Antimalarial Dihydroisocoumarins Produced by *Geotrichum* sp., an Endophytic Fungus of *Crassocephalum crepidioides*

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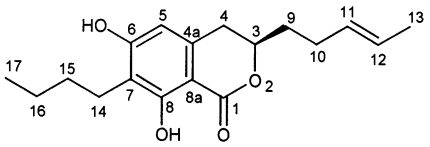
Three novel dihydroisocoumarin derivatives (**1**−**3**) with antimalarial, antituberculous, and antifungal activities have been isolated by bioassay-guided fractionation from an endophytic fungus, *Geotrichum* sp., collected from *Crassocephalum crepidioides*. Structures were established as 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**1**), 7-but-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**2**), and 7-butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one (**3**) using spectroscopic data.

Approximately a quarter of all known biologically active natural products have been obtained from fungi, whose members are estimated to be around 1.5 million.[1−3] Endophytic fungi residing in the intercellular spaces of vascular plants are believed to be a fruitful source of biologically active secondary metabolites; however, they are relatively unexplored chemically.[4,5]

In our search for bioactive compounds from microorganisms, the crude extract of *Geotrichum* sp. (Endomycetaceae), an endophytic fungus isolated from *Crassocephalum crepidioides* S. Moore (Compositae), was found to be active against *Plasmodium falciparum* (K1, multi-drug-resistant strain) with an IC_{50} value of 0.63 μg/mL and against *Candida albicans* with an IC_{50} of 19.19 μg/mL. Bioassay-guided fractionation by Sephadex LH-20 and reversed-phase HPLC resulted in the isolation of three new dihydroisocoumarins (**1**−**3**).



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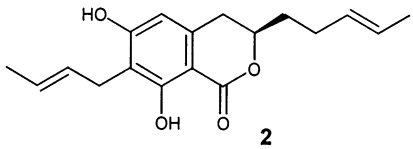

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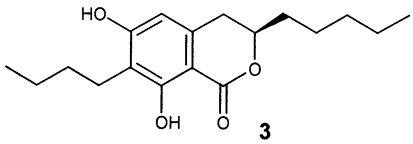

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The crude EtOAc extract of fungal culture broth was passed through a Sephadex-LH20 column and was further purified by reversed-phase HPLC to obtain pure compounds, **1**−**3**. Compound **1** was isolated as a colorless solid, and its molecular formula was determined by accurate mass measurement (ESITOFMS) as $C_{18}H_{24}O_4$. In the ^{1}H NMR spectrum, the lone aromatic proton at δ 6.22 revealed a pentasubstituted aromatic ring, while the sharp singlet signal at δ 11.42 indicated the presence of an intramolecular hydrogen bond of a phenolic hydrogen to a carbonyl group. ^{13}C and DEPT NMR spectra revealed two methyl, six methylene, four methine, and six quaternary carbons. The ^{13}C carbonyl carbon signal at δ 170.5 indicated the presence of a lactone moiety whose carbonyl carbon was attached to the aromatic ring at C-8a (δ 138.2) and the lactone oxygen was connected to the C-3 methine carbon (δ 78.5). Correlations of the methylene protons (H-4, δ 2.84) to H-3 (δ 4.53) and the aromatic proton (H-5) to the C-4 methylene carbon (δ 32.9), respectively, in the COSY and HMBC experiments supported a dihydroisocoumarin skeleton. The COSY spectrum also revealed the presence of a butyl group and a *trans*-pentenyl group. The position of the butyl group was established to be at C-7 by the HMBC correlations of H-14 (δ 2.65) to C-7 (δ 114.8) and to two other aromatic carbons, with a hydroxyl group, at C-6 (δ 160.1) and C-8 (δ 162.2), respectively. A correlation of H-3 to H-9 (δ 1.94) in the COSY spectra as well as HMBC correlations of H-9 to C-3 and C-4 conclusively placed the pentenyl group at C-3 in the molecule. The doublet signal of the C-13 methyl group confirmed the position of the C-11−C-12 olefinic bond of the pentenyl moiety. The absolute configuration at C-3 was concluded to be *R* due to the fact that the circular dichroic spectrum of **1** showed a negative Cotton effect ascribed to the K-absorption band at 274 nm.[6−9] On the basis of the above observations, the structure of **1** was established as 3-(*R*)-7-butyl-6,8-dihydroxy-3-pent-11-enylisochroman-1-one.

Compound **2** had a molecular formula of $C_{18}H_{22}O_4$ as determined by ESITOFMS. ^{13}C and ^{1}H NMR spectra of **2** were very similar to those of **1** except for the marked differences in chemical shift values corresponding to C-15 and C-16. In the NMR spectra of **2**, the methylene signals of C-15 and C-16 were missing, and two additional olefinic protons (J = 15.6 Hz) were assigned to be at H-15 and H-16. Along with the doublet signal of the methyl group at C-17 and the COSY peak between H-17 and H-16, the NMR data led to the conclusion that **2** was 7-but-15-enyl-

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Table 1. ^{1}H and ^{13}C NMR Data (δ) for Compounds **1**, **2**, and **3** in $CDCl_3$[a]

	1		**2**		**3**	
no.	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		170.5	-	170.3		170.4
3	4.53 m	78.5	4.54 m	78.5	4.50 m	79.2
4	2.84 m	32.9	2.84 m	32.9	2.81 m	32.9
4a		138.2	-	139.0		138.3
5	6.22 s	106.0	6.24 s	106.4	6.19 s	105.8
6		160.1		161.2		159.8
7		114.8		111.5		114.6
8		162.2		161.7		162.2
8a		101.6		101.7		101.8
9	1.94 m	34.5	1.95 m	34.5	1.86 m	34.7
10	2.22 m	27.7	2.21 br m	27.8	1.70 m	31.5
11	5.45 (15.2)	129.5	5.47 (15.2)	129.5	1.63 m	24.5
12	5.50 (15.2)	126.4	5.47 (15.2)	126.4	1.41 m	22.5
13	1.66 d (6.0)	17.9	1.67 d (6.0)	17.9	0.95 t (7.2)	14.0
14	2.65 t (7.6)	22.3	2.43 d (5.2)	25.7	2.63 t (7.5)	22.3
15	1.54 m	30.9	5.65 (15.6)	128.8	2.53 m	30.9
16	1.42 qn (7)	22.8	5.65 (15.6)	127.7	1.33 m	22.8
17	0.95 t (7.2)	14.0	1.72 d (5.6)	17.8	0.91 t (6.7)	14.0
8-OH	11.42 s		11.56 s		11.47 s	

[a] Coupling constants in parentheses.

Table 2. Bioactivities of Compounds **1**, **2**, and **3**

compound	antimalarial[a] (IC_{50}, μg/mL)	anti-TB[b] (MIC, μg/mL)	antifungal[c] (IC_{50}, μg/mL)
1	4.7	25	19
2	inactive	50	inactive
3	2.6	inactive	33

[a] *Plasmodium falciparum* K1 (multi-drug-resistant strain). [b] *Mycobacterium tuberculosis* H27Ra. [c] *Candida albicans*.

6,8-dihydroxy-3-pent-11-enylisochroman-1-one. The structure of **2** was confirmed by the HMBC correlations between the doublet H-14 (δ 3.43, J 5.24 Hz) to the aromatic carbon at C-7 (δ 111.5) and the olefinic carbons C-15 and C-16.

The molecular formula of **3** was determined as $C_{18}H_{26}O_4$ from ESITOFMS data. ^{1}H and ^{13}C NMR data of **3** were analogous to those of **1** except that two additional methylene carbons replaced those of the olefin signals. The COSY spectrum between H_3-13 and H-12 and between H_2-9 and H-3, H-10 confirmed the position of the pentyl side chain at C-3 and thus led to the assignment of 7-butyl-6,8-dihydroxy-3-pentylisochroman-1-one for compound **3**.

The configurations of dihydroisocoumarins **2** and **3** at C-3 were established by comparison of their optical rotation values with that of **1**. Optical rotations measured for the three compounds were all negative. Hence, the configurations of **1**−**3** at C-3 were all proposed to be *R*.

The crude EtOAc extracts showed activity against *P. falciparum* K-1 as well as the fungus *C. albicans* and showed moderate potency against *Mycobacterium tuberculosis*. The activities of the purified individual dihydroisocoumarin (**1**−**3**) against the test organisms were compiled in Table 2.

Experimental Section

General Experimental Procedures. Melting points were determined using an Electrothermal melting point apparatus and were uncorrected. The ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were taken at the Central Instrumental Facility of Mahidol University on a Bruker DPX300 spectrometer, with TMS (δ_H 0 ppm) and $CDCl_3$ (δ_C 77.0 ppm) as internal references, respectively. HPLC was carried out on an HP1100 system using a Merck Lichrospher-C_{18} column (5 μm, 4.6 × 250 mm) with UV detection at 215 nm. Optical rotations were measured with a JASCO J-810 spectropolarimeter. IR spectra were recorded on an FT-IR system 2000 (Perkin-Elmer) spectrometer. TLC was performed on silica gel UV_{254} plates (Merck). ESITOFMS data were recorded on a Micromass LCT mass spectrometer.

Fungal Isolation. Fresh stems of *Crassocephalum crepidioides* S. Moore were collected from apparently healthy plants in the forest area of Songkhla Province, Southern Thailand. The stems were washed in running H_2O and surface sterilized by successive soaking in 70% EtOH for 1 min and sodium hypochlorite solution (5.25%) for 5 min and then rinsed twice with water. Aseptically, the stem rods were cut open and 2 × 5 mm^2 pieces of internal tissues were excised and placed on agar in Petri plates. After incubation at 25 °C, individual hyphal tips of the emerging fungi were removed and placed on potato dextrose agar (PDA). During the first two weeks of incubation, the cultures were periodically checked for purity and successively subcultured by hyphal tipping method until pure cultures were obtained. A fungal isolate, Ccre7, grew on PDA as a white filamentous fungus colony and produced arthoconidia upon maturation. The taxonomic identification of Ccre7 as *Geotrichum* sp. was performed based on fungal morphology and analysis of the DNA sequence of the ribosomal RNA gene region. Primers NS5 and ITS4 were used to amplified the ITS1−5.8S−ITS2 region from total DNA extracted from Ccre7.[10,11] The amplified DNA was purified and directly subjected to sequencing reactions using primers ITS2, ITS3, ITS4, and ITS5. BLAST was used to search for similar sequences in the GenBank. DNA sequences, analyzed by CLUSTALW and PAUP phylogenetic analysis programs, revealed that Ccre7 was a fungal member of the genus *Geotrichum*. The DNA sequence of ITS1−5.8S−ITS2 of Ccre7 has been submitted to GenBank (Accession Number AF481862). A subculture of the *Geotrichum* sp. Ccre7 has been deposited at the BIOTEC Culture Collection under the designation BCC 8964.

Bioassay Procedures. In vitro antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multi-drug-resistant strain), which was cultured continuously according to the method of Trager and Jensen.[12] Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based on the method described by Desjardins et al.[13] An IC_{50} value of 0.16 μg/mL (0.31 μM) was observed for the standard sample, chloroquine diphosphate, in the same test system. The antifungal activity was assessed against *C. albicans*, employing the colorimetric method.[14] In our system, the IC_{50} value of the standard drug, amphotericin B, was 0.01 μg/mL. Growth inhibitor activity against *Mycobacterium tuberculosis* H37Ra strain was determined using the Microplate Alamar Blue Assay (MABA).[15] The MIC values of the standard drugs isoniazid and kanamycin sulfate are 0.050 and 2.5 μg/mL, respectively, in our system.

Extraction and Isolation. *Geotrichum* sp. was grown on a PDA plate as an inoculum for 7 days at 25 °C. Six pieces of 5 × 5 mm blocks of the well-grown culture were inoculated into 200 mL of malt Czapek broth in a 1 L Erlenmeyer flask. The fungus was grown by still culture at 25 °C for 21 days. Fungal cultures (10 L) were filtered to remove mycelia and subsequently extracted twice with equal volumes of EtOAc. Evaporation of organic extracts in vacuo afforded 2.33 g of an oily orange-red residue. The crude extract was redissolved in methanol, loaded into a Sephadex LH-20 column (3 × 86 cm), and eluted by an isocratic system of MeOH. The eluate was collected in 15 mL fractions and combined to nine fractions on the basis of their TLC profiles. On the basis of bioactivity data, two fractions (6 and 7) were subsequently purified on a 4.6 × 250 mm Merck LiChrospher-C_{18} column with a gradient of MeCN−H_2O, 50:50 to 70:30, as the mobile phase with a flow rate of 1.0 mL/min to yield compound **1** (37 mg, t_R 20 min), compound **2** (19 mg, t_R 15 min), and compound **3** (6 mg, t_R 17 min).

7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (1): colorless solid (CH_2Cl_2); mp 158−160 °C; $[\alpha]^{25}_D$ −28.3° (*c* 0.25, MeOH); CD (*c* 3.42 × 10^{-4} M, MeOH) $\Delta\epsilon$ (nm) 0 (291.3), −7.65 (274.3), 0 (256.8), 2.61 (249.5), 0 (242.7); UV (MeOH) λ_{max} (log ϵ) 220 (4.5), 274 (4.2) nm; IR ν_{max} (KBr) 3185, 2956,

1618, 1510, 1449, 1387, 1125 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 305.1756 $[M + H]^+$ (calcd for $C_{18}H_{25}O_4$, 305.1755).

7-But-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (2): colorless solid (CH_2Cl_2); mp 138−140 °C; $[\alpha]^{25}_D$ −28.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.1), 268 (3.7) nm; IR ν_{max} (KBr) 3150, 1616, 1503, 1449, 1384, 1266,-1198 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 303.1568 $[M + H]^+$ (calcd for $C_{18}H_{23}O_4$, 303.1597).

7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one (3): colorless solid (CH_2Cl_2); mp 146−147 °C; $[\alpha]^{25}_D$ −20.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 217 (4.6), 269 (4.2) nm; IR ν_{max} (KBr) 3188, 1618, 1503, 1444, 1389, 1307, 1126 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 307.1902 $[M + H]^+$ (calcd for $C_{18}H_{27}O_4$, 307.1901).

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