JOURNAL OF NATURAL PRODUCTS

Article

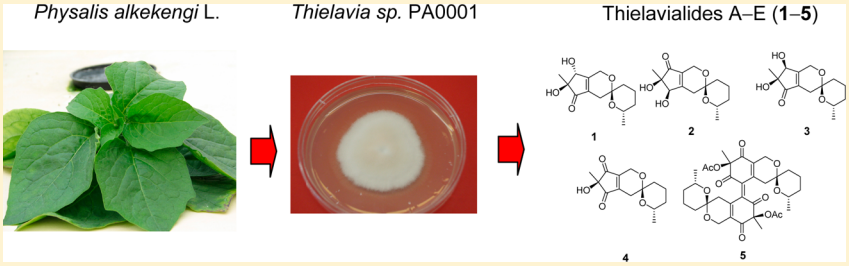
pubs.acs.org/jnp

Thielavialides A−E, *Nor*-Spiro-azaphilones, and a Bis-spiro-azaphilone from *Thielavia* sp. PA0001, An Endophytic Fungus Isolated from Aeroponically Grown *Physalis alkekengi*†

E. M. Kithsiri Wijeratne, Patricia Espinosa-Artiles, Raphael Gruener, and A. A. Leslie Gunatilaka*

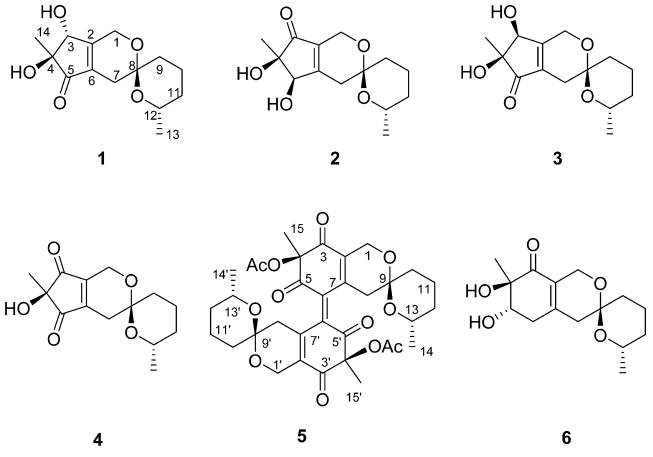
Southwest Center for Natural Products Research and Commercialization, School of Natural Resources and the Environment, College of Agriculture and Life Sciences, The University of Arizona, 250 East Valencia Road, Tucson, Arizona 85706, United States

Supporting Information



ABSTRACT: Four new *nor*-spiro-azaphilones, thielavialides A−D (**1**− **4**), a new bis-spiro-azaphilone, thielavialide E (**5**), together with pestafolide A (**6**), were isolated from the endophytic fungal strain, *Thielavia* sp. PA0001, occurring in the healthy leaf tissue of aeroponically grown *Physalis alkekengi*. The structures and relative configurations of **1**−**5** were established on the basis of their MS and NMR data. Possible biosynthetic pathways to thielavialides A−E (**1**− **5**) from pestafolide A (**6**), some involving a Favorskii-like rearrangement, are proposed.

Endophytic fungi are a diverse group of ubiquitous organisms that colonize interior organs of living plants at some stage during their life cycle without any apparent negative effect on their hosts.[2] In their symbiotic association, the host plant (macrophyte) protects and feeds the endophyte, which in return produces bioactive secondary metabolites to enhance the growth and competitiveness of the host and to protect it from herbivores and plant pathogens.[3] In our ongoing search for bioactive and/or novel small-molecule natural products from plants and their associated microorganisms, we have investigated fungal strains inhabiting plants and mosses found in their natural environments.[1,4] We have now extended our search to include fungal endophytes of plants growing under environmentally controlled conditions. Herein we report the isolation and characterization of four new *nor*-spiro-azaphilones, thielavialides A−D (**1**−**4**), a new bis-spiro-azaphilone, thielavialide E (**5**), and the known spiro-azaphilone, pestafolide A (**6**), from *Thielavia* sp. PA0001 (Chaetomiaceae), a fungal strain inhabiting the leaf tissue of *Physalis alkekengi* L. (bladder cherry; Solanaceae) grown under aeroponic conditions in a greenhouse.[5] Previous investigations of *Thielavia* species have afforded thielavins A−E,[6−9] thielocins A1α−A4β and B1−B3,[10,11] hydroxybenzoates,[12] metabolite k4610178 with testosterone 5α-reductase-inhibitory activity,[13] and antibiotics 15352A−B.[14] Pestafolide A (**6**) with modest antifungal activity has previously been encountered in the endophytic fungus, *Pestalotiopsis foedan*.[15] To the best of our knowledge, this constitutes the first report of chemical investigation of an endophytic fungus isolated from an aeroponically grown plant.



RESULTS AND DISCUSSION

Thielavialide A (**1**), obtained as an optically active colorless gum, was determined to have the molecular formula $C_{14}H_{20}O_5$ by a combination of HRESIMS (m/z 269.1383 $[M + H]^+$, calcd 269.1384), ^{13}C NMR, and HSQC data, indicating 5 degrees of unsaturation. Its IR spectrum had a strong absorption band at 1713 cm^{-1} characteristic of an α,β-unsaturated carbonyl moiety of a pentacyclic ring.[16] Analysis of the ^1H, ^{13}C NMR, and

Received: March 12, 2014
Published: June 2, 2014

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data for Thielavialides A−E (1−4) in $CDCl_3$

	1		2		3		4	
position	δ_C (type)	δ_H (J in Hz)	δ_C (type)	δ_H (J in Hz)	δ_C (type)	δ_H (J in Hz)	δ_C (type)	δ_H (J in Hz)
1	59.0 (CH_2)	a: 4.26, ddd (18.8, 3.6, 2.0)	55.7 (CH_2)	a: 4.13, ddd (15.6, 6.0, 3.2)	59.5 (CH_2)	a: 4.35, d (18.4)	55.9 (CH_2)	a: 4.29, dt (18.4, 3.6)
		b: 4.56, dd (18.8, 2.0)		b: 4.33, dt (15.6, 2.4)		b: 4.44, dt (18.4, 2.0)		b: 4.58, dd (18.4, 2.0)
2	166.4 (C)		134.3 (C)		165.2 (C)		153.2 (C)	
3	76.9 (CH)	4.74, brd (2.8)	202.9 (C)		78.2 (CH)	4.78, brs	200.3 (C)	
4	81.4 (C)		80.8 (C)		80.7 (C)		72.3 (C)	
5	204.6 (C)		78.4 (CH)	4.59, brs	204.6 (C)		198.9 (C)	
6	132.4 (C)		165.6 (C)		132.9 (C)		152.7 (C)	
7	31.7 (CH_2)	a: 2.17, ddd (17.6, 5.6, 2.8)	36.1 (CH_2)	a: 2.63, dd (19.6, 3.2)	31.8 (CH_2)	a: 2.23, dq (17.6, 3.2)	32.9 (CH_2)	a: 2.44, ddd (19.6, 4.0, 2.0)
		b: 2.36, dt (17.6, 2.4)		b: 2.35, ddd (19.6,3.6, 2.0))		b: 2.31, brd (17.6)		b: 2.53, ddd (19.2, 3.6, 0.4)
8	95.4 (C)		95.8 (C)		95.4 (C)		95.4 (C)	
9	33.9 (CH_2)	a: 1.53, m	34.0 (CH_2)	a: 1.55, m	33.8 (CH_2)	a: 1.53, m	33.7 (CH_2)	a: 1.58, dd (13.2, 4.8)
		b: 1.72, m		b: 1.75, m		b: 1.72, dt (13.6, 2.0)		b: 1.75, m
10	19.1 (CH_2)	a: 1.62, m	19.0 (CH_2)	a: 1.65, m	19.0 (CH_2)	a: 1.62, m	18.8 (CH_2)	a: 1.66, m
		b: 1.89, qt (13.2, 4.0)		b: 1.90, qt (12.8,4.0)		b: 1.89, qt (13.6, 4.0)		b: 1.90, ddd (14.0, 4.0, 4.0)
11	32.2 (CH_2)	a: 1.20, m	32.1 (CH_2)	a: 1.20, m	32.2 (CH_2)	a: 1.20, m	31.9 (CH_2)	a: 1.23, m
		b: 1.60, m		b: 1.59, m		b: 1.60, m		b: 1.61, m
12	67.1 (CH)	3.76, dqd (12.0, 6.4, 2.0)	67.3 (CH)	3.77, dqd (12.0, 6.4, 2.0)	67.1 (CH)	3.77, dqd (12.0, 6.4, 2.0)	67.5 (CH)	3.76, dqd (12.0, 6.4, 2.0)
13	21.7 (CH_3)	1.08, d (6.4)	21.7 (CH_3)	1.08, d (6.4)	21.7 (CH_3)	1.10, d (6.4)	21.6 (CH_3)	1.08 d (6.4)
14	21.2 (CH_3)	1.28, s	21.0 (CH_3)	1.29, s	21.1 (CH_3)	1.29, s	20.8 (CH_3)	1.42, s
OH-3		2.80, brd (6.0)				2.62, brs		
OH-4		3.01, brs		2.65, brs		2.28, brs		2.58, brs
OH-5				2.52, brd (7.2)				

HSQC data (Table 1) revealed that **1** contained a tertiary methyl (δ_H 1.28 s; δ_C 21.2), a secondary methyl [δ_H 1.08 d (J = 6.4 Hz); δ_C 21.7], five methylenes of which one is oxygenated [δ_H 4.26 ddd (J = 18.8, 3.6, 2.0 Hz), 4.56 dd (J = 18.8, 2.0 Hz); δ_C 59.0], and two oxymethine groups [δ_H 4.74 brd (J = 2.8 Hz); δ_C 76.9 and 3.76 dqd (J = 12.0, 6.4, 2.0 Hz); δ_C 67.1]. It also contained five quaternary carbons consisting of one monooxygenated (δ_C 81.4), one dioxygenated (δ_C 95.4), and two olefinic (δ_C 166.4 and 132.4) carbons, and a ketone carbonyl (δ_C 204.6). The presence of the spin system $-CH_2CH_2CH_2CH(O)CH_3$ in thielavialide A (**1**) was evident from its ^{1}H−^{1}H COSY data (Figure 1). The connectivity of this spin system, two methylenes, tertiary methyl, and the remaining atoms in **1** was established by the analysis of its HMBC spectrum to constitute a tricyclic ring system containing a cyclopentenone ring and two six-membered spiro-ketal rings (Figure 1). Thus, HMBC correlations of H_2-1 to C-2, C-6, and C-8, and H_2-7 to C-2, C-6, and C-8 suggested the presence of a dihydropyran moiety in **1** and those of H_2-9 to C-7 and H_2-10 to C-8 established the connectivity between C-8 of this dihydropyran moiety and terminal methylene (CH_2-9) of the spin system, $-CH_2CH_2CH_2CH(O)CH_3$. The oxymethine proton at δ 3.76 (H-12) showed an HMBC correlation to the dioxygenated carbon (C-8), establishing the connectivity between C-8 and C-12 via the oxygen atom of the above spin system. Connectivity of C-2 to C-3 and C-5 to C-6 was established by the HMBC correlations of H_2-1 to C-2 and C-3, and H_2-7 to C-5 and C-6, respectively. HMBC correlations of H_3-14 to C-3, C-4, and C-5 provided evidence for the placement of the tertiary methyl group (CH_3-14) on C-4. The ^{1}H NMR of **1** had two D_2O exchangeable protons due to OH groups. One of these protons (δ 3.01) showed HMBC correlations to C-3, C-4, and C-5, suggesting that the OH bearing this proton is attached to C-4. The remaining D_2O exchangeable proton at δ 2.80 showed HMBC correlations to C-2, C-3, and C-4, placing this OH at C-3. Thus, the planar structure of thielavialide A was established as that depicted in **1**. The relative stereoconfiguration of **1** was established by the analysis of its ^{1}H NMR J-values, NOEDIFF data combined with three-dimensional molecular modeling. The large coupling constant (12.0 Hz) observed for H-11b and H-12 suggested that these protons had a *trans* diaxial relationship. NOE correlations of H-12 to H-10_{ax} and H-11_{eq} (H-11a) indicated that they were cofacial and hence CH_3-13 should have opposite orientation. NOE correlations of H-1a (δ 4.26) to H-3 (δ 4.74) and H-12 (δ 3.76) suggested that these three protons were cofacial. These data confirmed that the relative stereochemistry

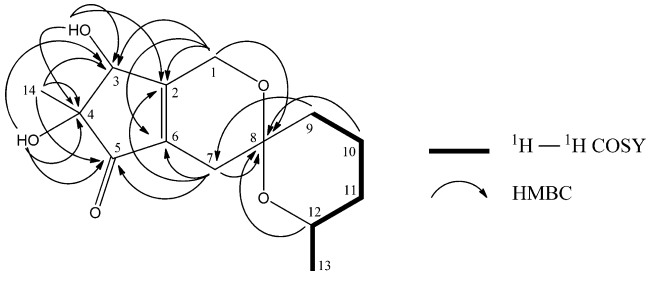


Figure 1. Selected COSY and HMBC correlations for **1**.

dx.doi.org/10.1021/np500237h | J. Nat. Prod. 2014, 77, 1467−1472

of the two hexacyclic rings of **1** was similar to that of pestafolide A (**6**).[15] NOE correlations observed for H-3 (δ 4.74) with OH-4 (δ 3.01) and H-1a (δ 4.26) suggested that these protons were cofacial. Hence OH-3 and CH_3-14 should be on the opposite side of the molecule. The relative configuration of thielavialide A (**1**) was thus established as that shown in Figure 2.

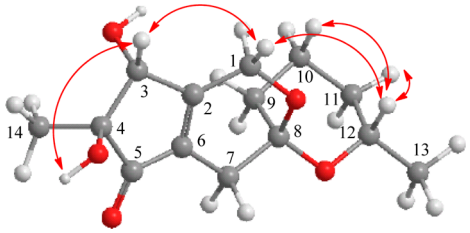


Figure 2. Selected NOE correlations for **1**.

The HRESIMS of thielavialide B (**2**), also obtained as an optically active colorless gum, displayed a molecular ion consistent with the molecular formula $C_{14}H_{20}O_5$ (*m*/*z* 269.1382 [M + H]$^+$, calcd 269.1384). Its ^{1}H and ^{13}C NMR data (Table 1) closely resembled those of **1** except for the differences observed for the methylene carbons, C-1 (δ 59.0 for **1**; 55.7 for **2**) and C-7 (δ 31.7 for **1**; 36.1 for **2**). Because **1** and **2** have the same molecular formula, these changes in chemical shifts may be explained as due to the structural differences in cyclopentenone rings of these two compounds. Oxidation of the hydroxymethine group at C-3 in **1** to a carbonyl group in **2** and the reduction of the carbonyl group in **1** to a hydroxymethine group in **2** would result in differences observed in their NMR spectra. This was confirmed by the HMBC correlations of H-7b (δ_H 2.35) to the hydroxymethine carbon C-5 (δ 78.4) of **2** (Supporting Information, Figure S1). Application of NOEDIFF data suggested that the relative stereoconfiguration of two six-membered spiro-ketal moieties in **2** were similar to that of **1**. The orientation of the OH at C-5 of **2** was determined to be opposite to that of H-1a by NOE correlations observed between H-12/H-1a, H-1b/H-7b, and H-7b/H-5 (Supporting Information, Figure S1). The foregoing suggested that thielavialide B is isomeric with **1** and has the structure as depicted in **2**.

Thielavialide C (**3**) obtained as an optically active colorless gum, was determined to have the same molecular formula ($C_{14}H_{20}O_5$) as **1** and **2** by a combination of HRESIMS (*m*/*z* 269.1385 [M + H]$^+$, calcd 269.1384) and NMR data. Its ^{1}H and ^{13}C NMR data (Table 1) were almost identical with those of **1** except for minor differences at C-3 [δ_H 4.74 brd (J = 2.8 Hz), δ_C 76.9 for **1**; δ_H 4.78 brs, δ_C 78.2 for **3**), suggesting that **1** and **3** are stereoisomeric at C-3. The presence of carbonyl group at C-5 in **3** was supported by the observed HMBC correlations of H-7a to C-5 (δ_C 204.6) (Supporting Information, Figure S2). NOEDIFF data indicated that the relative stereoconfiguration of the two spiro-ketal rings in **3** were similar to that of **1**. In addition, NOE correlations between H-12/H-1a, H-1b/H-3, and H_3-14/H-3 (Supporting Information, Figure S2) revealed that H-3 and H-1b are cofacial and therefore the OH at C-3 should be cofacial with H-1a, confirming the structure **3** proposed for thielavialide C.

The molecular formula of thielavialide D (**4**), obtained as an optically active white amorphous solid, was determined to be $C_{14}H_{18}O_5$ on the basis of HRESIMS (*m*/*z* 267.1224 [M + H]$^+$, calcd 267.1227), ^{13}C NMR, and HSQC data, accounting for 6 degrees of unsaturation. Its IR spectrum showed characteristic absorption bands for hydroxyl (3449 cm^{-1}) and 4-cyclopentene-1,3-dione (1702 cm^{-1}) moieties.[16] The ^{1}H and ^{13}C NMR data (Table 1) and the molecular weight difference of 2 Da between **1**−**3** and **4** suggested that **4** is probably an oxidation product of **1**, **2**, or **3**. The major difference in NMR spectra of **1**−**3** and **4** was found to be the absence of the hydroxymethine group at C-3 or C-5 of **4**. Compared with **1**−**3**, which contain a single carbonyl group, the ^{13}C NMR of thielavialide D (**4**) showed the presence of two carbonyl groups at δ_C 200.3 and 198.9, exhibiting HMBC correlations to H-1 and H-7, respectively (Supporting Information, Figure S23). As expected, oxidation of thielavialide A (**1**) with MnO_2 afforded a product which was shown to be identical ($[\alpha]_D^{25}$, LRMS, and ^{1}H NMR) with **4**. The foregoing suggested that thielavialide D has the structure depicted in **4** with the same relative stereochemistry as that of **1**.

Thielavialide E (**5**), obtained as an optically active white amorphous solid, analyzed for $C_{34}H_{40}O_{12}$ by a combination of HRESIMS (*m*/*z* 641.2590 [M + H]$^+$, calcd 641.2593) and NMR data and accounted for 15 degrees of unsaturation. Its UV spectrum displayed a λ_{max} at at 310 nm, indicating the presence of an $\alpha,\beta,\gamma,\delta$-conjugated ketone chromophore.[17] The absorption bands at 1736 and 1697 cm^{-1} in its IR spectrum suggested that it contained ester and α,β-unsaturated ketone carbonyls. Analysis of ^{1}H, ^{13}C, and HSQC NMR data of **5** revealed the presence of an acetate (δ_H 2.12 s; δ_C 20.5), a tertiary methyl (δ_H 1.70 s; δ_C 20.0), a secondary methyl [δ_H 1.18 d (J = 6.4 Hz); δ_C 22.1], five methylenes of which one is oxygenated [δ_H 4.44 dd (J = 17.6, 2.4 Hz), 4.33 ddd (J = 17.6, 4.4, 2.4 Hz); δ_C 58.8], an oxygenated methine (δ_H 3.83 m; δ_C 67.1), eight quaternary carbons of which one is dioxygenated (δ_C 95.6), one is mono-oxygenated (δ_C 84.8), and three are olefinic (δ_C 139.8, 137.7 and 136.2), two ketone carbonyls (δ_C 192.8 and 192.6), and an ester carbonyl (δ_C169.4) moieties. These ^{1}H and ^{13}C NMR data accounted for only half the number of signals expected for its molecular formula ($C_{34}H_{40}O_{12}$), suggesting **5** to be a symmetrical dimer. Analysis of 2D NMR data including ^{1}H−^{1}H COSY, HSQC, and HMBC (Supporting Information, Figures S27−S29) suggested that it is a dimer derived from monomers structurally related to pestafolide A (**6**), which co-occurs with **5** in the same extract. The above 2D NMR data of **5** also indicated that there are some similarities with those of the known atropisomeric bis-spiro-azaphilones, cochliodones A and B.[17] The molecular formula of **5** ($C_{34}H_{40}O_{12}$) suggested that it may be a monosaturated analogue of cochliodone A or B ($C_{34}H_{38}O_{12}$). This was further supported by comparison of their ^{1}H and ^{13}C NMR data. The oxygenated methine group present in cochliodones A [δ_H 7.78 (s); δ_C 157.6 (CH)] and B [δ_H 7.75 (s); δ_C 157.2 (CH)][17] was replaced in **5** with an oxygenated methylene [δ_H 4.44 (dd, J = 17.6, 2.4 Hz) and 4.33 (ddd, J = 17.6, 4.4, 2.4 Hz); δ_C 58.8 (CH_2)] moiety. In addition, this oxygenated methylene group of **5** exhibited HMBC correlations to the dioxygenated carbon (C-9; δ_C 95.6), carbonyl carbon (C-3; δ_C192.8), and two olefinic quaternary carbons (C-2/C-7 and C-2′/C-7′; δ_C 139.8 and 136.2) (Figure 3). The ^{13}C NMR chemical shifts and quaternary nature of C-6/C-6′ [δ_C 137.7 (C)] suggested that the two monomer units are connected at these carbons by a double bond. The orientation of the two monomeric units and possible steric hindrance due the equatorial protons at C-8/C8′ and the oxygen atoms of C-5/C-5′ carbonyl groups in these resulted in

dx.doi.org/10.1021/np500237h | J. Nat. Prod. 2014, 77, 1467−1472

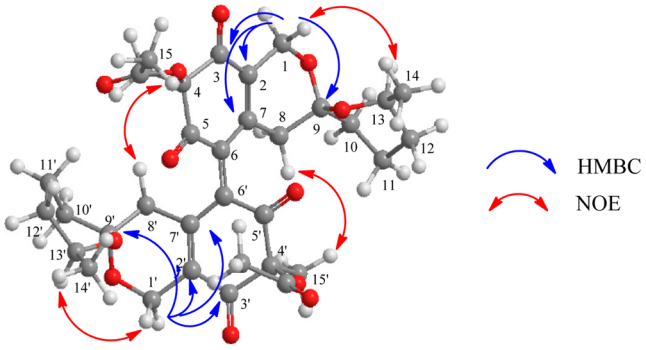
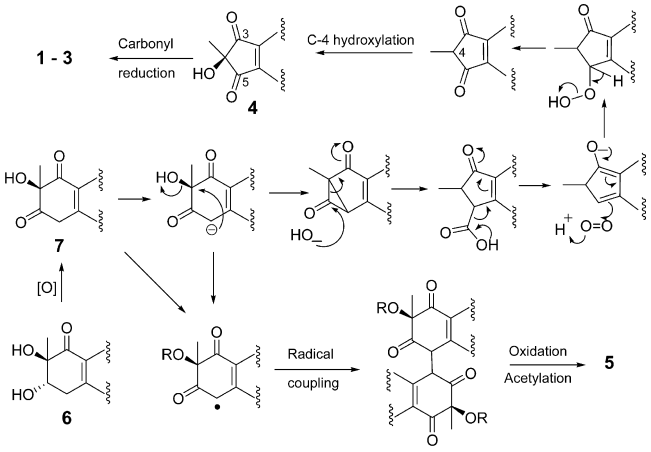


Figure 3. Selected HMBC and NOE correlations for **5**.

four possible MM2 energy-minimized stereoisomers as predicted by three-dimensional molecular modeling [two twisted *trans* isomers (**5a** and **5b**) and two twisted *cis* isomers (**5c** and **5d**, Supporting Information, Figure S3]. As we failed to obtain crystals suitable for X-ray analysis, we utilized NMR data to differentiate between these four stereoisomers of **5**. Irradiation of one of the oxygenated methylene protons at δ 4.33 (H-1b/H-1b′) during the NOEDIFF experiment led to a significant enhancement of H-13/H-13′, indicating that these two protons were cofacial as those in pestafolide A (**6**).[15] One of the methylene protons at δ 2.96 (H-8a/H-8′a) showed a long-range NOE with the tertiary methyl protons at δ_H 1.70 (H_3-15/H_3-15′)] similar to that shown by related protons of naphthylisoquinoline alkaloids.[18] Close examination of structures **5a**–**5d** indicated that in the same monomeric unit these two protons (H-8a and H_3-15 or H-8′a and H_3-15′) are located ca. 5 Å from each other, suggesting that the NOEs observed are caused by these protons in different monomeric units (Figure 3). This is possible only with the twisted *trans* and the twisted *cis* isomers, **5a** and **5c**, respectively (Supporting Information, Figure S3). Careful analysis of ^{1}H NMR data indicated that one of the two protons H-8 or H-8′ of **5** was shifted downfield (δ_H 2.96) compared to the other (δ_H 2.26), while these two protons showed very close chemical shifts (δ_H 2.32 and 2.20) in pestafolide A (**6**).[15] This suggested the possible existence of magnetic anisotropy or a steric compression due to some group(s) present in **5**. Examination of the isomeric structures **5a** and **5c** revealed that the C-5 (or C-5′) carbonyl group was close (2.4 Å) to H-8a (or H-8′a) in the *trans* isomer **5a**. In the *cis* isomer **5c**, the distance between C-5 carbonyl and H-8a (or C-5′ carbonyl and H-8′a) was found to be considerably larger (3.9 Å) (Supporting Information, Figure S3), suggesting that thielavialide E has the structure with the probable relative configuration as depicted in **5** and in Figure 3. The metabolite **6** was identified as pestafolide A by comparison of its $[\alpha]_D$ and NMR data with those reported.[15]

All compounds were tested in our in-house cytotoxicity,[19] cell migration inhibition,[20] and heat-shock induction[21] assays and were found to be inactive up to a concentration of 10.0 μM. Biosynthetically, thielavialides may be formed from pestafolide A (**6**), which was found to co-occur in *Thielavia* sp. PA0001. A probable biosynthetic pathway to thielavialides A–D (**1**–**4**) from **6** involving a Favorskii-like rearrangement of its oxidation product, 5-dehydropestafolide A (**7**), is depicted in Scheme 1. Favorskii-like rearrangements have previously been invoked in biosynthesis of the microbial metabolites aspyrone,[22] asterredione,[23] and enterocin.[24] As depicted in Scheme 1, biosynthesis of thielavialide E (**5**) may involve a radical

Scheme 1. Proposed Biosynthetic Pathway to Thielavialides A–E (1–5) from Pestafolide A (6) Involving a Favorskii-Like Rearrangement and Radical Coupling



oxidative coupling of the intermediate **7** or its acetate. Similar oxidative coupling has recently been proposed for the biosynthesis of the related bis-spiro-azaphilone, cochliodone A.[25]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured at 25 °C with a JASCO Dip-370 digital polarimeter using MeOH as solvent. UV spectra were recorded in $CHCl_3$ on a Shimadzu UV-1601 UV–vis spectrometer. FT-IR spectra for KBr discs were recorded on a Shimadzu FTIR-8300 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ with a Bruker AVANCE III instrument at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR using residual $CHCl_3$ as internal standard; the chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. Low-resolution mass spectra were measured in the APCI positive ion and negative ion modes on a Shimadzu LCMS-QP8000α spectrometer with 4 kV detector voltage and 400 °C probe temperature. High-resolution mass spectra were measured in the ESI positive-ion mode on a Bruker 9.4T FT-ICR spectrometer with the collision energy of 6 eV. Column chromatography was performed on Baker silica gel 40 μm flash chromatography packing (J. T. Baker) or Sephadex LH-20 (25–100 μm; GE Healthcare). Analytical and preparative thin-layer chromatography (TLC) were performed on precoated 0.20 mm thick plates of silica gel 60 F_{254} (Merck); spraying with a solution of anisaldehyde and concd H_2SO_4 in glacial acetic acid and water followed by heating was used to visualize the spots on analytical TLC.

Fungal Isolation and Identification. In June 2007, healthy leaves of *Physalis alkekengi* were collected from aeroponically grown plants in a greenhouse at the Southwest Center for Natural Products Research and Commercialization (SCNPRC), University of Arizona, Tucson, Arizona, USA. The leaves were cut into 2 cm × 2 cm segments, washed in tap water, surface-sterilized by agitating sequentially in 95% EtOH for 30 s, 0.5% NaOCl for 2 min, and 70% EtOH for 2 min and then surface-dried under sterile conditions before cutting into 2 mm × 2 mm pieces.[26] These pieces of leaves were plated on 2% malt extract agar (MEA) in a Petri plate. The plate was sealed with Parafilm and incubated under ambient light/dark conditions at room temperature (ca. 21.5 °C). The emergent fungal colonies were isolated and transferred into 2% MEA, vouchered in sterile water, and deposited as living voucher at SCNPRC under accession no. PA0001. Total genomic DNA was extracted directly from the isolate,[26] and PCR amplification of the nuclear ribosomal internally transcribed spacer region (ITSrDNA) was performed in 25 μL reaction volumes containing 12.5 μL of EmeraldAmp GT PCR Mastermix from Clontech, 6.5 μL of PCR-quality water, 2.5 μL of each primer (5 μM),

dx.doi.org/10.1021/np500237h | J. Nat. Prod. 2014, 77, 1467–1472

and 1 μL of template. Primers ITS5 and LR3 were used as primers to amplify ITSrDNA (see http://biology.duke.edu/fungi/mycolab/primers.htm). Cycling reactions were run on Eppendorf Matercycler epgradient S under the following conditions: 30 cycles of 98 °C for 10 s, 46 °C for 30 s, and 72 °C for 120 s, followed by one cycle of 72 °C for 300 s. A vivid green dye stain (from the EmeraldAmp Mastermix) was used to detect DNA bands on a 1% agarose gel. All products yielded single bands. PCR products were cleaned using GFX PCR DNA and Gel Band Purification Kit from GE Healthcare. Amplified DNA was sequenced for both forward and reverse reads at the University of Arizona Genetics Core facility. The sequencing reads were submitted to BLAST searches of the NCBI GenBank database for identification. The BLAST search revealed 99% similarity with *Thielavia terrestris* NRRL 8126 accession number CP003011.1. Because PA0001 was not 100% similar to this, it was designated as *Thielavia* sp. PA0001. The sequence is deposited in GenBank under accession no. KJ813011.

Culturing and Isolation of Metabolites. A seed culture of *Thielavia* sp. PA0001 grown on PDA for 2 weeks was used for inoculation. Mycelia were scraped out and vortexed with sterile PDB (90 mL) and filtered through a 100 μm filter to separate spores from the mycelia. Absorbance of the spore solution was measured (at 600 nm) and adjusted to 0.6. This spore solution was used to inoculate 4 × 2.0 L Erlenmeyer flasks, each holding 1.0 L of the medium (PDB) containing 0.25 mM $CuSO_4$ and incubated at 160 rpm and 28 °C. One week after inoculation, the glucose level in the medium was monitored using glucose strips (URISCAN glucose strip). On day 49, the strip gave a blue color for the glucose test, indicating the absence of glucose in the medium. Mycelia were then separated by filtration, and the filtrate (pH = 5.74) was neutralized by adding 1.0 M aq NaOH solution and extracted with EtOAc (3 × 2 L). The EtOAc extract was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the crude extract as a brown semisolid (373.2 mg). A portion (340.0 mg) of this was chromatographed over a column of silica gel (10.0 g) made up in CH_2Cl_2 and eluted with CH_2Cl_2 containing increasing amounts of MeOH. Fractions (100, 6.0 mL each) were collected, and those having similar TLC profiles were combined to give 10 major fractions [A (20.0 mg), B (6.7 mg), C (34.6 mg), D (32.3 mg), E (47.8 mg), F (87.2 mg), G (30.2 mg), H (18.1 mg), I (32.6 mg), and J (35.6 mg)]. Only those fractions containing isolable compounds were subjected to further purification. Fraction A (26.0 mg) was purified by preparative TLC developed with hexanes/EtOAc (4:6) to give **5** (2.8 mg, R_f = 0.65). Repeated preparative TLC of fraction B (6.7 mg) yielded **6** (4.5 mg). Fraction C (34.6 mg) was subjected to repeated preparative TLC (silica gel) to give **4** (4.1 mg). Fraction E (47.8 mg) was subjected to gel permeation chromatography over column of Sephadex LH-20 (3.0 g) made up in hexanes/CH_2Cl_2 (1:4) and eluted with hexanes/CH_2Cl_2 (1:4, 100 mL), CH_2Cl_2/acetone (1:4, 100 mL), CH_2Cl_2/acetone (3:2, 100 mL), and finally with CH_2Cl_2/MeOH (1:1, 50 mL). Thirty fractions (6.0 mL each) were collected, and fractions having similar TLC profiles were combined to give three major fractions [E-1 (15.7 mg), E-2 (19.8 mg), and E-3 (28.4 mg)]. Fraction E-2 was subjected to repeated preparative TLC (silica gel) to give **1** (3.8 mg), **2** (1.7 mg), and **3** (1.5 mg).

*Thielavialide A (**1**).* Colorless gum; $[\alpha]_D^{25}$ −109.4 (*c* 0.35, MeOH); UV λ_{max} (log ε) 236 (3.42) nm; IR (KBr) ν_{max} 3449, 2947, 1713, 1663, 1454, 1383, 1229, 1178, 1120, 1080, 1047, 959, 914 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 269.1383 $[M + H]^+$ (calcd for $C_{14}H_{21}O_5$, 269.1384).

*Thielavialide B (**2**).* Colorless gum; $[\alpha]_D^{25}$ −60.4 (*c* 0.15, MeOH); UV λ_{max} (log ε) 237 (3.34) nm; IR (KBr) ν_{max} 3447, 2947, 1713, 1663, 1452, 1379, 1254, 1229, 1182, 1123, 1078, 1032, 959, 914 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 269.1382 $[M + H]^+$ (calcd for $C_{14}H_{21}O_5$, 269.1384).

*Thielavialide C (**3**).* Colorless gum; $[\alpha]_D^{25}$ −46.2 (*c* 0.15, MeOH); UV λ_{max} (log ε) 236 (3.48) nm; IR (KBr) ν_{max} 3447, 2945, 1713, 1663, 1452, 1379, 1285, 1254, 1227, 1182, 1078, 1032, 959, 916, 914 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 269.1385 $[M + H]^+$ (calcd for $C_{14}H_{21}O_5$, 269.1384).

*Thielavialide D (**4**).* White amorphous solid; $[\alpha]_D^{25}$ −67.1 (*c* 0.32, MeOH); UV λ_{max} (log ε) 246 (3.28) nm; IR (KBr) ν_{max} 3449, 2947, 1702, 1662, 1654, 1450, 1378, 1232, 1174, 1118, 1084, 1049, 962, 916 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 267.1224 $[M + H]^+$ (calcd for $C_{14}H_{19}O_5$, 267.1227).

*Thielavialide E (**5**).* White amorphous solid; $[\alpha]_D^{25}$ −78.9 (*c* 0.2, MeOH); UV λ_{max} (log ε) 341 (1.42), 310 (2.62), 235 (0.74), 210 (2.23) nm; IR (KBr) ν_{max} 2935, 2864, 1736, 1697, 1468, 1238, 1199, 1024 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.44 (2H, dd, *J* = 17.6, 2.4 Hz, H-1a, H-1′a), 4.33 (2H, ddd, *J* = 17.6, 4.4, 2.4 Hz, H-1b, H-1′b), 3.83 (2H, m. H-13, H-13′), 2.96 (2H, dd, *J* = 18.4, 2.4 Hz, H-8a, H-8′a), 2.26 (2H, ddd, *J* = 18.4, 4.4, 2.4 Hz, H-8b, H-8′b), 2.12 (6H, s, H_3-17, H_3-17′), 1.91 (2H, m, H-11a, H-11′a), 1.70 (6H, s, H_3-15, H_3-15′), 1.65 (2H, m, H-12a, H-12′a), 1.64 (2H, m, H-11b, H-11′b), 1.63 (2H, m, H-10a, H-10′a), 1.63 (2H, m, H-10b, H-10′b), 1.28 (2H, dt, *J* = 2.8, 12.4 Hz, H-12b, H-12′b), 1.18 (6H, d, *J* = 6.4 Hz, H_3-14, H_3-14′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.8 (C, C-3, C-3′), 192.6 (C, C-5, C-5′), 169.4 (C, C-16, C-16′), 139.8 (C, C-2/C7, C-2′/ C-7′), 137.7 (C, C-6, C-6′), 136.2 (C, C-2/C-7, C-2′/C-7′), 95.6 (C, C-9, C-9′), 84.8 (CH, C-4, C-4′), 67.1 (CH, C-13, C-13′), 58.8 (CH_2, C-1, C-1′), 37.6 (CH_2, C-8, C-8′), 32.8 (CH_2, C-10, C-10′), 32.5 (CH_2, C-12, C-12′), 22.1 (CH_3, C-14, C-14′), 20.5 (CH_3, C-15, C-15′), 20.0 (CH_3, C-17, C-17′), 18.7 (CH_2, C-11, C-11′); HRESIMS *m*/*z* 641.2590 $[M + H]^+$ (calcd for $C_{34}H_{41}O_{12}$, 641.2593).

*Pestafolide A (**6**).* Colorless oil; $[\alpha]_D^{25}$ −19.5 (*c* 0.23, MeOH) [lit.[15] −19.0 (*c* 0.05, MeOH)]; ^1H and ^{13}C NMR data were fully consistent with those reported.[15]

*Oxidation of thielavialide A (**1**).* To a solution of **1** (0.5 mg) in $CHCl_3$ was added activated MnO_2 (2.0 mg) and stirred at 25 °C. After 6 h (TLC control), MnO_2 was filtered off and washed with $CHCl_3$. Combined $CHCl_3$ layer was evaporated under reduced pressure to give the oxidation product (0.4 mg, 80.6%) as a white amorphous solid; $[\alpha]_D^{25}$ −66.8 (*c* 0.3, MeOH); LRMS (+ve mode) *m*/*z* 267 $[M + 1]^+$; LRMS (−ve mode) *m*/*z* 265 $[M - 1]^-$. ^1H NMR data were identical with those of thielavialide D (**4**).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Selected HMBC and NOE correlations, MM2 energy-minimized stereoisomeric structures, ^1H and ^{13}C NMR spectra, $^1H-^1H$ COSY spectra, HSQC and HMBC spectra of thielavalide compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (520) 621-9932. Fax: (520) 621-8378. E-mail: leslieg@cals.arizona.edu.

Notes

The authors declare no competing financial interest.

†Studies on Arid Lands Plants and Microorganisms, Part 26.

■ ACKNOWLEDGMENTS

Financial support for this work was provided by grant R01 CA090265 funded by the National Cancer Institute (NCI) and grant P41 GM094060 funded by National Institute of General Medical Sciences (NIGMS). We thank Dr. Yaming Xu for helpful discussions and Manping X. Liu for her help with the biological assays.

■ REFERENCES

(1) For Part 25 of Studies on Arid Lands Plants and Microorganisms, see: Xu, Y.; Espinosa-Artiles, P.; Liu, M. X.; Arnold, A. E.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2013**, *76*, 2330−2336.

(2) Wilson, D. *Oikos* **1995**, *73*, 274−276.

(3) Carroll, G. C. *Ecology* **1988**, *69*, 2−9.

(4) (a) Gunatilaka, A. A. L. *J. Nat. Prod.* **2006**, *69*, 509−526. (b) Wang, X.-N.; Bashyal, B. P.; Wijeratne, E. M. K.; U'Ren, J. M.; Liu, M. X.; Gunatilaka, M. K.; Arnold, A. E.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2011**, *74*, 2052−2061.
(5) (a) Xu, Y.; Marron, M. T.; Seddon, E.; McLaughlin, S. P.; Ray, D. T.; Whitesell, L.; Gunatilaka, A. A. L. *Bioorg. Med. Chem.* **2009**, *17*, 2210−2214. (b) Xu, Y.; Gao, S.; Bunting, D. P.; Gunatilaka, A. A. L. *Phytochemistry* **2011**, *72*, 518−522.
(6) Endo, A.; Kitahara, N.; Takahashi, H.; Kotani, K. *Jpn. Kokai Tokkyo Koho*, JP 54098392 A 19790803, 1979.
(7) Matsutani, S.; Sakazaki, R.; Hinoo, H.; Terui, Y.; Tanaka, K.; Matsumoto, K.; Yoshida, T. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1992**, *34*, 142−149.
(8) Matsumoto, K.; Tanaka, K.; Matsutani, S.; Sakazaki, R.; Hinoo, H.; Utani, N.; Tanomoto, T.; Kawamura, Y.; Nakamoto, S.; Yoshida, T. *J. Antibiot.* **1995**, *48*, 106−112.
(9) Kitahara, N.; Haruyama, H.; Hata, T.; Takahashi, S. *J. Antibiot.* **1983**, *36*, 599−600.
(10) Yoshida, T.; Arita, H.; Shoji, J.; Inoue, K.; Matsutani, S.; Kawamura, Y. Eur. Pat. Appl. EP 395418 A1 19901031, 1990.
(11) Yoshida, T.; Nakamoto, S.; SakaZaki, R.; Matsumoto, K.; Terui, Y.; Sato, T.; Arita, H.; Matsutani, S.; Inoue, K.; Kudo, I. *J. Antibiot.* **1991**, *44*, 1467−1470.
(12) Yoshida, T.; Matsutani, S.; Kawamura, Y.; Matsumoto, K. *Jpn. Kokai Tokkyo Koho*. JP 04159252 A 19920602, 1992.
(13) Sugano, M.; Hatano, E.; Hamada, T.; Hosoya, T.; *Jpn. Kokai Tokkyo Koho*. JP 09221493 A 19970826, 1997.
(14) Rothstein, D.; Mani, N.; Jiang, Z.-D. PCT Int. Appl. WO 9800019 A1 19980108, 1998.
(15) Ding, D.; Liu, S.; Guo, L.; Zhou, Y.; Che, Y. *J. Nat. Prod.* **2008**, *71*, 615−618.
(16) Fortunato, B.; Giorgini, M. G. *J. Mol. Struct.* **1977**, *37*, 27−34.
(17) Phonkerd, N.; Kanokmedhakul, S.; Kanokmedhakul, K.; Soytong, K.; Prabpai, S.; Kongsearee, P. *Tetrahedron* **2008**, *64*, 9636−9645.
(18) Bringmann, G.; Koppler, D.; Scheutzow, D.; Porzel, A. *Magn. Reson. Chem.* **1997**, *35*, 297−301.
(19) O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. *Eur. J. Biochem.* **2000**, *267*, 5421−5426.
(20) Zhan, J.; Burns, A. M.; Liu, M. X.; Faeth, S. H.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2007**, *70*, 227−232.
(21) Turbyville, T. J.; Wijeratne, E. M. K.; Liu, M. X.; Burns, A. M.; Seliga, C. J.; Luevano, L. A.; David, C. L.; Faeth, S. H.; Whitesell, L.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2006**, *69*, 178−184.
(22) Tanabe, M.; Uramoto, M.; Hamasaki, T.; Cary, L. *Heterocycles* **1976**, *5*, 355−365.
(23) Wijeratne, E. M. K.; Turbyville, T. J.; Zhang, Z.; Bigelow, D.; Pierson, L. S., III; VanEtten, H. D.; Whitesell, L.; Canfield, L. M.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2003**, *66*, 1567−1573.
(24) Xiang, L.; Kalaitzis, J. A.; Nilsen, G.; Chen, L.; Morre, B. S. *Org. Lett.* **2002**, *4*, 957−966.
(25) Nakazawa, T.; Ishiuchi, K.; Sato, M.; Tsunematsu, Y.; Sugimoto, S.; Gotanda, Y.; Noguchi, H.; Hotta, K.; Watanabe, K. *J. Am. Chem. Soc.* **2013**, *135*, 13446−13455.
(26) U'Ren, J. M.; Lutzoni, F.; Miadlikowska, J.; Arnold, A. E. *Microbiol. Ecol.* **2010**, *60*, 340−353.