Cytotoxic Nor-chamigrane and Chamigrane Endoperoxides from a Basidiomycetous Fungus

Supichar Chokpaiboon,[†] Damrong Sommit,[‡] Thapong Teerawatananond,[§] Nongnuj Muangsin,[§] Taridaporn Bunyapaiboonsri,[⊥] and Khanitha Pudhom*[,§]

Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand, Department of Chemistry, Faculty of Science, Mahanakorn University of Technology, Bangkok 10530, Thailand, Research Centre for Bioorganic Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, and National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Pathumthani 12120, Thailand

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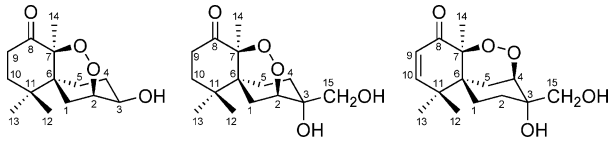
A new nor-chamigrane endoperoxide, merulin A (**1**), and two new chamigrane endoperoxides, merulins B and C (**2**, **3**), were isolated from the culture broth extract of an endophytic fungus of *Xylocarpus granatum*. Their structures were elucidated on the basis of spectroscopic, mainly NMR and MS, data. X-ray crystallographic analysis confirmed the structure of **1**. Compounds **1** and **3** displayed significant cytotoxicity against human breast (BT474) and colon (SW620) cancer cell lines.

Endophytic fungi have been the source of a wide range of structurally interesting and biologically active compounds.[1] As part of ongoing efforts to discover novel bioactive secondary metabolites from fungal sources in Thailand,[2] we investigated the endophytic fungus XG8D, which was isolated from the mangrove plant, *Xylocarpus granatum* König (Meliaceae), as the EtOAc extract of this strain showed potent cytotoxic activity against human breast (BT474) and colon (SW620) cancer cell lines. The fungus strain XG8D was classified as a member of the family Meruliaceae (order Polyporales, subclass Incertae sedis, class Agaricomycetes, phylum Basidiomycota) from rDNA sequences and LUS phylogeny. Herein we report the isolation and structural elucidation of three new chamigrane endoperoxides (**1**−**3**). To the best of our knowledge, sesquiterpenes possessing the chamigrane skeleton are produced by several species of marine algae of the genus *Laurencia.*[3−9] This is the first report of chamigranes from an endophytic fungus.

The fungus XG8D was fermented in corn steep liquor containing medium (5 L) under static conditions. An EtOAc extract of the culture broth was subjected to chromatographic fractionation using Sephadex LH20, silica gel, and preparative HPLC (ODS column) to furnish compounds **1** (142.0 mg), **2** (10.0 mg), and **3** (37.4 mg).



1 **2** **3**

Compound **1** was obtained as colorless crystals and was named merulin A. Its molecular formula was established as $C_{14}H_{22}O_4$ by HRESIMS data (*m*/*z* 277.1414 [M + Na]$^+$, calcd 277.1416), indicating four degrees of unsaturation. IR absorptions implied the presence of OH (3515 cm^{-1}) and carbonyl (1719 cm^{-1}) groups. Analysis of ^{13}C and DEPT NMR spectra of **1** revealed three methyl carbons (δ_C 21.5, 24.7, and 26.2), five methylene carbons (δ_C 25.6, 30.5, 32.1, 35.6, and 35.8), two methine carbons (δ_C 69.4 and 79.1), three quaternary carbons (δ_C 37.3, 41.2, and 90.1), and one ketone carbonyl carbon (δ_C 208.4). Two methines (δ_C 69.4 and 79.1) and one quaternary carbon (δ_C 90.1) were

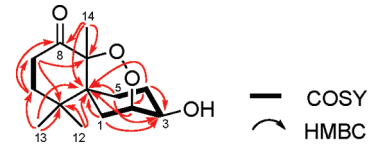


Figure 1. ^{1}H−^{1}H COSY and key HMBC correlations of **1**.

ascribed to those bearing oxygen atoms. Since a carbonyl group of **1** accounted for one out of four degrees of unsaturation, the remaining degrees of unsaturation indicated a tricyclic system. The ^{1}H−^{1}H COSY spectrum revealed two discrete spin systems, including −CH_2−CH−CH−CH_2−CH_2− (from H-1 to H-5) and −CH_2−CH_2− (from H-9 to H-10), as drawn with bold lines in Figure 1. The HMBC study established the connectivity of these two fragments, methyl groups, carbonyl groups, and quaternary carbons. Correlations from a methyl singlet (Me-14) at δ_H 1.40 to C-6, C-7, and C-8 indicated that the C-14 methyl group was connected to the oxygenated quaternary carbon (C-7) and also confirmed the bonding of C-6, C-7, and a ketone group at C-8, which in turn correlated to methylene protons at C-9 and C-10. The cross-peaks of Me-13 and Me-14 to C-6, along with correlations of H_2-1 and H_2-5 to C-6, completed the connectivity of the two rings through the spiro carbon C-6. However, NMR data ultimately proved to be insufficient to solve the complete structure of **1**. The connection between C-2 and C-7 through an endoperoxide linkage could not be confirmed by NMR. Thus, a single-crystal X-ray diffraction study was performed. The X-ray structure of **1** (Figure 2) revealed that **1** is an unprecedented nor-chamigrane containing an endoperoxide linkage and a 6,6,6-tricyclic framework. It also allowed determination of its relative configuration, which was also in good accordance with its relative configuration in solution as assigned by the NOESY spectrum.

Merulin B (**2**) was isolated as a white powder, and the molecular formula was determined to be $C_{15}H_{24}O_5$ by HRESIMS (*m*/*z* 285.1704 [M + H]$^+$, calcd 285.1702) with four degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **2** (see Table 1) were strikingly similar to those of **1**, with the only difference being the presence of a −CH_2−OH unit instead of −H at C-3. This was confirmed by the HMBC correlations from H_2-15 to C-2, C-3, and C-4. The relative configuration of **2** was similar to that of **1**, as established by NOESY spectroscopic data. A key NOESY correlation between H-4ax and H_2-15 indicated the equatorial position for the −CH_2−OH unit at C-3 (Figure 3).

Merulin C (**3**) was isolated as a white powder having the molecular formula $C_{15}H_{22}O_5$ as established by HRESIMS (*m*/*z*

* To whom correspondence should be addressed. Tel: 66-2-218-7639. Fax: 66-2-218-7598. E-mail: Khanitha.P@chula.ac.th.
[†] Program in Biotechnology, Chulalongkorn University.
[‡] Mahanakorn University of Technology.
[§] Department of Chemistry, Chulalongkorn University.
[⊥] BIOTEC.

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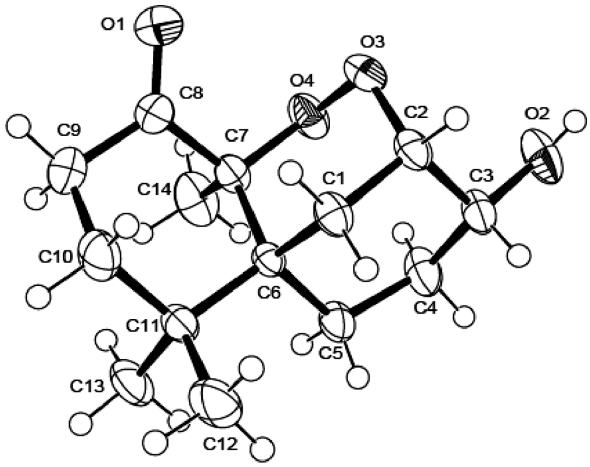
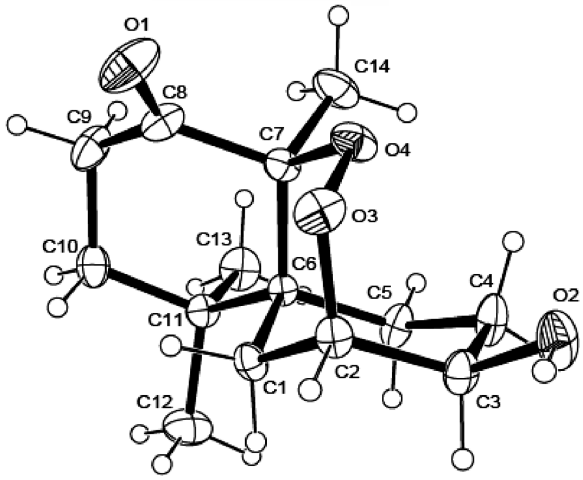


(a) top view



(b) side view

Figure 2. ORTEP diagram of **1**.

305.1366 [M + Na]$^+$, calcd 305.1365, implying five degrees of unsaturation). The ^{1}H and ^{13}C NMR spectra of **3** (Table 1) were quite similar to those of **2**, except for the appearance of a new pair of doublets at δ_H 5.75 and 6.27 (J = 10.4 Hz) due to olefinic protons, instead of two methylenes groups at C-9 and C-10 in **2**.

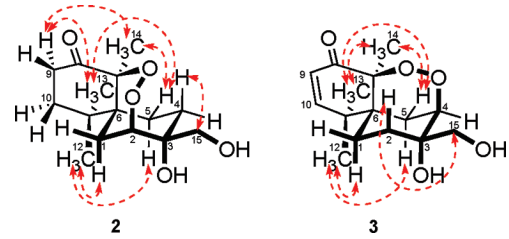


Figure 3. Selected NOESY correlations of **2** and **3**.

All of the NMR data implied that **3** was an α,β-unsaturated ketone derivative of **2**, which was confirmed by 2D NMR (^{1}H−^{1}H COSY, HMQC, and HMBC experiments). However, analysis of NOESY spectroscopic data revealed a difference between their relative configurations. Proton H-5eq of **2**, which had NOESY correlations with H_3-13 and H_3-14, exhibited a COSY correlation with H-4 (oxygenated methane) instead of the methylene protons (H_2-4). Therefore, the endoperoxide linkage of **3** was proposed to be attached to C-4 instead of C-2, as observed in **2**. A key NOESY correlation between H-2ax and H_2-15 indicated the equatorial orientation of the −CH_2−OH unit at C-3 (Figure 3).

Cytotoxic activity of compounds **1**−**3** against human breast (BT474) and colon (SW620) cancer cell lines was evaluated. Compounds **1** and **3** showed cytotoxicity against BT474 cell lines with IC_{50} values of 4.98 and 1.57 μg/mL, respectively. They were also active against SW620 cell lines with IC_{50} values of 4.84 and 4.11 μg/mL, respectively. Compound **2** was inactive against both cell lines tested (IC_{50} > 10 μg/mL).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter at 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Bruker vector 22 Fourier transform spectrophotometer. HRESIMS spectra were obtained with a Bruker micrOTOF. The NMR spectra were recorded on a Bruker AD500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR using TMS (trimethylsilane) as internal standard. Preparative HPLC was performed using a GL Science column (length 250 mm, i.d. 20 mm) packed with C18 (3 μm) stationary phase.

Fungal Material. The fungus XG8D used in the present study was isolated from leaves of *Xylocarpus granatum* collected in Samutsakorn Province, Thailand, in July 2008. The fungus was identified on the basis of both morphology of fungi grown on potato dextrose agar (PDA)

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**3** in $CDCl_3$

	1		**2**		**3**	
position	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C
1	2.05 (ddd, 13.5, 4.2, 3.9), H-1eq 1.56 (dd, 13.5, 1.8), H-1ax	30.5	2.05 (dd, 13.3, 1.8), H-1ax 1.78 (ddd, 13.3, 4.1, 3.4), H-1eq	27.2	1.97 (ddd, 14.5, 13.1, 7.0), H-1ax 1.65 (m), H-1eq	27.0
2	4.14 (ddd, 4.2, 3.8, 1.8)	79.1	4.03 (br s)	78.5	2.27 (ddd, 13.3, 13.1, 6.3), H-2ax 1.54 (dd, 13.3, 7.0), H-2eq	32.5
3	3.77 (ddd, 10.1, 7.9, 3.8)	69.4		71.5		71.9
4	2.17 (m), H-4ax and H-4eq	32.1	2.07 (13.9, 13.5, 6.5), H-4ax 1.65 (dd, 13.9, 6.2), H-4eq	31.4	4.14 (d, 4.2)	78.9
5	2.11 (m), H-5eq 1.59 (m), H-5ax	25.6	1.96 (m), H-5eq 1.81 (ddd, 13.5, 13.0, 6.2), H-5ax	22.8	2.29 (ddd, 13.1, 4.2, 3.6), H-5eq 1.96 (dd, 13.1, 1.7), H-5ax	23.0
6		41.2		42.2		40.6
7		90.1		90.3		87.8
8		208.4		208.6		197.6
9	2.69 (ddd, 15.4, 14.9, 6.7), H-9α 2.45 (ddd, 15.4, 4.7, 2.4), H-9β	35.6	2.65 (ddd, 15.4, 14.8, 6.7), H-9α 2.41 (ddd, 15.4, 4.6, 2.3), H-9β	35.6	5.82 (d, 10.4)	123.6
10	2.01 (ddd, 14.9, 14.8, 4.7), H-10β 1.58 (m), H-10α	35.8	2.02 (ddd, 14.8, 14.4, 4.6), H-10β 1.55 (ddd, 14.4, 6.7, 2.3), H-10α	35.5	6.32 (d, 10.4)	155.1
11		37.3		37.4		40.2
12	0.98 (s)	26.2	1.00 (s)	26.4	1.09	27.0
13	1.26 (s)	24.7	1.23 (s)	24.4	1.26	23.8
14	1.41 (s)	21.5	1.35 (s)	21.4	1.82	23.9
15			3.94 (d, 11.1) 3.43 (d, 11.1)	66.5	3.99 (d, 11.0) 3.45 (d, 11.0)	66.3

at 25 °C and analysis of the DNA sequences of the internal transcribed spacer (ITS) region of rRNA gene and large subunit ribosomal (LSU) phylogeny. Phylogenetic analysis based on the LSU data demonstrated that this unidentified fungus showed a close relationship with a suitable taxa from the family Meruliaceae (order Polyporales, subclass Incertae sedis, class Agaricomycetes, phylum Basidiomycota, kingdom Fungi), while the molecular data based on the ITS region of this fungus was insufficiently sensitive for genus identification and was unresolved in its phylogeny due to the limitation of the number of taxa in the family Meruliaceae from NCBI for data comparing. However, searching the ITS database on nucleotide collection using Megablast (optimizing for highly similar sequences with all GenBank data from NCBI+EMBL+DDBJ+PDB sequences) showed that it was defined as 89−99% homology to the best matching sequence (10 taxa from the order Polyparales) over the whole length of the sequence. The DNA sequences 28SrDNA and ITS of the XG8D fungus have been submitted to GenBank with the accession number HM060640 and HM060641, respectively.

Fermentation and Isolation. The endophytic fungus XG8D was cultured in 1000 mL Erlenmeyer flasks (×25) containing 200 mL of corn steep-containing medium at 30 °C for 21 days under static conditions. The fungal cells were separated from the broth by filtration, and the culture broth was subsequently extracted with EtOAc (1 L × 3), yielding 4.10 g of crude extract. The extract was subjected to Sephadex LH-20 column chromatography (CC) and eluted with MeOH to afford six fractions (I−VI). Fraction V was chromatographed on silica gel (using 5% MeOH in CH_2Cl_2 as the eluent) and preparative reversed-phase HPLC (using acetonitrile−H_2O (40:60) as the eluent) to yield **1** (142.0 mg) and **3** (37.3 mg), respectively. Fraction VI was subjected to flash CC on silica gel (using 5% MeOH in CH_2Cl_2) and then rechromatographed on a silica gel column eluted with EtOAc−hexane (2:1) to afford **2** (10.0 mg).

Merulin A (1): colorless crystals (MeOH); mp 214−217 °C; $[\alpha]^{25}_D$ +230 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.38) nm; IR (KBr) ν_{max} 3515, 2970, 2928, 1719, 1454, 1294, and 1075 cm^{-1}; ^1H and ^{13}C ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 277.1414 $[M + Na]^+$ (calcd for $C_{14}H_{22}O_4Na$, 277.1416).

X-ray Crystallographic Analysis of Merulin A (1). Crystal data: colorless crystal; $C_{14}H_{22}O_4$, M_r = 254.32, orthorhombic, $P2_12_12_1$, a = 7.3526(10) Å, b = 12.4749(3) Å, c = 13.9626(3) Å, $\alpha = \beta = \gamma$ = 90°, Z = 41, and V = 1280.69(4) $Å^3$, Mo Kα radiation, λ = 0.71073 Å. The intensity data were collected at 293 K to a maximum 2θ value of 66.50°. Of the 15 534 reflections collected, 4861 were unique (R_{int} = 0.0282). The crystal structure was solved by direct methods and using the SHELXS97[10] program. Refinements were made by full-matrix least-squares on all F^2 data using SHELXL97[11] to final R values [$I > 2\sigma(I)$] of R_1 = 0.0422, wR_2 = 0.1158 and goodness of fit on F^2 = 1.059. All non-hydrogen atoms were anisotropically refined. 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 (deposition number CCDC 761514). Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/data_request/cif, or 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.

Merulin B (2): white solid; mp 94−96 °C; $[\alpha]^{25}_D$ +170 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.14) nm; IR (KBr) ν_{max} 3406, 2966, 1728, 1444, 1374, 1354, 1126, 1055, and 1010 cm^{-1}; ^1H and ^{13}C ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 285.1704 $[M + H]^+$ (calcd for $C_{15}H_{25}O_5$, 285.1702).

Merulin C (3): white solid; mp 144−146 °C; $[\alpha]^{25}_D$ +203 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 205 (2.82) nm; IR (KBr) ν_{max} 3396, 3019, 2965, 1685, 1451, 1373, 1091, and 1029 cm^{-1}; ^1H and ^{13}C ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 305.1366 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_5Na$, 305.1365).

Cytotoxicity Bioassays. Stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock. After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbance at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC_{50} is the concentration of agent that inhibits cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductal carcinoma ATCC No. HTB 20 (BT474) and colon adenocarcinoma ATCC No. CCL 227 (SW-620). Both cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, and were cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25% (w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin. Doxorubicin was used as a positive control with IC_{50} values of 0.09 and 0.53 μg/mL against SW-620 and BT-474 cell lines, respectively.

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Supporting Information Available: HRESIMS, 1D and 2D NMR spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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