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Bioactive meroterpenoids and alkaloids from the fungus *Eurotium chevalieri*

Kwanjai Kanokmedhakul [a], Somdej Kanokmedhakul [a,*], Ruchiruttikorn Suwannatrai [a], Kasem Soytong [b], Samran Prabpai [c,d], Palangpon Kongsaeree [c,d]

[a] Natural Products Research Unit, Department of Chemistry, and Center for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok 10520, Thailand
[c] Department of Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[d] Center for Excellence in Protein Structure and Function, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

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ABSTRACT

Five new meroterpenoids, chevalones A–D (**1**–**4**), aszonapyrone B (**8**), and a new sequiterpene alkaloid, eurochevalierine (**5**), together with four known compounds, sequiterpene (**6**), terpenoid pyrrolobenzoxazine named CJ-12662 (**7**), meroterpenoid, aszonapyrone A (**9**), and ergosterol were isolated from the fungus *Eurotium chevalieri*. The structures were established on the basis of spectroscopic evidence. The configurations of **1** and **5** were determined by X-ray analysis. The biosynthetic pathway of **1**–**3**, **8**, and **9** were proposed. Chemical transformation of aszonapyrone A (**9**) was also studied. Compounds **4**, **5**, and **7** exhibited antimalarial activity against *Plasmodium falciparum*, while **3**, **5**, and **7** showed antimycobacterial activity against *Mycobacterium tuberculosis*. In addition, compounds **2**–**7** showed cytotoxicity against cancer cell lines.

1. Introduction

Eurotium chevalieri Mangin imperfect stage of *Aspergillus chevalieri* (Mangin) Thom and Church belongs to Phylum Ascomycota.[1] Colonies reach 7.00 cm diameter on potato dextrose agar in 10 days at a temperature of 30 °C, with gray in the central areas and ascomata abundant on medium in a continuous yellow layer. There are eight ascospores per ascus, ascospores lens-shaped, roughened, and with equatorial crests 0.8 μm apart, 4.5–4.8×3.2–3.4 μm. These characteristics are similar to those reported by Blaser (1976).[2] Previous investigation on secondary metabolites from the fungus genus *Eurotium* species[3–10] including *E. chevalieri*[11–13] resulted in the isolation of numerous types of compounds, such as alkaloids, indole derivatives, phenolic compounds, anthraquinones, and diketopiperazines. In our continuing search for bioactive constituents from fungi, we noted that hexane, EtOAc, and MeOH extracts of the fungus *E. chevalieri* showed antimycobacterial activity against *Mycobacterium tuberculosis* with MIC values of 12.5, 3.13, and 6.26 μg/mL, respectively. In addition, the EtOAc extract of the strain also exhibited cytotoxicity against cancer cell lines, NCI-H187 and KB. Investigation of the extracts of dried fungal biomass of *E. chevalieri* cultured in two different periods of time have led to the isolation of six new compounds (**1**–**5**, and **8**) and four known compounds, **6**, **7**, **9**, and ergosterol (Fig. 1). We report herein the isolation, characterization, chemical transformation of **9** and proposed biosynthesis of **1**–**3**, **8**, and **9**, as well as bioactivity of these compounds.

2. Results and discussion

The fungus *E. chevalieri* was stationary cultured at 30 °C for 40 days in potato dextrose broth (PDB). The hexane, EtOAc, and MeOH extracts from the air-dried fungal biomass was separated on silica gel column chromatography and preparative TLC to yield compounds **1**–**7**. The PDB sample cultured for 30 days produced three compounds, **8**, **9**, and ergosterol. Structures of the known compounds were identified by physical and spectroscopic data measurements (^{1}H and ^{13}C NMR, 2D NMR, and MS) and by comparing the data obtained with published values, as diterpene (**6**),[14] terpenoid pyrrolobenzoxazine (**7**),[14] and aszonapyrone A (**9**) [mp 240–242 °C (lit.,[15] 242–244 °C)].

Compound **1** was obtained as colorless crystals, and its molecular formula $C_{26}H_{38}O_4$, was deduced from HRMS *m*/*z* 437.2669

* Corresponding author. Tel.: +66 43 202222x12243; fax: +66 43 202373; e-mail address: somdej@kku.ac.th (S. Kanokmedhakul).

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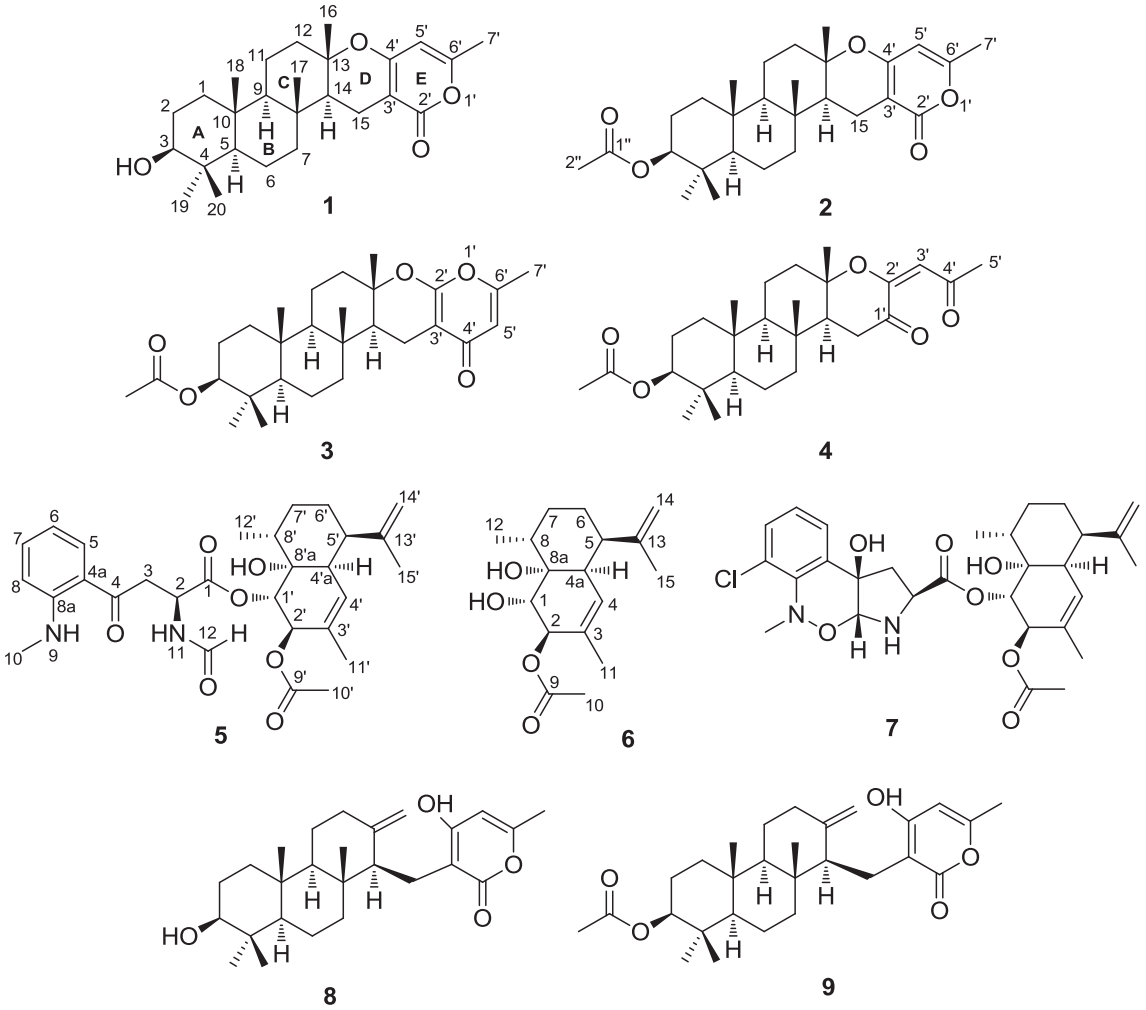

Fig. 1. Structures of compounds **1**–**9**.

$[M+Na]^+$, implying eight degrees of unsaturation. The IR spectrum showed absorption bands of hydroxyl (3467 cm^{-1}) and a conjugated carbonyl (1686 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra revealed 26 signals attributable to six methyl, seven methylene, five methine (including alkene), and eight quaternary carbons. The ^1H NMR spectrum of **1** (Table 1) showed five singlet signals of methyl groups at δ 0.77 (H-20), 0.82 (H-18), 0.86 (H-17), 0.95 (H-19), 1.18 (H-16), an oxymethine proton at δ 3.18 (1H, dd, J=11.2, 4.4 Hz, H-3), six methylene protons at δ 0.75–2.05 and three methine protons at δ 0.75–1.41, providing a most useful indicator for the presence of a tricyclic terpane.[16] The methylene protons at δ 2.42 (dd, J=16.8, 4.8 Hz, H-15), a vinyl proton at δ 5.66 (1H, s, H-5′) and a singlet signal at δ 2.16 (3H), which was assigned to a methyl group adjacent to an alkene carbon were part of rings D and E. The ^{13}C NMR (Table 2) showed signals characteristic for two oxygenated olefinic carbons, at δ 163.2 (C-4′) and 159.7 (C-6′) of an α-pyrone ring, while the other olefinic carbons at C-3′ and C-5′ appeared at δ 97.8 and 100.6, respectively. Two oxygenated carbons, C-3 and C-13 showed resonances at δ 78.7 and 80.5, respectively. The COSY exhibited a cross coupling network between H-1/H-2/H-3, H-5/H-6/H-7, H-9/H-11/H-12, H-14/H-15, and H-5′/H-7′ (allylic coupling) allowed formulation of five fragments (Fig. 2). The HMBC spectrum exhibited correlation of an olefinic proton H-5′ to C-3′, C-4′, C-6′, and C-7′; and H-7′ to C-5′ and C-6′ to complete the assignment of the α-pyrone ring (E) and its substituents. The cross-peaks of correlations of H-15 to C-8, C-14, C-13, C-2′, C-3′, and C-4′ extended the connectivity between two fragments, C and E through the D ring junction. The correlations of H-7 to C-9, C-5, C-8, and C-17; and H-14 to C-13, C-9, C-8, C-7, C-16, and C-17 extended between two fragments to include the B/C ring junction. In addition, the HMBC spectrum showed correlation of methylene protons H-1 to C-3, C-5, C-9, C-10, and C-18 and H-5 to C-6, C-18, and C-20 to complete the planar structure of compound **1** (Fig. 2).

The relative stereochemistry was determined by a combination of coupling constant and analysis of the NOESY spectral data (Fig. 3). The 1,3-diaxial NOESY correlation was observed between H-5 and H-3. The J values of 4.4 and 11.2 Hz for the coupling of H-3 to H-2 also supported the axial position of H-3. There was no correlation between H-5 and the methyl group at C-10 (H-18) suggesting the *trans* A/B ring junction. A 1,3-diaxial correlation was observed for H-14 and H-9, and H-14 and H-12, while the correlation between H-14 and H-16 was not observed, indicating the *trans* C/D ring junction. Finally, the relative configuration of **1** was confirmed by a single-crystal X-ray diffraction analysis (Fig. 4). From the above spectroscopic analysis it was found that most of the constitutive structure of **1** was similar to taondiol,[16] except for the structure of ring E. The structure of **1** was established as a new meroterpenoid type as described by Geris and Simpson,[17] and has been named chevalone A.

Compound **2** was obtained as colorless crystals, and its molecular formula, $C_{28}H_{40}O_5$, was deduced from the HRMS m/z 479.2771 $[M+Na]^+$, implying nine degrees of unsaturation. The IR spectrum showed the presence of ester (1733 cm^{-1}), conjugate lactone (1702 cm^{-1}) and alkene (1652 and 1588 cm^{-1}) groups. The ^1H and

Table 1
^{1}H NMR data of compounds **1**–**4** and **8** ($CDCl_3$, 400 MHz)

Position	1	2	3	4	8
1	1.72 m[b]	1.71 m[b]	1.72 m[b]	1.73 m[b]	1.67 m[b]
	0.96 m[b]	1.06 m[b]	1.06 m[b]	1.09 m[b]	0.97 m
2	1.64 m[b]	1.66 m[b]	1.63 m[b]	1.69 m[b]	1.56 m[b]
	1.56 m[b]		1.60 m[b]		
3	3.18 dd (11.2, 4.4)[a]	4.44 dd (11.4, 4.8)	4.45 dd (11.4, 5.2)	4.48 dd (11.5, 4.7)	3.14 dd (10.0, 6.4)
5	0.75 m[b]	0.88 m[b]	0.85 m[b]	0.90 m[b]	0.83 m
6	1.55 m[b]	1.57 m[b]	1.55 m[b]	1.60 m[b]	1.58 m[b]
	1.45 m[b]	1.47 m[b]	1.45 m[b]	1.44 m	
7	1.82 tt (13.2, 3.2)	1.83 tt (13.08, 3.08)	1.88 m	1.65 m[b]	1.87 m
	1.02 m[b]	1.01 m[b]	1.03 m[b]	1.05 m[b]	1.42 m
9	0.89 dd (12.2, 2.0)	0.92 m	0.93 m	1.01 m[b]	1.07 m
11	1.69 m[b]	1.69 m[b]	1.69 m[b]	1.76 m[b]	1.61 m
	1.33 m[b]	1.33 m	1.33 m		1.29 m
12	2.05 td, (12.4, 3.0)	2.06 m[b]	2.09 m	2.15 m[b]	2.24 m
	1.59 m[b]	1.61 m[b]	1.66 m[b]	1.78 m[b]	1.92 m
14	1.41 m[b]	1.43 m[b]	1.51 m[b]	2.08 m[b]	2.48 m
15	2.42 dd (16.8, 4.8)	2.42 dd (16.7, 4.6)	2.53 dd (16.4, 4.8)	2.50 m	2.58 m
	2.14 m[b]	2.15 m[b]	2.15 dd (16.4, 3.6)		2.42 m
16	1.18 s	1.18 s	1.27 s	1.35 s	4.81 br s, 4.62 br s
17	0.86 s	0.87 s	0.87 s	0.87 s[b]	0.74 s
18	0.82 s	0.84 s[b]	0.83 s[b]	0.85 s[b]	0.81 s
19	0.95 s	0.85 s[b]	0.85 s[b]	0.85 s[b]	0.94 s
20	0.77 s	0.84 s[b]	0.83 s[b]	0.85 s[b]	0.74 s
3′				5.91 s	
5′	5.66 s	5.69 s	5.96 s	2.35 s	5.87 s
7′	2.16 s	2.17 s	2.18 s		2.14 s
2″		2.03 s	2.02 s	2.05 s	

[a] Figures in parentheses are coupling constants in Hz.
[b] Overlapping with other protons.

Table 2
^{13}C NMR data of compounds **1**–**4** and **8** ($CDCl_3$, 100 MHz)

Position	1	2	3	4	8
1	38.3 t[a]	38.0 t	37.9 t	37.9 t	38.6 t
2	27.2 t	23.5 t	23.5 t	23.4 t	26.7 t
3	78.7 d	80.6 d	80.5 d	80.4 d	78.4 d
4	38.8 s	37.7 s	37.7 s	37.7 s	38.7 s
5	55.3 d	55.4 d	55.4 d	55.4 d	55.3 d
6	17.8 t	17.7 t	17.7 t	17.5 t	18.5 t
7	40.9 t	40.8 t	40.9 t	39.9 t	40.0 t
8	37.2 s	37.2 s	37.2 s	37.1 s	39.8 s
9	60.3 d	60.2 d	60.1 d	59.8 d	60.0 d
10	37.1 s	37.0 s	37.0 s	36.9 s	37.4 s
11	18.6 t	18.6 t	18.6 t	18.6 t	23.3 t
12	40.2 t	40.2 t	40.0 t	40.4 t	38.1 t
13	80.5 s	80.4 s	84.1 s	80.3 s	148.9 s
14	51.9 d	51.9 d	52.2 d	51.8 d	53.4 d
15	16.8 t	16.8 t	15.3 t	32.8 t	18.7 t
16	20.5 q	20.5 q	20.4 q	21.9 q	105.7
17	16.3 q	16.0 q	16.0 q	15.5 q	14.6 q
18	16.0 q	16.3 q	16.4 q	16.4 q	15.8 q
19	27.9 q	27.9 q	27.9 q	27.9 q	27.5 q
20	15.2 q	16.4 q	16.3 q	16.3 q	14.9 q
1′				195.5 s	
2′	165.3 s	165.3 s	162.5 s	154.0 s	167.3 s
3′	97.8 s	97.7 s	98.4 s	108.9 d	102.6 s
4′	163.2 s	163.2 s	180.5 s	198.4 s	166.1 s
5′	100.6 d	100.6 d	111.8 d	31.8 q	105.5 d
6′	159.7 s	159.7 s	160.4 s		159.6 s
7′	19.6 q	19.6 q	19.2 q		18.8 q
1″		170.9 s	170.9 s	170.9 s	
2″		21.2 q	21.2 q	21.2 q	

[a] Multiplicities were determined by analysis of the DEPT and HSQC spectra.

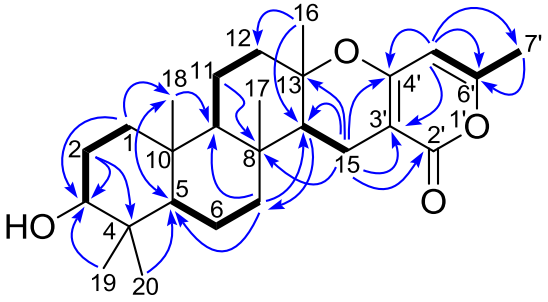

Fig. 2. COSY (bold line) and selected HMBC (arrow line) correlations of **1**.

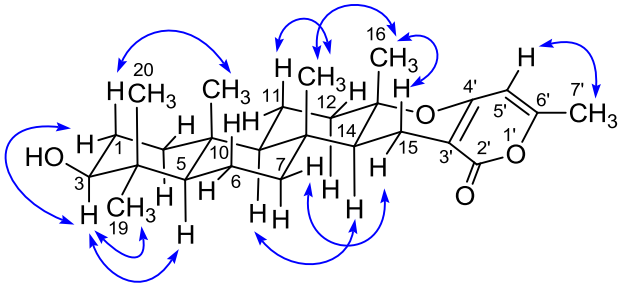

Fig. 3. NOESY correlations of **1**.

^{13}C NMR spectra of **2** (Tables 1 and 2) were similar to those of **1**, except for an appearance of an acetoxy group at C-3 (δ_H 2.03, δ_C 21.2 and 170.9). The relative stereochemistry was determined by a combination of coupling constant and analysis of the NOESY spectrum, which was similar to those of **1**. Thus, **2** was identified as an acetyl derivative of **1** and was named chevalone B.

Compound **3** was obtained as a white solid, and its molecular formula, $C_{28}H_{40}O_5$, was deduced from the HRMS *m*/*z* 479.2773 $[M+Na]^+$, implying nine degrees of unsaturation. The IR spectrum showed the presence of ester (1734 cm^{-1}), conjugate ketone (1669 cm^{-1}), and alkene (1637 and 1596 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectra of **3** (Tables 1 and 2) were similar to those of **2**, except for the α-pyrone, which was displaced by γ-pyrone, δ_H 5.96 (s, H-5′), 2.18 (H-7′) and δ_C 162.5 (C-2′), 98.4 (C-3′), 111.8 (C-5′),

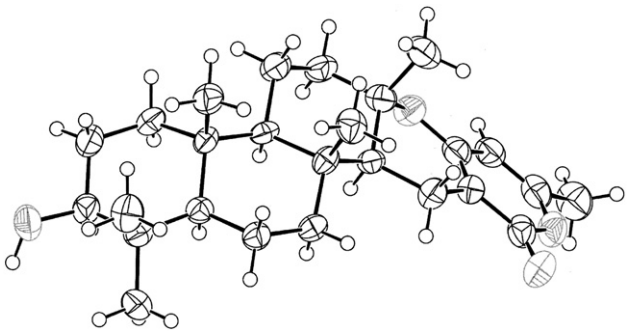

Fig. 4. ORTEP plot of the crystal structure of **1**.

160.4 (C-6′), 180.5 (C-4′), and 19.2 (C-7′). The HMBC spectrum clearly demonstrated correlations of H-5′ to C-3′, C-4′, C-6′, and C-7′; methyl protons at C-6′ (H-7′) to C-5′ and C-6′; as well as methylene protons (H-15) to C-14, C-13, C-2′, C-3′, and C-4′, confirming the formation of a γ-pyrone ring. The relative stereochemistry of **3** was assigned by a combination of coupling constant and analysis of the NOESY spectral data, which was similar to those of **1** and **2**. On the basis of the above evidence, **3** was a new meroterpenoid, which has been named chevalone C.

Compound **4** was obtained as a white solid and its molecular formula, $C_{27}H_{40}O_5$, was deduced from the HRMS *m/z* 467.2773 $[M+Na]^+$, implying eight degrees of unsaturation. The IR spectrum showed the presence of ester carbonyl (1729 cm^{-1}), conjugated ketone (1702 cm^{-1}), and alkene (1653 and 1594 cm^{-1}) groups. The ^{13}C NMR and DEPT spectrum showed twenty seven carbon signals attributable to seven methyl (including acetoxy group), seven methylene, five methine (including alkene carbon) and eight quaternary (including three carbonyl function) carbons. The ^1H and ^{13}C NMR spectra of **4** (Tables 1 and 2) were similar to those of **2**, except that the α-pyrone ring was replaced by the α,β unsaturated diketones, δ_H 5.91 (H-3′) and 2.35 (H-5′), δ_C 198.4 (C-4′), 195.5 (C-1′), 154.0 (C-2′), 108.9 (C-3′), and 31.8 (C-5′). The HMBC spectrum showed correlation of a vinyl proton, H-3′ to C-1′, C-2′, and C-5′; methylene protons, H-15 to C-13, C-1′, and C-2′; methyl protons (H-5′) to C-3′ and C-4′ to confirm the formation of ring D and its substituents. The relative stereochemistry was assigned by a combination of coupling constant and analysis of the NOESY spectral data and by comparison to those of **1**, **2**, and **3**. On the basis of the above evidence, **4** was a new meroterpenoid and we named it chevalone D.

Compound **5** was obtained as yellow needles, and its molecular formula, $C_{29}H_{38}N_2O_7$, was deduced from the HRMS *m/z* 527.2842 $[M+H]^+$, implying twelve degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3331 cm^{-1}), NH (3440 cm^{-1}), ester carbonyls (1754 and 1725 cm^{-1}), aromatic ketone (1670 cm^{-1}), amide (1644 cm^{-1}), and aromatic (1569 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra showed twenty nine signals attributable to five methyl, four methylene (including terminal alkene), twelve methine (including aromatic, alkene, and formamide), and eight quaternary carbons. The four carbonyl groups were confirmed by ^{13}C NMR spectrum showing signals at δ 199.8, 170.2, 169.8, and 160.8. The ^1H and ^{13}C NMR spectral data of **5** (Table 3) indicated a structure relating to a known terpenoid pyrrolobenzoxazine (**7**) especially, a terpenoid unit.[14] The ^1H NMR spectrum of **5** displayed a singlet signal at δ 8.17, which correlated to a carbon at δ_C 160.8 (H-12) in the HSQC spectrum revealing a formamide unit.[18] Four resonance signals at δ 7.66 (d, *J*=8.1 Hz, H-5), 6.56 (t, *J*=7.6 Hz, H-6), 7.38 (t, *J*=7.7 Hz, H-7), and 6.66 (d, *J*=8.7 Hz, H-8) indicated the presence of a 1,2-disubstituted aromatic group. A sharp doublet signal at δ 2.90 (*J*=5.0 Hz) and a broad quartet signal at δ 8.65 were assigned to a methyl group and a proton in the substituents at N-9 of the secondary amine, respectively. This was checked by mixing a sample of **5** with D_2O, wherein the signal at δ 8.65 disappeared, while the signal at δ 2.90 showed as a singlet. The triplet of doublet signals at δ 5.02 (*J*=7.6, 3.8 Hz), which coupled to methylene protons at δ 3.80 (dd, *J*=17.7, 3.8 Hz) and 3.60 (dd, *J*=17.7, 3.7 Hz) at C-3, and a proton in the substituents at N-11 of secondary amide δ 6.70 (d, *J*=7.4 Hz) was assigned to a methine proton at C-2. The COSY spectrum demonstrated correlation between H-3/H-2/H-11/H-12, H-5/H-6/H-7/H-8 of four aromatic protons, and also H-9/H-10 supported the partial units for the alkaloid structure of **5** (Table 3). The HMBC spectrum demonstrated correlation of the methyl group at N-9 (H-10) to C-8a and C-8; H-9 to C-8, C-10, and C-4a confirmed the methyl amino group located at C-8a. Complete HMBC correlations of **5** are shown in Fig. 5 and Table 3. Finally, the X-ray structure of **5** (Fig. 6) proved the presence of all protons in space clearly supporting the NOESY spectral data (Table 3). The X-ray structure of **5** showed stereochemistry of the terpenoid unit similar to those reported for compound **6**, which has been proven to be the absolute configuration.[14] Therefore, it can be concluded that compound **5**, especially the terpenoid unit, has absolute configuration similar to that of **6**. It also implies the *S* absolute configuration at C-2 in the alkaloid unit. From the above evidence, compound **5** is a new sesquiterpene alkaloid, which has been named eurochevalierine.

Table 3
NMR data of compound **5** ($CDCl_3$, 400 MHz)

No	δ_H	δ_C	COSY	HMBC	NOESY
1		170.2 s[b]			
2	5.02 td (7.6, 3.8)[a]	47.6 d	H-3, 11	C-1, 3, 4, 12	H-3
3	3.80 dd (17.7, 3.8) 3.60 dd (17.7, 3.7)	40.4 t	H-2	C-1, 2, 4	H-2, 5
4		199.8 s			
4a		116.1 s			
5	7.66 d (8.1)	131.8 d	H-6, 7	C-4, 4a, 8a, C-7	H-3, 6
6	6.56 t (7.6)	114.4 d	H-5, 7	C-5, 8, 4a	H-5
7	7.38 t (7.7)	136.3 d	H-5, 6, 8	C-5, 8a	H-8
8	6.66 d (8.7)	111.7 d	H-7	C-4, 4a, 6	H-7, 10
8a		152.5 s			
9	8.65 (NH) br q		H-10	C-8, 4a, 10	H-10
10	2.90 d (5.0)	29.3 q	H-9	C-8, 8a	H-9, 8
11	6.70 (NH) d (7.4)	—	H-2, 12	C-12	H-12
12	8.17 s	160.8 d	H-11	C-2	H-11, 1′
1′	5.41 br s	74.9 d	H-2′	C-1, 2′, 3′, C-8′, 4′a	H-12′
2′	5.14 br s	72.6 d	H-1′, 4′, H-4′a, 11′	C-1′, 3′, 4′, C-9′, 11′, 8′a	H-11′
3′		129.4 s			
4′	5.32 br s	127.5 d	H-2′, 11′, H-4′a	C-11′, 8′a	H-11′, H-15′
4′a	2.27 m	39.4 d	H-2′, 4′, H-11′		H-6′, OH-8′a
5′	2.30 m	40.7 d	H-7′, 14′, H-15′		—
6′	1.49 m 1.28 m	25.7 t			H-4′a
7′	1.52 m 1.42 m	30.1 t	H-5′, 8′	C-5′, 8′, 8′a	H-12′
8′	1.95 m	30.6 d	H-7′, 12′		H-6′, 7′, 12′
8′a		72.3 s			
9′		169.8 s			
10′	2.09 s	20.7 q		C-9′	
11′	1.62 s	19.7 q	H-4′, 4′a, H-2′	C-2′, 3′, 4′	H-2′, 4′
12′	0.93 d (6.6)	14.8 q	H-8′	C-8′, 8′a	H-1′, 7′, 8′
13′		146.9 s			
14′	4.82 br s 4.60 br s	110.9 t	H-5′, 15′	C-5′, 15′	H-15′
15′	1.24 s	21.4 q	H-5′, 14′	C-5′, 13′, 14′	H-4′, 14′
OH-8a	2.39 s			C-8′, 8′a	H-4′a

[a] Figures in parentheses are coupling constants in Hz.
[b] Multiplicities were determined by analysis of the DEPT and HSQC spectra.

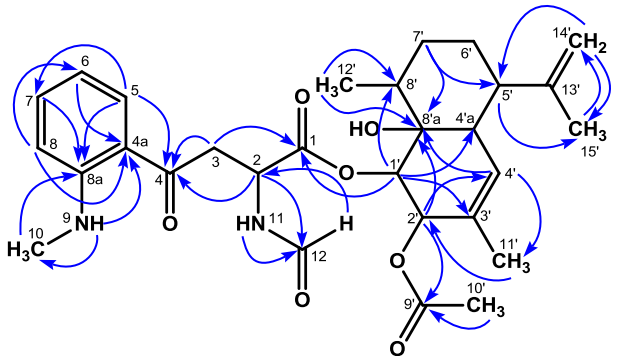

Fig. 5. Selected HMBC (H→C) of **5**.

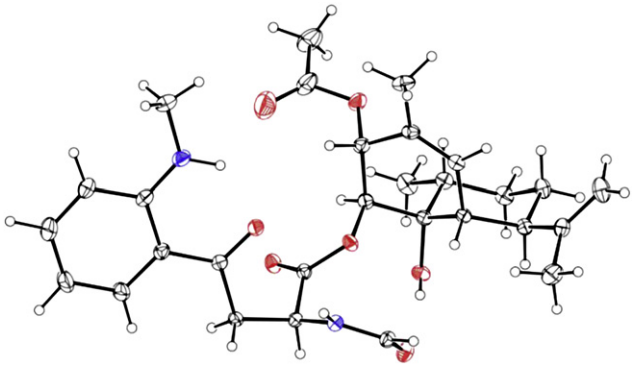

Fig. 6. ORTEP plot of the crystal structure of **5**.

We assumed that **5** should be derived from the ring opening of a terpenoid pyrrolobenzoxazine, CJ-12663, which has previously been isolated from *Aspergillus fischeri* var. *thermomutatus* ATCC 18618.[14,19] Since CJ-12663 could not be isolated from *E. chevalieri*, its chloro-derivative compound **7**, could be employed for this experiment by ring-opening with CH_2Cl_2 in the presence of *p*-toluenesulfonic acid. However, the attempt was not satisfied due to the decomposition and limitation of the sample. Therefore, the fungus was re-cultured for 30 days, and only aszonapyrone A (**9**), aszonapyrone B (**8**) and ergosterol have been isolated.

Compound **8** was obtained as colorless crystals, and its molecular formula $C_{26}H_{38}O_4$, was deduced from HRMS *m*/*z* 415.2849 $[M+H]^+$, implying nine degrees of unsaturation. The ^1H and ^{13}C NMR spectral data of **8** (Tables 1 and 2) were similar to those of aszonapyrone A (**9**), which has been reported from the fungus *Aspergillus zonatus*,[15] except for the acetoxy group at C-3, which was displaced by a hydroxyl group. A terminal alkene appeared at δ_H 4.81 (s) and 4.62 (s) and δ_C 105.7 and 148.9. To prove that **8** was a new isolated compound not derived from **9** during the isolation process, compound **9** was stirred in various solvents (CH_2Cl_2, MeOH, EtOAc, and hexane) with or without silica gel for 3 days, and no hydrolysis product **8** occurred. Thus, the new compound **8** was named aszonapyrone B.

With **8** and **9** in hand, the biosynthetic pathway of meroterpenoids **1**–**3** and aszonapyrones **8** and **9** drew our attention. According to Gonzales et al., the biosynthesis of taondiol, the closest compound to **1**, was proposed to occur by a synchronous cyclization of the terpene unit.[16] However, the finding of compounds **8** and **9** has led us to believe that **1** and other related compounds, **2** and **3** occurred by cyclization of **8** or **9**. Biosynthesis of **8** might be proposed via the cyclization of terpene units to form tricyclic rings A–C similar pathway to breviane skeleton,[20] followed by deprotonation. Cyclization of **8** should provide **1**, which transforms to **2** by acetylation. Acetylation of **8** should give **9**, which could transform to **2** and **3** by cyclization (Fig. 7).

To prove the structures of **2** and **3**, chemical transformation of **9** was performed by reaction in $CHCl_3$ in the presence of *p*-toluenesulfonic acid at room temperature overnight, yielding compounds **2** (8.7%), **3** (39.5%), and a deacetylated product, compound **12** (3.2%). These products were identical (mp, IR, NMR, and behavior on TLC) to the isolated compounds **2** and **3**. The occurrence of α-pyrone derivative **2** was formed by cyclization of the intermediate **10**. The γ-pyrone derivative **3** was proposed to be formed from

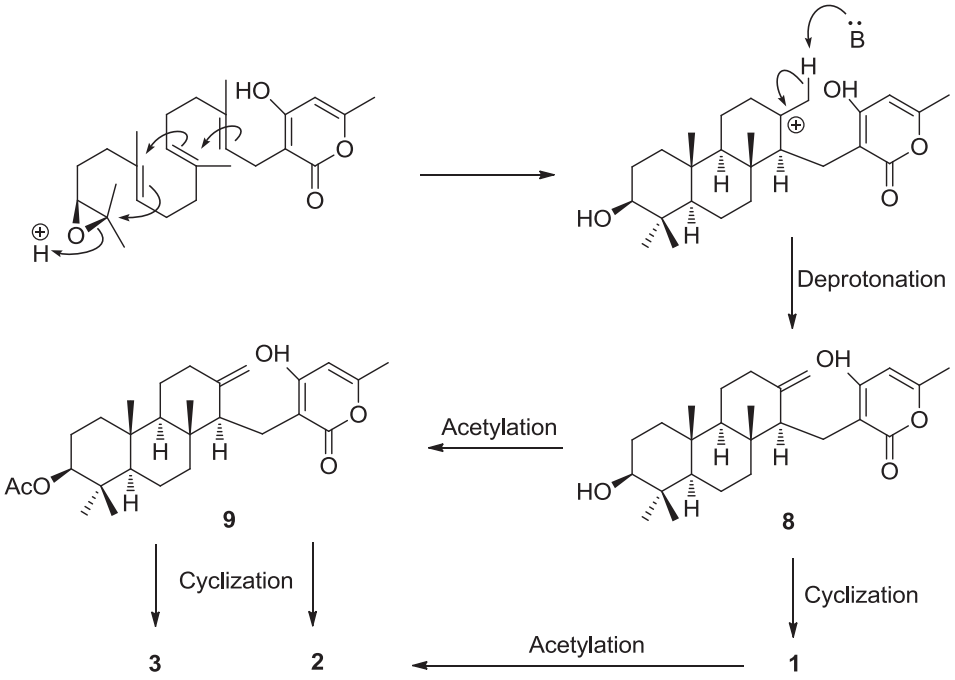

Fig. 7. Proposed biosynthesis for **1**–**3**, **8** and **9**.

tautomerization of α-pyrone **9** in acid conditions to form an intermediate **11**, followed by bond rotation and then cyclization to give **3**. Also some of **3** was hydrolyzed to yield compound **12** (Fig. 8).

The results of bioactivity tests on the isolated compounds are shown in Table 4. Compounds **4**, **5**, and **7** exhibited antimalarial activity against *Plasmodium falciparum* with IC_{50} values of 3.1, 3.4, and 6.5 μg/mL, respectively, while **3**, **5**, and **7** showed antimycobacterial activity against *M. tuberculosis* with MIC values of 6.3, 50.0, and 12.5 μg/mL, respectively. Compounds **3**, **4**, **5**, and **7** had respective IC_{50} values against the BC1 cell line of 8.7, 7.8, 5.9, and 7.6 μg/mL. In addition, compounds **2** and **5** exhibited cytotoxicity against two cancer cell lines, KB and NCI-H187 with IC_{50} values of 2.9 and 3.9 and 9.8 and 9.2 μg/mL, respectively. Among these, compound **5** was active against all tests. Its related structure **7** was also active against malaria, TB and BC1. However, **6**, which is part of **5** and **7**, was not active for all tests.

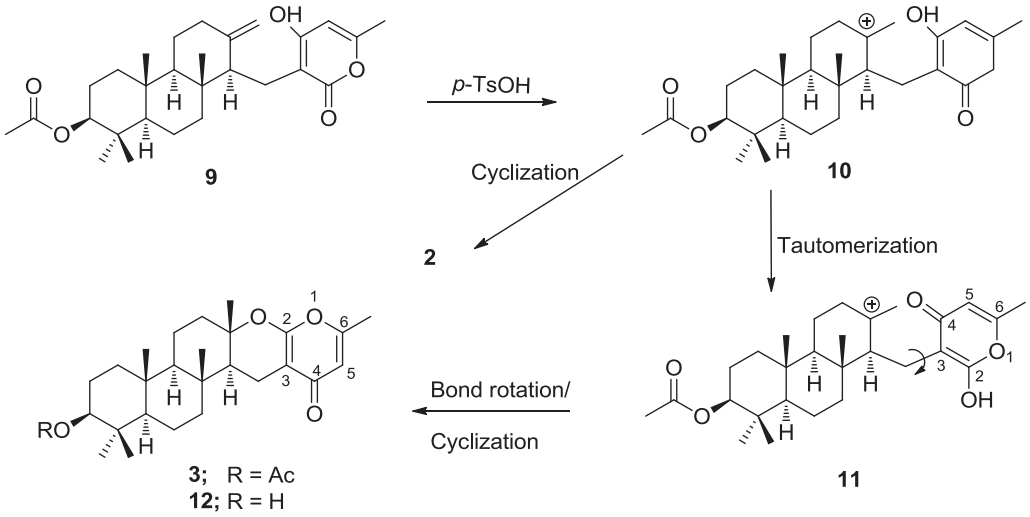

Fig. 8. Proposed mechanism for the formation of **2**, **3**, and **12** from **9**.

Table 4
Biological activities of isolated compounds **1**–**7**

Compound	Antimalarial (IC_{50}, μg/mL)	anti-TB (MIC, μg/mL)	Cytotoxicity (IC_{50}, μg/mL)		
			BC1[a]	KB[b]	NCI-H187[c]
1	Inactive	Inactive	Inactive	Inactive	Inactive
2	Inactive	Inactive	Inactive	2.9	9.8
3	Inactive	6.3	8.7	Inactive	Inactive
4	3.1	Inactive	7.8	Inactive	Inactive
5	3.4	50.0	5.9	3.9	9.2
6	Inactive	Inactive	Inactive	Inactive	Inactive
7	6.5	12.5	7.6	Inactive	Inactive
Dihydroartemisinin	0.001				
Isoniazid		0.05			
Kanamycin sulfate		2.5			
Ellipticine			0.36	0.26	0.32

[a] Human breast cancer cells.
[b] Human epidermoid carcinoma in the mouth.
[c] Small cell lung cancer.

3. Experimental section

3.1. General

Melting points were determined using a Gallenkamp melting point apparatus and were uncorrected. Optical rotations were obtained using a JASCO DIP-1000 digital polarimeter. UV spectra were measured on an Agilent 8453 UV–vis spectrophotometer. IR spectra were taken on a Perkin–Elmer Spectrum One spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Varian Mercury Plus 400 spectrometer, using residual $CHCl_3$ as an internal standard. HRMS spectra were obtained using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate masses. Column chromatography and preparative TLC were carried out on silica gel 60 (230–400 mesh) and PF_{254}, respectively.

3.2. Fungal material

The fungus was collected from rhizosphere soil of para rubber tree, 40 cm soil depth at Surathani Province, Thailand, in 2007 and was identified by one of the authors (K. S.). A voucher specimen (EuC01) was deposited at the Department of Plant Pest Management, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The fungus was cultivated in Potato Dextrose Broth by standing at 25–28 °C for 30 to 40 days.

3.3. Extraction and isolation

3.3.1. Cultivation for 40 days. Dried fungal biomass of *E. chevalieri* (140 g) cultured for 40 days was ground into powder and then extracted successively with hexane (3×700 mL), EtOAc (3×700 mL), and MeOH (3×700 mL). Removal of solvents under reduced pressure gave crude hexane (13.9 g, 9.9%), EtOAc (6.8 g, 4.8%), and MeOH (6.3 g, 4.5%) extracts, respectively.

The hexane extract (13.9 g) was subjected to silica gel column chromatography (CC), eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH, to give ten combined fractions, HF_1–HF_{10}. Fraction HF_5 was dissolved in MeOH, the solid precipitate was filtered out and recrystallized from EtOAc–hexane to yield colorless crystals of **2** (10.0 mg). Further separation of the residue by silica gel flash column chromatography (FCC), eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH, yielded eight combined fractions, $HF_{5.1}$–$HF_{5.8}$. Fraction $HF_{5.3}$ was further purified by FCC, eluted with a gradient system of hexane–CH_2Cl_2 and CH_2Cl_2–EtOAc, to give six fractions, $HF_{5.3.1}$–$HF_{5.3.6}$. Fraction $HF_{5.3.5}$ was dissolved in EtOAc, the precipitate was filtered out and was recrystallized from CH_2Cl_2–hexane to give colorless crystals of **6** (5.0 mg). Fraction $HF_{5.4}$ was filtered and a solid was recrystallized from EtOAc–hexane to yield an additional amount of **2** (94.6 mg). The residue was further separated by FCC, eluted with a gradient system of EtOAc–CH_2Cl_2 and MeOH–EtOAc, to give an additional amount of **2** (105.3 mg), a bright green solid of **4** (25.9 mg) and colorless crystals of **6**

(131.4 mg). Fraction $HF_{5.5}$ was dissolved in MeOH, the solid precipitate was filtered out and further recrystallized from EtOAc–hexane to yield an additional amount of **2** (326.6 mg). The filtrate was further separated by FCC, eluted with a gradient system of hexane–EtOAc, to yield an additional amount of **4** (10.4 mg). Fraction $HF_{5.6}$ was purified by FCC, eluted with a gradient system of hexane–CH_2Cl_2 (50–100%) and CH_2Cl_2–EtOAc, to give colorless needles of **1** (4.7 mg). Fraction HF_6 was subjected to silica gel CC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH, to yield four combined fractions, $HF_{6.1}$–$HF_{6.4}$. Fraction $HF_{6.2}$ was dissolved in MeOH, the solid precipitate was filtered out and was recrystallized from EtOAc–hexane to give an additional amount of **2** (6.5 mg). Fraction $HF_{6.3}$ was filtered and a solid was recrystallized from EtOAc to yield an additional amount of **1** (24.0 mg). The residue was further separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc, to give colorless crystals of **7** (32.4 mg). Fraction $HF_{6.4}$ was subjected to FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and EtOAc–MeOH, to give five combined fractions, $HF_{6.4.1}$–$HF_{6.4.5}$. Fraction $HF_{6.4.3}$ was recrystallized from MeOH to yield yellow needles of **5** (48.6 mg). Fraction $HF_{6.4.4}$ was separated by FCC, eluted with a gradient system of hexane–EtOAc to give an additional amount of **7** (55.5 mg). Fraction HF_8 was recrystallized from hexane–CH_2Cl_2 to yield a white solid of **3** (77.7 mg).

The EtOAc extract (6.8 g) was subjected to silica gel FCC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH, to give five combined fractions, EF_1–EF_5. Fraction EF_2 was separated by FCC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH, to yield four combined fractions, $EF_{2.1}$–$EF_{2.4}$. Fraction $EF_{2.2}$ was further separated by FCC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH (10–20%) to yield nine combined fractions, $EF_{2.2.1}$–$EF_{2.2.9}$. Fractions $EF_{2.2.4}$, $EF_{2.2.6}$ and $EF_{2.2.8}$ were recrystallized from EtOAc–hexane and gave an additional amount of **2** (204.7 mg), **4** (5.1 mg) and **6** (10.9 mg), respectively. Fraction $EF_{2.3}$ was separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc to yield an additional amount of **1** (17.6 mg). Fraction EF_3 was further separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc, to yield five combined fractions, $EF_{3.1}$–$EF_{3.5}$. Fraction $EF_{3.2}$ gave an additional amount of **1** (14.2 mg). Fraction $EF_{3.3}$ was separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc to yield four combined fractions, $EF_{3.3.1}$–$EF_{3.3.4}$. Fraction $EF_{3.3.2}$ yielded an additional amount of **5** (166.5 mg). Fraction $EF_{3.3.4}$ was recrystallized from MeOH to give an additional amount of **7** (28.8 mg). Fraction EF_4 was separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and EtOAc–MeOH, to yield an additional amount of **3** (38.5 mg).

The crude MeOH extract (6.3 g) was subjected to silica gel FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and EtOAc–MeOH to give five combined fractions, MF_1–MF_5. Fraction MF_2 was separated by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and MeOH–EtOAc (20–40%) to yield seven combined fractions, $MF_{2.1}$–$MF_{2.7}$. Fraction $MF_{2.2}$ yielded an additional amount of **2** (50.4 mg). Fraction $MF_{2.3}$ was filtered and the solid was recrystallized from EtOAc–CH_2Cl_2, to give an additional amount of **1** (9.6 mg). The filtrate was purified by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and EtOAc–MeOH, to give an additional amount of **2** (32.7 mg) and **3** (7.4 mg).

3.3.2. Cultivation for 30 days. Dried fungal biomass of *E. chevalieri* (300 g) cultured for 30 days was ground into powder and then extracted successively with hexane (3×700 mL), EtOAc (3×700 mL), and MeOH (3×700 mL). Removal of solvents under reduced pressure gave crude hexane (26.8 g, 8.9%), EtOAc (18.4 g, 6.1%), and MeOH (15.5 g, 5.2%) extracts, respectively. The hexane extract contained only long chain fatty acids while the MeOH extract contained mostly sugar. The EtOAc extract was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH to give six combined fractions, E_1–E_6. Fraction E_2 was recrystallized from EtOAc–hexane to yield ergosterol (59.8 mg). Fraction E_3 was dissolved in a mixture of MeOH–EtOAc to give a white precipitate of **9** (199.2 mg). Fraction E_4 was dissolved in a mixture of MeOH–EtOAc to give an additional amount of **9** (315.4 mg). The filtrate was further purified by FCC, eluted with a gradient system of hexane–EtOAc and EtOAc–MeOH to yield seven subfractions, $E_{4.1}$–$E_{4.7}$. Subfraction $E_{4.3}$ was recrystallied from MeOH–EtOAc to give an addition amount of **9** (207.7 mg). Subfraction $E_{4.4}$ was recrystallied from MeOH–EtOAc to give colorless needles of **8** (34.2 mg). Fraction E_5 was purified by FCC, eluted with a gradient system of CH_2Cl_2–EtOAc and EtOAc–MeOH to yield an additional amount of colorless needles of **8** (36.2 mg).

*3.3.3. Chevalone A (**1**).* Colorless crystals; mp 303–305 °C; R_f (50% EtOAc–hexane) 0.43; $[\alpha]_D^{24}$ −121.6 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 208 (4.28), 286 (3.83); IR (KBr) ν_{max}: 3467, 2988, 2944, 2871, 2845, 1686, 1644, 1574, 1446 cm^{-1}; ^1H and ^{13}C NMR see Tables 1 and 2; HRMS (ESI): $[M+Na]^+$, found 437.2669. $C_{26}H_{38}O_4$ Na requires 437.2668.

*3.3.4. Chevalone B (**2**).* Colorless crystals; mp 162–165 °C; R_f (50% EtOAc–hexane) 0.60; $[\alpha]_D^{23}$ −50.8 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 208 (4.30), 286 (4.06); IR (KBr) ν_{max}: 2943, 2868, 1733, 1702, 1652, 1588, 1447, 1406, 1379, 1239 cm^{-1}; ^1H and ^{13}C NMR see Tables 1 and 2; HRMS (ESI): $[M+Na]^+$, found 479.2771. $C_{28}H_{40}O_5Na$ requires 479.2773.

*3.3.5. Chevalone C (**3**).* White solid; mp 118–119 °C; R_f (50% EtOAc–hexane) 0.15; $[\alpha]_D^{23}$ −120.6 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 207 (4.12), 245 (3.87), 259 (3.90); IR (KBr) ν_{max}: 2941, 2860, 1734, 1669, 1637, 1596, 1427, 1386, 1240, 1188 cm^{-1}; ^1H and ^{13}C NMR see Tables 1 and 2; HRMS (ESI): $[M+Na]^+$, found 479.2773. $C_{28}H_{40}O_5Na$ requires 479.2773.

*3.3.6. Chevalone D (**4**).* White solid; mp 227–229 °C; R_f (50% EtOAc–hexane) 0.65; $[\alpha]_D^{22}$ −46.0 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 204 (4.05), 298 (4.20); IR (KBr) ν_{max}: 2944, 2846, 1729, 1702, 1653, 1594, 1369, 1258 cm^{-1}; ^1H and ^{13}C NMR see Tables 1 and 2; HRMS (ESI): $[M+Na]^+$, found 467.2773. $C_{27}H_{40}O_5Na$ requires 467.2773.

*3.3.7. Compound **5**.* Yellow needles; mp 140–142 °C; R_f (50% EtOAc–hexane) 0.36; $[\alpha]_D^{22}$ −171.2 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 203 (4.30), 230 (4.35), 260 (3.83); IR (KBr) ν_{max}: 3440, 3331, 3078, 2959, 2928, 1754, 1725, 1670, 1644, 1569, 1523, 1427, 1387, 1218, 1161 cm^{-1}; ^1H and ^{13}C NMR see Table 3; HRMS (ESI): $[M+H]^+$, found 527.2842. $C_{29}H_{39}N_2O_7$ requires 527.2759.

*3.3.8. Compound **6**.* Colorless crystals; mp 160–163 °C (from CH_2Cl_2–hexane) (lit.[14] 171–173 °C, from from Et_2O); R_f (50% Et_2O/hexane) 0.21 (lit[14] 0.22); $[\alpha]_D^{22}$ −51.2 (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max}: 3445, 3325, 2974, 2958, 2930, 1733, 1641, 1451, 1404, 1369 cm^{-1}; HRMS (ESI): $[M+H]^+$, found 295.1926. $C_{17}H_{27}O_4$ requires 295.1928.

*3.3.9. Compound **7**.* White solids; mp 121–125 °C; R_f (50% EtOAc–hexane) 0.43; $[\alpha]_D^{24}$ −78.1 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 208 (4.32), 248 (3.72), 287 (3.31); IR (KBr) ν_{max} 3436, 2971, 2922, 1745, 1642, 1439, 1372, 1238, 1177 cm^{-1}; HRMS (ESI): $[M+Na]^+$, found 583.1836 and 585.2085. $C_{29}H_{37}N_2O_7ClNa$ requires 583.2186 and 585.2186.

*3.3.10. Compound **8**.* White solids; mp 173–175 °C; R_f (20% EtOAc–CH_2Cl_2) 0.23; $[\alpha]_D^{24}$ −87.3 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 207 (4.1), 290 (4.3); IR (KBr) ν_{max} 3290, 2944, 2922, 1677, 1652, 1581, 1449, 1408, 1257, 1248, 1040 cm^{-1}; ^1H and ^{13}C NMR see

Tables 1 and 2; HRMS (ESI): $[M+H]^+$, found 415.2849. $C_{26}H_{39}O_4$ requires 415.2848.

3.3.11. Cyclization of ***9****.* To a solution of **9** (31.1 mg) in dry $CHCl_3$ (3 mL) was added *p*-toluenesulfonic acid (9.4 mg) and the solution was stirred at room temperature for 12 h. Water was added and the reaction mixture was extracted with $CHCl_3$ (10 mL×3). The organic layer was combined, washed with 10% aq $NaHCO_3$ and then water and dried over anhydrous Na_2SO_4. The filtrate was evaporated to dryness and the residue was separated by preparative TLC (50% EtOAc–hexane) to give **2** (2.7 mg, 8.7%, R_f 0.58); **3** (12.3 mg, 39.5%, R_f 0.15) and **12** (0.9 mg, 3.2%, R_f 0.10). IR and NMR spectra of the products were identical to the compounds isolated **2** and **3** (Tables 1 and 2).

3.4. X-ray crystallographic analyses of 1 and 5

Colorless crystals of **1** and **5** were obtained in the mixture of EtOAc/hexane by slow evaporation. X-ray diffraction data were measured on a Bruker–Nonius kappaCCD diffractomer with graphite monochromated Mo Kα radiation (λ=0.71073 Å) at 298(2) K. The structure was solved by direct methods by SIR97,[21] and refined with full-matrix least-squares calculations on F2 using SHELXL-97.[22]

3.4.1. X-ray data of ***1****.* $C_{26}H_{38}O_4$, MW=414.59, orthorhombic, dimensions: 0.25×0.15×0.10 mm^3, D=1.218 g/cm^3, space group $P2_12_12_1$, Z=4, a=7.6809(5), b=11.5023(8), c=25.594(2) Å, V=2261.2(3) $Å^3$, reflections collected/unique: 14,182/1924 (R_{int}=0.0382), number of observations [$>2\sigma(I)$] 1728, final R indices [$I>2\sigma(I)$]: R_1=0.0466, wR_2=0.1179.

3.4.2. X-ray data of ***5****.* $C_{29}H_{38}N_2O_7$, MW=526.63, orthorhombic, dimensions: 0.15×0.10×0.10 mm^3, D=1.247 g/cm^3, space group $P2_12_12_1$, Z=4, a=9.97890(10), b=11.2446(2), c=24.9984(4) Å, V=2805.04(7) $Å^3$, reflections collected/unique: 14,818/7140 (R_{int}=0.034), number of observations [$>2\sigma(I)$] 6132, final R indices [$I>2\sigma(I)$]: R_1=0.0545, wR_2=0.1533.

Crystallographic data of compounds **1** and **5** have been deposited at the Cambridge Crystallographic Data Center under the reference numbers CCDC 812972 and 812973. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk).

3.5. Bioassay

3.5.1. Antimalarial assay. Antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multidrug resistant strain), using the method of Trager and Jensen.[23] Quantitative assessment of malarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al.[24] The inhibitory concentration (IC_{50}) represents the concentration, which causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound, dihydroartemisinin, exhibited an IC_{50} value of 1.0 ng/mL.

3.5.2. Antimycobacterial assay. Antimycobacterial activity was assessed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[25] The standard drugs, isoniazid and kanamycin sulfate, showed respective MIC values of 0.05 and 2.5 μg/mL.

3.5.3. Cytotoxicity assay. Cytotoxicity assays against human breast cancer (BC1), human epidermoid carcinoma (KB) and human small cell lung cancer (NCI-H187) cell lines were performed employing the colorimetric method as described by Skehan et al.[26] The reference substance was ellipticine, which showed IC_{50} values of 0.26, 0.36, and 0.32 μg/mL, respectively.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.05.066.

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