11-Hydroxymonocerin from the Plant Endophytic Fungus *Exserohilum rostratum*

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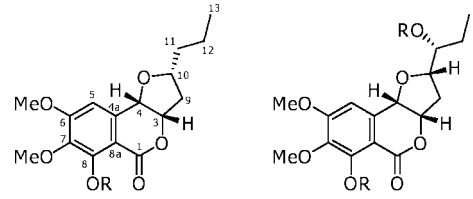
A new analogue of monocerin, 11-hydroxymonocerin (**2**), along with monocerin (**1**) and 12-hydroxymonocerin (**3**) were isolated from cultures of *Exserohilum rostratum*, a fungal strain endophytic in *Stemona* sp. The structure of **2** was determined by analysis of NMR and MS data and by comparison of spectroscopic data to those of **1**. Monocerin (**1**) and 11-hydroxymonocerin (**2**) displayed activity against *Plasmodium falciparum* (K1, multidrug-resistant strain) with IC_{50} values of 0.68 and 7.70 μM, respectively. None of the compounds were cytotoxic against any of the tumor cell lines tested.

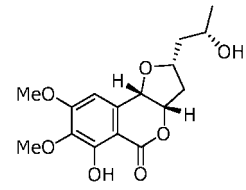
Endophytic fungi have proven to be a rich source of bioactive secondary metabolites.[1−5] *Exserohilum rostratum* has been isolated from both marine and terrestrial sources and is known as a marine invertebrate pathogen[6,7] and a cause of foot and root diseases,[8] and its chemical study has led to the identification of phytotoxins useful as herbicides.[9] In the course of our search for biologically active metabolites from endophytic fungi from Thai medicinal plants, a subculture of an isolate of *E. rostratum*, obtained from leaves of a *Stemona* sp., was cultivated in yeast extract sucrose (YES) broth. An EtOAc extract of the culture showed significant antimalarial activity against *Plasmodium falciparum* (K1, multidrug-resistant strain). Herein, we report the isolation and structural elucidation of a new isocoumarin derivative, 11-hydroxymonocerin (**2**), together with known monocerin (**1**) and 12-hydroxymonocerin (**3**). The compounds were evaluated for antiplasmodal (potential antimalarial) and cytotoxic activities. This is a first report of the potential antimalarial activity of monocerin (**1**) and its analogues.

The culture broth of *E. rostratum* grown in YES broth for 3 weeks was extracted with EtOAc, and the crude extract was purified by column chromatography to yield compounds **1**−**3**.

Monocerin (**1**), the major metabolite, was characterized by analyses of its spectroscopic data and by comparison with data reported in the literature.[10] Monocerin has been isolated as an antifungal, insecticidal, and phytotoxic secondary metabolite from several fungal species including *Helminthosporium monoceras*,[11] *Exserohilum turcum*,[12−14] *Fusarium larvarum*,[15,16] and *Microdochium bolleyi*.[10]

Compound **2** was isolated as a white solid and was optically active. The molecular formula was determined to be $C_{16}H_{20}H_7$ by analysis of its HRESIMS (*m*/*z* 347.1128 $[M + Na]^+$, Δ −0.1 mmu), implying seven double-bond equivalents. The IR spectrum showed a strong broadened OH absorption band at 3431 cm^{-1} and characteristic absorption bands for ester carbonyl (1665 cm^{-1}) and aromatic ring (1521, 1455, and 1276 cm^{-1}) groups. The ^1H NMR spectrum showed a chelated OH signal (δ_H 11.24, OH-8) and signals for an aromatic proton (δ_H 6.61), four oxygen-bearing methine groups (δ_H 5.10, 4.63, 4.02, and 3.53), and two OCH_3 groups (δ_H 3.97 and 3.91). Analysis of ^{13}C NMR and HSQC experiments revealed the presence of a conjugated ester carbonyl (δ_C 167.6) strongly hydrogen-bonded with an OH, six aromatic carbons of which three were oxygenated (δ_C 158.7, 156.2, and 137.4), and one protonated (δ_C 104.6), four oxymethine (δ_C 81.8, 80.9, 75.0, and 74.6), two methylene (δ_C 35.9 and 25.9), and a methyl (δ_C 9.9) carbon. The ^1H and ^{13}C NMR spectra were very similar to those of monocerin (**1**), except for the marked differences in chemical shift values corresponding to position 11. In the ^1H NMR spectrum of **2**, the signal attributable to an oxygen-bearing methine at δ_H 3.53 replaced those corresponding to the methylene signal of **1** at δ_H 1.55 and 1.66.





Treatment of **2** with acetic anhydride resulted in formation of diacetate **5**, and the ^1H NMR spectrum of **5** revealed two acetate methyl singlets at δ_H 2.09 and 2.40, respectively. The shift in the signal corresponding to H-11 (δ_H 4.92) indicated that one OH group was attached to C-11 in **2**. The proposed structure was confirmed by a proton spin system from H-4 to H_3-13, established by $^1H-^1H$ correlation observed in the COSY spectrum and by HMBC correlations (Figure 1) of H-4 to δ_C 130.6 (C-4a), 101.9 (C-8a), 104.5 (CH-5), and 80.9 (CH-3) as well as H-3 to δ_C 38.9 (CH_2-9). The full assignments and connectivity were determined by $^1H-^1H$ COSY correlations as indicated by bold lines and HMBC correlations shown by arrows (Figure 1). By analysis of NOESY data, the compound exhibited NOEs between H-3 and H-4 and between

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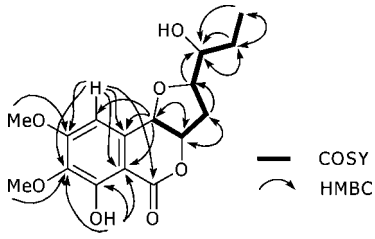
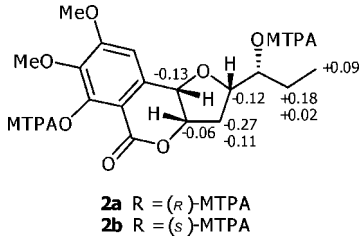


Figure 1. Key HMBC and COSY correlations for **2**.



2a R = (*R*)-MTPA
2b R = (*S*)-MTPA

Figure 2. $\Delta\delta$ values (in ppm) $= \delta_S - \delta_R$ for (*S*)- and (*R*)-MTPA esters in **2a** and **2b**.

Table 1. In Vitro Antimalarial Activity of Compounds **1**, **2**, **4**, and **5** against *P. falciparum*

compound	IC_{50} (μM)
1	0.68
2	7.70
4	0.82
5	9.10
dihydroartemisinin[a]	4×10^{-3}

[a] Standard antimalarial drug.

H-4 and H-10, indicating that these protons are all on the same face of the ring system.

The absolute configuration of **2** was assigned by application of the modified Mosher method.[17,18] Treatment of **2** with (*S*)- and (*R*)-MTPA Cl afforded the (*R*)-MTPA ester (**2a**) and (*S*)-MTPA ester (**2b**), respectively. The difference in chemical shift values ($\Delta\delta = \delta_S - \delta_R$) for the diastereomeric esters **2b** and **2a** was calculated in order to assign the absolute configuration at C-11. Calculation for all relevant signals suggested the *R* absolute configuration at C-11, as shown in Figure 2. Therefore, compound **2** was determined to be 11(*R*)-hydroxymonocerin.

Compound **3** had the same molecular formula as **2**, $C_{16}H_{20}H_7$. Comparison of the optical rotation and its NMR spectroscopic data with those in the literature[10] indicated that compound **3** is 12(*R*)-hydroxymonocerin.

Monocerin (**1**), 11-hydroxymonocerin (**2**), and their acetylated products **4** and **5** were evaluated for antiplasmodial activity against the multidrug-resistant K1 strain of *Plasmodium falciparum*. Monocerin exhibited antiplasmodial activity (IC_{50} value of 0.68 μM). Activity of the 11-hydroxy analogue (**2**) was 10-fold lower, indicating that an additional OH group in the *n*-propyl chain reduces the activity (Table 1). Conversion of the OH groups to acetyl esters (**4** and **5**) did not show any significant effect on their activity. The compounds were also tested for cytotoxicity against five human tumor cell lines: BT474, CHAGO, Hep-G2, KATO-3, and SW-620. None were cytotoxic at a concentration of 20 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength 589 nm, and UV data were recorded on a Varian 50Probe UV/vis spcectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. HRESIMS spectra were obtained using a Micromass LCT mass spectrometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz NMR for ^{13}C NMR using TMS (tetramethylsilane) as internal standard.

Isolation of Endophytic Fungi. Healthy leaves and roots of a *Stemona* sp. were collected from Amphur Bangban, Ayutthaya Province, Thailand, in June 2007. Plant samples were washed in tap water and air-dried. The cleaned leaf and root fragments were surface-sterilized as described by Schulz and co-workers[19] with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, 6% NaOCl solution for 5 min, and sterile distilled H_2O for 1 min (two times). Then, the surface-sterilized fragments were cut into small pieces (ca. 5 mm in length) using a sterile blade and placed on sterile water agar plates for further incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile pipet and transferred onto a potato dextrose agar (PDA) plate. After incubation at 30 °C for 7−14 days, culture purity was determined from colony morphology.

Identification of Endophyte. The fungal endophyte isolate (stem3) was identified on the basis of both morphology of the fungus grown on banana leaf agar at 25 °C and analysis of the DNA sequences of the ITS region of the rRNA gene. The fungus grew on PDA as brown filamentous colonies, and they developed characteristic brown spores. A GenBank search for sequences similar to their ITS region revealed *Exserohilum rostratum* in the family Pleosporaceae as the closest matches, with sequence identity of 99%. The culture of isolate stem3 (accession number EU571210) has been deposited at the Department of Microbiology, Mahidol University, Thailand.

Fermentation, Extraction, and Isolation. The endophytic fungus *Exserohilum rostratum* was cultured in 1000 mL Erlenmeyer flasks (×25) containing 200 mL of yeast extract sucrose (YES) broth at 30 °C for 21 days under static conditions. The fungal cells were separated from the broth by filtration, and a culture broth was subsequently extracted with EtOAc (equal volume × 3), yielding 4.10 g of crude extract. The extract was subjected to SiO_2 column chromatography (CC) eluted with hexane−EtOAc and MeOH−CH_2Cl_2 mixtures of increasing polarity to afford eight fractions (I−VIII). Fraction II was rechromatographed over silica gel eluted with EtOAc−hexane (2:3) to afford monocerin (**1**, 764.2 mg). Fraction IV was further subjected to flash CC on silica gel (benzene−EtOAc, 1:1) to yield compound **2** (165.4 mg). Fraction V was rechromatographed by flash CC on SiO_2 eluted with benzene−EtOAc (3:2), followed by preparative TLC (MeOH−CH_2Cl_2, 1:19) to give compound **3** (3.6 mg).

Monocerin (1): colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.25 (1H, s, 8-OH), 6.57 (1H, s, H-5), 5.03 (1H, brs, H-3), 4.52 (1H, brs, H-4), 4.08 (1H, m, H-10), 3.92 (3H, s, 6-OCH_3), 3.86 (3H, s, 7-OCH_3), 2.58 (1H, m, H-9β), 2.12 (1H, dd, J = 14.4, 5.2 Hz, H-9α), 1.66 (1H, m, H-11a), 1.55 (1H, m, H-11b), 1.38 (2H, m, H_2-12), 0.88 (3H, t, J = 7.0 Hz, H_3-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.8 (C, C-1), 158.7 (C, C-6), 156.2 (C, C-8), 137.2 (C, C-7), 131.2 (C, C-4a), 104.9 (CH, C-5), 101.9 (C, C-8a), 81.3 (CH, C-3), 78.7 (CH, C-10), 74.7 (CH, C-4), 60.7 (CH_3, 7-OCH_3), 56.2 (CH_3, 6-OCH_3), 39.0 (CH_2, C-9), 38.0 (CH_2, C-11), 19.1 (CH_2, C-12), 13.9 (CH_3, C-13); HRESIMS *m/z* 331.1151 $[M + Na]^+$ (calcd for $C_{16}H_{20}O_6$ Na, 331.1158).

11-Hydroxymonocerin (2): white solid; mp 118−121 °C; $[\alpha]^{20}_D$ +50.0 (*c* 0.1, in EtOH); UV (EtOAc) λ_{max} (log ε) 308 (3.78), 273 (4.23) nm; IR (KBr) ν_{max} 3431, 2930, 1665, 1455, 1276, 1117 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.24 (1H, s, 8-OH), 6.61 (1H, s, H-5), 5.10 (1H, brs, H-3), 4.63 (1H, brs, H-4), 4.02 (1H, m, H-10), 3.97 (3H, s, 6-O*CH$_3$*), 3.91 (3H, s, 7-O*CH$_3$*), 3.53 (1H, brs, H-11), 2.57 (1H, m, H-9β), 2.27 (1H, dd, J = 14.4, 5.0 Hz, H-9α), 1.53 (1H, m, H-12a), 1.45 (1H, m, H-12b), 1.02 (3H, t, J = 6.8 Hz, H_3-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.6 (C, C-1), 158.7 (C, C-6), 156.2 (C, C-8), 137.4 (C, C-7), 130.6 (C, C-4a), 104.6 (CH, C-5), 101.9 (C, C-8a), 81.8 (CH, C-10), 80.9 (CH, C-3), 75.0 (CH, C-11), 74.6 (CH, C-4), 60.8 (CH_3, 7-O*CH$_3$*), 56.3 (CH_3, 6-O*CH$_3$*), 35.9 (CH_2, C-9), 25.9 (CH_2, C-12), 9.9 (CH_3, C-13); HRESIMS *m/z* 347.1106 $[M + Na]^+$ (calcd for $C_{16}H_{20}O_7$ Na, 347.1107).

Acetylation of 1 and 2. Acetic anhydride (0.3 mL) and DMAP (catalytic amount) were added to a solution of monocerin (**1**) (10 mg) in pyridine, and the mixture was left stirring at room temperature for 5 h. After removing the solvent under reduced pressure, acetate **4** was purified by CC on silica gel with EtOAc−hexane (3:1); 95% yield (10.8 mg): colorless oil; $[\alpha]^{20}_D$ −3.0 (*c* 0.1, in EtOH); ^{1}H NMR of **4** (400 MHz, $CDCl_3$) δ 6.89 (1H, s, H-5), 4.96 (1H, brs, H-3), 4.54 (1H, d, J = 3.2 Hz, H-4), 4.11 (1H, m, H-10), 3.94 (3H, s, 6-O*CH$_3$*), 3.82

(3H, s, 7-OCH_3), 2.51 (1H, m, H-9β), 2.39 (3H, s, 8-OCOCH_3), 2.10 (1H, dd, J = 14.4, 5.6 Hz, H-9α), 1.65 (1H, m, H-11a), 1.53 (1H, m, H-11b), 1.34 (2H, brm, H_2-12), 0.88 (3H, t, J = 7.2 Hz, H_3-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.8 (C, 8-O*C*OCH$_3$), 159.5 (C, C-1), 157.5 (C, C-6), 145.5 (C, C-8), 142.6 (C, C-7), 132.2 (C, C-4a), 109.8 (C, C-8a), 109.5 (CH, C-5), 79.4 (CH, C-3), 78.4 (CH, C-10), 74.0 (CH, C-4), 60.7 (CH_3, 7-OCH_3), 55.8 (CH_3, 6-OCH_3), 38.5 (CH_2, C-9), 37.6 (CH_2, C-11), 20.5 (CH_3, 8-OCOCH_3), 18.6 (CH_2, C-12), 13.5 (CH_3, C-13); HRESIMS m/z 373.1260 $[M + Na]^+$ (calcd for $C_{18}H_{22}O_7$ Na, 373.1263).

In a similar fashion, compound **2** (10 mg), acetic anhydride (0.3 mL), and DMAP (catalytic amount) in pyridine (1 mL) were allowed to react at room temperature for 5 h, and the reaction mixture was processed as described above for **4** to afford diacetate **5** (12.1 mg, 96% yield): $[\alpha]^{20}_D$ +24.0 (*c* 0.1, in EtOH); ^{1}H NMR of **5** (400 MHz, $CDCl_3$) δ 6.90 (1H, s, H-5), 4.98 (1H, brs, H-3), 4.92 (1H, m, H-11), 4.59 (1H, d, J = 3.6 Hz, H-4), 4.26 (1H, m, H-10), 3.97 (3H, s, 6-OCH_3), 3.84 (3H, s, 7-OCH_3), 2.48 (1H, m, H-9β), 2.40 (3H, s, 11-OCOCH_3), 2.21 (1H, dd, J = 14.4, 6.0 Hz, H-9α), 2.09 (3H, s, 8-OCOCH_3), 1.62 (2H, m, H_2-12), 0.87 (3H, t, J = 7.6 Hz, H_3-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5 (C, 11-O*C*OCH$_3$), 169.5 (C, 8-O*C*OCH$_3$), 160.0 (C, C-1), 158.0 (C, C-6), 146.0 (C, C-8), 143.2 (C, C-7), 132.2 (C, C-4a), 110.4 (C, C-8a), 110.0 (CH, C-5), 78.9 (CH, C-3), 78.7 (CH, C-10), 74.7 (CH, C-11), 74.4 (CH, C-4), 61.2 (CH_3, 7-OCH_3), 56.3 (CH_3, 6-OCH_3), 35.6 (CH_2, C-9), 23.7 (CH_2, C-12), 21.0 (CH_3, 8-OCOCH_3), 21.0 (CH_3, 11-OCOCH_3), 9.9 (CH_3, C-13); HRESIMS m/z 431.1321 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_9$ Na, 431.1318).

Preparation of (*R*)-MTPA Ester (2a) and (*S*)-MTPA Ester (2b). A reaction mixture of **2** (2 mg), (*S*)- or (*R*)-MTPA Cl (20 μL), and DMAP (catalytic amount) in pyridine (0.25 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure, the (*R*)- and (*S*)-MTPA esters (**2a** and **2b**) were purified by mini-column chromatography on silica gel with EtOAc (1:4).

Compound 2a: ^{1}H NMR of **2***R*-MTPA ester (400 MHz, $CDCl_3$) δ 6.57 (1H, s, H-5), 5.25 (1H, m, H-11), 5.03, (1H, m, H-3), 4.37 (1H, d, J = 3.6 Hz, H-4), 4.24 (1H, m, H-10), 3.98 (3H, s, 6-OCH_3), 3.91 (3H, s, 7-OCH_3), 2.56 (1H, m, H-9β), 2.15 (1H, dd, J = 14.0, 6.2 Hz, H-9α), 2.10 (1H, m, H-12a), 1.74 (1H, m, H-12b), 1.17 (3H, t, J = 7.2 Hz, H_3-13).

Compound 2b: ^{1}H NMR of **2***S*-MTPA ester (400 MHz, $CDCl_3$) δ 6.58 (1H, s, H-5), 5.23 (1H, m, H-11), 4.97, (1H, m, H-3), 4.50 (1H, d, J = 3.2 Hz, H-4), 4.12 (1H, m, H-10), 3.96 (3H, s, 6-OCH_3), 3.94 (3H, s, 7-OCH_3), 2.29 (1H, m, H-9β), 2.12 (1H, m, H-12a), 2.04 (1H, dd, J = 14.2, 5.8 Hz, H-9α), 1.92 (1H, m, H-12b), 1.26 (3H, t, J = 7.0 Hz, H_3-13).

Biological Assays. Antimalarial activity in vitro was determined by means of the microculture radioisotope technique based on the method decribed by Desjardins.[20] The parasite *P. falciparum* (K1, multidrug-resistant strain) was cultured continuously according to the method of Trager and Jensen.[21] An IC_{50} value of 4.0×10^{-3} μM (n = 3) was observed for the positive control, dihydroartemisinin. Cytotoxicity was assessed against human cell cultures, BT474 (breast carcinoma), CHAGO (lung carcinoma), Hep-G2 (hepatocarcinoma), KATO-3 (gastric carcinoma), and SW-620 (colon carcinoma), using the MTT [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide] colorimetric method.[22]

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