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ชื่อโครงการ: องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพจากพืช ราและเห็ด

Chemical constituents and their bioactivities from plants, fungi and mushroom

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ชื่อหัวหน้าโครงการ นายสมเดช กนกเมธากุล

1. การดำเนินงาน ☑ ได้เปลี่ยนแปลงแผนที่วางไว้ดังนี้คือ

1.1 เปลี่ยนการวิจัยเห็ด *Leucocoprinus fragilissimus* เป็นรา Aspergillus terreus เนื่องจากเมื่อทำการแยกสารแล้ว ไม่พบสารที่น่าสนใจ แม้ว่าจะได้ทำการเลี้ยงเส้นใยเห็ดเพิ่มขึ้นแล้วก็ตาม

1.2 เพิ่มการวิจัยเชื้อรา ในสกุล *Penicillium* sp. และ *Neosartorya spinosa* KKU-1NK1

2. สรุปผลการดำเนินงาน

2.1 กิจกรรมที่วางแผนไว้

2.1.1 เลี้ยงเห็ด *Leucocoprinus fragilissimus* ได้เส้นใยแห้ง 200 กรัม และเชื้อรา 3 ชนิดคือ *Aspergillus terreus* *Penicillium multicolor* CM01 และ *Neosartorya spinose* KKU-1NK1 ในอาหาร เหลว PDB กรองเส้นใยและผึ่งลมให้แห้ง

2.1.2 สกัดเส้นใยแห้งด้วยตัวทำละลายอินทรีย์ 3 ชนิดคือ hexane, EtOAc และ MeOH เพื่อแยก เป็นส่วนสกัดหยาบของ hexane, EtOAc และ MeOH ตามลำดับ จำนวน 9 ส่วนสกัด

2.1.3 ทดสอบฤทธิ์ทางชีวภาพของส่วนสกัดหยาบทั้งหมดกับเชื้อมาลาเรีย วัณโรค และเซลล์มะเร็ง 3 ชนิดที่ศูนย์พันธุวิศวกรรมและเทคโนโลยีแห่งชาติ

2.1.4 แยกสารจากสารสกัดหยาบทั้ง 3 ส่วน คือ hexane, EtOAc และ MeOH ที่ได้จากเห็ด และรา 2 ชนิด (9 ส่วนสกัด) โดยวิธีทางโครมาโทรกราฟี

2.1.5 วิเคราะห์หาโครงสร้างทางเคมีของสารที่แยกได้ด้วยวิธีทางสเปกโทรสโกปี

2.1.6 ส่งสารบริสุทธิ์ที่แยกได้ไปทดสอบฤทธิ์ทางชีวภาพ กับเชื้อมาลาเรีย วัณโรค และเซลล์มะเร็ง

2.1.7 วิเคราะห์ข้อมูล สรุปผล รายงานผลการวิจัย

2.1.8 เตรียมต้นฉบับสำหรับส่งไปตีพิมพ์ในวารสารระดับนานาชาติ

2.2 กิจกรรมที่ทำได้จริง

2.2.1 การเลี้ยงเชื้อรา

เลี้ยงเห็ด *Leucocoprinus fragilissimus* ได้เส้นใยแห้ง 200 กรัม และเชื้อรา 3 ชนิดคือ *Aspergillus terreus* *Penicillium multicolor* CM01 และ รา *Neosartorya spinose* KKU-1NK1 ในอาหาร เหลว PDB กรองเส้นใยและผึ่งลมให้แห้ง ได้เส้นใยแห้ง 160 กรัม 180 กรัม และ 75 กรัม ตามลำดับ

2.2.2 การสกัด

ทำการสกัดเส้นใยแห้งของเห็ด และราทั้ง 3 ชนิด ด้วยตัวทำละลาย จากขั้วน้อยไปมากคือ hexane จำนวน 3 ครั้งๆ ละ 0.5-1 ลิตร (ขึ้นอยู่กับปริมาณของเส้นใย) ทำการกรองแยก เอาเส้นใยออก นำส่วนเส้นใยที่กรองได้ ไปสกัดต่อ ด้วย EtOAc และ MeOH โดยวิธีการเหมือนกับการสกัดด้วย hexane นำสารละลายที่กรองได้ไประเหยตัวทำละลายออก ได้สารสกัดหยาบ ของราแต่ละชนิด รวมได้ 9 ส่วนสกัดหยาบ

2.2.3 การทดสอบฤทธิ์ทางชีวภาพ

นำส่วนสกัดหยาบทั้ง 9 ส่วนจากข้อ 2.2.2 ไปทำการทดสอบฤทธิ์ทางชีวภาพเบื้องต้นกับเชื้อ มาลาเรีย และวัณโรค (ทดสอบเพียง 2 ชนิด เนื่องเป็นส่วนสกัดหยาบและมีค่าใช้จ่ายในการทดสอบสูง) ผลการทดสอบฤทธิ์ทางเภสัชวิทยาพบว่าสารสกัดหยาบของรา *A. terreus* *Penicillium multicolor* CM01 และ *N. Spinose* KKU-1NK1 มีฤทธิ์ทางชีวะภาพอยู่ในเกณฑ์ที่สามารถนำไปศึกษาต่อได้

2.2.4 การแยกสารให้บริสุทธิ์

2.2.4.1 เห็ด *Leucocoprinus fragilissimus*

ทำการแยกสารจากส่วนสกัดหยาบทั้ง 3 ส่วน ด้วยวิธีทางคอลัมน์โครมาโทรกราฟีแบบต่างๆ ได้สารบริสุทธิ์ในปริมาณน้อย และจากผลของการตรวจวัดสเปคตราด้วยเครื่องมือ NMR พบว่าสารที่ได้ เป็นกลุ่มสารที่ไม่น่าสนใจ และแตกต่างจากที่คาดการณ์ไว้มาก จึงไม่ดำเนินการต่อ

2.2.4.2 รา *Aspergillus terreus*

Introduction

Aspergillus terreus belongs to the family Trichocomaceae. Several of its varities have been reported as being sources of various bioactive secondary metabolites, such as 4*R*-(hydroxymethyl)-4*R*-demethylterritrem B (Peng et al. 1997) and terreulactone A (Kim et al. 2002) which inhibit acetylcholinesterase (AChE), butyrolactone V has moderate antimalarial activity against *Plasmodium falciparum* K1 (Haritakun et al. 2010), terremides A and B have antibacterial activity against *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (Wang et al. 2011), butyrolactone I has anti-H1N1 activity (Wang et al. 2011), and asperterrestide A inhibits the influenza virus strains A/WSN/33 (H1N1) and A/Hong Kong/8/68 (H3N2), while also being cytotoxic toward human carcinoma U937 and MOLT4 cell lines (He et al. 2013). Recently, several alkaloids and polyketide derivatives isolated from the mycelial extract of a mixed culture of two marine algaderived fungal strains of the genus *Aspergillus* exhibited selective anti-proliferative activities in four different human cancer cell lines tested (Ebada et al. 2014). More recently, a new cyclicpeptide, 14-hydroxy-cyclopeptine isolated from *Aspergillus* sp. SCSIOW2 has been reported to inhibit nitric oxide production (Zhou et al. 2015). Therefore, the search for bioactive compounds from the fungus *A. terreus* is currently of interest. As part of our search for bioactive compounds from fungi, we have found that the crude EtOAc extract of the fungal biomass of *A. terreus*, isolated from the garbage component in organic fertilizer, has shown cytotoxicity against NCI-

H187 with 67.7% inhibition at 50 μg/ml. We report herein the isolation, structural elucidation, and bioactivity of a new lumazine peptide, penilumamide E (**1**), together with thirteen known compounds from the fungus *A. terreus*.

Results and Discussion

Compound **1** was obtained as colorless needles and its molecular formula deduced as $C_{20}H_{20}N_6O_7$ by HR-ESI-TOF-MS *m/z* 479.1296 $[M+Na]^+$ (calcd. *m/z* 479.1291), implying fourteen degrees of unsaturation. The UV spectrum showed maxima absorption at 223, 251 and 318 nm of an aromatic ring. The IR absorptions indicated the presence of secondary amide (3388 cm^{-1}), hydroxyl (3260 cm^{-1}), ester and amide (1702 cm^{-1} overlapping), and aromatic (1582 and 1491 cm^{-1}) groups. The ^{13}C NMR spectrum of **1** (Table S1) was assigned by the DEPT and HMQC techniques as having 20 carbon signals attributable to five carbonyl (149.8, 160.4, 163.2, 168.2 and 169.0), five sp^2 quaternary (125.7, 115.7, 139.7, 140.1 and 150.7), five sp^2 methine (120.4, 123.1, 130.7, 134.4 and 148.1), two sp^3 methine (60.8 and 67.2) and three methyl (19.6, 28.9 and 52.0) carbons. The ^1H NMR spectrum (Table S1) showed signals for five aromatic protons at δ 9.37 (s, H-7), 7.91 (d, *J* = 7.2 Hz, H-4″), 7.05 (t, *J* = 7.2 Hz, H-5″), 7.47 (t, *J* = 7.2 Hz, H-6″), 8.59 (d, *J* = 7.2 Hz, H-7″), two methine groups at δ 4.63 (d, *J* = 3.6 Hz, H-2′) and 4.54 (m, H-4′), a methyl ester at δ 3.66 (s, H-9″), a methyl amine at δ 3.65 (s, H-9) and a methyl group at δ 1.31 (d, *J* = 6.0 Hz , H-5′). Information from the COSY and HMBC spectra indicating the structure of **1** contained three main units of lumazine, threonine and anthranilic acid. The COSY spectrum (Figure S9) showed correlations between NH-1'↔H-2'↔H-4'↔H-5' and H-4″↔H-5″↔H-6″↔H-7″. The HMBC spectrum (Figure S8) clearly demonstrated the correlations of H-7 to C-4a (δ 125.7), C-6 (δ139.6) and C-8a (δ150.7); methoxy protons (H-9) to C-2 (δ149.8) and C-8a indicated the lumazine unit. The correlations of H-2′ to C-10 (δ163.2), C-3′ (δ169.0), C-4′ (δ67.2) and C-5′ (δ 19.6), H-4′ to C-3′ and C-5′, H-5′ to C-4′ and C-2′ (δ60.8) indicated the presence of a threonine unit. Furthermore, the correlations of H-4″ to C-2″ (δ140.1), C-6″ (δ134.4) and C-8″ (δ168.2), H-5″ to C-3″ (δ115.7) and C-7″ (δ120.2), H-6″ to C-2″ and C-4″ (δ130.7), H-7″ to C-3″ (δ115.7) and C-5″ (δ123.1), H-9″ to C-8″ confirmed the anthranilic acid methyl ester unit. The connections between a lumazine unit and a threonine unit were indicated by the correlations of H-2′ to C-10 and NH-1′ to C-10, while the connection between a threonine unit and an anthranilic acid methyl ester unit were confirmed from the correlations of NH-1″ to C-3′ and C-7″. From analysis of ^1H and ^{13}C NMR spectral data, compound **1** has a core structure the same as the known compound, penilumamide (Meyer et al. 2010), except for the methionine sulfoxide unit at C-2' which is replaced by a threonine unit. In addition, the N-methyl group at N-3 of the lumazine unit in penilumamide was replaced by a hydrogen atom. Moreover, the structure of **1** was also compared to the recent report for penilumamides B-D isolated from *Aspergillus* sp. XS-20090B15 (Chen et al. 2014). The NOESY spectrum (Figure S10) displayed

correlations between H-4′ and H-5′, H-4′′ and H-5′′, H-5′′ and H-6′′, H-6′′ and H-7′′ indicating the relative configurations at C-2′ and C-4′. Finally, the X-ray crystallographic technique was carried out to confirm the structure of compound **1** (Figure 2 and Tables S2 and S3). On the basis of the above data, **1** was defined as a new lumazine peptide which has been named penilumamide E. The lumazine peptide group is a rare natural product which has been isolated from the marine-derived fungus, *Penicillium* sp. CNL-338 (Meyer et al. 2010) and *Aspergillus* sp. XS-20090B15 (Chen et al. 2014). This is the first report of this type of compound from *A. terreus* isolated from soil.

The known compounds were identified on the basis of spectroscopic data and by comparing to data reported in literature as 3,4,5-trimethoxy-2-(2-(nicotinamido) benzamido)benzoate (**2**) (Zhou et al. 2011), *epi*-aszonalenin A (**3**) (Rank et al. 2006), butyrolactone I (**4**), butyrolactone II (**5**) (Rao et al. 2000), butyrolactone V (**6**) (Haritakun et al. 2010), terretonin (**7**) (Springer et al. 1979), terretonin A (**8**) (Li et al. 2005), territrem A (**9**) (Lee et al. 1992), territrem B (**10**) (Lee et al. 1992), territrem C (**11**) (Lee et al. 1992), ergosterol (**12**) (Kwon et al. 2002), 24(*R*)-5α,8α-epidioxyergosta-6-22-diene-3β-ol (**13**) (Jinming et al. 2001) and D-mannitol (**14**) (Pouchert et al. 1993).

Among the isolated compounds, only **10** exhibited antimalarial activity against *Plasmodium falciparum* with IC_{50} values of 2.83 μg/ml. Compounds **4** and **6** showed weak cytotoxicity against the cholangiocarcinoma (CCA) cell line, KKU-100, while **4** and **11** exhibited weak cytotoxicity against HepG2 cells. Compound **1** showed non-toxic to KKU-100 and HepG2 cell lines, while **9** and **11** showed non-toxic to KKU-100 cell line at 500 μg/ml. However, compounds **6**, **7, 8** and **9** significantly induced an increase in cell number (proliferation) of HepG2 cells at certain concentrations (0.2-100 μg/ml) (Table 1). In addition, these isolated compounds showed non-cytotoxicity ($<$50 μg/ml) against the three cancer cell lines tested (KB, NCI-H187 and MCF7).

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เนื่องจากงานวิจัยนี้เสร็จสมบูรณ์แล้ว และตีพิมพ์เผยแพร่ ชื่อเรื่อง “A new lumazine peptide penilumamide E from the fungus *Aspergillus terreus*” ในวารสาร Natural Products Research (IF = 1.828, Q2) จึงนำเสนอแบบครบถ้วนในภาคผนวก

2.2.4.3 รา *Penicillium multicolor* CM01

Introduction

The members of the genus *Penicillium* members are known for their ability to produce bioactive secondary metabolites, for example, spiroditerpenoids (Li et al. 2009) alkaloids (Dilip de Silva et al. 2009; Du et al. 2009), spiroazaphilones (Ariza et al. 2001) and polyphenols (Lu et al. 2010). One of the specific metabolites repeatedly isolated from this genus is azaphilone, with various substitution groups on the main skeleton. Various strains of *P. multicolor* have been reported to produce azaphilone metabolites such as sclerotiorin (Birkinshaw et al. 1952), isochromophilone I-VI (Matsuzaki et al. 1995, Arai et al. 1995) and 8-*O*-methylsclerotiorinamine (Nam et al. 2000). Previous reports have shown that azaphilones exhibited broad activities in many biological tests, such as cholesteryl ester transfer protein (Tomoda et al. 1999), endothelin receptor binding, (Pairet et al. 1995) and the inhibition of gp120-CD4 binding (Matsuzaki et al. 1998). In our search for bioactive compounds from fungi isolated from Thai soil, an EtOAc extract of *P. multicolor* CM01 showed cytotoxicity against the KB cell line with an IC_{50} value of 10.4 μg/mL. We report herein the isolation, characterization and bioactivity of two new compounds (**1** and **2**), a new natural product (**3**) and eleven known compounds (**4**-**14**) from *P. multicolor* CM01.

Results and discussion

Chromatographic separation of the EtOAc extract of *P. multicolor* CM01 gave fourteen compounds, **1**-**14** (Figure 1). Their structures were determined by spectroscopic data (IR, UV, ^{1}H and ^{13}C NMR, 2D-NMR, and MS). The known compounds were identified by physical properties and spectroscopic data measurements as well as by comparing the data obtained with published values, as sclerotiorin (**4**) (Curtin and Reilly, 1940), ochrephilone (**5**) (Seto and Tanabe, 1974), isochromophilone II (**6**) (Omura et al. 1993), isochromophilone III (**7**), isochromophilone IV (**8**), isochromophilone VI (**9**) (Arai et al. 1995), sclerotioramine (**10**) (Wang et al. 2010), isochromophilone VIII (**11**) (Yang et al. 1996), (*S*)-6-((1*S*,2*S*)-1,2-dihydroxypentyl)-4-methoxy-5,6-dihydro-2*H*-pyran-2-one (**12**) (Eade et al. 1957), ergosterol (**13**) (Bok et al. 1999) and ergosterol peroxide (**14**) (Rösecke and König, 2000). Although compound **3** has previously been synthesized, this is the first isolation from a natural source.

Compound **1** had the molecular formula $C_{22}H_{28}O_4$ deduced from HRESITOFMS (*m*/*z* 357.2074 $[M + H]^+$), indicating nine degrees of unsaturation. The UV spectrum exhibited an absorption maximum at 251 nm. The IR spectrum showed characteristics of hydroxyl (3400 cm^{-1}), ketone (1711 cm^{-1}) and alkene (1607 cm^{-1}) groups. The ^{13}C NMR, HSQC and DEPT spectra revealed the presence of six sp^2 quaternary (including two carbonyl groups), six sp^2 methine, one sp^3 quaternary, two methine, two methylene, and five methyl carbons. The ^{1}H NMR spectrum of **1** showed three olefinic proton signals at δ 7.37 (s, H-1), 6.04 (s, H-4), and 5.36 (s, H-5), two methyl signals at δ 1.26 (s, H-18) and 2.04 (s, H-3′), two nonequivalent methylene proton signals at δ 3.04 (dd, *J* = 3.2, 18.4 Hz, H_b-1′), and 2.34 (dd, *J* = 7.6, 18.4 Hz, H_a-1′), and a methine proton signal at δ 3.42 (dd, *J* = 3.2, 7.6 Hz, H-8). These data, combined with the conjugated carbonyl at δ_C 198.1 (C-6) and ketone carbonyl at δ_C 207.3 (C-2′),

suggested the presence of an azaphilone skeleton and a 2-oxypropyl moiety for **1**, analogous to isochromophilone II (**6**) (Quang et al. 2006). The distinct difference in the NMR spectra between **1** and **6** was that the chlorine atom at C-5 in **6** was replaced by an olefinic proton (H-5) in **1**. The HMBC correlations (Figure 2) of H-4 to C-4a, C-5, C-8a; and H-5 to C-4, C-4a, C-7, and C-8a indicated that H-4 and H-5 were in close proximity. The 3,5-dimethyl-1,3-heptadienyl unit was established by COSY correlations of H-9/H-10, H-17/H-12/H-13/H-16 and H-13/H-14/H-15 (Figure 2). The HMBC spectrum exhibited correlations of H-9 and H-10 to C-3 and H-4 to C-9, confirming the connection of this unit at C-3. The structure of **1** was completely characterized by 2D NMR. The absolute configuration of C-7 was concluded to be *R* on the basis of the CD spectrum, which revealed the positive 234 nm ($\Delta\varepsilon$ +1.64) and 325 nm ($\Delta\varepsilon$ +19.17) and negative 253 nm ($\Delta\varepsilon$ -3.42) and 379 nm ($\Delta\varepsilon$ -9.13) Cotton effects, as reported for cohaerin F [228 nm ($\Delta\varepsilon$ +0.9), 318 nm ($\Delta\varepsilon$ +0.8), 254 nm ($\Delta\varepsilon$ -0.7), 370 nm ($\Delta\varepsilon$ +1.0)] (Quang et al. 2006). The NOESY correlations observed for H-8 to H_3-18 indicated that these protons were located on the same side. Thus, the absolute configuration of C-8 was *R*. The absolute configuration on C-13 of the side chain moiety was then assigned to be *S* by comparison of the ^{1}H and ^{13}C NMR data with those reported for isochomophilone II (**6**) (Omura et al. 1993) and rotiorinol A (Kanokmedhakul et al. 2006). Since the absolute configurations of C-7 and C-8 in isochrophilone II (**6**) have not been previously reported (Omura et al. 1993) we then measured the specific rotation and CD spectrum of our isolated **6**. The results showed that the specific rotation of **1** [-58.61 (*c* 0.14, $CHCl_3$)] was comparable with the isolated **6** [-60.65 (*c* 0.11, $CHCl_3$)], whereas the CD spectrum of **6 [**232 ($\Delta\varepsilon$ +4.62), 255 ($\Delta\varepsilon$ -5.82), 325 ($\Delta\varepsilon$ +18.21), 379 ($\Delta\varepsilon$ -8.61)] is similar to that of **1**. Finally, the absolute configuration of **1** was solved to be 7*R*, 8*R*, and 13*S*. Thus, the structure of **1** was deduced as a new azaphilone and it was named dechloroisochromophilone II.

Compound **2** was obtained as a yellow solid and its molecular ion peak at *m/z* 353.1541 $[M+H]^+$, 355.1530 [M+H+2] in the HRESITOFMS spectrum corresponds to the molecular formula $C_{19}H_{25}ClO_4$. The intensive ratio 3:1 of isotope peaks ([M+H]:[M+H+2]) supports the presence of a chlorine atom in the molecule. The molecular formula revealed seven degrees of unsaturation. The IR spectrum showed absorption bands of hydroxyl (3445 cm^{-1}) and conjugated ketone (1668 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data and 2D NMR techniques (COSY, HSQC, HMBC and NOESY) of **2** indicated a structure similar to isochromophilone III (**7**) (Arai et al., 1995). However, there were some differences in chemical shifts between **2** and **7** at δ_H 4.50, 4.24 and 4.85, 3.82 (for H_2-1); 4.11 and 3.46 (for H-8); 3.05 and 3.12 (for H-8a), respectively. While, the ^{13}C NMR spectra between **2** and **7** signals were slightly different at δ_C 68.2 and 68.7 (for C-1); 77.3 and 75.0 (for C-7); 73.6 and 73.5 (for C-8); and 36.9 and 37.6 (for C-8a), respectively. The NOESY spectrum of **2** showed the correlations between H-8 and H-8a; H_b-1 and H-8a; indicating the stereochemistry of those protons. The absolute configuration of C-7 was assigned to be *R* on the basis of the CD spectrum, which revealed the positive 215 nm ($\Delta\varepsilon$ +3.40), 276 nm ($\Delta\varepsilon$ +2.64)

and 347 nm ($\Delta\varepsilon$ +3.98), and negative 247 nm ($\Delta\varepsilon$ -4.75) Cotton effects, agree well with those of **1** and cohaerin F (Quang et al. 2006). Also the absolute configuration on C-13 of the side chain was assigned to be *S* by comparison of the NMR data with isochomophilone II (**6**) (Omura et al. 1993). From the basis of data above compound **2** was deduced as 7*R*, 8*S*, and 8a*R* which was an epimer of isochromophilone III (7*R*, 8*R*, and 8a*R*). Therefore, **2** was a new azaphilone in terms of stereochemistry which we named *epi*-isochromophilone III.

Compound **3** had the molecular formula, $C_6H_8O_4$ deduced from the HRESITOFMS (*m/z* 145.0464 $[M+H]^+$), indicating three degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3400 cm^{-1}), conjugate lactone (1683 cm^{-1}) and alkene (1620 cm^{-1}) groups. The ^{13}C NMR and HSQC spectra showed six carbon signals attributable to a methoxy group (δ 56.3, 4-O*C*H_3), oxymethylene (δ 63.5, C-6), two methine (δ 91.9, C-3 and 70.3, C-5), and two quaternary (δ 166.2, C-2 and 171.9, C-4) carbons. The ^1H NMR showed resonances for an olefinic proton at δ 5.15 (s, H-3), methine proton at δ 4.26 (dd, *J* = 3.6, 4.0 Hz, H-5), two nonequivalent methylene protons at δ 4.39 (dd, *J* = 3.6, 12.2 Hz, H_b-6), 4.33 (dd, *J* = 4.0, 12.2 Hz, H_a-6) and a methoxy group at δ 3.79 (s, 4-O*C*H_3). The HMBC spectrum showed correlations of H-3 to C-2 and C-4; 4-O*C*H_3 to C-4; H-5 to C-4 and C-6; and H-6 to C-2 and C-4 confirming the location of the methoxy and carbonyl groups. However, the absolute stereochemistry of **3** could not be determined due to lack of the sample. On the basis of the above evidence, **3** was identified as 5-hydroxy-4-methoxy-5,6-dihydro-2*H*-pyran-2-one which has previously been reported as a synthetic compound (CAS Registry Number 1332747-94-0, SciFinder database). This is the first report of **3** isolated as a new natural product.

The isolated compounds were tested for their bioactivities and results are shown in Table 1. Azaphilones **9** and **10** with 1,4-dihydropyridine (N in the ring) showed antimycobacterial activity against *Mycobacterium tuberculosis* with MIC value of 6.2 and 50.0 μg/mL, respectively, while other azaphilones with pyran ring (O in the ring) were inactive. However, both of N and O analogues showed activities against other tests. Compounds **2**, **8**, **10** and **11** exhibited antimalarial activity against *Plasmodium falciparum* with respective IC_{50} values of 7.8, 3.5, 2.1 and 5.9 μg/mL. Compounds **2**, **4**, and **7-11** also showed cytotoxicity against three cancer cell lines (KB, MCF-7 and NCI-H187) with IC_{50} values ranging from 2.2 to 35.2 μg/mL. In addition, compounds **1-12** were also screened for AChE inhibitory activities. Among these, **1** and **7** were the most active compounds with MIC of 10 ng (0.03 nmol), whereas compounds **5**, **6** and **11** were five times and **8** was ten times less active than **1** and **7** (Table 2). This leads us to note that the configuration at C-8 (*R*) might play an important role in AChE inhibition.

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เนื่องจากงานวิจัยนี้เสร็จสมบูรณ์แล้ว และตีพิมพ์เผยแพร่ ชื่อเรื่อง ."Bioactive azaphilones from the fungus *Penicillium multicolor* CM01" ในวารสาร Phytochemistry Letters (IF = 1.575, Q2) จึงนำเสนอแบบครบถ้วนในภาคผนวก

2.2.4.3 รา *Neosartorya spinosa* KKU-1NK1

Introduction

The fungus *Botryotrichum piluliferum* belongs to the family Chaetomiaceae.[1] Colonies grown on potato dextrose agar were white when young and turned to pale brown when mature, at 30 ^{o}C in 7 d with septate mycelia, branches and setae. Conidia were chain-like on hyaline conidiophores, globose, 12.50 – 15.50 µm diameter, with irregularly thick walls 3.0-3.5µm. Teleomorphs were not found in this isolate. It was morphologically identified according to Domsch et al.,[1] and Downing.[2] The fungus *B. piluliferum* has been reported as one of the seed borne fungi of chili pepper.[3] Previous chemical investigation on the genus *Botryotrichum* have been reported to contain asterriquinone CT2 from *Botryotrichum* spp.[4] and botryolides A **-** E,[5] decarestrictine D,[5] and sterigmatocystin[5] from *Botryotrichum* sp. (NRRL38180).[5] However, no reports on the chemical constituents and bioactivity of *B. piluliferum* have been found. Many fungi such as *Aspergillus* species,[6] *Aschersonia coffeae* Henn. BCC 28712,[7] *Penicillium chrysogenum,*[8] including *Botryotrichum* sp. (NRRL 38180)[5] have been reported to produce mycotoxins. Mycotoxins have been reported as mutagenic and having carcinogenic effects in animals and humans.[9,10] Our continuing investigation on bioactive metabolites from fungi isolated from Thai soil, crude *n*-hexane and EtOAc extracts of *B. piluliferum* displayed cytotoxicity against the KB cell line with 85.5% and 59.7% inhibition, respectively, at a concentration of 50 μg/mL. Moreover, the EtOAc extract presented cytotoxicity toward the MCF-7 cell line with 57.5% inhibition. Herein, we report the isolation, structural elucidation, and bioactivities of eleven mycotoxins from *B. piluliferum* were pesented.

Results and discussion

The chromatographic separation of biomass powder of *B. piluliferum* gave two new sterigmatocystin derivatives, **1** and **2,** and nine known compounds, **3**-**11**. Their structures were identified by spectroscopic data and by comparing the data obtained to those of related known compounds published in literature. They were oxisterigmatocystins E and F, **1** and **2**, oxisterigmatocystins G and H, **3** and **4,** sterigmatocystin, **5**,[16] N-0532B, **6**,[17] *O*-methylsterigmatocystin, **7**,[18] N-0532A, **8**,[17] 6-*O*-methylversicolorin A, **9**, 6,8-*O*-dimethylversicolorin A, **10**,[19] and 8-*O*-methylaverufin, **11**.[7] Compounds **3**, **4**, and **9** were isolated from the natural source for the first time.

Compounds **1** and **2** had the molecular formula, $C_{21}H_{17}ClO_{8}$, derived from ^{13}C NMR and HR-ESI-TOFMS, signifying 13 indices of hydrogen deficiency. The IR spectra of compounds **1** and **2** showed absorption bands for ester (1749/1752 cm^{-1}), aromatic ketone (1664/1659 cm^{-1}), and aromatic (1418/1452 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra of these two compounds indicated 21 carbon

signals attributable to three methyl (two methoxy and an acetoxy groups), a methylene, three sp^2 methine, three sp^3 methine and eleven sp^2 quaternary (including two carbonyls) carbons. The ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) were agree with those of isolated N-0532A, **8**, [17] except that the double bond at C-3′ was saturated by a proton at C-3′ [$\delta_{H/C}$ 2.55 (m, H_2-3′)/37.1] and an acetoxy group at C-4′ [$\delta_{H/C}$ 2.11(s)/21.2, δ_C 169.8]. The three resonances of aromatic protons appeared at δ 6.38 (s, H-2), 7.08 (d, *J* = 9.0 Hz, H-5) and 7.58 (d, *J* = 9.0 Hz, H-6). Correlations of H-1′/H-2′/H_2-3′/H-4′ in the COSY spectrum confirmed the lack of a double bond at C-3′ of the bishydrofuran unit. The HMBC also indicated the connectivity of an acetoxy group through C-4′ by showing correlations of H_3-6′ to C-5′ and C-4′, and H-4′ to C-5′.

The ^1H and ^{13}C NMR spectroscopic data of **2** (Table 1) were similar to those of **1**, except that the resonance of methyl protons of the acetoxy group at C-4′ of **2** (δ 1.69) appeared at a higher field than that of **1** (δ 2.11). The coupling constants between H-1′ and H-2′ (*J* = 6.0 Hz) and the NOESY correlations of the two protons of **1** and **2** revealed a *cis* ring fusion, the same as in the sterigmatocystin, **5**,[18,20,21] which allowed assignment of the absolute configurations at both C-1′ and C-2′ as *S*. The assignment of configurations at C-4′ of **1** and **2** as *R* and *S* were determined by comparing the ^1H NMR resonances of an acetoxy group to those reported for related analogues, dothistromin pentaacetate[22] and oxisterigmatocystin D.[23] The methyl protons of the 4′-acetoxy group of **2** appeared at a higher field (δ_H 1.69) than in **1** (δ_H 2.11) agreeing with that reported for *endo* dothistromin pentaacetate (δ_H 1.67)[22] which was due to the strong shielding effect of xanthone. On the other hand, the 4′-acetoxy group of **1** showed a resonance at δ_H 2.11, suggesting the *exo* arrangement (Figure 3), which correspond to that of oxisterigmatocystin D (δ_H 2.07).[23] Furthermore, the optical rotation values of **1** was different from **2** suggesting the difference configurations at C-4′ of the two compounds. Based on the above evidence, the structure of **1**, oxisterigmatocystin F was determined as a new sterigmatocystin derivative. Compound **2**, oxisterigmatocystin F was identified as the C-4′ epimer of **1**.

Compounds **3** and **4** processed a molecular formula, $C_{21}H_{18}O_8$, from ^{13}C NMR and HR-ESI-TOFMS, indicating 13 indices of hydrogen deficiency. IR spectra of both, **3** and **4** showed bands for ester (1752/1752 cm^{-1}), aromatic ketone (1659/1659 cm^{-1}), and aromatic (1462/1471 cm^{-1}) groups. Their ^{13}C NMR and DEPT spectra displayed 21 carbon signals attributable to three methyls (two methoxy groups and an acetoxy group), a methylene, four sp^2 methine, three sp^3 methine and ten sp^2 quaternary (including two carbonyl) carbons. The ^1H NMR, ^{13}C NMR and DEPT spectroscopic data of **3** (Table 1) were similar to those of **1**, except for the presence of an additional sp^2 methine proton at C-7 [δ_H 6.75 (d, *J* = 8.4 Hz, H-7)]. This information, together with absence of Cl isotope in MS data established that the chlorine atom was displaced by the sp^2 methine proton. The COSY spectrum showed correlations of H-5/H-6/H-7, indicating trisubstitution of the aromatic ring. The HMBC spectrum of **3** clearly demonstrated correlations of H-7 to C-8a and C-5, H-6 to C-8 and C-10a, and H-5 to C-7, C-8a and C-

10a, confirming the structure of **3**. Since the resonances of acetoxyl groups of **3** (δ_H 2.10) and **4** (δ_H 1.67) appeared at low and high fields in the same manner of those of **1** and **2**, the configurations at C-4′ of **3** and **4** were assigned as *R* and *S*, respectively. Moreover, the optical rotation values of **3** and **4** were comparable to those of **1** and **2**, respectively. However, compounds **3** and **4** have been previously reported as synthetic mixture products during the preparation of *O*-methylsterigmatocystin (**7**).[24] This is the first isolation of compounds **3** and **4** from a natural source and they have been named oxisterigmatocystin G, **3**, and oxisterigmatocystin H, **4**. Their spectroscopic data was also reported.

Compound **9** had the molecular formula $C_{19}H_{12}O_7$, deduced from ^{13}C NMR and HR-ESI-TOFMS, requiring 14 of hydrogen deficiency. The IR spectrum displayed absorption bands for aromatic ketones (1629 and 1613 cm^{-1}) and aromatic (1579 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra displayed 19 carbon signals for a methoxy, five sp^2 methine, two sp^3 methine and eleven sp^2 quaternary (including two carbonyl) carbons. Careful examination of 1D and 2D NMR data indicated that the structure of **9** was similar to that of 6,8-*O*-dimethylversicolorin A, which has been previously reported as a methylation product of versicolorin A.[25] The HMBC spectrum revealed correlations of hydroxyl proton at C-8 to C-7, C-8 and C-8a confirming the position of the hydroxyl group at C-8. Besides, the correlations of H-4 to C-2, C-3, C-10, and C-9a, H-5 to C-7, C-8a, and C-10, H-7 to C-5, C-6, C-8, and C-8a, H-1′ to C-3, C-2′, C-3′, and C-4′, H-3′ to C-1′ and C-2′, H-4′ to C-2′, methoxyl protons at C-6 to C-6, and hydroxyl proton (OH-1) to C-1, C-2 and C-9a indicated the structure of **9**. The absolute configuration of **9** was assigned to be the same as that of 6,8-*O*-dimethylversicolorin A, **10,** by comparing their optical rotations, -320 (*c* 0.12, dioxane) for **10**[19] and -312.8 (*c* 0.1, $CHCl_3$) for **9**. Thus, this is the first report of 6-*O*-methylversicolorin A, **9**, isolated from a natural source.

Compounds **1**, **3** and **4** showed antimalarial activity against *P. falciparum* with IC_{50} values of 7.9, 14.7 and 23.9 μM, respectively. Compounds **1**-**4** exhibited cytotoxicity against three cancer cell lines (KB, MCF-7 and NCI-H187) with IC_{50} values ranging from 3.5 to 78.6 μM. Compound **3** showed significant cytotoxicity against KB cells with IC_{50} value of 3.5 μM, which is lower than the control drug, ellipticine. Furthermore, **3** was cytotoxic against the MCF-7 cell line with IC_{50} value of 6.9 μM, which is lower than the control drug, doxorubicin. However, **3** was highly cytotoxic toward the normal cell line (Vero cell) with IC_{50} value of 1.6 μM, which is lower than the control drug, ellipticine 2.5 μM. Among compounds **1**-**4**, the influence of an *exo*- versus an *endo*- arrangement was noticed. The *exo*-arrangements in **1** and **3** showed both higher antimalarial activity and cytotoxicity against cancer cells than their *endo*- analogues, **2** and **4**. It should be noted that the missing double bond at C-3′ and the presence of an acetoxy group at C-4′ of sterigmatocystin derivatives **1-4** would play an important role for cytotoxicity enhancement comparing to derivatives **5-8**. Moreover, the double bond at C-3′ and C-4′ of sterigmatocystin derivatives **5-8** exhibited no cytotoxicity (>100 μM) toward KB and MCF-7, which corresponds to the results for related structures in a previous report.[7] In addition, compounds **9** and **10**

showed strong cytotoxicity against NCI-H187 cell line with IC_{50} values of 2.1 and 0.38 μM, respectively, which were lower than the control drug, ellipticine. By comparison between compounds **9** and **10**, the presence of a methoxy group at C-8 led to a decrease in cytotoxicity toward normal cells. Interestingly, compound **10** was not cytotoxic against Vero cells and should be further studied in detail. However, most of the isolated compounds showed cytotoxicity against *Vero* cells with IC_{50} values in the range from 0.65 to 12.3 μM. Sterigmatocystin, **5**, has been reported to contaminate food stuffs such as wheat, rice, coffee beans, corn and red pepper. Furthermore, it has been discovered in cheese contaminated with *Aspergillus versicolor*.[9] Based on our results, most isolated compounds from *B. piluliferum* are mycotoxins with structures metabolically related to the aflatoxin carcinogen. These mycotoxins could be responsible for the toxicity of the fungus *B. piluliferum*. As mentioned above, the fungus *B. piluliferum* was found in the seeds of chili pepper,[3] and so the contamination of *B. piluliferum* in the food chain and agricultural soil should be monitored.

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เนื่องจากงานวิจัยนี้เสร็จสมบูรณ์แล้ว และตีพิมพ์เผยแพร่ ในชื่อเรื่อง “Chevalone C analogues and globoscinic acid derivatives from the fungus Neosartorya spinosa KKU-1NK1” ในวารสาร Phytochemistry (IF = 3.186, Q1) จึงนำเสนอแบบครบถ้วนในภาคผนวก

3. สรุปผลการดำเนินงานของโครงการ

โครงการนี้ได้ดำเนินไปเป็นระยะเวลา 1 ปี โดยยุติโครงการในปี พ.ศ. 2529 เนื่องจากหัวหน้าผู้วิจัย (ศาสตราจารย์สมเดช กนกเมธากุล) ได้รับทุนส่งเสริมการวิจัย เมธีวิจัยอาวุโสในช่วงปี พ.ศ. 2529-2562 ผลงานวิจัยอยู่ในเกณฑ์ที่ดี สามารถตีพิมพ์ผลงานในวานสารนานาชาติ 3 เรื่องคือ

3.1 Chaiyosang, B.; Kanokmedhakul, K.; Boonmak, J.; Youngme, S.; Kukongviriya.pan, V.; Soytong, K.; Kanokmedhakul, S. “A new lumazine peptide penilumamide E from the fungus *Aspergillus terreus*” *Nat. Prod. Res*. **2016**, *30*, 1017-2016. (IF = 1.828, Q2)

3.2 Hemtasin, C.; Kanokmedhakul, S.;* Moosophon, P.; Soytong, K.; Kanokmedhakul, K.; “Bioactive azaphilones from the fungus Penicillium multicolor CM01” *Phytochemistry Letters* **2016**, *16*, 56-60. (IF = 1.575, Q2)

3.3 Rajachan, O.A.; Kanokmedhakul, K.; Sanmanoch W.; Boonlue, S.; Hannongbua, S.; Saparpakorn, P.; Kanokmedhakul, S. “Chevalone C analogues and globoscinic acid derivatives from the fungus Neosartorya spinosa KKU-1NK1” *Phytochemistry* **2016**, *132* , 68-75. (IF = 3.186, Q1)

4. ความเห็นและข้อเสนอแนะ ไม่มี

5. งานวิจัยที่ต้องดำเนินต่อไป ไม่มี

ลงนาม

(หัวหน้าโครงการวิจัยผู้รับทุน)

วันที่ 31 กรกฎาคม 2561

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A new lumazine peptide penilumamide E from the fungus *Aspergillus terreus*

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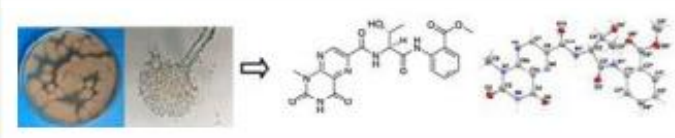
ABSTRACT

A new rare lumazine peptide, penilumamide E (**1**), together with 13 known compounds (**2–14**) were isolated from the fungus *Aspergillus terreus*. Their structures were identified by spectroscopic techniques. The relative configuration of **1** was confirmed by single-crystal X-ray diffraction analysis. Compound **10** exhibited antimalarial activity against *Plasmodium falciparum* with IC_{50} values of 2.83 µg/mL. Compounds **4** and **6** showed weak cytotoxicity against cholangiocarcinoma (CCA) cell lines. In addition, **4** and **11** exhibited weak cytotoxicity against human hepatoma cell line.



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Aspergillus terreus; lumazine peptide; penilumamide E; *epi*-aszonalenin A; butyrolactone; antimalarial

1. Introduction

Aspergillus terreus Thom belongs to the family Trichocomaceae. Several of its varieties have been reported as being sources of various bioactive secondary metabolites, such as 4*R*-(hydroxymethyl)-4*R*-demethylterritrem B (Peng 1997) and terreulactone A (Kim et al. 2002) which inhibit acetylcholinesterase (AChE), butyrolactone V has moderate antimalarial activity against *Plasmodium falciparum* K1 (Haritakun et al. 2010), terremides A and B have antibacterial activity against *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (Wang et al. 2011), butyrolactone I has anti-H1N1 activity (Wang et al. 2011) and asperterrestide A inhibits the influenza virus strains A/WSN/33 (H1N1) and A/Hong Kong/8/68 (H3N2), while

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also being cytotoxic toward human carcinoma U937 and MOLT4 cell lines (He et al. 2013). Recently, several alkaloids and polyketide derivatives isolated from the mycelial extract of a mixed culture of two marine alga-derived fungal strains of the genus *Aspergillus* exhibited selective antiproliferative activities in four different human cancer cell lines tested (Ebada et al. 2014). More recently, a new cyclicpeptide, 14-hydroxy-cyclopeptine isolated from *Aspergillus* sp. SCSIOW2 has been reported to inhibit nitric oxide production (Zhou et al. 2015). Therefore, the search for bioactive compounds from the fungus *A. terreus* is currently of interest. As part of our search for bioactive compounds from fungi, we have found that the crude EtOAc extract of the fungal biomass of *A. terreus*, isolated from the garbage component in organic fertilizer, has shown cytotoxicity against NCI-H187 with 67.7% inhibition at 50 µg/mL. We report herein, the isolation, structural elucidation and bioactivity of a new lumazine peptide, penilumamide E (**1**), together with 13 known compounds from the fungus *A. terreus* (Figure 1).

2. Results and discussion

Compound **1** was obtained as colourless needles and its molecular formula was deduced as $C_{20}H_{20}N_6O_7$ by HR-ESI-TOF-MS *m/z* 479.1296 $[M + Na]^+$ (calcd. *m/z* 479.1291), implying fourteen degrees of unsaturation. The UV spectrum showed maxima absorption at 223, 251 and 318 nm of an aromatic ring. The IR absorptions indicated the presence of secondary amide (3388 cm^{-1}), hydroxyl (3260 cm^{-1}), ester and amide (1702 cm^{-1} overlapping) and aromatic (1582 and 1491 cm^{-1}) groups. The ^{13}C NMR spectrum of **1** (Table S1) was assigned by the DEPT and HMQC techniques as having 20 carbon signals attributable to five carbonyl (149.8, 160.4, 163.2, 168.2, and 169.0), five sp^2 quaternary (125.7, 115.7, 139.7, 140.1 and 150.7), five sp^2 methine (120.4, 123.1, 130.7, 134.4 and 148.1), two sp^3 methine (60.8 and 67.2) and three methyl (19.6, 28.9 and 52.0) carbons. The ^1H NMR spectrum (Table S1) showed signals for five aromatic protons at δ 9.37 (s, H-7), 7.91 (d, J = 7.2 Hz, H-4″), 7.05 (t, J = 7.2 Hz, H-5″), 7.47 (t, J = 7.2 Hz, H-6″) and 8.59 (d, J = 7.2 Hz, H-7″), two methine groups at δ 4.63 (d, J = 3.6 Hz, H-2′) and 4.54 (m, H-4′), a methyl ester at δ 3.66 (s, H-9″), a methyl amine at δ 3.65 (s, H-9) and a methyl group at δ 1.31 (d, J = 6.0 Hz, H-5′). Information from the COSY and HMBC spectra indicated that the structure of **1** contained three main units of lumazine, threonine and anthranilic acid. The COSY spectrum (Figure S9) showed correlations between NH-1′↔H-2′↔H-4′↔H-5′ and H-4″↔H-5″↔H-6″↔H-7″. The HMBC spectrum (Figure S8) clearly demonstrated the correlations of H-7 to C-4a (δ 125.7), C-6 (δ 139.6) and C-8a (δ 150.7); methoxy protons (H-9) to C-2 (δ 149.8) and C-8a indicated the lumazine unit. The correlations of H-2′ to C-10 (δ 163.2), C-3′ (δ 169.0), C-4′ (δ 67.2) and C-5′ (δ 19.6), H-4′ to C-3′ and C-5′, H-5′ to C-4′ and C-2′ (δ 60.8) indicated the presence of a threonine unit. Furthermore, the correlations of H-4″ to C-2″ (δ 140.1), C-6″ (δ 134.4) and C-8″ (δ 168.2), H-5″ to C-3″ (δ 115.7) and C-7″ (δ 120.2), H-6″ to C-2″ and C-4″ (δ 130.7), H-7″ to C-3″ (δ 115.7), and C-5″ (δ 123.1), H-9″ to C-8″ confirmed the anthranilic acid methyl ester unit. The connections between a lumazine unit and a threonine unit were indicated by the correlations of H-2′ to C-10 and NH-1′ to C-10, while the connection between a threonine unit and an anthranilic acid methyl ester unit were confirmed from the correlations of NH-1″ to C-3′ and C-7″. From analysis of ^1H and ^{13}C NMR spectral data, compound **1** has a core structure the same as the known compound, penilumamide (Meyer et al. 2010), except for the methionine

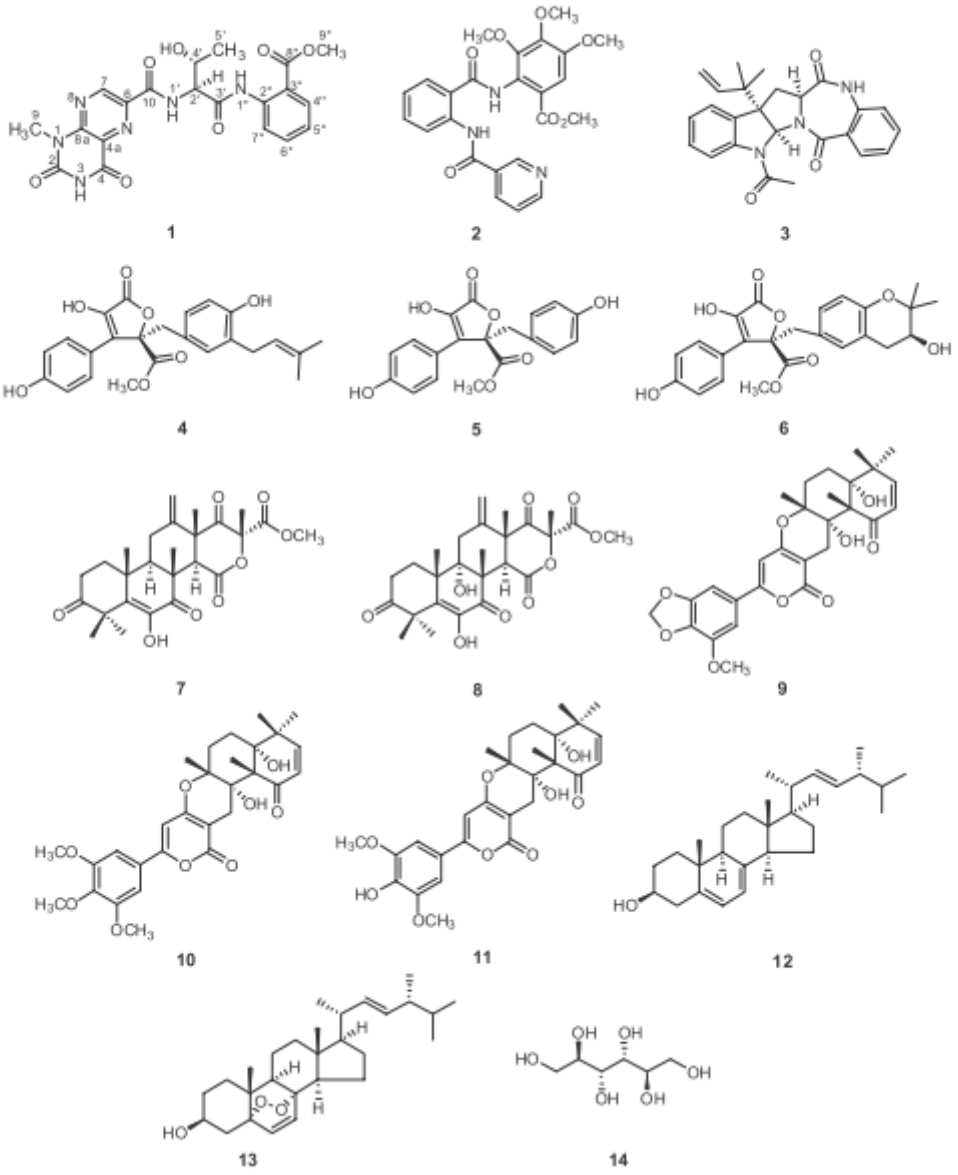

Figure 1. Structures of compounds **1**–**14**.

sulfoxide unit at C-2′ which is replaced by a threonine unit. In addition, the N-methyl group at N-3 of the lumazine unit in penilumamide was replaced by a hydrogen atom. Moreover, the structure of **1** was also compared to the recent report for penilumamides B-D isolated from *Aspergillus* sp. XS-20090B15 (Chen et al. 2014). The NOESY spectrum (Figure S10) displayed correlations between H-4′and H-5′, H-4″ and H-5″, H-5″ and H-6″, and H-6″ and H-7″ indicating the relative configurations at C-2′ and C-4′. Finally, the X-ray crystallographic technique was carried out to confirm the structure of compound **1** (Figure 2 and Tables S2 and S3). On the basis of the above data, **1** was defined as a new lumazine peptide which has been named penilumamide E. The lumazine peptide

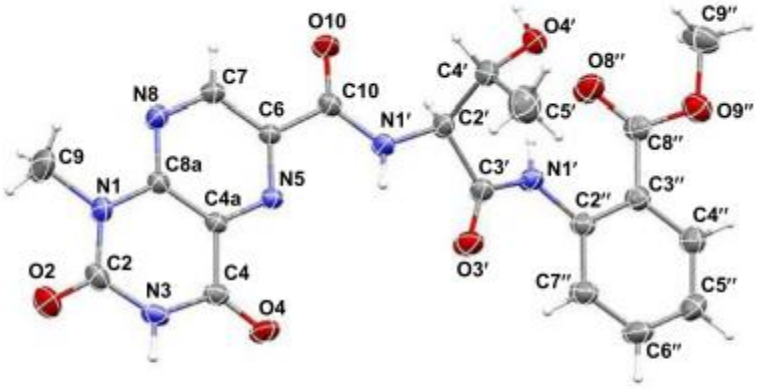

Figure 2. X-ray crystallographic structure of penilumamide E (**1**).

Table 1. Effects of compounds on KKU-100 and HepG2 cells.

Compound	KKU-100[a]	HepG2[b]
	IC_{50} (μg/mL)	IC_{50} (μg/mL)
1	Non-toxic	Non-toxic
2	507 ± 517	640 ± 710
4	149 ± 82	93 ± 67
6	345 ± 188	Proliferative[c]
7	Proliferative[c]	Proliferative[c]
8	566 ± 356	Proliferative[c]
9	Non-toxic	Proliferative[c]
11	Non-toxic	164 ± 141
Cisplatinum[d]	8.4 ± 2.2	4.1 ± 2.1

[a]Human cholangiocarcinoma.
[b]Human hepatoma.
[c]Increase in cell number.
[d]Positive control substance.

group is a rare natural product which has been isolated from the marine-derived fungus, *Penicillium* sp. CNL-338 (Meyer et al. 2010) and *Aspergillus* sp. XS-20090B15 (Chen et al. 2014). This is the first report of this type of compound from *A. terreus* isolated from soil.

The known compounds were identified on the basis of spectroscopic data and by comparing to data reported in literature as 3,4,5-trimethoxy-2-(2-(nicotinamido) benzamido) benzoate (**2**) (Zhou et al. 2011), *epi*-aszonalenin A (**3**) (Rank et al. 2006), butyrolactone I (**4**), butyrolactone II (**5**) (Rao et al. 2000), butyrolactone V (**6**) (Haritakun et al. 2010), terretonin (**7**) (Springer et al. 1979), terretonin A (**8**) (Li et al. 2005), territrem A (**9**) (Lee et al. 1992), territrem B (**10**) (Lee et al. 1992), territrem C (**11**) (Lee et al. 1992), ergosterol (**12**) (Kwon et al. 2002), 24(*R*)-5α, 8α-epidioxyergosta-6-22-diene-3β-ol (**13**) (Jinming et al. 2001) and D-mannitol (**14**) (Pouchert & Behnke 1993).

Among the isolated compounds, only **10** exhibited antimalarial activity against *P. falciparum* with IC_{50} values of 2.83 μg/mL. Compounds **4** and **6** showed weak cytotoxicity against the cholangiocarcinoma (CCA) cell line, KKU-100, while **4** and **11** exhibited weak cytotoxicity against HepG2 cells. Compound **1** proved to be non-toxic to KKU-100 and HepG2 cell lines, while **9** and **11** proved to be non-toxic to KKU-100 cell line at 500 μg/mL. However,

compounds **6**, **7, 8** and **9** significantly induced an increase in cell number (proliferation) of HepG2 cells at certain concentrations (0.2–100 µg/mL) (Table 1). In addition, these isolated compounds showed non-cytotoxicity (<50 µg/mL) against the three cancer cell lines tested (KB, NCI-H187 and MCF7).

3. Experimental

3.1. General experimental procedures

Melting points were determined using Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). Optical rotations were determined on a JASCO DIP-1000 digital polarimeter (JASCO, Inc., Maryland, USA). UV spectra were recorded using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Agilent Technologies, USA). NMR spectra were recorded in $CDCl_3$ and CD_3OD as solvents on a Varian Mercury Plus 400 spectrometer (Varian, Inc., USA); the internal standards were referenced from the residue of those solvents. HR-ESI-TOF-MS spectra were acquired using a Micromass Q-TOF-2 spectrometer (Bruker, Germany). Column chromatography was carried out on Merck silica gel 60 (230–400 mesh) (Merck, Darmstadt, Germany). TLC was performed with precoated Merck silica gel 60 PF254 (Merck, Darmstadt, Germany); the spots were visualised under UV light (254 and 366 nm) and further by spraying with anisaldehyde and then heating until charred.

3.2. Fungal material

3.2.1. Collection, isolation and taxonomy

The fungus *A. terreus* was collected from the garbage component at an organic fertilizer factory, Bangkok, Thailand in 2012. The fungus was isolated and identified by K. Soytong. A voucher specimen (AsT01-KMIL) was deposited at the Department of Plant Production Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The fungus was cultured in conical flasks (500 mL, 75 flasks) with potato dextrose broth (PDB) (200 mL/flask) and incubated in a standing condition at 25–28 °C for 30 days. The culture broth was filtered to give a wet fungal biomass, which was then air-dried at room temperature.

3.3. Extraction and isolation

Air dried biomass *A. terreus* (160 g) was extracted exhaustively three times by hexane, EtOAc and MeOH to obtain 12.3 g (7.7%), 9.6 g (6.0%) and 15.7 g (9.8%) of crude extracts, respectively. The hexane extract was filtered out to afford colourless needles of **12** (222.8 mg). The filtrate was separated on silica gel flash column chromatography (FCC), eluted with a gradient system of hexane:EtOAc and EtOAc:MeOH to obtain eight subfractions, H_1–H_8. Fraction H_5 was further purified by preparative TLC using hexane:EtOAc (40:60) as an eluent to afford a white solid of **13** (8.0 mg, $R_f = 0.33$). Fraction H_6 was purified by preparative TLC using CH_2Cl_2:MeOH (1:99) as an eluent to yield colourless needles of **10** (3.9 mg, $R_f = 0.45$). Fraction H_7 was purified by preparative TLC using CH_2Cl_2:MeOH (1:99) as an eluent to yield colourless needles of **2** (5.2 mg, $R_f = 0.48$). The EtOAc extract was subjected to silica gel FCC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH to obtain seven

fractions E_1–E_7. Fraction E_4 was purified by FCC, eluted with an isocratic system of CH_2Cl_2 (100) to give five subfractions, $E_{4.1}$–$E_{4.5}$. Subfraction $E_{4.2}$ was purified by preparative TLC using EtOAc:hexane (40:60) as an eluent to give **8** as colorless crystals (11.6 mg, R_f = 0.68). Subfraction $E_{4.5}$ was purified by preparative TLC using MeOH:CH_2Cl_2 (5:95) as an eluent to give **4** as colourless crystals (10.1 mg, R_f = 0.44). The solid in E_5 was filtered out to yield a white solid of **7** (55.7 mg), and the filtrate was subjected to FCC, eluted with an isocratic system of MeOH:CH_2Cl_2(1:99) to give seven subfractions, $E_{5.1}$–$E_{5.7}$. Subfraction $E_{5.1}$ was subjected to FCC, eluted with an isocratic system of EtOAc:hexane (15:85) to give colourless needles of **9** (14.8 mg, R_f = 0.44). Subfraction $E_{5.3}$ was purified by preparative TLC using EtOAc:hexane (40:60) as an eluent to give colourless crystals of **3** (6.7 mg, R_f = 0.45), an additional amount of **10** (3.0 mg) and **2** (6.7 mg). The solid in subfraction $E_{5.5}$ was filtered out to give an additional amount of **4** (76.5 mg). Subfraction $E_{5.7}$ was purified by FCC, eluted with an isocratic system of MeOH:CH_2Cl_2 (1:99) to give four subfractions, $E_{5.7.1}$–$E_{5.7.4}$. Subfraction $E_{5.7.2}$ was purified by preparative TLC using MeOH:CH_2Cl_2 (10:90) as an eluent to yield colourless crystals of **6** (32.6 mg, R_f = 0.37). Subfraction $E_{5.7.4}$ was purified by preparative TLC using MeOH:CH_2Cl_2 (10:90) as eluent to give colourless crystals of **5** (2.0 mg, R_f = 0.32) and an additional amount of **6** (20.2 mg). Fraction E_6 was purified by FCC, eluted with an isocratic system of MeOH:CH_2Cl_2 (1:99) to give six subfractions, $E_{6.1}$–$E_{6.5}$. Subfraction $E_{6.2}$ was purified by preparative TLC using EtOAc:hexane (80:20) as an eluent to yield colourless needles of **11** (9.0 mg, R_f = 0.34). Subfraction $E_{6.4}$ was purified by preparative TLC using MeOH:CH_2Cl_2 (10:90) as an eluent to yield colourless crystals of **1** (6.9 mg, R_f = 0.61). The solid in the MeOH extract was filtered out to give a white solid of **14** (767.5 mg). The filtrate was separated on FCC, eluted with a gradient system of hexane–EtOAc and EtOAc -MeOH to give five fractions, M_1–M_5. Fraction M_2 was purified by preparative TLC using MeOH:CH_2Cl_2 (3:97) as eluent to yield an additional amount of **8** (7.3 mg). Fraction M_3 was purified by preparative TLC using EtOAc:hexane (40:60) as an eluent to yield an additional amount of **10** (13.0 mg).

Penilumamide E (**1**): colourless needles ($CHCl_3$:MeOH, 8:2); mp 246–247 °C; R_f = 0.61 (5% MeOH:CH_2Cl_2); $[\alpha]_D^{23}$ + 46 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.53), 251 (4.39), 318 (3.99) nm; IR (thin film) ν_{max}cm^{-1}: 3388, 3260, 2955, 2957, 2849, 1702, 1582, 1491, 1444, 1265; ^{1}H and ^{13}C NMR (see Table S1); HR-ESI-TOF-MS *m/z* 479.1296 $[M + Na]^+$ (calcd. *m/z* 479.1291).

4. Conclusions

Investigation of the fungus *A. terreus* Thom gave a new rare lumazine peptide, penilumamide E (**1**), and 13 known compounds, methyl 3,4,5-trimethoxy-2-(2-(nicotinamido)benzamido) benzoate (**2**), *epi*-aszonalenin A (**3**), butyrolactone I (**4**), butyrolactone II (**5**), butyrolactone V (**6**), terretonin (**7**), terretonin A (**8**), territrems A-C (**9–11**), ergosterol (**12**), 24(*R*)-5α, 8α-epidioxyergosta-6-22-diene-3β-ol (**13**) and D-mannitol (**14**). It was found that compound **10** exhibited antimalarial activity against *P. falciparum* with IC_{50} values of 2.83 µg/mL. Compounds **4** and **6** showed weak cytotoxicity against cholangiocarcinoma (CCA) cell lines. In addition, **4** and **11** exhibited weak cytotoxicity against human hepatoma cell line.

Supplementary material

Supplementary material relating to this article is available online, alongside Tables S1–S3 and Figures S1–S10.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Bioactive azaphilones from the fungus *Penicillium multicolor CM01*

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ABSTRACT

Two new azaphilones, dechloroisochromophilone II (**1**) and *epi*-isochromophilone III (**2**), a new natural product, 5-hydroxy-4-methoxy-5,6-dihydro-2*H*-pyran-2-one (**3**), together with eleven known compounds, **4**–**14** were isolated from the fungus, *Penicillium multicolor* CM01. Their structures were elucidated by spectroscopic methods. Compounds **2**,**8**, **10** and **11** exhibited antimalarial activity against *Plasmodium falciparum* (IC_{50} 2.1–7.8 μg/mL), while compounds **9** and **10** showed antimycobacterial activity against *Mycobacterium tuberculosis* (MIC 6.2 and 50.0 μg/mL, respectively). Compounds **2**, **4**, and **7**–**11** showed cytotoxicity against three cancer cell lines, KB, MCF-7 and NCI-H187 (IC_{50} 2.2–35.2 μg/mL). In addition, compounds **1**, **5**–**8** and **11** showed a minimum inhibition requirement to acetylcholinesterase (AChE) assay in the range of 0.03–0.25 nM.

1. Introduction

The members of the genus *Penicillium* members are known for their ability to produce bioactive secondary metabolites, for example, spiroditerpenoids (Li et al., 2009) alkaloids (Dilip de Silva et al., 2009; Du et al., 2009), spiroazaphilones (Ariza et al., 2001) and polyphenols (Lu et al., 2010). One of the specific metabolites repeatedly isolated from this genus is azaphilone, with various substitution groups on the main skeleton. Various strains of *P. multicolor* have been reported to produce azaphilone metabolites such as sclerotiorin (Birkinshaw, 1952), isochromophilone I–VI (Matsuzaki et al., 1995; Arai et al., 1995) and 8-*O*-methylsclerotiorinamine (Nam et al., 2000). Previous reports have shown that azaphilones exhibited broad activities in many biological tests, such as cholesteryl ester transfer protein (Tomoda et al., 1999), endothelin receptor binding, (Pairet et al., 1995) and the inhibition of gp120-CD4 binding (Matsuzaki et al., 1998). In our search for bioactive compounds from fungi isolated from Thai soil, an EtOAc extract of *Phallus multicolor* CM01 showed cytotoxicity against the KB cell line with an IC_{50} value of 10.4 μg/mL. We report herein the isolation, characterization and bioactivity of two new compounds (**1** and **2**), a new natural product (**3**) and eleven known compounds (**4**–**14**) from *P. multicolor* CM01 (Fig. 1).

2. Results and discussion

Chromatographic separation of the EtOAc extract of *P. multicolor* CM01 gave fourteen compounds, **1**–**14** (Fig. 1). Their structures were determined by spectroscopic data (IR, UV, ^{1}H and ^{13}C NMR, 2D-NMR, and MS). The known compounds were identified by physical properties and spectroscopic data measurements as well as by comparing the data obtained with published values, as sclerotiorin (**4**) (Curtin and Reilly, 1940), ochrephilone (**5**) (Seto and Tanabe, 1974), isochromophilone II (**6**) (Omura et al., 1993), isochromophilone III (**7**), isochromophilone IV (**8**), isochromophilone VI (**9**) (Arai et al., 1995), sclerotioramine (**10**) (Wang et al., 2010), isochromophilone VIII (**11**) (Yang et al., 1996), (*S*)-6-((1*S*,2*S*)-1,2-dihydroxypentyl)-4-methoxy-5,6-dihydro-2*H*-pyran-2-one (**12**) (Eade et al., 1957), ergosterol (**13**) (Bok et al., 1999) and ergosterol peroxide (**14**) (Rösecke and König, 2000). Although compound **3** has previously been synthesized, this is the first isolation from a natural source. Compound **1** had the molecular formula $C_{22}H_{28}O_4$ deduced from HRESITOFMS (*m*/*z* 357.2074 $[M+H]^+$), indicating nine degrees of unsaturation. The UV spectrum exhibited an absorption maximum at 251 nm. The IR spectrum showed characteristics of hydroxyl (3400 cm^{-1}), ketone (1711 cm^{-1}) and alkene (1607 cm^{-1}) groups. The ^{13}C NMR, HSQC

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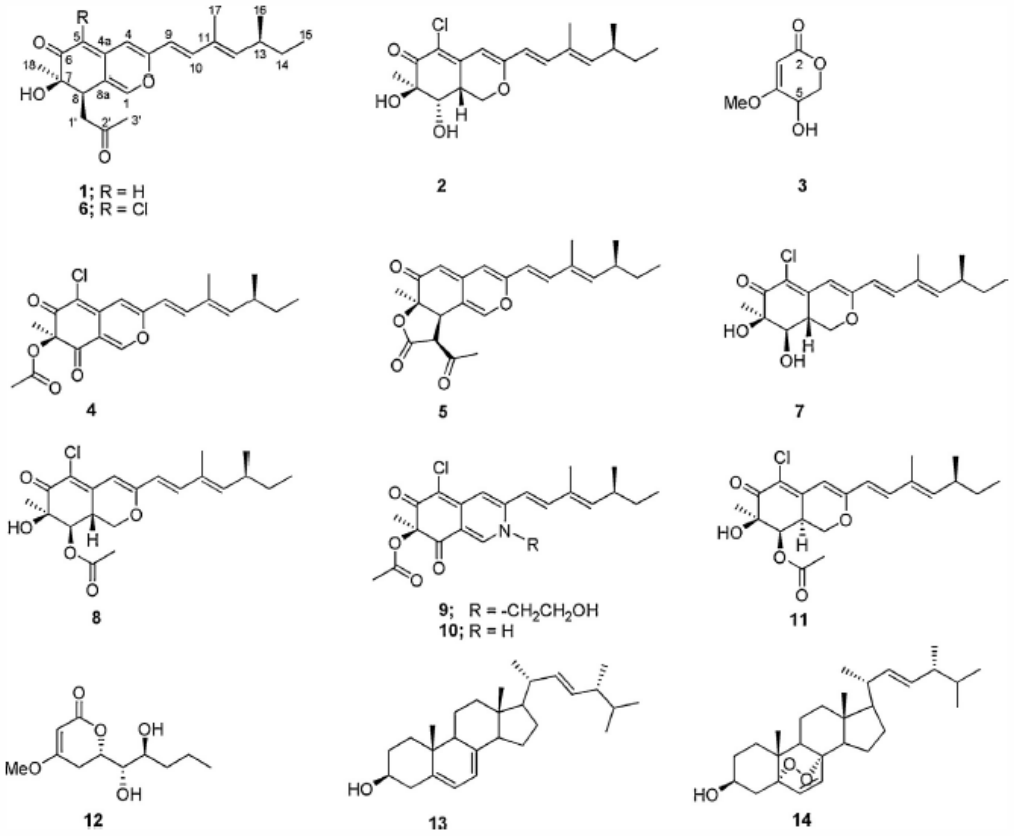

Fig. 1. Structures of compounds **1–14**.

and DEPT spectra revealed the presence of six sp^2 quaternary (including two carbonyl groups), six sp^2 methine, one sp^3 quaternary, two methine, two methylene, and five methyl carbons. The ^{1}H NMR spectrum of **1** showed three olefinic proton signals at δ 7.37 (s, H-1), 6.04 (s, H-4), and 5.36 (s, H-5), two methyl signals at δ 1.26 (s, H-18) and 2.04 (s, H-3′), two nonequivalent methylene proton signals at δ 3.04 (dd, J = 3.2, 18.4 Hz, H_b-1′), and 2.34 (dd, J = 7.6, 18.4 Hz, H_a-1′), and a methine proton signal at δ 3.42 (dd, J = 3.2, 7.6 Hz, H-8). These data, combined with the conjugated carbonyl at δ_C 198.1 (C-6) and ketone carbonyl at δ_C 207.3 (C-2′), suggested the presence of an azaphilone skeleton and a 2-oxypropyl moiety for **1**, analogous to isochromophilone II (**6**) (Quang et al., 2006). The distinct difference in the NMR spectra between **1** and **6** was that the chlorine atom at C-5 in **6** was replaced by an olefinic proton (H-5) in **1**. The HMBC correlations (Fig. 2) of H-4 to C-4a, C-5, C-8a; and H-5 to C-4, C-4a, C-7, and C-8a indicated that H-4 and H-5 were in close proximity. The 3,5-dimethyl-1,3-heptadienyl unit was established by COSY correlations of H-9/H-10, H-17/H-12/H-13/H-16 and H-13/H-14/H-15 (Fig. 2). The HMBC spectrum exhibited correlations of H-9 and H-10 to C-3 and H-4 to C-9, confirming the connection of this unit at C-3. The structure of **1** was completely characterized by 2D NMR. The absolute configuration of C-7 was concluded to be *R* on the basis of the CD spectrum, which revealed the positive 234 nm (Δe + 1.64) and 325 nm ($\Delta\varepsilon$ + 19.17) and negative 253 nm ($\Delta\varepsilon$ − 3.42) and 379 nm ($\Delta\varepsilon$ − 9.13) Cotton effects, as reported for cohaerin F [228 nm ($\Delta\varepsilon$ + 0.9), 318 nm ($\Delta\varepsilon$ + 0.8), 254 nm ($\Delta\varepsilon$ − 0.7), 370 nm ($\Delta\varepsilon$ + 1.0)] (Quang et al., 2006). The NOESY correlations observed for H-8 to H_3-18 indicated that these protons were located on the same side. Thus, the absolute configuration of C-8 was *R*. The absolute configuration on C-13 of the side chain moiety was then assigned to be *S* by comparison of the ^{1}H and ^{13}C NMR data with those reported for isochomophilone II (**6**) (Omura et al., 1993) and rotiorinol A (Kanokmedhakul et al., 2006). Since the absolute configurations of C-7 and C-8 in isochrophilone II (**6**) have not been previously reported (Omura et al., 1993) we then measured the specific rotation and CD spectrum of our isolated **6**. The results showed that the specific rotation of **1** [−58.61 (*c* 0.14, $CHCl_3$)] was comparable with the isolated **6** [−60.65 (*c* 0.11, $CHCl_3$)], whereas the CD spectrum of **6** [232 ($\Delta\varepsilon$ + 4.62), 255 ($\Delta\varepsilon$ − 5.82), 325 ($\Delta\varepsilon$ + 18.21), 379 ($\Delta\varepsilon$ − 8.61)] is similar to that of **1**. Finally, the absolute configuration of **1** was solved to be 7*R*, 8*R*, and 13*S*. Thus, the structure of **1** was deduced as a new azaphilone and it was named dechloroisochromophilone II.

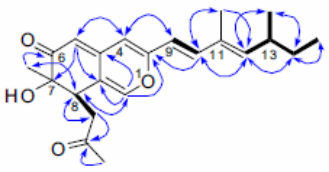

Fig. 2. COSY (bold lines) and selected HMBC correlations (H → C) of **1**.

Compound **2** was obtained as a yellow solid and its molecular ion peak at *m/z* 353.1541 $[M+H]^+$, 355.1530 [M + H + 2] in the

HRESITOFMS spectrum corresponds to the molecular formula $C_{19}H_{25}ClO_4$. The intensive ratio 3:1 of isotope peaks ([M+H]: [M+H+2]) supports the presence of a chlorine atom in the molecule. The molecular formula revealed seven degrees of unsaturation. The IR spectrum showed absorption bands of hydroxyl (3445 cm^{-1}) and conjugated ketone (1668 cm^{-1}) groups. The ^1H and ^{13}C NMR data and 2D NMR techniques (COSY, HSQC, HMBC and NOESY) of **2** indicated a structure similar to isochromophilone III (**7**) (Arai et al., 1995). However, there were some differences in chemical shifts between **2** and **7** at δ_H 4.50, 4.24 and 4.85, 3.82 (for H_2-1); 4.11 and 3.46 (for H-8); 3.05 and 3.12 (for H-8a), respectively. While, the ^{13}C NMR spectra between **2** and **7** signals were slightly different at δ_C 68.2 and 68.7 (for C-1); 77.3 and 75.0 (for C-7); 73.6 and 73.5 (for C-8); and 36.9 and 37.6 (for C-8a), respectively. The NOESY spectrum of **2** showed the correlations between H-8 and H-8a; H_b-1 and H-8a; indicating the stereochemistry of those protons. The absolute configuration of C-7 was assigned to be *R* on the basis of the CD spectrum, which revealed the positive 215 nm ($\Delta\varepsilon$+3.40), 276 nm ($\Delta\varepsilon$+2.64) and 347 nm ($\Delta\varepsilon$+3.98), and negative 247 nm ($\Delta\varepsilon$ −4.75) Cotton effects, agree well with those of **1** and cohaerin F (Quang et al., 2006). Also the absolute configuration on C-13 of the side chain was assigned to be *S* by comparison of the NMR data with isochomophilone II (**6**) (Omura et al., 1993). From the basis of data above compound **2** was deduced as 7*R*, 8*S*, and 8a*R* which was an epimer of isochromophilone III (7*R*, 8*R*, and 8a*R*). Therefore, **2** was a new azaphilone in terms of stereochemistry which we named *epi*-isochromophilone III.

Compound **3** had the molecular formula, $C_6H_8O_4$ deduced from the HRESITOFMS (*m/z* 145.0464 $[M+H]^+$), indicating three degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3400 cm^{-1}), conjugate lactone (1683 cm^{-1}) and alkene (1620 cm^{-1}) groups. The ^{13}C NMR and HSQC spectra showed six carbon signals attributable to a methoxy group (δ 56.3, 4-OCH_3), oxymethylene (δ 63.5, C-6), two methine (δ 91.9, C-3 and 70.3, C-5), and two quaternary (δ 166.2, C-2 and 171.9, C-4) carbons. The ^1H NMR showed resonances for an olefinic proton at δ 5.15 (s, H-3), methine proton at δ 4.26 (dd, *J* = 3.6, 4.0 Hz, H-5), two nonequivalent methylene protons at δ 4.39 (dd, *J* = 3.6, 12.2 Hz, H_b-6), 4.33 (dd, *J* = 4.0, 12.2 Hz, H_a-6) and a methoxy group at δ 3.79 (s, 4-OCH_3). The HMBC spectrum showed correlations of H-3 to C-2 and C-4; 4-OCH_3 to C-4; H-5 to C-4 and C-6; and H-6 to C-2 and C-4 confirming the location of the methoxy and carbonyl groups. However, the absolute stereochemistry of **3** could not be determined due to lack of the sample. On the basis of the above evidence, **3** was identified as 5-hydroxy-4-methoxy-5,6-dihydro-2*H*-pyran-2-one which has previously been reported as a synthetic compound (CAS Registry Number 1332747-94-0, SciFinder database). This is the first report of **3** isolated as a new natural product.

The isolated compounds were tested for their bioactivities and results are shown in Table 1. Azaphilones **9** and **10** with 1,4-dihydropyridine (N in the ring) showed antimycobacterial activity against *Mycobacterium tuberculosis* with MIC value of 6.2 and 50.0 μg/mL, respectively, while other azaphilones with pyran ring (O in the ring) were inactive. However, both of N and O analogues showed activities against other tests. Compounds **2**, **8**, **10** and **11** exhibited antimalarial activity against *Plasmodium falciparum* with respective IC_{50} values of 7.8, 3.5, 2.1 and 5.9 μg/mL. Compounds **2**, **4**, and **7–11** also showed cytotoxicity against three cancer cell lines (KB, MCF-7 and NCI-H187) with IC_{50} values ranging from 2.2 to 35.2 μg/mL. In addition, compounds **1–12** were also screened for AChE inhibitory activities. Among these, **1** and **7** were the most active compounds with MIC of 10 ng (0.03 nmol), whereas compounds **5**, **6** and **11** were five times and **8** was ten times less active than **1** and **7** (Table 2). This leads us to note that the configuration at C-8 (*R*) might play an important role in AChE inhibition.

Table 1
Biological activities of compounds **1–5** and **7–12**.

compound	anti-TB MIC(μg/mL)	antimalarial IC_{50} (μg/mL)	cytotoxicity IC_{50} (μg/mL)		
			KB[a]	MCF−7[b]	NCI-H187[c]
1	>50	>10	>50	>50	>50
2	>50	7.8	6.9	10.6	6.2
3	>50	>10	>50	>50	>50
4	>50	>10	7.9	>50	>50
5	>50	>10	>50	>50	>50
7	>50	>10	8.5	30.2	14.4
8	>50	3.5	3.1	23.5	4.6
9	50.0	>10	11.9	>50	35.2
10	6.2	2.1	2.2	16.5	5.5
11	>50	5.9	10.6	25.7	8.4
12	>50	>10	>50	>50	>50
dihydroartemisinin		0.001			
isoniazid	0.2–0.4				
ellipticine			0.3		0.4
doxorubicin			0.2	1.2	

[a] Human epidermoid carcinoma of the mouth.
[b] Human breast adenocarcinoma (MCF-7).
[c] Human small cell lung cancer.

3. Experimental

3.1. General experimental procedures

Melting points were determined using an Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and were uncorrected. UV spectra were measured on an Agilent 8453 UV–vis spectrophotometer (Agilent Technologies, USA). Optical rotations were obtained using a JASCO P-1030 digital polarimeter, and CD spectra were obtained using a JASCO J-810 apparatus (USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Agilent Technologies, USA). NMR spectra were recorded in $CDCl_3$ on a Varian Mercury Plus 400 spectrometer (Varian, Inc., USA), using residual $CHCl_3$ as an internal standard. HRESITOFMS were recorded on a Micromass Q-TOF-2 spectrometer (Bruker, Germany). Column chromatography (CC) and preparative TLC were carried out on silica gel 60 (230–400 mesh) and PF_{254} (Merck, Darmstadt, Germany), respectively

Table 2
Acetylcholinesterase inhibitory activity of compounds **1-12**.

compound	minimum inhibitory requirement	
	ng	nmol
1	10	0.03
2	>1000	>2.83
3	>1000	>6.94
4	>1000	>2.56
5	50	0.13
6	50	0.13
7	10	0.03
8	100	0.25
9	>1000	>2.30
10	>1000	>2.56
11	50	0.13
12	>1000	>4.34
galanthamine	1	0.003

3.2. Fungal material

The fungi *P. multicolor* CM01 was collected from soil at Chiang Mai Province, Thailand, in 2008 and was identified by K. Soytong. A voucher specimen Pm-CM01 was deposited at the Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok 10520, Thailand. The fungi was cultured in conical flasks (1L, 70 flasks) with potato dextrose broth (200 mL/flask) and incubated in standing conditions at 25–28 °C for 40 days. The culture broth was filtered to give a wet biomass and then air dried at room temperature.

3.3. Extraction and isolation

Air dried fungal biomass of *P. multicolor* CM01 (180 g) was ground and extracted successively at room temperature with EtOAc (500 mL × 3) and MeOH (500 mL × 3) to give crude EtOAc (13.5 g, 7.5%) and MeOH (2 g, 1.1%) extracts. The crude EtOAc extract was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of hexane:EtOAc and EtOAc:MeOH to give five combined fractions, M_1-M_5. Fraction M_1 was recrystallized from CH_2Cl_2 to give compound **13** (342 mg). Fraction M_2 was purified by silica gel FCC, eluted with an isocratic system of hexane:EtOAc (4:1) to give compound **4** (1.1 g). Fraction M_3 was subjected to silica gel FCC, eluted with CH_2Cl_2:EtOAc (19:1), to give two subfractions, $M_{3.1}$ and $M_{3.2}$. Subfraction $M_{3.1}$ was purified by preparative TLC using hexane:EtOAc (3:1) as eluent, to give compound **7** (1.6 mg). Subfraction $M_{3.2}$ was then purified by preparative TLC using hexane:EtOAc (1:1) as eluent, to afford compound **10** (89.3 mg). Fraction M_4 was subjected to silica gel FCC, eluted with a gradient system of CH_2Cl_2:EtOAc to give four ative TLC using CH_2Cl_2:EtOAc (4:1) as eluent, to give compounds **2** (13.2 mg) and **6** (2.5 mg). Subfractions $M_{4.2}$ was then purified by preparative TLC, using CH_2Cl_2:EtOAc (17:3) as eluent, to afford compound **5** (7.0 mg). Subfractions $M_{4.3}$ was separated by silica gel FCC, eluted with an isocratic system of CH_2Cl_2:acetone (3:1) to give compound **8** (30.4 mg). Subfractions $M_{4.4}$ was purified by preparative TLC using CH_2Cl_2:acetone (3:1) as an eluent, to give compounds **1** (62.0 mg) and **11** (38.8 mg). Fraction M_5 was subjected to silica gel FCC, eluted with a gradient system of CH_2Cl_2:EtOAc to give three subfractions, $M_{5.1}$-$M_{5.3}$. Subfraction $M_{5.1}$ was then purified by preparative TLC using hexane:EtOAc (1:7) as eluent, to afford compound **3** (3.5 mg). Subfraction $M_{5.2}$ was purified by preparative TLC using hexane:EtOAc (1:7) as eluent, to give compound **12** (27.3 mg). Subfraction $M_{5.3}$ was further purified by preparative TLC using CH_2Cl_2:acetone (3:1) as an eluent, to afford compounds **9** (34.9 mg) and **14** (12.9 mg).

3.3.1. Dechloroisochromophilone II (**1**)

Yellow brown solid; R_f = 0.38 (EtOAc-hexane; 1:1); mp 74–76 °C; $[\alpha]_D^{26}$ –58 (*c* 0.14, $CHCl_3$); CD (*c* 0.0076, MeOH) nm 234 (+1.64), 253 (−3.42), 325 (+19.17), 379 (−9.13); UV ($CHCl_3$) λ_{max} (log ε) 251 (3.74) nm; IR (film) ν_{max}: 3400, 2964, 1711, 1607, 1541, 1216, 1168 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.37 (s, H-1), 6.04 (s, H-4), 5.36 (s, H-5), 3.42 (dd, *J* = 3.2, 7.6 Hz, H-8), 5.91 (d, *J* = 16.0 Hz, H-9), 6.92 (d, *J* = 16.0 Hz, H-10), 5.57 (d, *J* = 10.0 Hz, H-12), 2.42 (m, H-13), 1.42–1.35 (m, H-14$_b$), 1.31–1.22 (m, H-14$_a$), 0.83 (t, *J* = 7.6 Hz, H_3-15), 0.97 (d, *J* = 6.8 Hz, H-16), 1.77 (s, H-17), 1.26 (s, H_3-18), 3.04 (dd, *J* = 3.2, 18.4 Hz, H_b-1′), 2.34 (dd, *J* = 7.6, 18.4 Hz, H_a-1′), 2.04 (s, H_3-3′); ^{13}C NMR (100 MHz, $CDCl_3$): δ 145.9 (C-1), 156.5 (C-3), 107.8 (C-4), 146.0 (C-4a), 103.4 (C-5), 198.1 (C-6), 73.0 (C-7), 40.5 (C-8), 119.9 (C-8a), 116.4 (C-9), 140.9 (C-10), 131.7 (C-11), 146.7 (C-12), 34.9 (C-13), 30.1 (C-14), 11.9 (C-15), 20.2 (C-16), 12.3 (C-17), 26.8 (C-18), 41.5 (C-1′), 207.3 (C-2′), 30.4 (C-3′); HRESITOFMS *m/z* 357.2074 $[M+H]^+$ (calcd for $C_{22}H_{28}O_4$ + H, 357.2066).

3.3.2. epi-*isochromophilone III* (**2**)

Yellow solid; R_f = 0.54 (EtOAc:hexane; 1:1); mp 96–97 °C; $[\alpha]_D^{26.3}$ +49 (*c* 0.11, MeOH); CD (*c* 0.0048, MeOH) nm 215 (+3.40), 247 (-4.75), 276 (+2.64), 347 (+3.98); UV ($CHCl_3$) λ_{max} (log ε) 232 (3.40), 256 (3.43), 290 (3.53) nm; IR (film) ν_{max} 3445, 2962, 1668, 1609, 1555, 1460 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.50 (dd, *J* = 4.0, 12.0 Hz, H_b-1), 4.24 (dd, *J* = 12.0, 16.0 Hz, H_a-1), 6.08 (s, H-4), 4.11 (d, *J* = 4.0 Hz, H-8), 3.05 (ddd, *J* = 4.0, 4.0, 16.0 Hz, H-8a), 5.98 (d, *J* = 16.0 Hz, H-9), 6.99 (d, *J* = 16.0 Hz, H-10), 5.61 (d, *J* = 7.2 Hz, H-12), 2.54 (sept, *J* = 6.3 Hz, H-13), 1.44–1.34 (m, H_b-14), 1.32–1.24 (m, H_a-14), 0.84 (t, *J* = 7.6 Hz, H_3-15), 0.98 (d, *J* = 6.8 Hz, H_3-16), 1.80 (s, H_3-17), 1.37 (s, H_3-18); ^{13}C NMR (100 MHz, $CDCl_3$): 68.2 (C-1), 162.9 (C-3), 102.1 (C-4), 145.6 (C-4a), 115.4 (C-5), 192.8 (C-6), 77.3 (C-7), 73.6 (C-8), 36.9 (C-8a), 118.9 (C-9), 141.5 (C-10), 132.2 (C-11), 147.1 (C-12), 34.9 (C-13), 30.1 (C-14), 11.8 (C-15), 20.2 (C-16), 12.3 (C-17), 23.4 (C-18); HRESITOFMS *m/z* 353.1541 $[M+H]^+$ and 355.1530 [M+H+2] (calcd for $C_{19}H_{25}ClO_4$ + H, 353.1521 and $C_{19}H_{25}ClO_4$ + H + 2, 355.1511).

3.3.3. 5-Hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one (**3**)

Yellow solid; R_f = 0.20 (EtOAc:hexane; 7:3); mp 115–117 °C; $[\alpha]_D^{28}$ –216 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 235 (3.87) nm; IR (film) ν_{max} 3400, 2958, 1683, 1620, 1458 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 5.15 (s, H-3), 3.79 (s, 4-OCH_3), 4.26 (dd, *J* = 3.6, 4.0 Hz, H-5), 4.39 (dd, *J* = 3.6, 12.2 Hz, H_b-6), 4.33 (dd, *J* = 4.0, 12.2 Hz, H_a-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2 (C-2), 91.9 (C-3), 171.9 (C-4), 56.3 (4-OCH_3), 70.3 (C-5), 63.5 (C-6); HRESITOFMS *m/z* 145.0464 $[M+H]^+$ (calcd for $C_6H_8O_4$ + H, 145.0456).

3.4. Biological activity procedures

3.4.1. Antimalarial assay

Antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multidrug-resistant strain), using the method of Trager and Jensen (1976). Quantitative assessment of activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al. (1979). The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound was dihydroartemisinin (Table 1).

3.4.2. Antimycobacterial assay

Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the microplate Alamar Blue assay (MABA) (Collins and Franzblau, 1997). The standard drug, isoniazid, was used as the reference compound (Table 1).

3.4.3. Cytotoxicity assay

Cytotoxic assays against human epidermoid carcinoma (KB), human breast cancer (MCF-7) and human small cell lung cancer (NCI-H187) were performed employing the colorimetric method as described by Skehan et al. (1990). The reference substances were ellipticine and doxorubicin (Table 1).

3.4.4. AChE bioautography assay

The acetylcholinesterase (AChE) inhibition activity was screened using bioautographic enzyme assay on TLC as described by Marston et al. (2002). The positive control was galanthamine (Table 2).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.03.004.

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Chevalone C analogues and globoscinic acid derivatives from the fungus *Neosartorya spinosa* KKU-1NK1

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ABSTRACT

Four meroterpenoids, 1-hydroxychevalone C, 1-acetoxychevalone C, 1,11-dihydroxychevalone C, and 11-hydroxychevalone C and two ester epimers, 2*S*,4*S*-spinosate and 2*S*,4*R*-spinosate, together with seven known compounds, chevalones B, C, and E, tryptoquivaline, nortryptoquivaline, tryptoquivaline L, and quinadoline A were isolated from the fungus *Neosartorya spinosa*. Their structures were established based on spectroscopic data analyses. The theoretical ECD spectra of epimers, 2*S*,4*S*-spinosate and 2*S*,4*R*-spinosate were calculated to support the experimental results of their CD spectra. 1-hydroxychevalone C exhibited antimycobacterial activity against *Mycobacterium tuberculosis* with a MIC value of 26.4 μM. 1-Acetoxychevalone C and tryptoquivaline showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values of 6.67 and 2.65 μM, respectively. In addition, 1-hydroxychevalone C, 1-acetoxychevalone C, 1,11-dihydroxychevalone C and quinadoline A showed cytotoxicity against KB and NCI-H187 cancer cell lines with IC_{50} values in the range of 32.7–103.3 μM.

1. Introduction

Neosartorya species are the sexual states of the *Aspergillus* species, which belong to the family Trichocomaceae and are distributed in soil worldwide (Samson et al., 2007). According to the literature, investigation of secondary metabolites from the *Neosartorya* species has not been well explored, unlike *Aspergillus*. However, previous reports on phytochemical investigations of the *Neosartorya* species have resulted in isolation of xanthones (Tan et al., 2012), alkaloids (Wong et al., 1993; Eamvijarn et al., 2012), indole alkaloids (Buttachon et al., 2012), γ-lactone derivatives (Yang et al., 2010), pyripyropene analogues (Eamvijarn et al., 2012), polyketides (Liu et al., 2015), and meroterpeneoids (Eamvijarn et al., 2013; Yim et al., 2014), respectively. Some of them were also reported with their bioactivities. In a previous publication, the isolation of a new meroterpenoid, tatenoic acid, and five known compounds, were reported from the fungus *N. tatenoi* (Yim et al., 2014). In a continuing search for bioactive compounds from this genus, the crude EtOAc extract from the fungal biomass of *N. spinosa* showed antimycobacterial activity against *Mycobacterium tuberculosis*, with 50.0% inhibition at a concentration of 50 μg/mL. The crude EtOAc extract of culture broth of this fungus exhibited cytotoxicity against MCF-7 and NCI-H187 cell lines with 98.7 and 93.3% (50 μg/mL) values, respectively. Moreover, the investigation of secondary metabolites from the fungus *N. spinosa* has not been previously reported. Herein, reported are the isolation, characterization, and bioactivities of four new compounds, **1**–**4** and seven known compounds **5**–**7** and **10**–**13** from the fungal biomass, and also two new compounds **8** and **9** from the culture broth of the fungus *N. spinosa* KKU-1NK1 (Fig. 1).

2. Results and discussion

The EtOAc extract of the dried fungal biomass of *N. spinosa* KKU-1NK1 was purified by chromatographic techniques, including silica

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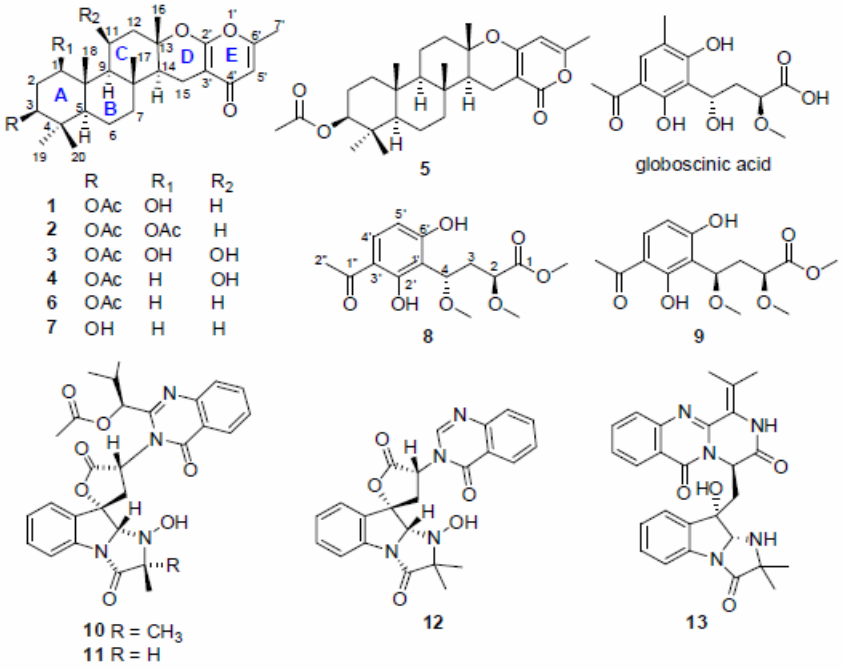

Fig. 1. The structures of isolated compounds (**1**–**13**) and globoscinic acid.

gel flash column chromatography and preparative TLC, to give four new meroterpenoids, 1-hydroxychevalone C (**1**), 1-acetoxychevalone C (**2**), 1,11-dihydroxychevalone C (**3**), and 11-hydroxychevalone C (**4**), together with seven known compounds, chevalones B and C (**5** and **6**) (Kanokmedhakul et al., 2011), chevalone E (**7**) (Prompanya et al., 2014), tryptoquivaline (**10**) and nortryptoquivaline (**11**) (Buechi et al., 1977), tryptoquivaline L (**12**) (Yamazaki et al., 1978), and quinadoline A (**13**) (Koyama et al., 2008). In addition, the EtOAc extract of culture broth of this fungus was also fractionated by chromatographic techniques to obtain two new epimers, 2*S*,4*S*-spinosate (**8**) and 2*S*,4*R*-spinosate (**9**). The structures of known compounds were identified by physical and spectroscopic data measurements (IR, 1D and 2D NMR), as well as by comparing the data obtained to published values.

Compound **1** had the molecular formula $C_{28}H_{40}O_6$, deduced from the HRESITOFMS (*m/z* 495.2719 $[M+Na]^+$), indicating nine degrees of unsaturation. The UV spectrum showed absorption maxima at 208, 245, and 259 nm. The IR spectrum displayed the presence of hydroxyl (3399 cm^{-1}), carbonyl ester (1730 cm^{-1}), and conjugated ketone (1665 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra indicated the presence of six methyl, an acetoxyl, six methylene, six methine (one alkene), seven quaternary (four aliphatic and three alkene) and two carbonyl carbon moieties. The ^1H NMR spectrum of **1** showed: five singlet resonances for six methyl groups at δ 0.84 (Me-19, Me-20), 0.90 (Me-17), 0.92 (Me-18), 1.28 (Me-16), and 2.21 (Me-7′); two oxymethine protons at δ 3.55 (1H, dd, J = 12.4, 4.4 Hz, H-1), and 4.48 (1H, dd, J = 12.4, 4.4 Hz, H-3); six methylene protons at δ 1.02–2.71; three methine protons at δ 0.77–1.52; and a singlet signal of a conjugated alkene at 6.06 (H-5′), indicating the core structure of a meroterpenoid (Kanokmedhakul et al., 2011). The singlet methine proton at δ_H 6.06 (H-5′) had correlations to C-3′ (98.6), C-4′ (180.7), C-6′ (160.9), and C-7′ (19.4), and also the methyl protons at δ_H 2.21 (H-7′) displayed correlations to C-5′ (111.8) and C-6′ in the HMBC spectrum, implying an γ-pyrone meroterpenoid type (Kanokmedhakul et al., 2011). The ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) were similar to those of the isolated chevalone C (**6**) (Kanokmedhakul et al., 2011), except for the presence of a hydroxyl group at C-1. Its COSY spectrum showed correlation of the methylene proton H-2 to oxymethine protons H-1 and H-3. The HMBC indicated correlations of H-1 to C-9 and C-18; H-2 to C-10, C-4, C-1, and C-3; H-3 to acetoxy carbonyl at C-3, C-19, and C-20; Me-18 to C-1, C-5, C-9, and C-10, which confirmed the position of the hydroxyl group at C-1 of ring A. The relative stereochemistry was determined by a combination of coupling constant and NOESY spectral data analyses. NOESY correlations of H-3 to H-1 and H-5, as well as J couplings of H-3 (J = 12.4, 4.4 Hz) to H-2, and H-1 (J = 12.4, 4.4 Hz) to H-2, supported the 1,3-diaxial positions of H-1, H-3, and H-5. The *trans* A/B ring junction was assigned by the absence of a NOESY correlation between H-5 and the methyl group at C-10. The proton H-1 also showed correlations to H-5 and H-9, which supported the 1,3-diaxial nature of these three protons. No correlation between H-9 and the methyl group at C-8 was observed, suggesting a *trans* B/C ring junction. Moreover, the methyl group at C-8 was correlated to the methyl groups at C-10, and C-13, indicating a cofacial orientation. Proton H-14 showed a correlation to H-9, but no correlation with methyl protons at C-13; therefore, H-14 and H-9 were 1,3-diaxial, indicating that the ring junction of C and D was also *trans*. From the above evidence, compound **1** was determined as a new meroterpenoid, 1-hydroxychevalone C.

Compound **2** had the molecular formula $C_{30}H_{42}O_7$, as obtained from the HRESITOFMS (*m/z* 537.2826 $[M+Na]^+$). Its IR spectrum displayed absorption bands of ester (1736 cm^{-1}) and conjugated ketone (1666 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra showed 30 carbon signals, including six methyl, two acetoxyl, six methylene, six methine (one alkene), seven quaternary (four aliphatic and three alkene), and three carbonyl carbons. The ^1H and ^{13}C NMR spectroscopic data of **2** (Table 1) were similar to those of **1**, except

Table 1
^{1}H and ^{13}C NMR spectral data of compounds **1**–**4**.[a]

Position	1		2		3		4	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	3.55 dd (12.4, 4.4)[b]	77.7 d	4.68 dd (12.4, 4.4)	79.6 d	3.66 dd (11.2, 4.8)	76.1 d	1.88 m 1.16 dd (14.4, 4.4)	38.2 d
2	1.90 m 1.69 m	34.5 t	2.00 m 1.68 m	30.0 t	1.81 m 1.95 m	33.5 t	1.72 m	23.5 t
3	4.48 dd (12.4, 4.4)	77.2 d	4.57 dd (12.4, 4.0)	76.3 d	4.45 dd (12.0, 4.0)	77.3 d	4.44 t (8.4)	80.8 d
4		37.9		38.0 s	–	37.7 s		38.0 s
5	0.77 brd (11.6)	53.3 s	0.88 m	53.2 d	0.81 m	54.3 d	0.83 m	56.4 d
6	1.60 m 1.54 m	17.6 t	1.68 m 1.29 m	17.4 t	1.69 m	18.3 t	1.57 m	18.3 t
7	1.89 m 1.02 m	40.9 t	1.88 m 1.06 m	40.8 t	1.90 m 1.04 m	42.6 t	1.90 m 1.05 m	43.2 t
8		38.3 s		38.3 s		38.1 s		37.8 s
9	1.11 brd (12.0)	61.1 d	1.15 br d (12.4)	60.2 d	0.93 br s	60.9 d	0.89 m	60.8 d
10		43.5 s		42.3 s		44.0 s		38.1 s
11	2.71 brd (14.4) 1.45 m	21.4 t	1.72 m 1.46 m	20.9 t	4.80 br s	69.8 d	4.63 br s	67.7 d
HO-11					2.83 br s			
12	2.09 m 1.75 m	40.4 t	2.03 m 1.71 m	40.1 t	2.35 m 1.93 m	46.7 t	2.23 dd (16.4, 12.8) 1.89 m	48.5 t
13		84.5 s		84.0 s		83.9 s		84.2 s
14	1.52 m	52.4 d	1.52 m	52.2 d	1.52 m	52.6 d	1.56 m	53.1 d
15	2.54 dd (16.4, 4.8) 2.12 m	15.6 t	2.54 dd (16.4, 4.4) 2.12 m	15.6 t	2.56 dd (16.4, 4.4) 2.31 m	15.4 t	2.58 dd (16.4, 4.8) 2.25 m	15.5 t
16	1.28 s	20.6 q	1.27 s	20.5 q	1.49 s	21.5 q	1.51 s	21.9 q
17	0.90 s	16.4 q	0.89 s	16.4 q	1.22 s	17.7 q	1.25 s	17.4 q
18	0.92 s	12.4 q	1.02 s	13.7 q	1.25 s	15.0 q	1.27 s	17.8 q
19	0.84 s	27.9 q	0.84 s	27.9 q	0.82 s	27.7 q	0.84 s	28.2 q
20	0.84 s	16.1 q	0.86 s	16.1 q	0.86	15.9 q	0.88 s	16.5 q
2′		163.0 s		163.0 s		162.5 s		162.5 s
3′		98.6 s		98.5 s		98.5 s		98.8 s
4′		180.7 s		180.5 s		180.7 s		180.7 s
5′	6.06 s	111.8 d	6.07 s	111.8 d	5.98 s	112.0 d	5.99 s	112.0 d
6′		160.9 s		161.0 s		160.7 s		160.7 s
7′	2.21 s	19.4 q	2.21 s	19.4 q	2.20 s	19.3 q	2.20 s	19.3 q
OAC-2			2.01 s	21.2 q 170.1 s				
OAC-3	2.04 s	21.3 q 170.9 s	2.01 s	21.8 q 170.4 s	2.05 s	21.3 q 171.0 s	2.04 s	21.4 q 171.1 s

[a] Recorded in $CDCl_3$.
[b] Values in parentheses are coupling constants in hertz.

that the hydroxyl group at C-1 in **1** was replaced by an acetoxyl group ($\delta_{H/C}$ 2.02/21.2 and δ_C 170.1). HMBC showed correlations of H-1 (4.68, dd, J = 12.4, 4.4 Hz) to acetoxy carbonyl at C-1, C-9, C-10, and C-18; Me-18 (1.02, s) to C-1, C-5, C-9, and C-10; and methylene protons H-2 (2.00, m and 1.68, m) to C-1, C-3, C-4, and C-10. The COSY spectrum exhibited a cross-peak of H-1/H-2/H-3 which supported the position of an acetoxy group located at C-1. The relative configuration of **2** was assigned by a combination of coupling constant and analysis of the NOESY spectral data which was the same as **1**. Therefore, **2** was a new meroterpenoid, 1-acetoxychevalone C.

Compound **3** had the molecular formula, $C_{28}H_{40}O_7$ deduced from the HRESITOFMS (m/z 489.2853 $[M+H]^+$). The ^{1}H and ^{13}C NMR spectral data of **3** (Table 1) were similar to those of **1**, except for the appearance of a hydroxyl group at C-11 (δ_H 4.80, brs/δ_C 69.8). The COSY spectrum confirmed the position of this hydroxyl group by showing correlations of H-9/H-11/H_2-12, and H-11/OH-11. The complete assignment of **3** was established based on extensive DEPT, COSY, HMQC, HMBC, and NOESY experiments. Thus, compound **3** was a new meroterpenoid, 1,11-dihydroxychevalone C.

Compound **4** had the molecular formula $C_{28}H_{40}O_6$, obtained from the HRESITOFMS (m/z 495.2720 $[M+Na]^+$). Its IR spectrum displayed presence of a hydroxyl (3353 cm^{-1}), ester (1730 cm^{-1}), and conjugated ketone (1666 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectroscopic data of **4** (Table 1) were similar to those of **3**, except for the missing hydroxy group at C-1. The HMBC displayed the correlations of the methylene proton H-1 ($\delta_{H/C}$ 1.88, m, H_a, 1.16, dd, J = 14.4, 4.4 Hz, H_b/38.2) to C-2, C-10, and C-18, as well as the COSY spectrum exhibiting the cross-peak of H-1/H-2/H-3, supporting the position of these methylene protons oriented at C-1. The relative configuration at C-11 was identified as that of **3**, based on the spectroscopic data analyses. Thus, **4** was identified as a new meroterpenoid, 11-hydroxychevalone C.

Compounds **8** and **9** were obtained as colorless oils and both had the same molecular formula, $C_{15}H_{20}O_7$, as deduced from HRESITOFMS (observed m/z 335.1106 $[M+Na]^+$ and m/z 335.1104 $[M+Na]^+$, respectively), indicating six degrees of unsaturation. Their IR spectra were almost identical with the presence of hydroxyl, aromatic ketone, ester, and aromatic groups. The ^{13}C NMR and DEPT spectra of the two isomers displayed 15 carbon signals attributable to four methyl (including two methoxy, an acetyl, and a methyl ester groups), a methylene, four methine (two aromatics), and six quaternary (including two carbonyl) carbons. The ^{1}H NMR spectrum of **8** showed two doublet signals at δ 7.57 (J = 8.8 Hz), and 6.39 (J = 8.8 Hz), which were assigned to H-4′ and H-5′ of aromatic protons, respectively. The ^{13}C NMR spectrum of **8** showed aromatic carbons at δ 163.3 (C-6′), 162.2 (C-2′), 132.1 (C-4′), 113.3 (C-3′), 111.2 (C-1′), and 108.9 (C-5′). Substituted groups on the aromatic ring were confirmed by analysis of the HMBC spectrum with correlations of H-4′ to C-6′, C-1″, C-2′, and C-3′; H-5′ to C-6′, C-3′, and C-1′;

6′-OH (9.27, brs) to C-1′, C-5′, and C-6′. Furthermore, a singlet methyl proton at $\delta_{H/C}$ 2.53/26.2 correlated to C-1″, C-3′, and C-4′, indicating the acyl group connected to an aromatic at C-3′, as well as the singlet downfield proton at 13.09 which was assigned to a chelated hydroxy proton at C-2′, which is in an *ortho* position to the acyl group. The COSY spectrum exhibited a cross-peak in the aliphatic region by H-2/H-3/H-4. The HMBC spectrum of **8** showed correlations between the methoxy protons at δ 3.45 (2-OMe) to C-2; δ 3.45 (4-OMe) to C-4; δ 3.73 (1-OMe) to carbonyl (C-1); H-2 to C-1, 2-OMe, C-3, and C-4; H_2-3 to C-1, C-2, C-1′, and C-4; and H-4 to C-2, C-3, C-1′, C-2′, and C-6′, confirming a methyl ester side-chain connecting to the aromatic ring at C-1′. Consequently, the ^{1}H and ^{13}C NMR spectroscopic data of the isomer **9** were similar to that of **8**, except for the chemical shift of the methine proton, H-2 of **8** (δ_H 4.07, dd, J = 7.2, 3.2 Hz) which appeared at a slightly lower field than **9** (δ_H 3.86, td, J = 5.2, 2.0 Hz). Unexpectedly, NOE correlations between H-2 and H-4 were observed in both isomers. This corresponded to the calculated distance between H-2 and H-4 from their optimized structures, which were 2.914 Å and 2.319 Å, respectively (Fig. 2). This information, however could not allow assignment of their orientations. Therefore, the absolute configurations of **8** and **9** were further characterized through comparison of experimental ECD and electronic circular dichroism calculations. The ECD calculations were done for four possible conformations using Gaussian 09 with TD-CAM-B3LYP/6−311++G**//B3LYP/6-31G*. The solvent effect was included using Polarizable Continuum Model (PCM) with methanol solvent (Frisch et al., 2009) (see supporting information). In addition, molecular orbital analysis was performed in order to find the transitions responsible for the observed Cotton effects (see supporting information). The ECD calculated spectrum of **8** generated for the 2*S*, 4*S* diastereomer showing a Cotton effect at 215 nm ($\Delta\varepsilon$ 49.7), 231 ($\Delta\varepsilon$ −6.4), 270 ($\Delta\varepsilon$ −6.9) and 307 ($\Delta\varepsilon$ 2.9), which agrees well with its experimental data (Figs. 3 and 4). The ECD calculated spectrum of **9** generated for the 2*S*, 4*R* diastereomer showed a Cotton effect at 215 nm ($\Delta\varepsilon$ −45.8), 230 ($\Delta\varepsilon$ −9.4), 271 ($\Delta\varepsilon$ 1.4) and 307 ($\Delta\varepsilon$ −3.4) which was also in good agreement with its experimental data (Figs. 3 and 4). Thus, **8** and **9** were identified as two new epimers, which have been named 2*S*,4*S*-spinosate and 2*S*,4*R*-spinosate, respectively.

Since 1995, the only analogue of these spinosates is globoscinic acid isolated from *Xylaria globosa*. However, the configuration of 2*S*,4*S*-globoscinic acid was reported with a negative sign of specific rotation [-92.5 (*c* 1.0, $CHCl_3$)] without ECD experiment support (Adeboya et al., 1995). According to our assignment, the specific rotation of 2*S*,4*S*-spinosate (**8**) [+90.8 (*c* 0.1, $CHCl_3$)] has an opposite sign to the 2*S*,4*S*-globoscinic acid. The difference in the specific rotation value might be affected by the free carboxylic acid and hydroxyl groups in 2*S*,4*S*-globoscinic acid.

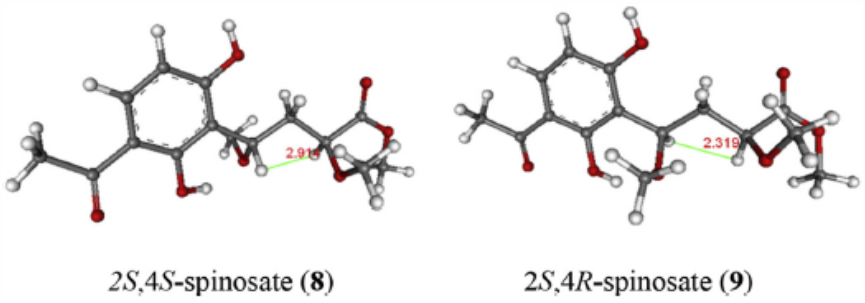

Fig. 2. Optimized structures of 2*S*, 4*S* and 2*S*, 4*R* configuration using B3LYP/6-31G(d), including the PCM model (methanol).

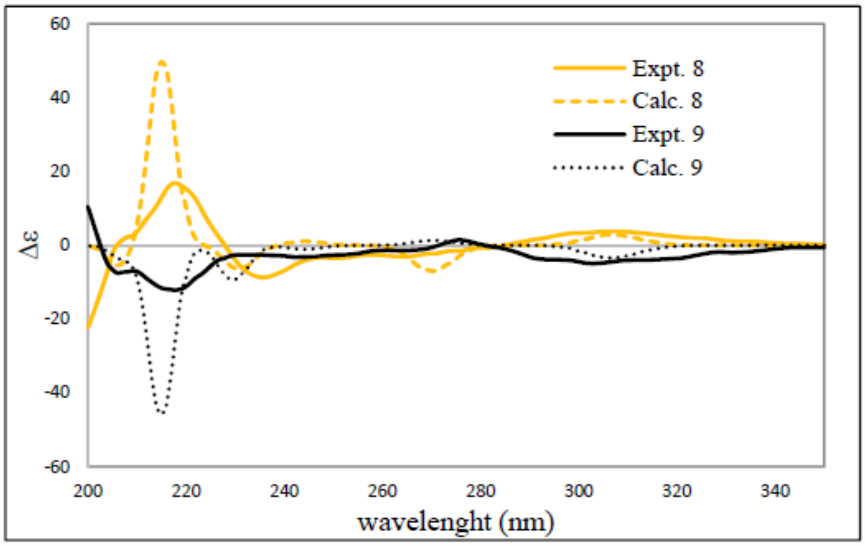

Fig. 3. Experimental and calculated ECD spectral data of compounds **8** and **9** measured in MeOH.

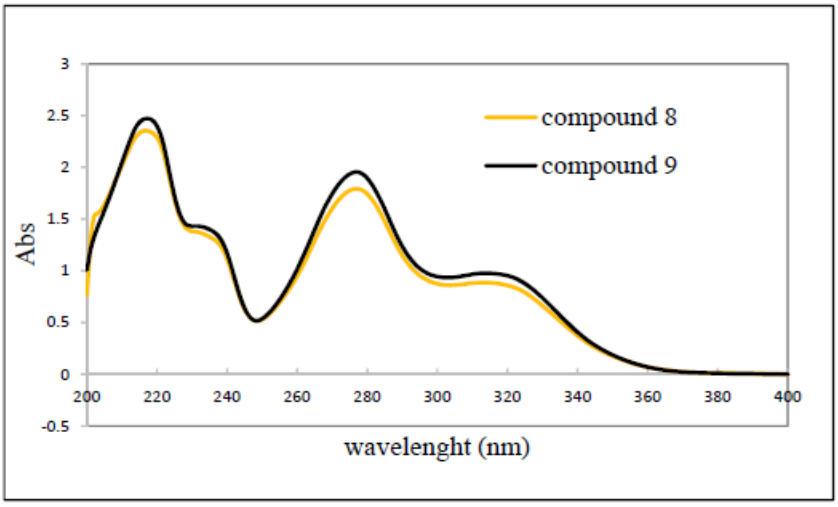

Fig. 4. UV spectral data of compounds **8** and **9** measured in MeOH.

All compounds, except for compound **11** (insufficient sample) were evaluated for their bioactivities and the results are shown in Table 3. Compounds **2** and **10** showed weak antimalarial activity against *P. falciparum* with IC_{50} values of 6.67 and 2.65 μM, respectively, while compound **1** exhibited antimycobacterial activity against *M. tuberculosis* with an MIC value of 26.5 μM. In addition, compounds **1**–**3** and **12** displayed cytotoxicity against three cancer cell lines, KB, MCF-7, and NCI-H187 with IC_{50} values in the range of 32.7–103.3 μM. However, **1**–**3** and **10** and **12** exhibited cytotoxicity against Vero cell lines with IC_{50} values in the range of 28.9–78.2 μM. Compound **5** has previously been reported for cytotoxicity against cancer cell lines KB (IC_{50} 6.1 μM) and NCI-H187 (IC_{50} 20.9 μM), while **6** showed antimycobacterial activity (MIC 14.3 μM) and cytotoxicity against BC (IC_{50} 19.7 μM) (Kanokmedhakul et al., 2011). In addition, compound **13** has previously been reported to possess antiviral activity against influenza A virus (H1N1) (IC_{50} 87 μM) (Peng et al., 2013).

3. Conclusions

Four new meroterpenoids, 1-hydroxychevalone C (**1**), 1-acetoxychevalone C (**2**), 1,11-dihydroxychevalone C (**3**), and 11-hydroxychevalone C (**4**), two new ester epimers, 2*S*,4*S*-spinosate (**8**) and 2*S*,4*R*-spinosate (**9**), three known meroterpenoids, chevalones B, C, and E (**5**, **6**, and **7**) and four known indole alkaloids, tryptoquivaline (**10**), nortryptoquivaline (**11**), tryptoquivaline L (**12**), and quinadoline A (**13**) were isolated from the fungus *N. spinosa* for the first time. The configurations of **8** and **9** were assigned by the combination of their ECD and theoretical computational ECD spectra. Compound **1** exhibited moderate antimycobacterial activity, while **2** and **10** showed weak antimalarial activity. Although compounds **1**–**3** and **12** showed cytotoxicity against KB and NCI-H187 cancer cell lines, they also exhibited cytotoxicity towards normal cells.

4. Experimental section

4.1. General experimental procedures

Melting points were obtained using a Gallenkamp melting point apparatus and were uncorrected. Optical rotations were determined using a JASCO DIP-1000 digital polarimeter. CD and UV spectra were measured using a JASCO J-810 apparatus. IR spectra were obtained using a Bruker Tenser 27 spectrophotometer. NMR spectra were recorded in $CDCl_3$ and CD_3OD as solvents on a Varian Mercury Plus 400 spectrometer and the internal standards were referenced from the residues of these solvents. Mass spectra were determined on Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source. TLC was performed with MERCK silica gel 60 PF_{254} TLC aluminum sheet; the spots were visualized under UV light (254 and 366 nm) and further by spraying with anisaldehyde then heating until charred. The internal column chromatography (CC) was carried out on MERCK silica gel 0.063–0.200 mm or less than 0.063 mm. Preparative layer chromatography (PLC) was carried out on glass (20 × 20 cm) supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography (MERCK).

4.2. Fungal material

The fungus *Neosartorya spinosa* KKU-1NK1 was collected from the forest soil around the Pha Nok Kao Silvicultural Station, Khon Kaen Province, Thailand, on July 2012. The specimen was isolated and identified by Sanmanoch (Sanmanoch et al., 2013) with accession number of AB185271.1 in the DNA Data Bank of Japan. The fungus was cultured in malt extract peptone broth (500 mL) in 2 L conical flasks (15 flasks) and incubated in a standing condition at room temperature for 3 weeks. The mycelia were harvested by filtration and dried in a hot air oven at 55 °C for 3 days, and the culture broth was immediately extracted with EtOAc.

4.3. Extraction and isolation

Dried biomass of *Neosartorya spinosa* (75 g) was ground and extracted successively at room temperature with EtOAc (500 mL x 3) to give a crude EtOAc extract (18 g). This was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of *n*-hexane-EtOAc and EtOAc-MeOH. On the basis of their TLC characteristics this gave seven fractions, Ns_1-Ns_7. Fraction Ns_3 was subjected to silica gel FCC, eluted with *n*-hexane-acetone

(70:30) to give **5** (640 mg). Fraction Ns_4 was purified by Sephadex LH-20, eluted with MeOH to give three subfractions, $Ns_{4.1}$-$Ns_{4.3}$. Subfraction $Ns_{4.2}$ was applied on a silica gel FCC, eluted with CH_2Cl_2-EtOAc (98:2) to give two subfractions, $Ns_{4.2.1}$ and $Ns_{4.2.2}$. Subfraction $Ns_{4.2.1}$ was purified by preparative TLC using *n*-hexane-acetone (80:20, x3) as eluent, to give **10** (96 mg, *Rf* 0.56). Subfraction $Ns_{4.3}$ was further purified by preparative TLC using CH_2Cl_2-acetone (95:5, x2) as eluent, to give **11** (3 mg, *Rf* 0.42). Fraction Ns_6 was subjected to silica gel FCC, eluted with *n*-hexane-acetone (90:10) to afford five subfractions, $Ns_{6.1}$-$Ns_{6.5}$. Recrystallization of subfraction $Ns_{6.1}$ from EtOAc gave **6** (1.06 g). Subfraction $Ns_{6.2}$ was purified by preparative TLC using CH_2Cl_2-MeOH (97:3, x5) as eluent, to yield **2** (8 mg, *Rf* 0.56). Subfraction $Ns_{6.3}$ was applied on Sephadex LH-20 CC, eluted with MeOH to give two subfractions, $Ns_{6.3.1}$ and $Ns_{6.3.2}$. Subfraction $Ns_{6.3.1}$ was further purified by Lichroprep RP-18 CC using MeOH-H_2O (50:50) as eluent, to give four subfractions, $Ns_{6.3.1.1}$-$Ns_{6.3.1.4}$. Purification of subfraction $Ns_{6.3.1.1}$ was performed by preparative TLC using CH_2Cl_2-MeOH (97:3) as eluent, to afford **13** (27 mg, *Rf* 0.46). Subfraction $Ns_{6.3.1.3}$ was further purified by preparative TLC using hexane-acetone (50:50, x5) as eluent, to yield **1** (11 mg, *Rf* 0.62). Purification of subfraction $Ns_{6.3.1.4}$ by preparative TLC using CH_2Cl_2-MeOH (99:1, x3) as eluent afforded **4** (7 mg, *Rf* 0.56). Subfraction $Ns_{6.3.2}$ was recrystallized from MeOH to give a white solid of **12** (264 mg). Subfraction $Ns_{6.4}$ was purified by Lichroprep RP-18 CC using MeOH-H_2O (60:40) as eluent, to give two subfractions, $Ns_{6.4.1}$ and $Ns_{6.4.2}$. Subfraction $Ns_{6.4.1}$ was further purified by preparative TLC using CH_2Cl_2-MeOH (97:3, x6) as eluent to yield **7** (4 mg, *Rf* 0.42). Purification of subfraction $Ns_{6.4.2}$ by preparative TLC using CH_2Cl_2-EtOAc (80:20, x6) as an eluent gave **3** (8 mg, *Rf* 0.58).

The fermentation broth of *N. spinosa* (12.5 L) was extracted with EtOAc (8.5 L x 3). The two layers were separated using a separatory funnel and the EtOAc extracts were combined and then concentrated under reduced pressure to yield a crude EtOAc extract (3.2 g). The EtOAc extract of culture broth of *N. spinosa* was separated on silica gel FCC and eluted with a gradient system of *n*-hexane-EtOAc and EtOAC-MeOH. From the TLC characteristics, it was divided into four fractions Nb_1-Nb_4. Fraction Nb_3 was purified by FCC and eluted with CH_2Cl_2-MeOH (99:1) and then the subfraction was further separated by preparative TLC using CH_2Cl_2-EtOAc (95:5, x4) as eluent to give compounds **8** (15 mg, *Rf* 0.68) and **9** (15 mg, *Rf* 0.64).

1-hydroxychevalone C (**1**): white solid; mp 156–158 °C; $[\alpha]^{23}_D$ −166.3 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (4.28), 245 (4.07), 259 (4.09) nm; IR (Neat) ν_{max} 3399, 2929, 2863, 1730, 1665, 1574, 1432, 1248 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 1; HRESITOFMS *m/z* 495.2719 $[M + Na]^+$ (calcd for $C_{28}H_{40}O_6$ + Na, 495.2723).

1-acetoxychevalone C (**2**): white solid; mp 167–169 °C; $[\alpha]^{24}_D$ −107.2 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (3.95), 245 (3.70), 260 (3.73) nm; IR (Neat) ν_{max} 2935, 2857, 1736, 1666, 1587, 1427, 1244, 1035 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 1; HRESITOFMS *m/z* 537.2826 $[M+Na]^+$ (calcd for $C_{30}H_{42}O_7$ + Na, 537.2828).

1,11-dihydroxychevalone C (**3**): white solid; mp 151–153 °C; $[\alpha]^{24}_D$ −77.2 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (4.04), 245 (3.83), 260 (3.88) nm; IR (Neat) ν_{max} 3377, 2940, 2857, 1730, 1664, 1570, 1244, 1190, 1036 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 1; HRESITOFMS *m/z* 489.2853 $[M+H]^+$ (calcd for $C_{28}H_{40}O_7$ + H, 489.2852).

11-hydroxychevalone C (**4**): white solid; mp 228–230 °C (decomp.); $[\alpha]^{24}_D$ −105.6 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (4.07), 246 (3.88), 260 (3.93) nm; IR (Neat) ν_{max} 3353, 2918, 2850, 1730, 1666, 1570, 1430, 1260 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 1; HRESITOFMS *m/z* 495.2720 $[M+Na]^+$ (calcd for $C_{28}H_{40}O_6$ + Na, 495.2723).

2*S*,4*S*-spinosate (**8**): colorless oil; $[\alpha]^{26}_D$ +90.1 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (4.66), 277 (4.53), 315 (4.21) nm; CD (*c* 0.00013, MeOH) nm: 308 (+3.8), 265 (−3.1), 236 (−8.7), 219 (+16.2); IR (Neat) ν_{max} 3250, 2932, 2832, 1750, 1621, 1498, 1369, 1253 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 2; HRESITOFMS *m/z* 335.1106 $[M + Na]^+$ (calcd for $C_{15}H_{20}O_7$ + Na, 335.1107).

2*S*,4*R*-spinosate (**9**): colorless oil; $[\alpha]^{26}_D$ −43.9 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (4.63), 277 (4.49), 315 (4.20) nm; CD (*c* 0.00013, MeOH) nm: 303 (−4.9), 277 (1.2), 242 (−3.2), 218(-12.1); IR (Neat) ν_{max} 3246, 2934, 2832, 1750, 1619, 1498, 1369, 1252 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$, 400 MHz), see Table 2; HRESITOFMS *m/z* 335.1104 $[M + Na]^+$ (calcd for $C_{15}H_{20}O_7$ + Na, 335.1107).

Table 2
^1H and ^{13}C NMR spectral data of compounds **8** and **9**.[a]

Position	8		9	
	δ_H	δ_C	δ_H	δ_C
1		173.0 s		172.7 s
OMe-1	3.73 s	52.0 q	3.75 s	52.0 q
2	4.07 dd (7.2, 3.2)[b]	77.1 d	3.86 td (5.2, 2.0)	77.0 d
OMe-2	3.45 s	58.2 q	3.45 s	58.3 q
3	2.20 ddd (14.0, 10.4, 3.2) 2.06 ddd (14.0, 10.4, 2.8)	37.7 t	2.33 ddd (14.0, 10.4, 5.2) 2.18 dt (5.2, 2.0)	37.6 t
4	5.13 dd (10.4, 2.8)	75.7 d	5.12 ddd (8.8, 4.8, 2.0)	75.3 d
OMe-4	3.45 s	58.4 q	3.38 s	58.1 q
1′		111.2 s		111.4 s
2′		162.2 s		162.2 s
OH-2′	13.09 s		13.10 s	
3′		113.3 s		113.3 s
4′	7.57 d (8.8)	132.1 d	7.59 d (8.8)	132.2 d
5′	6.39 d (8.8)	108.9 d	6.40 d (8.8)	109.0 d
6′		163.3 s		163.4 s
OH-6′	9.27 brs		9.24 s	
1″		202.8 s		202.9 s
1″-Me	2.53 s	26.2 q	2.54 s	26.3 q

[a] Recorded in $CDCl_3$.
[b] Values in parentheses are coupling constants in hertz.

Table 3
Biological activity of compounds **1**–**4** and **7**–**13**.

Compound	Antimalarial IC_{50} (μM)	Anti-TB MIC (μM)	Cytotoxicity IC_{50} (μM)			
			KB[a]	MCF-7[b]	NCI-H187[c]	Vero cells[d]
1	Inactive[e]	26.5	100.7	Inactive	40.0	39.1
2	6.67	Inactive	92.0	Inactive	32.7	28.9
3	Inactive	Inactive	Inactive	Inactive	39.9	78.2
4	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
7	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
8	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
9	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
10	2.65	Inactive	Inactive	Inactive	Inactive	66.5
12	Inactive	Inactive	103.3	Inactive	42.0	40.7
13	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Dihydroartemisinin	0.008					
Doxorubicin			2.06	14.53	0.16	1.39
Ellipticine			4.87		5.36	
Rifampicin		0.03				

[a] Human epidermoid carcinoma in the mouth.
[b] Human breast adenocarcinoma.
[c] Human small cell lung cancer.
[d] African green monkey kidney.
[e] >150 μM.

4.4. Methods of electronic circular dichroism (ECD) for **8** and **9**

For theoretical ECD spectra, four possible configurations at C-2 and C-4 of spinosates **8** and **9** were optimized by the B3LYP/6-31G(d) method and ECD spectra were calculated using the time-dependent density functional theory (TD-DFT) method with CAM-B3LYP functional and 6–311++G (d,p) basis set. Polarizable Continuum Model (PCM) solvation model using methanol was included in the calculations. All calculations were performed using the Gaussian09 program (Frisch et al., 2009).

4.5. Antimalarial assay

Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), using the method of Trager and Jensen (Trager and Jensen, 2005). Quantitative assessment of activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins (Desjardins et al., 1979). The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum* (Table 3).

4.6. Antimycobacterial assay

Antimycobacterial activity was assessed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The standard drug was rifampicin (Table 3).

4.7. Cytotoxicity assays

Cytotoxicity assays against human epidermoid carcinoma (KB), human breast adenocarcinoma (MCF-7), and human small cell lung cancer (NCI-H187) were performed employing the Resazurin microplate assay (REMA) described by O'Brien and co-workers (O'Brien et al., 2000). The reference substance was doxorubicin (Table 2). Cytotoxicity against primate cell line (Vero) was using the green fluorescent protein (GFP) detection method described by Hunt and co-workers (Hunt et al., 1999). The reference substance was doxorubicin (Table 3).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.09.008.

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