}C NMR data were in good agreement with those reported in the literature.[12,13]

Methylation of compound 3

To compound **3** (30 mg) in acetone (1 mL), K_2CO_3 (10 mg) and MeI (3.5 mL) were added, and the mixture was stirred at room temperature for overnight. Then, the reaction mixture was evaporated to dryness, dissolved in EtOAc (10 mL), and washed with H_2O (5 × 8 mL). The crude product was further purified by preparative TLC, developed with a solvent system of 50% EtOAc in hexane, to obtain a methylated product **5** (25 mg): $[\alpha]_D^{27}$ +23.0 (*c* 0.60, MeOH); UV (MeOH) λ_{max} (log ε) 290 (3.7) nm; FT-IR ν_{max} 3365, 2961, 2937, 1736, 1684, 1573, 1368, 1345, 1292, 1277, 1162, 1133, 1091 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 0.92 (3H, d, *J* = 6.5 Hz, 4′-Me), 0.95 (3H, t, *J* = 7.2 Hz, H-6′), 1.21 (3H, d, *J* = 6.8 Hz, H-17), 1.27 (3H, d, *J* = 7 Hz, 2′-Me), 1.47 (3H, s, H-16), 2.81 (1H, quin, *J* = 7 Hz, H-2′), 3.14 (1H, m, H-4), 3.66 (1H, m, H-1), 3.76 (1H, dd, *J* = 8.5 Hz, 2.3 Hz, H-3′), 3.95 (3H, s, 6-OMe), 7.14 (1H, s, H-7); HRESI-MS *m/z* 461.2532 $[M + H]^+$ (calcd for $C_{26}H_{37}O_7$, 461.2539).

Preparation of (*R*)- and (*S*)-MTPA esters of compounds 1 and 5

To compound **1** (*ca.* 6 mg) in NMR tube, 11.5 mg of (*S*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoic acid (MTPA) in $CDCl_3$ (0.3 mL) and 1.05 mg of 4-dimethylaminopyridine (DMAP) were added. The mixture was shaken vigorously and cooled to 0 °C, and 20 mg of *N*,*N*′-dicyclohexylcarbodiimide (DCC) was added. Completion of the reaction was indicated by analysis of ^1H NMR spectrum. A reaction mixture was purified by preparative TLC, developed with a solvent system of 30% EtOAC in hexane, to give **1a** (2.2 mg), or (*S*)-MTPA ester of **1**. (*R*)-MTPA ester (**1b**, 1.8 mg) was prepared in the same manner as that of (*S*)-MTPA ester.

(*S*)-MTPA ester 1a. $[\alpha]_D^{27}$ +13.2 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 245 (3.8), 275 (3.1) nm; FT-IR ν_{max} 3351, 2942, 2923, 1723, 1707, 1671, 1643, 1372, 1177, 1039 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 0.83 (3H, d, *J* = 6.8 Hz, H-8′), 0.91 (3H, t, *J* = 7.3 Hz, H-6′), 0.98 (3H, d, *J* = 6.5 Hz, H-17), 1.05 (3H, d, *J* = 6.94, H-7′), 1.07 (2H, m, H-13), 1.30 (2H, m, H-11), 1.35 (2H, m, H-5′), 1.35 (2H, m, H-12), 1.40 (3H, s, H-16), 1.65 (1H, m, H-4′), 1.75 (1H, m, H-14), 1.93 (1H, m, H-4a), 2.38 (1H, m, H-4b), 2.49 (1H, t, *J* = 10.4 Hz, H-9), 2.73 (1H, quin, *J* = 7.2 Hz, H-2′), 3.10 (1H, d, *J* = 18.0 Hz, H-7a), 3.42 (3H, brs, OCH_3 of MTPA) 3.64 (1H, d, *J* = 18.4 Hz, H-7b), 5.08 (1H, ddd, *J* = 10.8, 10.8, 4.2 Hz, H-10), 5.12 (1H, dd, *J* = 3.0, 3.6 Hz, H-5), 5.38 (1H, dd, *J* = 8.6, 3.0 Hz, H-3′), 7.41 (8H, m, aromatic signals of MTPA), 7.57 (2H, m, aromatic signals of MTPA); HRESI-MS *m/z* 907.3479 $[M + Na]^+$ (calcd for $C_{45}H_{54}F_6NaO_{11}$, 907.3468).

(*R*)-MTPA ester 1b. $[\alpha]_D^{27}$ +12.7 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 249 (3.9), 272 (3.2) nm; FT-IR ν_{max} 3349, 2946, 2922, 1726, 1702, 1674, 1647, 1368, 1170, 1042 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 0.72 (3H, d, *J* = 6.8 Hz, H-8′), 0.86 (3H, t, *J* = 6.0 Hz, H-6′), 0.92 (3H, d, *J* = 6.4 Hz, H-17), 1.02 (2H, m, H-12), 1.05 (2H, m, H-13), 1.11 (3H, d, *J* = 7.2 Hz, H-7′), 1.30 (2H, m, H-5′), 1.35 (2H, m, H-11), 1.40 (3H, s, H-16), 1.64 (1H, m, H-4′), 1.70 (1H, m, H-14), 1.93 (1H, m, H-4a), 2.31 (1H, m, H-4b), 2.46 (1H, t, *J* = 10.6 Hz, H-9), 2.76 (1H, quin, *J* = 7.2, H-2′), 3.01 (1H, d, *J* = 18.1 Hz, H-7a), 3.42

(3H, brs, OCH_3 of MTPA), 3.53 (1H, d, J = 16.4 Hz, H-7b), 5.01 (1H, ddd, J = 10.8, 10.7, 4.1 Hz, H-10), 5.10 (1H, dd, J = 3.0, 3.6 Hz, H-5), 5.32 (1H, dd, J = 8.6, 3.0 Hz, H-3′), 7.41 (10H, m, aromatic signals of MTPA), 7.57 (3H, m, aromatic signals of MTPA); HRESI-MS m/z 907.3482 $[M + Na]^+$ (calcd for $C_{45}H_{54}F_6NaO_{11}$, 907.3468).

(*R*)- and (*S*)-MTPA esters **5a** and **5b** were prepared from compound **5**, using the same method as those for **1a** and **1b**. To compound **5** (*ca.* 5.0 mg) in NMR tube, 7.0 mg of (*S*)- MTPA in $CDCl_3$ (0.3 mL) and 1.00 mg of DMAP were added. The mixture was shaken and cooled to 0 °C, then DCC (15 mg) was added to the reaction mixture. Completion of the reaction was indicated by analysis of ^1H NMR spectrum. A reaction mixture was purified by preparative TLC, eluted with 30% EtOAC in hexane, affording **5a** (2.3 mg), or (*S*)-MTPA ester of **5**. (*R*)-MTPA ester (**5b**, 2.0 mg) was prepared in the same manner as that of (*S*)-MTPA ester.

(*S*)-MTPA ester 5a. $[\alpha]_D^{27}$ +13.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 272 (3.3), 293 (3.4) nm; FT-IR ν_{max} 3347, 2965, 2940, 1731, 1710, 1681, 1577, 1356, 1341, 1290, 1273, 1160, 1134, 1089 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 0.93 (3H, d, J = 6.8 Hz, H-8′), 0.97 (3H, t, J = 7.3 Hz, H-6′), 1.20 (3H, d, J = 6.8 Hz, H-17), 1.32 (3H, d, J = 7.3 Hz, H-7′), 1.39 (3H, s, H-16), 1.50 (2H, m, H-5′), 1.60 (1H, m, H-3a), 1.60 (1H, m, H-2a), 1.70 (1H, m, H-3b), 1.85 (1H, m, H-2b), 1.85 (1H, m, H-4′), 2.43 (1H, ddd, J = 18.3, 9.8, 6 Hz, H-1a), 3.13 (1H, quin, J = 7.2 Hz, H-2′), 3.19 (1H, m, H-4), 3.50 (3H, s, OCH_3 of MTPA), 3.65 (1H, m, H-1b), 3.93 (3H, s, 6-OMe), 5.43 (1H, dd, J = 7.0, 4.5 Hz, H-3′), 7.09 (1H, s, H-7), 7.40 (3H, m, aromatic signals of MTPA), 7.64 (2H, m, aromatic signals of MTPA); HRESI-MS m/z 699.2735 $[M + Na]^+$ (calcd for $C_{36}H_{43}F_3NaO_9$, 699.2757).

(*R*)-MTPA ester 5b. $[\alpha]_D^{27}$ +15.0 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 275 (3.3), 291 (3.6) nm; FT-IR ν_{max} 3342, 2961, 2939, 1733, 1715, 1684, 1573, 1358, 1344, 1293, 1271, 1164, 1137, 1082 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 0.77 (3H, d, J = 6.7 Hz, H-8′), 0.89 (3H, t, J = 7.1 Hz, H-6′), 1.10 (1H, m, H-3a), 1.21 (3H, d, J = 6.8 Hz, H-17), 1.30 (1H, m, H-3b),1.30 (2H, m, H-5′), 1.35 (3H, d, J = 7.4 Hz, H-7′), 1.43 (3H, s, H-16), 1.60 (1H, m, H-2a), 1.68 (3H, s, H-15), 1.80 (1H, m, H-4′), 1.85 (1H, m, H-2b), 2.47 (1H, ddd, J = 17.2, 10.0. 6,0 Hz, H-1a), 3.12 (1H, quin, J = 7.1 Hz, H-2′), 3.20 (1H, m, H-4), 3.58 (3H, brs, OCH_3 of MTPA), 3.65 (1H, m, H-1b), 3.95 (3H, s, 6-OMe), 5.42 (1H, dd, J = 6.8, 4.7 Hz, H-3′), 7.12 (1H, brs, H-7), 7.39 (3H, m, aromatic signals of MTPA), 7.64 (2H, m, aromatic signals of MTPA); HRESI-MS m/z 699.2743 $[M + Na]^+$ (calcd for $C_{36}H_{43}F_3NaO_9$, 699.2757).

Bioassays

Cytotoxicity. Cytotoxic activity for HuCCA-1, HepG2, A549, and MOLT-3, cell lines was evaluated with MTT[23] and XTT[24] assays. Etoposide and doxorubicin were used as the reference drugs. Doxorubicin exhibited IC_{50} values of 0.4, 0.2, and 0.3 μg mL^{-1} against HuCCA-1, HepG2, and A549 cell lines, respectively, while etoposide showed cytotoxic activity toward MOLT-3 cell line with the IC_{50} value of 0.02 μg mL^{-1}.

Antimicrobial activity. Antibacterial activities of the purified compounds against *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* ATCC 33591 and *Escherichia coli* ATCC 25922 were determined using the Clinical and Laboratory Standards Institute (CLSI) M7-A4 method (National Committee for Clinical Laboratory Standards, 1997).[25] Suspension of each bacterial strain was prepared and diluted in Mueller-Hinton broth to 106 CFU mL^{-1}. A 50 μL bacterial inoculum was dispensed into each well containing 50 μL of serially diluted test compound. All compounds were tested in duplicates at up to a maximum concentration of 256 μg mL^{-1}. Sample solutions were prepared immediately before use. After incubation at 37 °C for 24 h, a 20 μL aliquot of *p*-iodonitrotetrazolium (INT) solution (1 mg mL^{-1}) was added into each well. The assay plates were further incubated for 1 h. Violet color developed in the well indicated growth of the test organism. No change in color indicated no growth, and thus no antibacterial activity of the test compound. Minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of the test bacteria. Ampicillin was used as a positive control, exhibiting respective MIC values of 0.125, 256, and 4.0 μg mL^{-1} against *S. aureus* ATCC 25923, *S. aureus* ATCC 33591 (methicillin resistant strain), and *E. coli* ATCC 25922.

Conclusions

We have isolated the novel tricyclic polyketide **3**, together with its congeners **1** and **2**, from the endophytic fungus *Dothideomycete* sp. CRI7. Compound **3** is the first polyketide furnished with the 6,6,6 ring system, similar to a terpenoid skeleton. The novel polyketide **3** is more likely derived from azaphilone derivatives **1** and **2** which are co-metabolites isolated from the same fungus.

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