A New Dihydrobenzofuran Derivative from the Endophytic Fungus *Botryosphaeria mamane* PSU-M76

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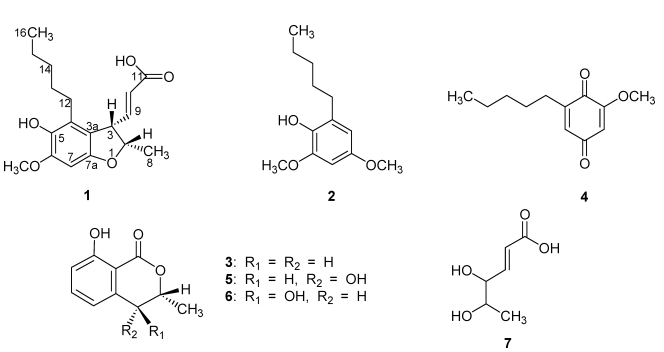
One new dihydrobenzofuran derivative, botryomaman (1), was isolated from the broth extract of the endophytic fungus *Botryosphaeria mamane* PSU-M76 along with six known compounds, 2,4-dimethoxy-6-pentylphenol, (*R*)-(−)-mellein, primin, *cis*-4-hydroxymellein, *trans*-4-hydroxymellein and 4,5-dihydroxy-2-hexenoic acid. The structures were assigned by spectroscopic methods. All compounds were tested for antibacterial activity against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* SK1. Primin showed the best activity against both strains with equal MIC values of 8 μg/ml.

Key words *Botryosphaeria mamane*; dihydrobenzofuran; phenol; mellein; benzoquinone; antibacterial activity

In the course of our ongoing search for antimicrobial substances from plants and endophytic fungi, the ethyl acetate extract from the culture broth of the endophytic fungus *Botryosphaeria mamane* PSU-M76 exhibited interesting antibacterial activity against *Staphylococcus aureus* ATCC 25923 (SA) and methicillin-resistant *S. aureus* SK1 (MRSA) with MIC values of 32 and 64 μg/ml, respectively. The endophytic fungus *B. mamane* PSU-M76 was isolated from the leaves of *Garcinia mangostana*. Chemical investigation of *B. mamane* has never been reported. Investigation of the extract of the culture filtrate led to the isolation of one new dihydrobenzofuran derivative, botryomaman (**1**), along with six known compounds, 2,4-dimethoxy-6-pentylphenol (**2**),[1] (*R*)-(−)-mellein (**3**),[2] primin (**4**),[3,4] *cis*-4-hydroxymellein (**5**),[5] *trans*-4-hydroxymellein (**6**),[5] and 4,5-dihydroxy-2-hexenoic acid (**7**).[6] Their structures were assigned by spectroscopic methods and comparison of the ^{1}H- and ^{13}C-NMR data with those reported in literature. All compounds were tested for antibacterial activity against both strains.

Botryomaman (**1**) was obtained as a white solid, melting at 149.6—150.0 °C with $[\alpha]_D^{29}$ +22.2° (c=0.2, MeOH). The UV spectrum showed a maximum absorption band at λ_{max} 300 nm. The IR spectrum exhibited absorption bands at 3392 cm^{-1} for a hydroxyl group and 1684 cm^{-1} for a carbonyl group of an α,β unsaturated carboxylic acid. The HR-EI-MS showed the molecular formula $C_{18}H_{24}O_5$. The ^{1}H-NMR spectrum displayed typical signals of two *trans*-olefinic protons of the α,β unsaturated carboxylic acid (δ 6.96, dd, J=15.5, 9.0 Hz and δ 5.79, d, J=15.5 Hz), one aromatic proton (δ 6.32, s), one oxymethine proton (δ 4.85, dq, J=9.0, 6.5 Hz), one methine proton (δ 3.90, t, J=9.0 Hz), one methoxyl group (δ 3.84, s), one pentyl side chain [δ 2.55 (m, 1H), 2.37 (m, 1H), 1.55 (m, 1H), 1.48 (m, 1H), 1.31 (m, 4H) and 0.87 (t, J=7.0 Hz, 3H)] and one secondary methyl group (δ 1.40, d, J=6.5 Hz). The carbonyl carbon signal at δ 168.8 in the ^{13}C-NMR spectrum supported the presence of the α,β unsaturated carboxylic acid. The aromatic proton at δ 6.32 was assigned as H-7. Irradiation of H-7 in the NOEDIFF experiment enhanced the signal intensity of the methoxyl group, thus indicating the attachment of the methoxyl group at C-6 (δ 146.8). HMBC correlations of H-7/C-3a (δ 118.2), C-5 (δ 137.9), C-6 and C-7a (δ 152.3) and those of the methylene protons of the pentyl moiety (δ 2.55, 2.37, H_2-12)/C-3a, C-4 (δ 126.2) and C-5 established the linkage of the pentyl group at C-4. In the COSY spectrum, the methine proton at δ 3.90 (H-3) was coupled with the oxymethine proton at δ 4.85 (H-2) and the downfield *trans*-olefinic proton (δ 6.96, H-9) of the α,β unsaturated carboxylic acid. H-2 was further coupled with the methyl protons at δ 1.40 (H_3-8). HMBC cross peaks of H-3/C-3a and C-7a established the linkage of the methine group carrying the 1-oxysubstituted ethyl group and *trans* α,β unsaturated carboxylic acid moiety at C-3a. According to the chemical shifts of both C-2 (δ 82.8) and C-7a and the molecular formula, these carbons were joined together with an ether linkage to form a dihydrobenzofuran skeleton. Since there was no other signal in the ^{1}H-NMR spectrum, the substituent at C-5 must be a hydroxyl group. This hydroxycarbon resonanted at much higher field due to the resonance effect of two oxysubstituents at C-6 and C-7a. Irradiation of H-3 in the NOEDIFF experiment enhanced signal intensity of H-2, indicating their *cis* relationship. Therefore, botryomaman (**1**) was determined as a new dihydrobenzofuran derivative, possibly derived from the condensation of the demethylated **2** and **7**.



All isolated compounds were tested for antibacterial activitiy against SA and MRSA. Primin (**4**) exhibited the best activity against SA and MRSA with equal MIC values of

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8 μg/ml, while other compounds were inactive with equal MIC values of >128 μg/ml. However, **4** was much less active than vancomycin which exhibited a MIC value of 1 μg/ml.

Experimental

General Experimental Procedures Melting points were measured on an electrothermal melting point apparatus (Electrothermal 9100). Infrared spectra (IR) were recorded as neat on a Perkin Elmer 783 FTS165 FT-IR spectrometer. Ultraviolet (UV) absorption spectra were measured in methanol solution on a SHIMADZU UV-160A spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometer in deuterochloroform solution with tetramethylsilane (TMS) as internal standard. Mass spectra were obtained on a MAT 95 XL mass spectrometer (Thermofinnigan). Optical rotations were measured in MeOH solution on a JASCO P-1020 polarimeter. Thin-layer chromatography (TLC) and precoated TLC were performed on silica gel GF_{254} (Merck). Column chromatography (CC) was carried out on silica gel (Merck) type 100 (70—230 Mesh ASTM) with a gradient system of CH_2Cl_2/MeOH or on Sephadex LH20 with MeOH, unless otherwise stated.

Fungal Material The endophytic fungus *B. mamane* PSU-M76 was isolated from the leaves of *G. mangostana*, collected in Suratthani Province, Thailand, in 2005. This fungus was deposited as PSU-M76 at the Department of Microbiology, Faculty of Science, Prince of Songkla University.

Fermentation and Isolation The endophytic fungus *B. mamane* PSU-M76, grown on potato dextrose agar (PDA) at 25 °C for 5 d, was inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) at room temperature for 4 weeks. The cultures were separated by filtration into the mycelia and filtrate. The filtrate was extracted three times with an equal volume of EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure to obtain a brown gum (320.0 mg). The crude extract was fractionated by CC over Sephadex LH20 to afford three fractions (A—C). Fraction B (220.9 mg) was further purified by CC over silica gel to give ten fractions (B1—B10). Fractions B2 and B3 contained **2** (3.0 mg) and **3** (4.8 mg), respectively. Fraction B5 (33.1 mg) was further separated by CC over silica gel to give three subfractions. The second subfraction gave **4** (11.8 mg). Fraction B7 (52.4 mg) was purified by CC over silica gel to afford three subfractions. The second subfraction (27.1 mg) was subjected to CC over Sephadex LH20 to give **5** (9.5 mg), and **6** (3.4 mg). Fraction B8 (16.1 mg) was subjected to precoated TLC using 40% EtOAc-light petroleum as a mobile phase (6 runs) to give **1** (3.8 mg). Fraction B10 (33.1 mg) was separated by CC over Sephadex LH20 to yield **7** (7.8 mg).

Botryomaman (**1**): white solid, mp 149.6—150.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.96 (1H, dd, J=15.5, 9.0 Hz, H-9), 6.32 (1H, s, H-7), 5.79 (1H, d, J=15.5 Hz, H-10), 4.85 (1H, dq, J=9.0, 6.5 Hz, H-2), 3.90 (1H, t, J=9.0 Hz, H-3), 3.84 (3H, s, 6-OCH_3), 2.55 (1H, m, H-12a), 2.37 (1H, m, H-12b), 1.55 (1H, m, H-13a), 1.48 (1H, m, H-13b), 1.40 (3H, d, J=6.5 Hz, H-8), 1.31 (4H, m, H-14, H-15), 0.87 (3H, t, J=7.0 Hz, H-16). ^{13}C-NMR ($CDCl_3$) δ: 168.8 (s, C-11), 152.3 (s, C-7a), 148.3 (d, C-9), 146.8 (s, C-6), 137.9 (s, C-5), 126.2 (s, C-4), 121.9 (d, C-10), 118.2 (s, C-3a), 92.0 (d, C-7), 82.8 (d, C-2), 56.2 (q, 6-OCH_3), 48.1 (d, C-3), 32.1 (t, C-14), 29.0 (t, C-13), 27.3 (t, C-12), 22.5 (t, C-15), 16.4 (q, C-8), 14.0 (q, C-16). FT-IR (neat) cm^{-1} 3392, 1684, 1605, 1554. UV λ_{max} (MeOH) nm (log ε) 300 (3.45). HR-EI-MS m/z 320.1604 $[M]^+$ (Calcd for $C_{18}H_{24}O_5$ 320.1624), EI-MS m/z (% relative intensity): 320 (100), 275 (9), 231 (16). $[\alpha]_D^{29}$ +22.2° (c=0.2, MeOH).

Antibacterial Assay The minimum inhibitory concentrations (MICs) were determined by the agar microdilution method.[7] The test substances were dissolved in DMSO (Merck, Germany). Serial 2-fold dilutions of the test substances were mixed with melted Mueller–Hinton agar (Difco) in the ratio of 1 : 100 in microtiter plates with flat-bottomed wells (Nunc, Germany). Final concentration of the test substances in agar ranged from 128 to 0.03 μg/ml. SA and MRSA were used as test strains. Inoculum suspensions (10 μl) were spotted on agar-filled wells. The inoculated plates were incubated at 35 °C for 18 h. MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Vancomycin was used as a positive control drug. Growth controls were performed on agar containing DMSO.

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