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Journal:	*Journal of Natural Products*
Manuscript ID:	np-2015-005616
Manuscript Type:	Note
Date Submitted by the Author:	24-Jun-2015
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Highly Oxygenated Chromones from Mangrove-Derived Endophytic Fungus *Rhytidhysteron rufulum*

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ABSTRACT: Five new highly oxygenated chromones, rhytidchromones A–E (**1**–**5**), were isolated from the culture broth of a mangrove-derived endophytic fungus, *Rhytidhysteron rufulum*, isolated from Thai *Bruguiera gymnorrhiza*. Their structures were determined by analysis of 1D and 2D NMR spectroscopic data. The structure of compound **1** was further confirmed by single-crystal X-ray diffraction analysis. Compounds were tested for cytotoxicity against human cancer cell lines. All compounds, except for **4**, displayed weak or moderate cytotoxicity against Kato-3 cell lines with IC_{50} values ranging from 16.0 to 23.3 μM. Compounds **1** and **3** was moderately active against MCF-7 cells with IC_{50} values of 19.3 and 17.7 μM, respectively.

Endophytic fungi, microorganisms residing in the healthy tissue of their host harmoniously without causing any apparent negative effect,[1–2] are known as an exceptional valuable resource for the discovery of structurally interesting and biologically active secondary metabolites, some of which are promising candidates for drug development or agrochemical applications.[3–6] Currently, increasing number of fungal endophytes have been isolated and their metabolites are receiving considerable attention, a number of structurally unique and biologically active compounds have been thus obtained from the cultures of them.[7–11] Among plant-derived fungi, those associated with marine habitat including mangrove plants have received much interest from natural product researchers due to the unique ecosystem.[12–15] Moreover, the largest mangrove formations are found in Thailand, other than Bangladesh, Brazil, Indonesia and India. This prompted us to embark on the study of bioactive metabolites from Thai mangrove-derived fungi.[16–19]

Recently, a chemical investigation of endophytic fungal strain BG2-Y was performed. This fungus was isolated from the leaves of the Thai mangrove plant *Bruguiera gymnorrhiza* and was identified as *Rhytidhysteron rufulum*. Five new chromones, namely rhytidchromones A–E (**1**–**5**), were obtained from the EtOAc extract of the culture broth grown in Sabouraud dextrose broth (SDB). These compounds contained the highly oxygenated side chain in their structure. Herein, we describe the isolation, structure elucidation, and cytotoxic activity against human cancer cell lines of these metabolites.

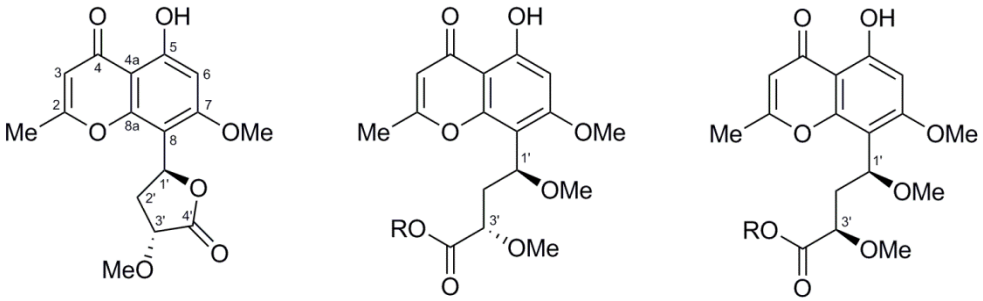


Figure1. Structures of compounds **1**–**5**.

RESULTS AND DISCUSSION

Rhytidchromone A (**1**), obtained as colorless crystals, was assigned a molecular formula of $C_{16}H_{16}O_7$ by the HRESIMS ion at *m/z* 343.0784 $[M + Na]^+$, implying nine degrees of unsaturation. The ^1H NMR spectrum (Table 1) contained signals for one phenolic proton bonded to a carbonyl group (δ_H 13.04 s), one aromatic proton (δ_H 6.36 s) attributed to a pentasubstituted

aromatic ring, one olefinic proton (δ_H 6.04 s) assigned to a trisubstituted olefin, two methoxy singlets (δ_H 3.64 and 3.89), and one methyl singlet (δ_H 2.35). Combined analysis of ^{13}C NMR and HSQC data further revealed the presence of one conjugated ketone carbonyl (δ_C 182.5), one ester carbonyl (δ_C 174.9), six quaternary carbons (δ_C 167.1, 163.4, 162.9, 155.7, 104.9, and 103.4), four methines (δ_C 108.7, 95.2, 76.1, and 69.2), two methoxys (δ_C 58.6 and 56.3), one methylene (δ_C 33.6), and one methyl (δ_C 20.4). In addition, the UV absorption maxima at 237, 255, 290, and 319 indicated that compound **1** should be a chromone derivative. According to its double bond equivalent (DBE), the seven units of unsaturation for a chromone skeleton and one unit for ester carbonyl, suggested that compound **1** contains an additional ring in the structure. The ^1H–^1H COSY spectrum revealing the existence of one isolated spin system, CH-1′–CH_2-2′–CH-3′, as well as the HMBC cross-peaks from H-1′ (δ_H 6.00, dd, J = 7.2, 9.6 Hz) and H-3′ (δ_H 4.29, t, J = 10.0 Hz) to an ester carbonyl at δ_C 174.9 (C-4′) led to the corroboration of the γ-lactone ring (Figure 2). Further HMBC correlations from H-1′ to C-7, C-8, and C-8a indicated that the C-1′ oxymethine of the γ-lactone ring was attached on C-8 of the chromone nucleus. One methoxy group (δ_H 3.64, δ_C 58.6) was located on C-3′ due to its HMBC correlation with C-3′, whereas another methoxy group (δ_H 3.89, δ_C 56.3) was placed on C-7 from its HMBC cross-peak to C-7. The singlet methyl proton (δ_H 2.35) showed HMBC correlations with C-2 and C-3, indicating the location of the methyl group at C-2. Additionally, observed HMBC correlations of 5-OH with C-4a, C-5, and C-6 verified the location of the hydroxy group on C-5. This assignment was also supported by the appearance of the hydroxy proton downfield due to a chelation effect. Thus, the structure of **1** was established as shown. The proposed structure of **1** was further confirmed as well as its relative configuration were established by single-crystal X-ray diffraction analysis using Mo Kα radiation, and a perspective ORTEP plot is depicted in Figure 3. To the best our knowledge, chromones possessing a γ-lactone ring as a side chain seem to be rare. The only precedents include lachnones C–D from a filamentous fungus *Lachnum* sp.[20]

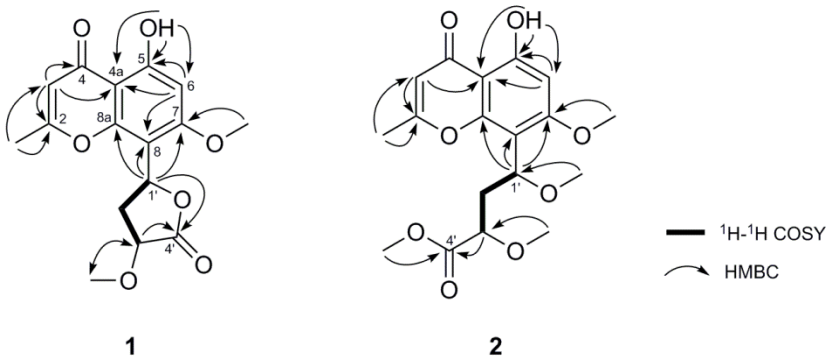


Figure 2. ^1H–^1H COSY and key HMBC correlations of **1** and **2**.

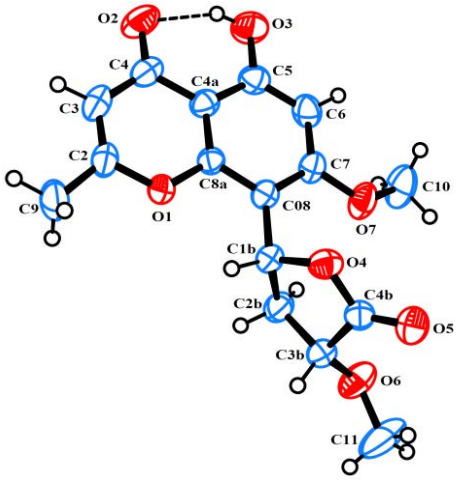


Figure 3. ORTEP drawing of compound **1**.

Rhytidchromone B (**2**) was obtained as a pale yellow gum. The HRESIMS afforded an $[M + Na]^+$ ion peak at *m/z* 389.1207, consistent with a molecular formula of $C_{18}H_{22}O_8$, indicating eight units of unsaturation. The ^1H and ^{13}C NMR spectroscopic data (Table 1) as well as the HMBC and ^1H–^1H COSY correlations (Figure 2) were similar to those of compound **1**. However, compound **2** had no the γ-lactone ring in the structure due to the lack of the HMBC correlation from H-1′ to an ester carbonyl at δ_C 172.8. This was correlated to its DBE, implying that the structure of **2** contained the oxygenated alkyl side chain in place of the γ-lactone ring in **1**. In addition, the additional proton signals of two methoxy groups were observed. The HMBC correlation of the methoxy protons at δ_H 3.17 with C-1′ allowed the attachment of this methoxy on the C-1′ carbon, while the methoxy protons at δ_H 3.73 displayed the HMBC cross-peak with the ester carbonyl carbon (δ_C 172.8), thus indicating the presence of the methyl ester at C-4′. The relative configurations of compound **1** were determined by on the basis of the NOESY data. In the NOESY spectrum (Figure S12, see Supporting information), the NOE correlation between 2-Me and 4′-OMe, in combination with the lack of correlation between 1′-OMe and 3′-OMe, indicated the different orientation of the methoxy groups at C-1′ and C-3′. Thus, rhytidchromone B (**2**) was established as shown.

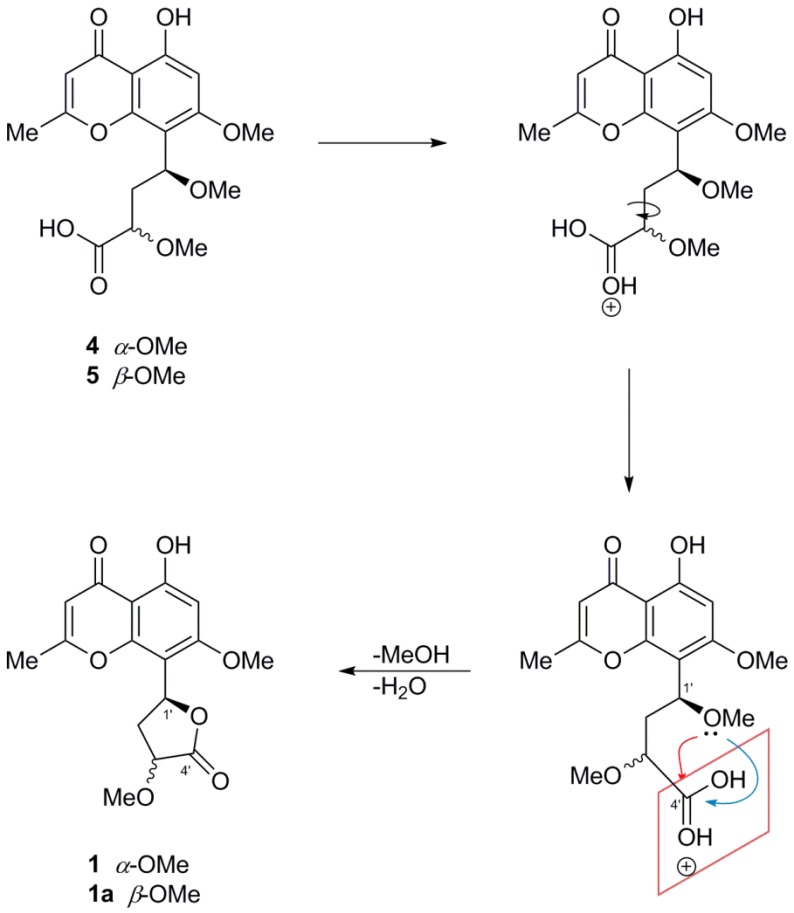
Rhytidchromone C (**3**), a pale yellow gum, had the molecular formula of $C_{18}H_{22}O_8$ from the HRESIMS ion peak at *m/z* 389.1211, being the same as that of compound **2**. The ^1H and ^{13}C NMR spectroscopic data (Table 1) of compound **3** were closely resembled those of compound **2**. Moreover, detailed 2D NMR analysis revealed that they shared the same planar structure. The obvious differences were the chemical shift of the proton signals for the side chain. This indicated compounds **2** and **3** differed in the configurations at C-1′ and/or C-3′. The observed NOE correlation (Figure S19, see Supporting information) between 1′-OMe and 3′-OMe as well

as between 2-Me and 4′-OMe supported the same orientation of the methoxy groups at C-1′ and C-3′. This assignment was supported by the opposite signs of their specific rotations ($[\alpha]^{20}_{D}$ – 24.8 for **2** vs +7.2 for **3**).

Rhytidchromone D (**4**), a pale yellow gum, had the molecular formula of $C_{17}H_{28}O_8$ as established by HRESIMS at *m/z* 375.1050. The NMR spectroscopic data (Table 2) of compound **4** were closely related to those of compound **2**, except for the disappearance of signals for a methoxy group. The location of three methoxy groups at C-7, C-1′, and C-3′ was confirmed by the HMBC experiment. Thus, compound **4** was identified as a carboxylic acid derivative of rhytidchromones B or C from its molecular formula, although the intensity of the carboxylic carbonyl in the ^{13}C NMR spectrum was quite low and no HMBC cross-peak from any proton to the carbonyl carbon was observed (Figure S21, see Supporting information). Finally, the configurations at C-1′ and C-3′ in compound **4** was identified as same as those in rhytidchromone B (**2**) from the almost identical chemical shift, splitting, and coupling patterns of H-1′, H_2-2′, and H-3′, as well as the same sign of their specific rotations ($[\alpha]^{20}_{D}$ –18.2 for **4** vs – 24.8 for **2**).

Rhytidchromone E (**5**) was obtained as a pale yellow gum. Its molecular formula of $C_{17}H_{28}O_8$ was deduced by the HRESIMS ion peak at *m/z* 375.1061, being the same that of compound **4**. The NMR spectroscopic data (Table 2) of compound **5** were very similar to those of compound **3**. The difference was the loss of signals for a methoxy group. Interpretation of 2D NMR spectroscopic data established the same planar structure as compound **4**. Similarly, the identical chemical shift, splitting, and coupling patterns of H-1′, H_2-2′, and H-3′ to those in compound **3** and the same sign of their specific rotations led to the designation of the same configurations at C-1′ and C-3′.

The existence of the carboxylic acid functionality as C-4′ in both rhytidchromones D (**4**) and E (**5**) could be verified by an additional evidence. Both compounds **4** and **5** were gradually converted into the mixture between **1** and **1a**, a 3′β-OMe epimer, in $CDCl_3$ over a few days (Figures S30 and S31, see Supporting information). The relative configuration of **1a** at C-1′ and C-3′ was determined by the NOE correlations. The NOESY spectrum (Figure S32, see Supporting information) of **1** and **1a** mixture showed correlations of H-1′ (δ_H 6.00)/H-2β′ (δ_H 2.86) and H-2′β/H-3′ (δ_H 4.29) for **1**, consistent with its ORTEP diagram. In the case of correlations for **1a**, the correlations of H-1′ (δ_H 6.25)/H-2β′ (δ_H 2.70) and H-2′α (δ_H 2.52)/H-3′ (δ_H 4.29), with the lack of correlation between H-2′β/H-3′, suggested the α-orientation of 3′-OMe. This indicated, upon acidity of $CDCl_3$, the carboxylic carbonyl is activated and the lactone ring is subsequently formed by the attack of the lone pair of 1′-OMe to the activated carbonyl carbon from both *re*- and *si*-face to afford compounds **1** and **1a** as shown in Scheme 1. Therefore the biosynthetic origin of **1** would probably be compounds **4** and **5** as evident.



Scheme 1. Proposed mechanism for compounds **1** and **1a**.

The isolated compounds **1**–**5** were evaluated for their cytotoxicity against human breast (MCF-7), liver (Hep-G2), cervical (CaSki), and gastric (KATO-3) cancer cell lines (Table 3) by MTT method. Compounds **1** and **5** showed weak cytotoxicity against MCF-7 and Kato-3 with IC_{50} values of 19.3 and 23.3 μM, respectively, while compound **2** and **4** was weakly and moderately active against only Kato-3 cells. Compound **5** showed moderate activity on MCF-7 and Kato-3 with IC_{50} values of 17.7 and 16.0 μM. Only compound **3** did not show any significant activity against all tested cell lines.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting point was determined on a melting point M565 apparatus and is uncorrected. Optical rotations were measured on a JASCO P-1010 polarimeter. UV spectra were recorded on a Spekol 1200 Analytic Jena spectrophotometer. IR spectra were recorded on a Bruker ALPHA FT-IR spectrometer. NMR spectra were acquired on a Varian Mercury-400 Plus NMR spectrometer with TMS as reference. HRESIMS were performed on a Bruker micrOTOF. Single-crystal X-ray diffraction analysis was performed on a Bruker APEX II diffractometer. Silica gel (Merck, 230-400 mesh), Sephadex LH20 (GE Healthcare), and ODS (WAKO®100C18) were used for open-column chromatography. Analytical TLC was performed using precoated silica gel 60 GF254 plates (Merck).

Fungal Material. The fungal strain BG2-Y was isolated from the healthy leaves of *B. gymnorrhiza* collected from Pak Nam Pran, Prachuab Kiri Khan Province, Thailand, in July 2012. The fungus was identified based on the internal transcribed spacer (ITS1-5.8S-ITS2) rDNA and on partial 28S rRNA analyses using universal fungal primers. It partial 28S rRNA sequence (GenBank accession number KP994148) showed affinity within class Dothideomycetes; sub-class Pleosporomycetidae; order Hysteriales and family Hysteriaceae. Its ITS sequence (GenBank accession number KP994147) of this isolate demonstated a high similarities of 98–99% and matched with *Rhytidhysteron rufulum* from GenBank (http://www.ncbi.nlm.nih.gov/) and CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/). Thus, the fungus was then identified as *Rhytidhysteron rufulum*.

Fermentation, Extraction, and Isolation. The fungal strain was cultured on potato dextrose agar at room temperature for 7 days. Then, six pieces (6 × 6 mm^2) of agar plugs were inoculated in a 1000 mL Erlenmeyer flask containing 200 mL of Sabouraud dextrose broth (SDB) (× 25). Ultimately, the fungus was statically cultured at room temperature for 21 days under static condition. After incubation, the fungal culture (5 L) was filtered to remove mycelia, and the culture broth was extracted twice with an equal volume of EtOAc. The combined EtOAc extract was concentrated under reduced pressure to afford a dark brown gum (4.62 g). The separated mycelia were successively extracted with hexane and EtOAc (× 3 for each). The EtOAc soluble part was evaporated under reduced pressure and dried under vacuum to yield a dark brown gum (0.87 g).

The EtOAc crude extract of culture broth (4.62 g) was subjected to Sephadex LH20 column chromatography and eluted with MeOH to yield five fractions (A1–A5). Fraction A3 (3.58 g) was chromatographed on a Sephadex LH20 column eluting with MeOH again to yield six subfractions (B1–B6). Subfraction B4 (0.36 g) was further isolated by silica gel column chromatography, eluting with a 1:1 mixture of EtOAc-hexane to yield nine fractions (C1–C9). Fraction C2 (72.0 mg) were subsequently purified by Sephadex LH20 column chromatography, eluting with MeOH to afford compound **1** (28.7 mg). Fraction A2 (0.36 g) was subjected to reverse phase ODS column chromatography with MeOH:H_2O (1:1) to yield seven subfractions

(D1–D7). Subfraction D6 (95.0 mg) was chromatographed over a silica gel column and eluted with MeOH-CH_2Cl_2 (1:19), then the major fraction was further purified by a reverse phase ODS column eluting with MeOH:H_2O (3:7) to yield compounds **2** (4.5 mg) and **3** (4.0 mg). Fraction B5 (1.63g) was subjected to a silica gel column eluting with EtOAc-hexane (2:3), and subsequently chromatographed on a Sephadex LH20 column eluting with MeOH to afford compound **4** (4.0 mg).

The mycelial EtOAc crude extract (0.87 g) was chromatographed on a Sephadex LH20 column eluting with MeOH to yield six fractions (E1–E6). Fraction E3 (0.18 g) was further fractionated by ODS column chromatography eluting with MeOH:H_2O (1:1) to give xx subfractions (F1–F7). Subfraction F7 (49.1 mg) was further purified by silica gel column chromatography eluting with MeOH-CH_2Cl_2 (1:19) to afford compound **5** (4.0 mg).

*Rhytidchromone A (**1**):* colorless crystals; mp 149–150 ^{o}C; UV (MeOH) λ_{max} (log ε) 237 (3.53), 255 (3.48), 290 (3.20), 319 (3.06); IR (neat) υ_{max} 3358, 1732, 1640, 1423, 1212 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 343.0784 $[M + Na]^+$ (calcd. for $C_{16}H_{16}O_7Na$, 343.0788).

*Rhytidchromone B (**2**):* pale yellow gum; UV (MeOH) λ_{max} (log ε) 246 (3.58), 265 (3.64), 291 (3.50); IR (neat) υ_{max} 3346, 1743, 1638, 1539, 1404, 1242 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 389.1207 $[M + Na]^+$ (calcd. for $C_{18}H_{22}O_8Na$, 389.1212).

*Rhytidchromone C (**3**):* pale yellow gum; UV (MeOH) λ_{max} (log ε) 245 (3.62), 266 (3.68), 292 (3.58); IR (neat) υ_{max} 3365, 1721, 1642, 1536, 1403, 1236 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 389.1211 $[M + Na]^+$ (calcd. for $C_{18}H_{22}O_8Na$, 389.1212).

*Rhytidchromone D (**4**):* pale yellow gum; UV (MeOH) λ_{max} (log ε) 247 (3.59), 265 (3.64), 289 (3.52); IR (neat) υ_{max} 3349, 1727, 1639, 1535, 1403, 1236 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 375.1056 $[M + Na]^+$ (calcd. for $C_{17}H_{28}O_8Na$, 375.1061).

*Rhytidchromone E (**5**):* pale yellow gum; UV (MeOH) λ_{max} (log ε) 244 (3.55), 260 (3.61), 289 (3.46); IR (neat) υ_{max} 3359, 1725, 1635, 1540, 1405, 1235 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 375.1050 $[M + Na]^+$ (calcd. for $C_{17}H_{28}O_8Na$, 375.1061).

*Compound **1a**:* ^{1}H NMR data δ_H 13.07 (brs, 5-OH), 6.38 (s, H-6), 6.25 (dd, *J* = 4.8, 6.8 Hz, H-1′), 6.06 (s, H-3), 4.19 (dd, *J* = 2.8, 6.4 Hz, H-3′), 3.89 (s, 7-OMe), 3.61 (s, 3′-OMe), 2.69 (ddd, *J* = 5.2, 6.4, 11.2 Hz, H-2′), 2.52 (m, H-2′).

X-ray Crystallograpic Analysis of Rhytidchromone A (1). All crystallographic data were collected at 298 K on a Bruker APEX II diffractometer with Mo Kα radiation (λ = 0.71073). The structures were solved by direct methods using SHELXS-97 and refined by full-matrix least-squares on all F^2 data using SHELXS-97 to final R values.[21–22] All hydrogen atoms

were added at calculated positions and refined using a rigid model. Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre with the deposition numbers CCDC 1406338. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/data_request/cif, by e-mailing data_request@ccdc.cam.ac.uk, or by contacting the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK: fax: +44 1223 336033. Crystal data of rhytidchromone (**1**): colorless crystal, $C_{16}H_{16}O_7$, M_r = 320.30, monoclinic, a = 8.0040(4) Å, b = 8.6152(4) Å, c = 11.7898(4) Å, space group $P2_1$, Z = 2, V = 766.45(6) Å^3, and μ (Mo Kα) = 0.11 mm^{-1}. Crystal dimensions: 0.50 × 0.20 × 0.20 mm. Independent reflections: 2347 (R_{int} = 0.016). The final R_1 values were 0.030, wR_2 = 0.060 ($I > 2\sigma(I)$).

Cytotoxic Assay. Cytotoxicity of isolated compounds against human breast (MCF-7), liver (Hep-G2), cervical (CaSki), and gastric (Kato-3) cancer cell lines was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide) assay according to previously described procedures.[23] Briefly, freshly trypsinized cell suspensions were seeded into 96-well culture plates at densities of 1×10^4 cells in the presence or absence of test compounds. After incubation at 37 °C for 72 h, 10 μL of MTT solution (5 mg in PBS 1 mL) was added to each well for 4 h. Cell-free supernatant was removed, and DMSO was then added to dissolve formazan crystals. Absorbance values were measured with a microplate reader at 540 nm. Experiments were operated in triplicate, and data are described as mean ± SD of three independent experiments. Doxorubicin was used as a positive control.

ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra of compounds **1**–**5**; CIF file for compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was financially supported by the Thailand Research Fund (Grant No. RSA5580023) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data for **1**–**3** ($CDCl_3$).

	1		**2**		**3**	
position	δ_C, type	δ_H, mult (*J* in Hz)	δ_C, type	δ_H, mult (*J* in Hz)	δ_C, type	δ_H, mult (*J* in Hz)
2	167.1, qC		166.9, qC		166.9, qC	
3	108.7, CH	6.04, s	108.4, CH	6.05, s	108.4, CH	6.04, s
4	182.5, qC		182.9, qC		182.9, qC	
4a	104.9, qC		105.3, qC		105.8, qC	
5	163.4, qC		162.5, qC		162.3, qC	
6	95.2, CH	6.36, s	95.3, CH	6.40, s	95.3, CH	6.38, s
7	162.9, qC		164.1, qC		164.0, qC	
8	103.4, qC		104.8, qC		104.7, qC	
8a	155.7, qC		156.1, qC		155.9, qC	
1′	69.2, CH	6.00, dd (7.2, 9.6)	70.6, CH	5.10, t (7.2)	70.2, CH	5.09, dd (3.9, 9.9)
2′	33.6, CH_2	2.55, ddd (7.2, 10.0, 12.8)	36.9, CH_2	2.30, m	37.4, CH_2	1.97, ddd (3.9, 9.7, 14.1)
		2.86, ddd (7.2, 9.6, 12.8)		2.60, dt (7.2, 14.0)		2.70, ddd (3.7, 9.9, 14.1)
3′	76.1, CH	4.29, t (10.0)	78.2, CH	3.63, dd (4.8, 12.8)	77.9, CH	4.06, dd (3.7, 9.7)
4′	174.9, qC		172.8, qC		173.3, qC	
2-Me	20.4, CH_3	2.35, s	20.5, CH_3	2.38, s	20.5, CH_3	2.38, s
5-OH		13.04, br s		13.01, br s		13.03, s
7-OMe	56.3, CH_3	3.89, s	56.2, CH_3	3.90, s	56.2, CH_3	3.89, s
1′-OMe			56.7, CH_3	3.17, s	56.6, CH_3	3.19, s
3′-OMe	58.6, CH_3	3.64, s	58.1, CH_3	3.29, s	58.4, CH_3	3.43, s
4′-OMe			51.8, CH_3	3.73, s	51.8, CH_3	3.71, s

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data for **4**–**5** ($CDCl_3$).

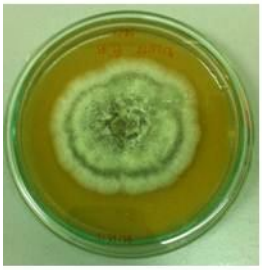
	4		**5**	
position	δ_C, type	δ_H, mult (*J* in Hz)	δ_C, type	δ_H, mult (*J* in Hz)
2	167.1, qC		167.0, qC	
3	108.4, CH	6.05, s	108.4, CH	6.05, s
4	182.8, qC		182.8, qC	
4a	105.1, qC		105.2, qC	
5	162.5, qC		162.5, qC	
6	95.3, CH	6.39, s	95.3, CH	6.37, s
7	164.0, qC		163.9, qC	
8	104.8, qC		104.8, qC	
8a	156.1, qC		156.0, qC	
1′	70.6, CH	5.16, t (6.8)	70.6, CH	5.10, dd (4.0, 10.0)
2′	36.0, CH_2	2.30, m	37.0, CH_2	2.00, ddd (4.0, 8.4, 14.0)
		2.64, m br		2.78, ddd (4.0, 10.0, 14.0)
3′	78.2, CH	3.68, br s	77.8, CH	4.05, dd (4.0, 8.4)
4′[a]	175.0, qC		175.0, qC	
2-Me	20.5, CH_3	2.38, s	20.5, CH_3	2.38, s
5-OH		13.05, br s		13.04, br s
7-OMe	56.2, CH_3	3.89, s	56.2, CH_3	3.88, s
1′-OMe	56.6, CH_3	3.18, s	56.5, CH_3	3.20, s
3′-OMe	58.1, CH_3	3.34, s	58.7, CH_3	3.48, s

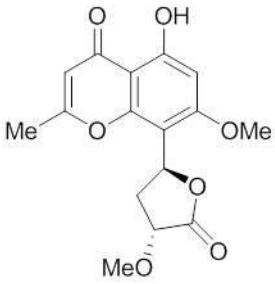
[a] ambiguous signal was determined.

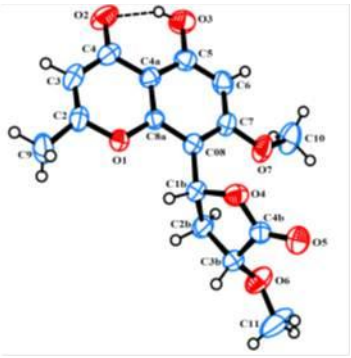
Table 3. Cytotoxicity of Compounds **1**–**5**.

	IC_{50} (μM)			
compound	MCF-7	Hep-G2	Kato-3	CaSki
1	19.3	>25	23.3	>25
2	>25	>25	21.4	>25
3	>25	>25	>25	>25
4	>25	>25	16.8	>25
5	17.7	>25	16.0	>25
doxorubicin	0.97	1.02	2.71	1.14

Graphical Abstract







Elsevier Editorial System(tm) for Phytochemistry
Manuscript Draft

Manuscript Number:

Title: Merulinols A-G, Chamigrane Sesquiterpenes from a Basidiomycetous Endophytic Fungus

Article Type: Full Length Article

Section/Category: Bioactive Products

Keywords: endophytic fungus; sequiterpene; chamigrane; cytotoxicity

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Abstract: Seven new chamigrane sesquiterpenes, merulinols A-G (1–7), were isolated from culture of the basidiomycetous fungus, a mangrove-derived endophyte. Their structures were elucidated mainly by 1D and 2D NMR. Structures of 1 and 2 were further confirmed by single-crystal X-ray diffraction analysis. Cytotoxicity of all compounds was evaluated in vitro against three human cancer cell lines, MCF-7, Hep-G2, and KATO-3. Compounds 3 and 4 exhibited activity against KATO-3 cells with IC50 values of 35.0 and 25.3 uM, respectively.

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4 July, 2015

Dear Professor Dr. Richard J. Robins,

I would like to submit the manuscript entitled "**Merulinols A-G, chamigrane sesquiterpenes from a basidiomycetous endophytic fungus**" for consideration to be published in Phytochemistry. This manuscript deals with seven new chamigrane type sesquiterpenes isolated from the extract of a cultured mangrove-derived fungal endophyte, XG8D. The structure and relative configuration of **1** and **2** was further confirmed by single-crystal X-ray diffraction analysis. Charmigrane sesquiterpenoids, mostly halogenated, are generally produced by the red alga in the genus *Laurencia* (Family Rhodomelaceae); however, those from fungi seem to be rare. In addition, the cytotoxicity of all isolated compounds against three human cancer cell lines was reported. So, I think this work is very suitable for the audiences of Phytochemistry.

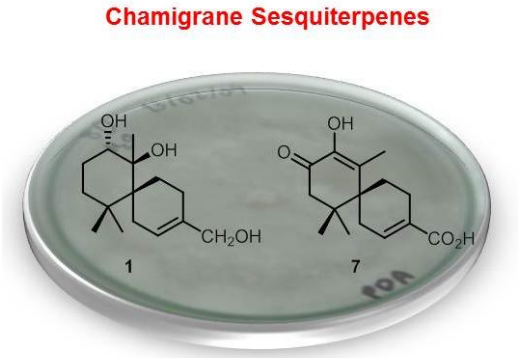
I am looking forward to hearing from your reply.

Sincerely yours,
Khanitha Pudhom
Corresponding author

Graphical Abstract

Merulinols A-G, Chamigrane Sesquiterpenes from a Basidiomycetous Endophytic Fungus

Khanitha Pudhom*, Siwattra Choodej, Thapong Teerawatananond



Highlights:

- Identification of a mangrove fungus as a prolific producer of secondary metabolites.
- Seven new chamigrane-type sequiterpenes of the chamigrane family were identified.
- Two new structures were confirmed by single-crystal X-ray diffraction analysis.
- Cytotoxicity of the compounds against three human cancer cells has been reported.

Merulinols A-G, Chamigrane Sesquiterpenes from a Basidiomycetous Endophytic Fungus

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ABSTRACT

Seven new chamigrane sesquiterpenes, merulinols A-G (**1**–**7**), were isolated from culture of the basidiomycetous fungus, a mangrove-derived endophyte. Their structures were elucidated mainly by 1D and 2D NMR. Structures of **1** and **2** were further confirmed by single-crystal X-ray diffraction analysis. Cytotoxicity of all compounds was evaluated in vitro against three human cancer cell lines, MCF-7, Hep-G2, and KATO-3. Compounds **3** and **4** exhibited activity against KATO-3 cells with IC_{50} values of 35.0 and 25.3 μM, respectively.

Keywords: endophytic fungus, sequiterpene, chamigrane, cytotoxicity

1. Introduction

Marine-associated microorganisms are recognized as a promising source of chemically diverse, structurally unique, and biologically active secondary metabolites (Rateb and Ebel, 2011; Chen et al., 2014). Among them, mangrove plants-derived fungi are of special interest and continue to attract considerable attention due to the extreme environment conditions (Anada and Sridhar, 2002; Debbab et al., 2013; Zhou et al., 2014; Meng et al., 2014; Peng et al., 2013; Zhang et al., 2012). Our research group has thus focused on the exploration of bioactive natural products from these type fungi collected from mangrove areas in Thailand (Pudhom et al., 2014; Pudhom and Teerawatananond, 2014). In a previous investigation, we described the isolation and characterization of unique sesquiterpene endoperoxides of the chamigrane type from an EtOAc extract of a marine-derived fungal strain XG8D, grown in corn-steep liquor containing medium (Chokpaiboon et al., 2010; Chokpaiboon et al., 2011). This fungus was obtained from leaves of a mangrove plant *Xylocarpus granatum*. Charmigrane sesquiterpenoids, mostly halogenated, are generally produced by the red alga in the genus *Laurencia* (Family Rhodomelaceae) (Eshworth, 1989; Brito et al., 2001; Davy et al., 2001; Kimura et al., 1999; Al-Massarani, 2014); however, those from fungi seem to be rare (Ebel, 2010). The only precedents include merulins A-D from our basidiomycetous endophytic fungus XG8D (Chokpaiboon et al., 2010; Chokpaiboon et al., 2011), steperoxide A from a higher fungus *Steccherinum ochraceum* (Liu et al., 2010), and teleperoxides A-D from a mangrove endophytic fungus, *Talaromyces flavus* (Li et al., 2011).

Moreover, some studies have shown that the production of secondary metabolites might be highly dependent on the fermentation conditions and modes (Lawrence, 1999; Zhang et al., 2012; Zhou et al., 2014). This prompted us to embark on the study of culture medium effect on metabolite production of our fungi. As a results, primary screening of cultivation conditions for XG8D fungal strain indicated that the alteration of culture medium greatly affected its chemical profiles. In the present study, chemical investigation of this strain grown in Sabouraud dextrose broth (SDB) led to the isolation of seven new chamigrane sequiterpenes, but not endoperoxide type, namely merulinols A-G (**1**–**7**). Details of the isolation, structure elucidation, and cytotoxic activity of these metabolites are reported herein.

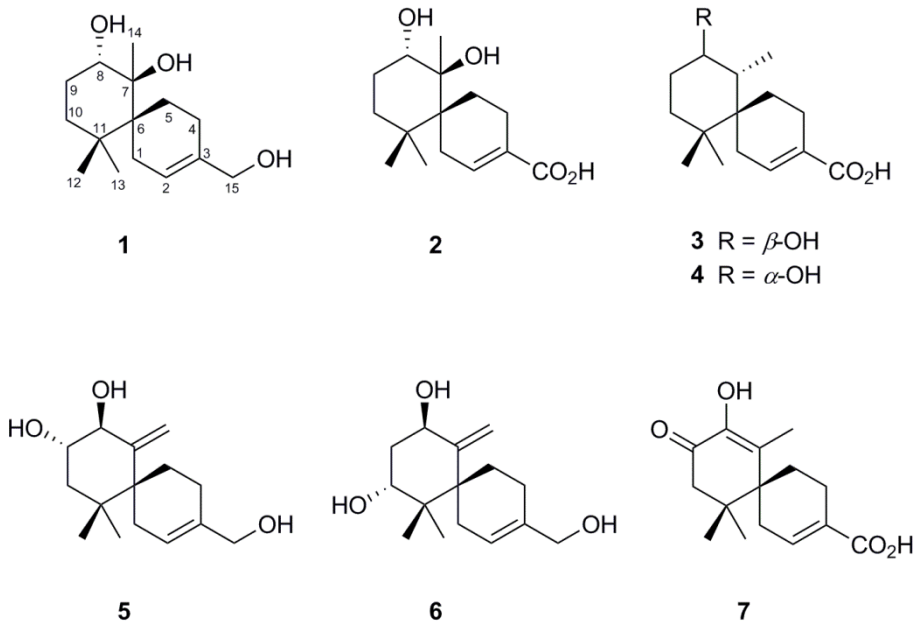


Fig. 1. Structures of isolated compounds **1**–**7**.

2. Results and discussion

The ethyl acetate extract of the endophytic basidiomycetous fungus cultured in SDB was subjected to chromatographic fractionation over Sephadex LH20 and silica gel to yield compounds **1**–**7** (Fig. 1).

Merulinol A (**1**) was obtained as colorless crystals and determined to have a molecular formula of $C_{15}H_{26}O_3$ by the HRESIMS ion at *m/z* 277.1771 $[M + Na]^+$, implying three degrees of unsaturation. Combined analysis of ^1H, ^{13}C (Table 1), and HSQC spectroscopic data revealed the presence of three singlet methyls including one downfield signal at δ_H 1.33, six sp^3 methylenes including one oxymethylene, one oxymethine, one olefinic methine, four quaternary carbons including one oxygenated and one olefinic carbon. These data accounted for all the NMR resonances of **1** and one of three unsaturations, indicated that **1** was a bicyclic compound. Analysis of ^1H–^1H COSY spectrum (Fig. 2) revealed the existence of three isolated spin systems, which were C-1–C-2, C-4–C-5, and C-8–C-10. Two singlet methyl groups, C-12 and C-13, were located on the same quaternary carbon, C-11, which was flanked by C-10 and C-6 due to HMBC correlations of H-10, H_3-12 and H_3-13 to C-11, as well as from H_3-12 and H_3-13 to C-6 (Fig. 2). Further HMBC cross-

peaks from a downfield singlet methyl at δ_H 1.33 to C-8 oxymethine, C-7 oxygenated quaternary carbon, and C-6 led to the attachment of this methyl group (C-14) on C-7 and the connection of C-6 and C-8 on C-7. Other two partial structures, C-1–C-2, C-4–C-5, were placed between C-6 and an olefinic carbon, C-3, due to HMBC correlations from H_2-1, H-2, H_2-4, H_2-5 to C-6 and from H_2-1 to C-3, and established a 6,6-bicyclic skeleton via a C-6 spiro carbon. The HMBC correlations from oxymethylene protons at δ_H 3.89 (br s) to both olefinic carbons, C-2 and C-3, and C-4 indicated that C-15 oxymethylene was located on C-3. Moreover, an exchangeable proton at δ_H 3.08 (s) was assigned to HO-7 by its correlations to C-6 and C-7. Therefore, the planar structure of merulinol A (**1**) was proposed as shown. The relative configuration of **1** was determined by NOESY experiment. The NOESY correlations of H_3-13/H_3-14, H_3-13/H_2-1, and H_3-14/H_2-1 supported these protons being on the same face, while the correlations of H_3-12/H_2-5 and H-8/HO-7 confirmed these protons being on the opposite face. The proposed structure of **1** and its relative configuration were further confirmed by single-crystal X-ray diffraction analysis using Mo Kα radiation, and a perspective ORTEP plot is depicted in Fig. 3.

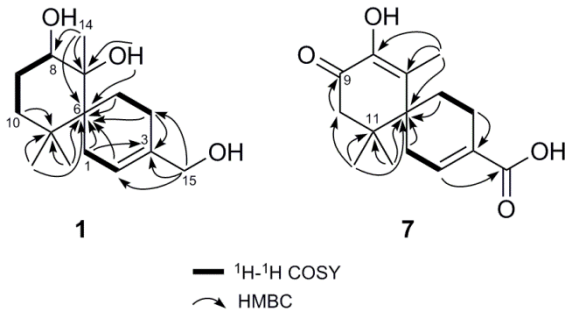


Fig. 2. ^{1}H-^{1}H COSY and selected HMBC correlations of **1** and **7**.

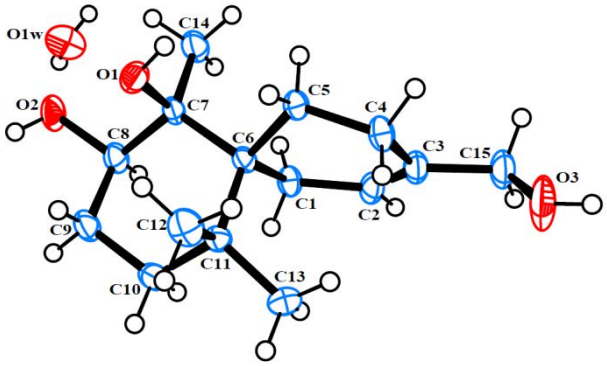


Fig. 3. ORTEP drawing of **1**.

Table 1

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data for compounds **1**–**4**.

position	**1** (acetone-d_6)			**2** (acetone-d_6)			**3** ($CDCl_3$)			**4** ($CDCl_3$)		
	δ_C, type		δ_H (J in Hz)	δ_C, type		δ_H (J in Hz)	δ_C, type		δ_H (J in Hz)[b]	δ_C, type		δ_H (J in Hz)[b]
1	29.2,	CH_2	2.19, br d (20)	28.0,	CH_2	2.13, m	26.9,	CH_2	2.15, br m	29.9,	CH_2	2.17, m
			2.34, br d (20)			2.33, m						2.40, m
2	124.0,	CH	5.16, br s	141.7,	CH	7.00, br s	144.1,	CH	7.18, br s	142.7,	CH	7.12, br s
3	137.7,	qC		130.8,	qC		130.0,	qC		129.1,	qC	
4	25.1,	CH_2	1.89, m	24.1,	CH_2	2.32, m	23.3,	CH_2	2.24, br m	22.6,	CH_2	2.27, m
									2.44, br m			2.35, m
5	25.8,	CH_2	2.05, m	24.2,	CH_2	1.83, m	29.1,	CH_2	1.63, m	27.5,	CH_2	1.50, m
						2.02, m						1.82, m
6	44.3,	qC		44.7,	qC		41.3,	qC		39.5,	qC	
7	78.3,	qC		79.3,	qC		44.9,	CH	1.65, m	36.8,	CH	1.95, m
8	76.9,	CH	3.56, br s	73.1,	CH	3.47, dd (12, 4)	72.5,	CH	3.33, td (12, 4)	70.8,	CH	3.91, dd (12, 6.8)
9	26.9,	CH_2	1.38, dq (12, 4)	28.0,	CH_2	1.52, dq (12, 3.2)	31.6,	CH_2	1.48, m	27.7,	CH_2	1.63, m
			2.10, m			1.79, m			1.79, dq (12, 4)			
10	34.7,	CH_2	1.00, br m	37.6,	CH_2	1.13, dt (14, 3.2)	36.6,	CH_2	1.23, dt (12, 4)	35.6,	CH_2	1.48, m
			1.86, m			1.63, td (14, 3.6)			1.60, m			
11	38.3,	qC		38.2,	qC		37.3,	qC		36.6,	qC	
12	30.5,	CH_3	0.85, s	30.4,	CH_3	0.76, s	27.9,	CH_3	0.78, s	25.7,	CH_3	0.84, s
13	26.3,	CH_3	1.17, s	25.1,	CH_3	1.22, s	22.5,	CH_3	0.99, s	26.3,	CH_3	0.95, s
14	25.2,	CH_3	1.33, s	23.9,	CH_3	1.26, s	12.8,	CH_3	1.01, d (6.8)	11.4,	CH_3	0.98, d (6.8)
15	66.9,	CH_2	3.89, br s	168.3,	qC		171.3,	qC		171.4,	qC	
7-OH			3.08, s									

Merulinol B (**2**) was also obtained as colorless crystals. HRESIMS analysis of the molecular ion (*m/z* 291.1564 $[M + Na]^+$) suggested the molecular formula of $C_{15}H_{24}O_4$, which was indicative of four degrees of unsaturation. ^{1}H NMR data (Table 1) of **2** was similar to those of **1**, indicating the presence of a closely related structural feature. However, signals for the oxymethylene group resonating at δ_H 3.89 (br s) and δ_C 68.0 (–CH_2OH, C-15) in the NMR spectra of **1** were absent, whereas a carbon shift in **2**, but absent in **1**, was observed at δ_C 169.3 and an olefinic proton resonating at much lower field (δ_H 7.00, br s, in **2**, δ_H 5.61, br s, in **1**), establishing an α,β-unsaturated carboxylic acid moiety of **2**. The presence of a carbonyl at C-15 was further verified by HMBC correlations from H-2 and H_2-4 to C-15.

Fortunately, the structure of merulinol B (**2**) was confirmed by single-crystal X-ray crystallographic analysis, as well as its relative configuration, identical to that in **1**, was established. The perspective ORTEP plot is shown in Fig. 4.

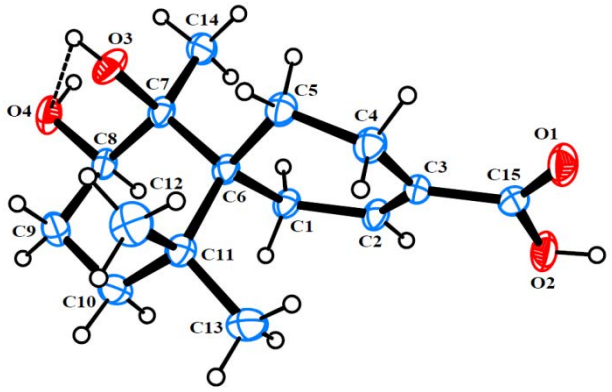
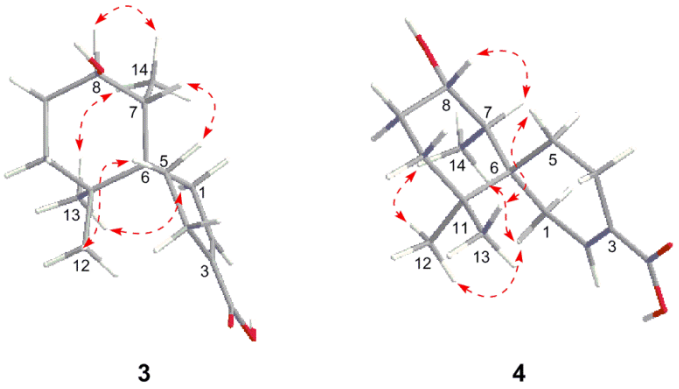


Fig. 4. ORTEP drawing of **2**.

Merulinol C (**3**), obtained as a pale yellow gum, was assigned a molecular formula of $C_{15}H_{24}O_3$ by HRESIMS (*m/z* 275.1612 $[M + Na]^+$), implying four degrees of unsaturation. The ^1H and ^{13}C NMR spectroscopic data of **3** (Table 1) closely resembled those of **2**. The obvious difference in the ^1H NMR spectra between **2** and **3** was the presence of a doublet methyl signal at δ_H 1.01 (J = 6.8 Hz) in **3**, replacing a singlet methyl signal (C-14) in **2**. In addition, the ^{13}C NMR signal of an oxyquaternary carbon (δ_C 77.9, C-7) in **2** was absent, while signals for a methine group (δ_H 1.66 m, δ_C 44.7) were observed. The location of the doublet methyl at C-14 was confirmed by its ^1H-^1H COSY correlation with H-7 and HMBC cross-peaks from H_3-14 to C-8, C-7, and C-6. The NOESY correlations of H_3-13/H_3-14, H_3-13/H_2-1, and H_3-14/H_2-1 (Fig. 5) supported that **3** shared the same relative configuration with that of **1** and **2**. Additionally the NOE correlation between H_3-14 and H-8, as well as the lack of correlation between H-7 and H-8, revealed the β-orientation of HO-8.

Merulinol D (**4**) was obtained as a pale yellow gum. Its molecular formula was established as $C_{15}H_{24}O_3$ by HRESIMS (*m/z* 275.1616 $[M + Na]^+$), being the same as that of **3**. Its ^1H and ^{13}C NMR spectra (Table 1) displayed resonances nearly identical with those of **3**. Interpretation of 2D NMR data established the same planar structure as **3**. The NOESY correlations of **4** indicated that **4** share the same configuration with **3**, except for that of C-8.

Observed NOE correlation between H-7 and H-8 (Fig. 5) suggested the α-orientation of HO-8. Therefore compound **4** was assigned as the C-8 epimer of **3**. This assignment was supported by the opposite signs of their specific rotations ($[\alpha]^{20}_{D}$ +6.4 for **3** vs –47.2 for **4**).



3 **4**

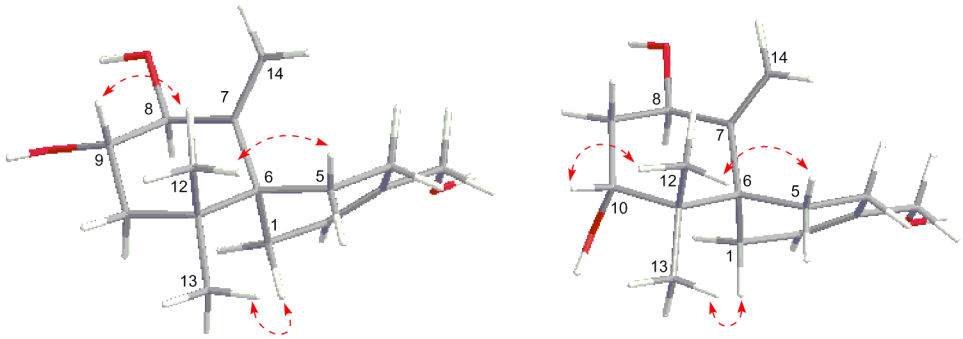
Fig. 5. Selected NOESY correlations of **3** and **4**.

Merulinol E (**5**), a pale yellow gum, was found to have a molecular formula of $C_{15}H_{24}O_3$ based on the HRESIMS ion at *m/z* 275.1622 $[M + Na]^+$, indicating four degrees of unsaturation. The ^{1}H NMR spectrum of **5** was very similar to that of **1**, except for the absence of one of the methyl singlets (δ_H 1.33) in **1** and the appearance of two singlet signals of methylene protons at δ_H 4.78 and 5.39, and a multiplet signal of methine proton at δ_H 3.45. The ^{13}C NMR (Table 2) and HSQC data of **5** also indicated the existence of an exomethylene (δ_H 4.78 s, 5.39 s, δ_C 109.6, 146.9) and an additional oxymethine (δ_H 3.45 m, δ_C 73.1]. Comparison of the ^{1}H and ^{13}C NMR data of **5** with those of **1** revealed that the only difference was the presence of a C-14 exomethylene instead of a C-14 methyl group in **1**. This was confirmed by the HMBC correlations from H_2-14 to C-6, C-7, and C-8. An additional methine in **5** was assigned as C-9 due to its ^{1}H-^{1}H COSY correlations with H-8 and H-10. Therefore the planar structure of **5** was established as shown. The large $^3J_{H\text{-}8,\ H\text{-}9}$ = 9.6 Hz showed that H-8 and H-9 have a *trans* diaxial relationship. The NOESY correlations of H-9/H_3-12 and H_2-5/H_3-12 indicated these protons being on the same face (Figure 5).

Merulinol F (**6**) shared the same molecular formula of $C_{15}H_{24}O_3$ with **5** as established by the HRESIMS ion at *m/z* 275.1615 $[M + Na]^+$. Analysis of ^{13}C (Table 2) and HSQC data revealed that **6** had a total of 15 resonances with two methyls, five methylenes (including one

oxygenated), two oxymethines, one exomethylene, one olefinic methine, and four quaternary carbons (including two olefinic), same as in **5**. The presence of the spin network of H-8/H_2-9 and H_2-9/H-10 in the COSY spectrum evidenced the location of the hydroxy groups at C-10 replacing C-9 in **5**. This was further confirmed by the HMBC cross-peaks from singlet protons of two methyls, H_3-12 and H_3-13, to C-10. The HO-8 group was established as being β-oriented on the basis of the key NOSEY correlation of H-8 with a methylene proton at δ_H 4.79, whereas the HO-10 group was assigned as α-orientation from the NOESY cross-peak of H-10 with H_2-5 (Fig. 6). The structure of **6** was thus established as shown.

Merulinol G (**7**) was obtained as a yellow gum. The molecular formula was determined to be $C_{15}H_{20}O_4$ by the HRESIMS ion at *m/z* 265.1431 $[M + H]^+$, implying six degrees of unsaturation. The ^1H, ^{13}C, and HSQC data (Table 2) indicated the structure of **7** contained three singlet methyls, four methylenes, one olefinic methine, five quaternary carbons (including two olefinic), and two carbonyls. Two signals at δ_C 198.3 and 170.9 were ascribed to one ketone and one carboxylic acid carbonyl, respectively. Comparison of the NMR data of **7** with those of **2** revealed that **7** had the same B ring with an α,β-unsaturated carboxylic acid moiety as found in **1**. The C-10 methylene was connected to C-11 by the HMBC correlations of H_3-12/C-11, H_3-13/C-11, H_3-12/C-10, and H_3-13/C-10 (Fig. 2). The location of the ketone carbonyl (δ_C 198.3) was assigned as C-9 due to its HMBC correlation with H_2-10. The remaining methyl (δ_H 1.85), resonating at much lower field than that in other compounds above, was determined as C-14, as well as an α,β-unsaturated ketone moiety was established from C-7 to C-9 by the HMBC cross-peaks from H_3-14 to a C-6 spiro carbon, and two quaternary olefinic carbons, C-7, and C-8. The C-8 resonance (δ_C 135.9) was deshielded, coupled with the HRESIMS data, leading to the attachment of the hydroxy group on this olefinic carbon. The NOESY data verified that **7** shared the same configuration as merulinols A-F. Consequently, the structure of **7** was established as shown.



5 **6**

Fig. 6. Selected NOESY correlations of **5** and **6**.

The cytotoxic activities of the isolated compounds **1**–**7** against MCF-7, Hep-G2, and KATO-3 cell lines were tested by an MTT method and doxorubicin was used as a positive control. Compounds **3** and **4** exhibited activities against KATO-3 cells with IC_{50} values of 35.0 and 25.3 μM, respectively. The other compounds displayed growth inhibition against three cell lines tested lower than 50% at a dose of 50 μM and were considered inactive.

Table 2

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compounds **5**–**7**.

position	**5** ($CDCl_3$)			**6** (acetone-d_6)			**7** ($CDCl_3$)		
	δ_C, type		δ_H (J in Hz)	δ_C, type		δ_H (J in Hz)	δ_C, type		δ_H (J in Hz[b]
1	29.5,	CH_2	2.05, m	30.7,	CH_2	1.93, m	30.1,	CH_2	2.43, d (20)
			2.15, m			2.20, d (16.8)			2.27, d (20)
2	121.9,	CH	5.63, br s	120.5,	CH	5.58, br s	142.4,	CH	7.26, br s
3	136.7,	qC		139.4,	qC		130.6,	qC	
4	23.6,	CH_2	1.72, m	24.7,	CH_2	1.44, td (12, 6.8)	22.3,	CH_2	2.10, m
			1.97, m			1.87, m			2.58, d (18)
5	26.8,	CH_2	1.52, td (12.8, 4.4)	27.7,	CH_2	2.39, dq (12, 2.8)	30.7,	CH_2	1.88, m
			1.90, m						
6	45.7,	qC		46.2,	qC		43.0,	qC	
7	147.1,	qC		150.4,	qC	4.36, br s	143.8,	qC	
8	74.5,	CH	4.08, d (9.6)	75.3,	CH	1.64, td (12.4, 4)	135.9,	qC	
9	73.3,	CH	3.45, m	39.7,	CH_2	1.90, m	192.3,	qC	
10	43.2,	CH_2	1.57, dd (13.2, 5.2)	68.9,	CH	4.16, dd (12, 4)	47.1,	CH_2	2.22, d (18)
			1.80, m						2.78, d (18)
11	37.5,	qC		42.8,	qC		40.8,	qC	
12	24.1,	CH_3	0.83, s	15.3,	CH_3	0.67, s	23.8,	CH_3	1.01, s
13	24.7,	CH_3	0.92, s	20.8,	CH_3	1.00, s	24.8,	CH_3	1.05, s
14	109.7,	CH_2	4.78, s	115.4,	CH_2	4.36, dd (1.6)	15.1,	CH_3	1.85, s
			5.39, s			4.79, dd (1.6)			
15	67.2,	CH_2	3.93, br s	66.5,	CH_2	3.82, br s	170.9,	qC	

3. Experimental section

3.1. General experimental procedures

Melting points were determined on a melting point M565 apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1010 polarimeter. UV spectra were recorded on a Spekol 1200 Analytic Jena spectrophotometer. IR spectra were recorded on a Bruker ALPHA FT-IR spectrometer. NMR spectra were acquired on a Varian Mercury-400 Plus NMR spectrometer. HRESIMS were measured on a Bruker micrOTOF. Silica gel (Merck, 230-400 mesh) and Sephadex LH20 (Amersham Biosciences) were used for open-column chromatography. Analytical TLC was performed using precoated silica gel 60 GF254 plates (Merck). Single-crystal X-ray diffraction analysis was performed on a Bruker APEX II diffractometer.

3.2. **Fungal Material**

The endophytic fungus XG8D used in this study was isolated from the healthy leaves of *Xylocarpus granatum* collected from Samutsakorn province, Thailand, in July 2008. The fungus was identified to the family Meruliaceae (order Polyporales, subclass Incertaesedis, class Agaricomycetes, phylum Basidiomycota) by analysis of the 28S rDNA and ITS data (GenBank accession nos. HM060640 and HM060641).

3.3. Fermentation, Extraction, and Isolation

The endopytic fungus XG8D was cultured on potato dextrose agar at room temperature for 10 days. Then, five pieces (0.5×0.5 cm^2) of agar plugs were inoculated in a 1000 mL Erlenmeyer flask containing 200 mL of Sabouraud dextrose broth at room temperature for 21 days. The fungal culture (10 L) was filtered to remove mycelia, and the culture broth was extracted twice with ethyl acetate (EtOAc). The combined EtOAc extract was concentrated under reduced pressure to afford a dark brown residue (12.60 g).

The EtOAc extract was subjected to column chromatography (CC) over Sephadex LH20 eluted with MeOH to obtain five fractions (A–E). Fraction E (4.68 g) was fractionated by CC over silica gel using a gradient of EtOAc–*n*-hexane (1:2 to 1:0) to give 11 fractions (E1–E11). Fraction E4 (379.4 mg) was separated by CC using MeOH–CH_2Cl_2 (1:19) to afford nine subfractions (E4.1–E4.9). Subfraction E4.8 was further purified using the same procedure as fraction E4 to provide **3** (5.2 mg), while subfraction E4.9 was eluted with

acetone-CH_2Cl_2 (1:2) on silica gel column to afford **4** (2.8 mg). Fraction E5 (256.0 mg) was subjected to CC over silica gel using EtOAc-CH_2Cl_2 (3:2) to give seven subfractions (E5.1–E5.7). Subfraction E5.3 was purified by CC over Sephadex LH20 eluted with MeOH to provide **7** (3.7 mg). Fraction E8 (198.2 mg) was chromatographed on silica gel (acetone–*n*-hexane, 1:3) to afford six subfractions (E8.1–E8.6). Subfraction E8.6 (52.6 mg) was further purified by a silica gel CC using EtOAc-CH_2Cl_2 (1:1) to yield a pale yellow solid, followed by recrystallization with MeOH to give **2** (16.2 mg). Subfraction E8.5 (75.3 mg) was subjected to CC over silica gel using MeOH–CH_2Cl_2 (1:19) to obtain five fractions (E8.5.1–E8.5.5). After solvent evaporation, a light yellow solid was formed from fraction E8.5.5, which was further recrystallized with MeOH to afford **1** (9.8 mg). Fraction E10 (239.4 mg) was subjected to CC over silica gel using MeOH–CH_2Cl_2 (1:19, then 1:9) to provide 14 subfractions (E10.1–E10.14). Subfraction E10.11 (12.8 mg) was further purified by a Sephadex LH20 CC using MeOH to afford **5** (5.6 mg), while subfraction E10.14 (10.1 mg) gave **6** (3.3 mg) by using the same procedure.

3.4. Merulinol A (1)

Colorless crystals; mp 126-129 ^{o}C, $[\alpha]^{20}_{D}$ –16.8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 213 (2.25), 239 (2.25) nm; IR (neat) ν_{max} 3359, 2924, 2853, 1738, 1658, 1632, 1468, 1367, 1216, 1054 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 277.1771 [M + Na]$^{+}$ (calcd for $C_{15}H_{26}O_3Na$, 277.1780).

3.5. Merulinol B (2)

Colorless crystals; mp 171-174 ^{o}C, $[\alpha]^{20}_{D}$ –24.8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.36) nm; IR (neat) ν_{max} 3412, 2925, 2857, 1685, 1655, 1421, 1367, 1249, 1031 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 291.1564 [M + Na]$^{+}$ (calcd for $C_{15}H_{24}O_4Na$, 291.1572).

3.6. Merulinol C (3)

Pale yellow gum; $[\alpha]^{20}_{D}$ +5.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.06) nm; IR (neat) ν_{max} 3441, 2925, 2855, 1737, 1685, 1457, 1367, 1217, 1024 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 275.1612 [M + Na]$^{+}$ (calcd for $C_{15}H_{24}O_3Na$, 275.1623).

3.7. Merulinol D (4)

Pale yellow gum; $[\alpha]^{20}_{D}$ –47.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 227 (2.98) nm; IR (neat) ν_{max} 3435, 2925, 2856, 1732, 1687, 1455, 1360, 1223, 1021 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 275.1616 $[M + Na]^{+}$ (calcd for $C_{15}H_{24}O_3Na$, 275.1623).

3.8. Merulinol E (5)

Pale yellow gum; $[\alpha]^{20}_{D}$ +6.4 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 219 (2.48) nm; IR (neat) ν_{max} 3386, 2969, 2928, 1738, 1724, 1461, 1367, 1217, 1057 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HRESIMS *m/z* 275.1622 $[M + Na]^{+}$ (calcd for $C_{15}H_{24}O_3Na$, 275.1623).

3.9. Merulinol F (6)

Pale yellow gum; $[\alpha]^{20}_{D}$ –25.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 217 (2.61) nm; IR (neat) ν_{max} 3392, 2945, 2896, 1729, 1692, 1488, 1369, 1229, 1052 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HRESIMS *m/z* 275.1615 $[M + Na]^{+}$ (calcd for $C_{15}H_{24}O_3Na$, 275.1623).

3.10. Merulinol G (7)

Pale yellow gum; $[\alpha]^{20}_{D}$ +127.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 218 (2.82), 280 (2.82) nm; IR (neat) ν_{max} 3361, 2923, 2852, 1738, 1658, 1468, 1366, 1217 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HRESIMS *m/z* 265.1431 $[M + H]^{+}$ (calcd for $C_{15}H_{21}O_4$, 265.1440).

3.11. X-ray Crystallograpic Analysis of Merulinols A (1) and B (2)

All crystallographic data were collected at 293 K on a Bruker APEX II diffractometer with Mo Kα radiation (λ = 0.71073). The structures were solved by direct methods using SHELXS-97 and refined by full-matrix least-squares on all F^2 data using SHELXS-97 to final R values (Sheldrick, 1997). All hydrogen atoms were added at calculated positions and refined using a rigid model. Crystallographic data for **1** and **2** have been deposited with the Cambridge Crystallographic Data Centre with the deposition numbers 832629 and 832630, respectively. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/data_request/cif, by e-mailing data_request@ccdc.cam.ac.uk, or by

contacting the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK: fax: +44 1223 336033.

Crystal Data for **1**: colorless crystal, $C_{30}H_{48}O_8$, M_r = 536.68, orthorhombic, a = 11.3273(7) Å, b = 11.8750(7) Å, c = 20.5308(13) Å, space group $P2_12_12_1$, Z = 4, D_x =1.291 Mg/m^3, and V = 2761.6(3) Å^3, μ (Mo Kα) = 0.09 mm^{-1}, and F(000) = 1168. Crystal dimensions: 0.40 × 0.30 × 0.18 mm. Independent reflections: 5564 (R_{int} = 0.112). The final R_1 values were 0.095, wR_2 = 0.268 ($I > 2\sigma(I)$).

Crystal Data for **2**: colorless crystal, $C_{15}H_{28}O_4$, M_r = 272.37, triclinic, a = 6.7631(7) Å, b = 7.1358(7) Å, c = 7.8934(9) Å, space group $P1$, Z = 1, D_x =1.245 Mg/m^3, and V = 363.14(7) Å^3, μ (Mo Kα) = 0.09 mm^{-1}, and F(000) = 150. Crystal dimensions: 0.50 × 0.40 × 0.35 mm. Independent reflections: 2997 (R_{int} = 0.064). The final R_1 values were 0.083, wR_2 = 0.218 ($I > 2\sigma(I)$).

3.12. **Cytotoxicity Assay.** Cytotoxicity of isolated compounds against human breast (MCF-7), liver (Hep-G2) and gastric (KATO-3) cancer cell lines was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide) assay according to previously described procedures (Xiao et al., 1997). Briefly, freshly trypsinized cell suspensions were seeded into 96-well culture plates at densities of 1 × 10^4 cells in the presence or absence of test compounds. After incubation at 37 ^{o}C for 72 h, 10 μL of MTT solution (5 mg in PBS 1mL) was added to each well for 4 h. Cell-free supernatant was removed, and DMSO was then added to dissolve formazan crystals. Absorbance values were measured with a microplate reader at 540 nm. Experiments were operated in triplicate, and data are described as mean ± SD of three independent experiments. Doxorubicin was used as a positive control.

Acknowledgements

This work was supported by the Thailand Research Fund (Grant No. RSA5580023). We thank Miss Sujitra Hantanong and Miss Supichar Chokpaiboon, Program in Biotechnology, Faculty of Science, Chulalongkorn University, for fungal cultivation and cytotoxicity assay, respectively.

Supplementary data

NMR spectra of compounds **1**–**7** and CIF files for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.ac.org.

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