Cytotoxic Metabolites from the Wood-Decayed Fungus *Xylaria* sp. BCC 9653

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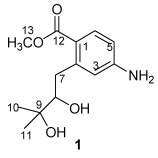
Chemical investigation of the wood-decayed fungus *Xylaria* sp. BCC 9653 has led to the isolation of a new methyl aminobenzoate (1) together with eleven known compounds. The structures were established by analysis of spectroscopic data. Cytochalasin D (2), one of the known metabolites, exhibited potent cytotoxicity against African green monkey kidney fibroblast (Vero) cells with an IC_{50} value of 0.19 μM.

Key words *Xylaria* sp.; methyl aminobenzoate; cytochalasin; mellein; 4-quinoline; cytotoxicity

The genus *Xylaria* is a rich source of biologically active secondary metabolites including antifungal sordaricins,[1] anti HIV-1 eremophilane sesquiterpenoids,[2] and antimalarial hexahydronaphthalenones.[3] We describe herein the isolation and structural elucidation of one new methyl benzoate derivative (**1**), along with eleven known compounds, cytochalasin D (**2**),[4,5] desacetylcytochalasin D (**3**),[6] cytochalasin O (**4**),[5] (*R*)-(−)-mellein methyl ether (**5**),[7] 5-carboxymellein (**6**),[8,9] (*R*)-(−)-5-hydroxymellein (**7**),[10,11] 4-quinolinecarbonitrile (**8**),[12] 4-quinolinecarboxaldehyde oxime (**9**),[13] cyclo(L-Pro-L-Tyr) (**10**),[14,15] cerevisterol (**11**),[16] and uracil (**12**)[17] from *Xylaria* sp. BCC 9653. Their structures were assigned by spectroscopic methods and comparison of the ^{1}H- and ^{13}C-NMR data with those reported in literatures. All isolated compounds were tested for antimalarial, antimycobacterial and cytotoxic activities.

Compound **1** was obtained as a colorless gum with $[\alpha]_D^{27}$ −19.17° (c=0.90, $CHCl_3$). The UV spectrum showed maximum absorption bands at 223, 251 and 334 nm, indicating the presence of a conjugation chromophore. Its IR spectrum showed absorption bands at 3371 and 1686 cm^{-1} for hydroxyl and carbonyl groups, respectively. The HR-EI-MS showed the molecular formula $C_{13}H_{19}NO_4$. The ^{1}H-NMR spectrum showed signals for three aromatic protons of a 1,2,4-trisubstituted benzene [δ 7.81 (d, J=8.1 Hz), 6.56 (d, J=1.5 Hz) and 6.52 (dd, J=8.1, 1.5 Hz)], one oxymethine proton (δ 3.62, dd, J=10.5, 2.4 Hz), nonequivalent methylene protons [δ 2.80 (dd, J=13.5, 2.4 Hz), and 2.50 (dd, J=13.5, 10.5 Hz)], one methoxyl group (δ 3.86, s) and two methyl groups (δ 1.29, s, 1.26, s). The aromatic protons at δ 7.81, 6.56 and 6.52 were attributed to H-6, H-3 and H-5, respectively, on the basis of their multiplicity and J values. The ester carbonyl resonance at δ 168.1 in the ^{13}C-NMR spectrum confirmed the presence of this functional group in the IR spectrum. ^3J HMBC correlations of both H_3-13 (δ 3.86) and H-6 with C-12 (δ 168.1) indicated the presence of a methyl ester moiety at C-1 (δ 109.5). The ^{1}H–^{1}H COSY spectrum revealed that the oxymethine proton, H-8 (δ 3.62), was coupled with the nonequivalent methylene protons, H_2-7 (δ 2.80, 2.50). In the HMBC spectrum, H_3-10 and H_3-11 correlated with C-8 (δ 78.6) and C-9 (δ 72.6), thus constructing a 2,3-dihydroxy-3-methylbutyl side chain. The attachment of this side chain at C-2 (δ 145.7) was established by HMBC correlations of H_2-7/C-1, C-2 and C-3 (δ 117.2). The substituent at C-4 (δ 150.6) was then assigned as an amino group according to the chemical-shift of C-4 and the molecular formula. Therefore, compound **1** was determined as a new methyl aminobenzoate.

Compound **1**, cytochalasin D (**2**), and desacetylcytochalasin D (**3**) exhibited cytotoxic activity against standard Vero cells with IC_{50} values of 26.13, 0.19 and 16.61 μM, respectively. Furthermore, cytochalasin D (**2**) displayed higher toxicity than the standard drug, ellipticine. The remaining compounds were non-cytotoxic at the concentration of 50 μg/ml. It should be noted that the acetoxyl group and the exocyclic double bond in **2** played an important role in the cytotoxic activity. Compounds **2**, **6** and **9** possessed weak antimycobacterial activity against *Mycobacterium tuberculosis* with respective MIC values of 394.3, 900.7 and 581.2 μM. All compounds gave no antimalarial activity against *Plasmodium falciparum*.



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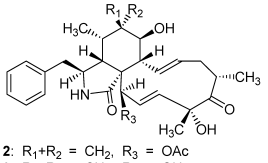
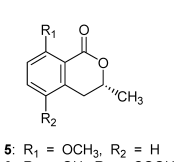
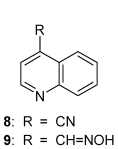


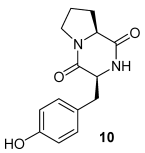
2: R_1+R_2 = CH_2, R_3 = OAc
3: R_1+R_2 = CH_2, R_3 = OH
4: R_1 = OH, R_2 = CH_3, R_3 = OAc



5: R_1 = OCH_3, R_2 = H
6: R_1 = OH, R_2 = COOH
7: R_1 = R_2 = OH



8: R = CN
9: R = CH=NOH



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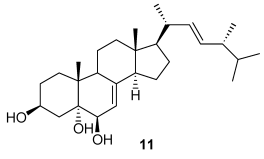

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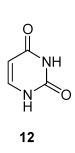

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Experimental

General Experimental Procedures Infrared spectra (IR) were recorded as neat on a Perkin Elmer 783 FTS165 FT-IR spectrometer. Ultraviolet (UV) spectra were determined 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™ 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 60 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, on reverse phase silica gel C-18 with a gradient system of MeOH/H_2O or on Sephadex LH20 with MeOH.

Fungal Material *Xylaria* sp. was collected on an unidentified wood in

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Bala-Hala Wildlife Sanctuary, Naratiwat Province, on 14 May 2001, by Mr. Prasert Srikitikulchai. This fungus was deposited at the BIOTEC Culture Collection as BCC 9653 on 16 July 2001.

Fermentation and Isolation *Xylaria* sp. BCC 9653 was maintained on potato dextrose agar at 25 °C, which was cut into pieces (1×1 cm), and inoculated into 2×250 ml Erlenmeyer flasks containing 25 ml of Difco™ potato dextrose broth (potato starch 4.0 g and dextrose 20.0 g, per liter) (10 pieces for each flask). After incubation at 25 °C for 5 d on a rotary shaker (200 rpm), each primary culture was transferred into a 1000 ml Erlenmeyer flask containing 250 ml of the same liquid medium (PDB), and incubated at 25 °C for 5 d on a rotary shaker (200 rpm). Each 25 ml portion of the secondary cultures was transferred into 20×1000 ml Erlenmeyer flasks each containing 250 ml of peptone yeast glucose medium (bacteriological peptone 5.0 g, yeast extract 20.0 g, glucose 10.0 g, KH_2PO_4 1.0 g and $MgSO_4 \cdot 7H_2O$ 0.5 g, per liter), and incubated on rotary shakers (200 rpm) at 25 °C for 14 d. 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 $MgSO_4$, and evaporated to dryness under reduced pressure to obtain a brown gum (560.0 mg). The broth extract was fractionated by CC over Sephadex LH20 to afford five fractions (A—E). Fraction B (405.0 mg) was further purified by CC over silica gel to give three fractions (B1—B3). Fraction B2 was separated by CC over silica gel to give five subfractions. The second subfraction contained **5** (1.8 mg). The third subfraction (116.2 mg) was purified by CC over silica gel to afford **2** (71.8 mg) and **4** (4.1 mg). The fourth subfraction (60.5 mg) was separated by CC over silica gel to give three subfractions. The last subfraction (22.8 mg) was subjected to CC over Sephadex LH20 to give **3** (2.0 mg). Fraction C (98.6 mg) upon CC over silica gel yielded five fractions (C1—C5). Fraction C2 contained **8** (1.2 mg). Fraction C4 (11.9 mg) was subjected to precoated TLC using 50% EtOAc-light petroleum as a mobile phase (3 runs) to give **1** (4.6 mg). Fraction C5 (7.0 mg) was purified on precoated TLC using 70% EtOAc-light petroleum as a mobile phase (7 runs) to give **10** (2.5 mg). Fraction D (34.4 mg) was separated by CC over reverse phase silica gel to give three fractions. The second fraction (6.7 mg) was subjected to precoated TLC using 2% MeOH–CH_2Cl_2 as a mobile phase (4 runs) to give **9** (5.2 mg). The wet mycelia was extracted twice with 500 ml of MeOH for 2 d. The aqueous MeOH layer was concentrated under reduced pressure. To the extract was added H_2O (50 ml), and the mixture was washed with hexane (700 ml). The aqueous layer was extracted three times with an equal volume of EtOAc (300 ml). The combined EtOAc extracts were dried over anhydrous $MgSO_4$ and then evaporated to dryness under reduced pressure to give a dark brown gum (522.1 mg). The mycelial extract was fractionated by CC over Sephadex LH20 to afford five fractions. The second fraction (223.8 mg) upon CC over silica gel gave three subfractions. The second subfraction (30.3 mg) was purified by CC over Sephadex LH20 to afford **11** (4.2 mg). The third fraction (87.3 mg) was further separated by CC over Sephadex LH20 to afford four subfractions. The second subfraction contained **12** (24.0 mg). The third (17.5 mg) and the last (11.3 mg) subfractions were further separated by CC over Sephadex LH20 to afford **6** (2.3 mg) and **7** (1.7 mg), respectively.

Compound **1**: Colorless gum, ^{1}H-NMR ($CDCl_3$) δ: 7.81 (1H, d, J=8.1 Hz, H-6), 6.56 (1H, d, J=1.5 Hz, H-3), 6.52 (1H, dd, J=8.1, 1.5 Hz, H-5), 3.86 (3H, s, H-13), 3.62 (1H, dd, J=10.5, 2.4 Hz, H-8), 2.80 (1H, dd, J=13.5, 2.4 Hz, H-7a), 2.50 (1H, dd, J=13.5, 10.5 Hz, H-7b), 1.29 (3H, s, H-10), 1.26 (3H, s, H-11). ^{13}C-NMR ($CDCl_3$) δ: 168.1 (s, C-12), 150.6 (s, C-4), 145.7 (s, C-2), 131.7 (d, C-6), 117.4 (d, C-5), 117.2 (d, C-3), 109.5 (s, C-1), 78.6 (d, C-8), 72.6 (s, C-9), 51.5 (q, C-13), 38.4 (t, C-7), 26.5 (q, C-10), 23.8 (q, C-11). FT-IR (neat) cm^{-1} 3371, 1686, 1618, 1554. UV λ_{max} (MeOH) nm (log ε) 223 (4.81), 251 (4.35), 334 (4.02). HR-EI-MS m/z 253.1293 $[M]^+$ (Calcd for $C_{13}H_{19}NO_4$ 253.1314), EI-MS m/z (% relative intensity): 253 (67), 195 (56), 163 (57), 135 (100), 120 (49), 100 (63). $[\alpha]_D^{27}$ −19.17° (c= 0.90, $CHCl_3$).

Biological Assays Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), using the microculture radioisotope technique based on the method described by Desjardins *et al.*[18] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound was dihydroartemisinin, showing an IC_{50} value of 4.5 nM. Cytotoxic assay against African green monkey kidney fibroblast (Vero) cells was performed employing the calorimetric method as described by Skehan and co-workers.[19] The standard compound was ellipticine, exhibiting the IC_{50} value of 2.07 μM. Antimycobacterial activity was performed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[20] Standard drugs, isoniazid and kanamycin sulfate, exhibited MIC values of 0.36 and 2.58 μM, respectively.

Acknowledgments W.P. thanks the Royal Golden Jubilee Ph.D. Program of the Thailand Research Fund (Grant No. PHD/0218/2547) for a financial support. Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC) and the Graduate School, Prince of Songkla University, are gratefully acknowledged for partial support.

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