Isocoumarin Glucosides from the Scale Insect Fungus *Torrubiella tenuis* BCC 12732

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Three new isocoumarin glucosides (**1**, **3**, and **4**), 6,8-dihydroxy-3-methylisocoumarin (**2**), and 6,8-dihydroxy-3-hydroxymethylisocoumarin (**5**) were isolated from the scale insect pathogenic fungus *Torrubiella tenuis* BCC 12732. Structures of these compounds were elucidated using NMR spectroscopic and MS spectrometric analyses. Compound **5** exhibited moderate anti-HSV-1 and antimycobacterial activities with IC_{50} and MIC values of 50 and 25 μg/mL, respectively.

Fungi present in the family Clavicipitaceae are known not only for their obligate symbiotic relationship with animals, plants, or even other fungi but also for their importance in traditional medicine. Clavicipitalean endophytes (*Neotyphodium* spp.), found in sleepy grass (*Achnatherum robustum*), were used as hypnotics and sleeping aids by Native Americans, *Balansia cyperi* as a remedy to hasten childbirth by the Amazonian Jívaro tribe, and *Cordyceps sinensis* as a valued medicine in ancient China.[1] Fungi in the genus *Torrubiella* and also a member of the family Clavicipitaceae are frequently found as pathogens in spiders (Araneida) and insects of the order Homoptera, particularly the scale insects (Cocidae or Lecaniidae).[2,3] *Torrubiella* species are linked to nine anamorphic genera including *Gibellula, Granulomanus, Akanthomyces, Paecilomyces, Hirsutella, Pseudogibellula, Lecanicillium, Simplicillium*, and *Verticillium*.[4] Although it is documented that fungi in these genera produce various biologically active compounds such as antimalarial compounds paecilodepsipeptide A (from *Paecilomyces cinnamonous*),[5] cyclohexadepsipeptides (enniatins H and I),[6] and diketopiperazines[7] (from *Verticillium hemipterigenum*) or antituberculous hirsutellones (from *Hirsutella nivea*),[8] reports of bioactive secondary metabolites from *Torrubiella* are few.[9] As a part of our ongoing research on bioactive compounds from fungi,[10] we investigated the constituents of the scale insect pathogenic fungus *Torrubiella tenuis* BCC 12732, guided by its strong cytotoxic activity against human epidermoid carcinoma (KB, $IC_{50} = 1.37$ μg/mL) and human breast cancer (BC, $IC_{50} = 0.44$ μg/mL) cell lines. The study led to the isolation of three new isocoumarin glucosides, **1**, **3**, and **4**, together with 6,8-dihydroxy-3-methylisocoumarin (**2**) and 6,8-dihydroxy-3-hydroxymethylisocoumarin (**5**).

Isocoumarins **1** and **3** were obtained from both the culture broth and mycelia extracts of BCC 12732, while compounds **2**, **4**, and **5** were present only in the culture broth extract (Figure 1). Column chromatography using silica gel and Sephadex LH-20 was used to purify compounds.

Compound **1** was obtained as a pale brown solid. Its molecular formula was determined by HRMS (ESITOF) in combination with ^{13}C NMR data as $C_{17}H_{20}O_9$. The IR spectrum showed absorption bands at 3424 and 1689 cm^{-1}, representing the alcohol and conjugated lactone functional groups, respectively. ^{1}H, ^{13}C, and 2D NMR data (COSY, NOESY, HMQC, and HMBC) in DMSO-d_6 revealed the presence of a sugar unit attached to a 6,8-dihydroxy-3-methylisocoumarin (**2**).[11] The ^{1}H NMR data of the isocoumarin moiety showed aromatic *meta*-coupled methine hydrogens at δ_H 6.60 and 6.57 ($J = 2.2$ Hz), a singlet methine at δ_H 6.52, one methyl singlet at δ_H 2.23, and a phenolic hydroxy group at δ_H 10.96. For the sugar moiety, the vicinal *trans*-coupling ($J_{1',2'} = 8.2$ Hz, $J_{2',3'} = 8.6$ Hz, $J_{3',4'}$ and $J_{4',5'} = 9.3$ Hz) proved the axial orientation of protons 1′−5′. The position of the methoxy group (δ_H 3.36) on the sugar unit was confirmed by HMBC correlation of the *O*-methyl protons to C-4′ (δ_C 79.3). These results indicated that the sugar unit was 4-*O*-methyl-β-glucopyranose. The position of attachment of the sugar unit at C-6 of the isocoumarin moiety was also established on the basis of HMBC correlation between the anomeric proton and C-6. The D-configuration of 4-*O*-methyl-β-glucopyranose was established by comparing the specific rotation of the aqueous layer of its acid hydrolysate ($[\alpha]^{25}_D$ +67.7, *c* 0.19, MeOH) with that of 4-*O*-methyl-D-glucopyranose ($[\alpha]^{20}_D$ +80, *c* 1.3, MeOH).[12] The ^{1}H and ^{13}C NMR data of the isocoumarin aglycone **2**, obtained by acid hydrolysis of **1**, were identical to those of 6,8-dihydroxy-3-methylisocoumarin.[11] Selected NOESY cross-coupling and HMBC correlations for compound **1** are presented in Figure 2.

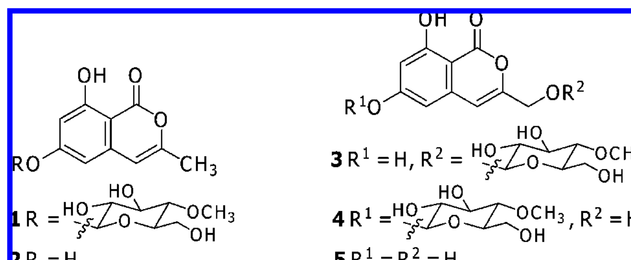

Figure 1. Structures of compounds **1**−**5** from BCC 12732.

Compounds **3** and **4** were obtained as off-white and pale brown solids, respectively. Related to compound **1**, the molecular formulas of compounds **3** and **4** were both established as $C_{17}H_{20}O_{10}$ on the basis of HRMS (ESITOF). The ^{1}H and ^{13}C NMR data of these two compounds were similar to those of compound **1** except for the lack of a methyl signal (δ_H 2.23) and the presence of an additional methylene group [δ_H 4.56 and 4.39 ($J = 14$ Hz), δ_C 66.3 and δ_H 4.28, δ_C 59.9 in compounds **3** and **4**, respectively]. The NMR data of both compounds **3** and **4** revealed the presence of a 6,8-dihydroxy-3-hydroxymethylisocoumarin unit[13] and a 4-*O*-methyl-β-glucopyranose moiety as present in compound **1**. The position of attachment of the sugar moiety on the isocoumarin unit was indicated by HMBC correlations of H-1′ to C-9 and H-9 to C-1′ in compound **3** and the correlation of H-1′ to C-6 in compound **4**. Acid hydrolysis of compound **3** yielded 4-*O*-methyl-D-glucopyranose[12] and 6,8-dihydroxy-3-hydroxymethylisocoumarin. However, due to the small quantity available, acid hydrolysis of compound **4** was not performed. Selected NOESY and HMBC correlations for compounds **3** and **4** are presented in Figure 2.

Compounds **2** and **5** were also isolated as co-metabolites from the culture broth extract of BCC 12732. Their spectroscopic data were identical to those of 6,8-dihydroxy-3-methylisocoumarin[12] and

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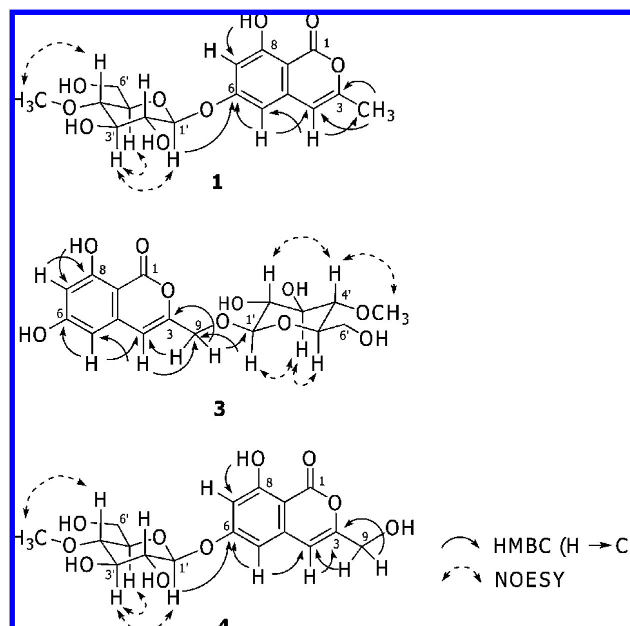

Figure 2. Selected NOESY and HMBC correlations of compounds **1**, **3**, and **4**.

6,8-dihydroxy-3-hydroxymethylisocoumarin,[13] respectively. These isocoumarins were reported as metabolites of fungi of the genus *Ceratocystis*[13−15] and are known for their effects on plant growth.[13,16,17]

Although, compounds **1−4** showed no biological activity (antimalarial, antimycobacterium, antivirus, and cytotoxicity against KB, MCF-7, NCI-H187, and Vero cells), compound **5**, however, displayed moderate growth inhibitory activity against both Herpes simplex virus-1 and *Mycobacterium tuberculosis* $H_{37}Ra$ with an IC_{50} value of 50 μg/mL and MIC value of 25 μg/mL, respectively. In addition, the strong cytotoxic activity found in the crude extract was absent in all pure materials. This unexpected observation could result from the amount of the cytotoxic compounds being too small to be detected during the separation process.

Experimental Section

General Experimental Procedures. Melting points were measured using an electrothermal IA9100 digital melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 digital polarimeter. UV and FT-IR spectra were recorded on a Varian Cary 1E UV−vis spectrophotometer and a Bruker VECTOR 22 spectrometer. NMR spectra were recorded on a Bruker AV500D spectrometer. ESITOFMS were obtained from a Micromass LCT mass spectrometer; accurate mass was determined by lock mass calibration.

Fungal Material. The fungus *T. tenuis* was isolated from a Homoptera scale insect collected in June 2002 from the underside of a dicotyledonous leaf from Doi Inthanon National Park, Chiang Mai Province, Thailand. The specimen was identified by Nigel L. Hywel-Jones, BIOTEC. This fungus was deposited in the BIOTEC Culture Collection (BCC) as BCC 12732 on May 6, 2003.

Fermentation and Isolation. *T. tenuis* BCC 12732 was maintained on potato dextrose agar at 25 °C, and the agar was cut into pieces (1 × 1 cm) and inoculated into 2 × 250 mL Erlenmeyer flasks containing 25 mL of potato dextrose broth (PDB, potato starch 4.0 g, dextrose 20.0 g, per liter). After incubation at 25 °C for 7 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1 L Erlenmeyer flask containing 250 mL of the same liquid medium (PDB) and incubated under the same conditions for 7 days. A 25 mL portion of the secondary culture was transferred into 20 × 1 L Erlenmeyer flasks containing 250 mL of liquid medium (PDB) and incubated at 25 °C for 19 days under static conditions.

After the mycelium was filtered, the culture broth was extracted with (3 × 5 L) EtOAc and evaporated to dryness, leaving a dark brown solid (2.78 g). The insoluble compound **1** (0.29 g) was obtained as a pale brown solid after trituration of the crude extract with MeOH followed by filtration. The resulting filtrate (2.48 g) was passed through a Sephadex LH-20 chromatographic column (3.5 × 22 cm) with MeOH as eluent to obtain five fractions (1-1−1-5). Fraction 1-3 contained compounds **3**, **4**, and **5**. Compound **3** was crystallized from MeOH as an off-white solid (0.17 g). The mother liquid (1.16 g) was fractionated using a silica gel column (3.5 × 20 cm, 5% step gradient elution from 0 to 60% MeOH/CH_2Cl_2, 200 mL each) to provide seven fractions (1-3-1−1-3-7). An additional amount of compound **3** (0.20 g) was obtained from fractions 1-3-3 and 1-3-4. Fraction 1-3-2 was further purified on a silica gel column (5% step gradient elution from 0 to 40% MeOH/CH_2Cl_2, 300 mL each) followed by Sephadex LH-20 with MeOH as eluent to furnish compound **5** (16.3 mg). Fractions 1-3-5 and 1-3-6 were combined and then subjected to silica gel column chromatography (5% step gradient elution from 0 to 40% MeOH/CH_2Cl_2, 100 mL each), followed by Sephadex LH-20 with MeOH as eluent to afford pure compound **4** (8.8 mg). Fraction 1-4 was subjected to silica gel column chromatography (10% step gradient elution from 10 to 100% EtOAc/*n*-hexane, 200 mL each) to provide nine fractions (1-4-1−1-4-9). Compound **2** (5 mg) and compound **5** (79.4 mg) were obtained from fractions 1-4-2 and 1-4-4, respectively.

Compounds **1** and **3** were isolated after the cells were macerated in MeOH for 3 days, followed by CH_2Cl_2 for 3 days. The MeOH and CH_2Cl_2 extracts were combined and evaporated under reduced pressure. Water (200 mL) was added, and the mixture was washed with *n*-hexane (3 × 200 mL), which was discarded, and then extracted with EtOAc (3 × 200 mL). The combined organic phase was evaporated to dryness to yield a dark brown solid (0.68 g). The crude extract was subjected to a Sephadex LH-20 column (3.5 × 29 cm) with MeOH as eluent to afford nine fractions (2-1−2-9). Compounds **1** (0.63 g) and **3** (23.7 mg) were obtained from fractions 2-7 and 2-8, respectively.

Isocoumarin (1): pale brown solid; mp 191.7−193.0 °C; $[\alpha]^{26}_{D}$ −63.41 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 235 (4.58), 242 (4.70), 255 sh (4.11), 274 (3.89), 286 (3.83), 328 (3.79) nm; IR (KBr) ν_{max} 3424, 1689, 1650, 1629, 1574, 1243, 1092, 1048, 1026, 998 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 10.96 (1H, br s, 8-OH), 6.60 (1H, d, J = 2.2 Hz, H-5), 6.57 (1H, d, J = 2.2 Hz, H-7), 6.52 (1H, s, H-4), 5.49 (1H, d, J = 5.2 Hz, 2′-OH), 5.32 (1H, d, J = 5.6 Hz, 3′-OH), 5.06 (1H, d, J = 8.2 Hz, H-1′), 4.72, (1H, t, J = 5.6 Hz, 6′-OH), 3.63 (1H, dd, J = 8.2, 5.6 Hz, H-6a′), 3.51 (1H, dd, J = 8.2, 5.6 Hz, H-6b′), 3.48 (1H, m, H-5′), 3.43 (1H, ddd, J = 9.3, 8.6, 5.6 Hz, H-3′), 3.36 (3H, s, 4′-OMe), 3.25 (1H, ddd, J = 8.6, 8.2, 5.2 Hz, H-2′), 3.05 (1H, dd, J = 9.3, 9.3 Hz, H-4′), 2.23 (3H, s, H-9); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 165.8 (C-1), 164.7 (C-6), 162.9 (C-8), 155.0 (C-4a), 139.9 (C-3), 104.8 (C-4), 103.4 (C-5), 102.3 (C-7), 100.4 (C-8a), 99.8 (C-1′), 79.3 (C-4′), 76.6 (C-3′), 76.1 (C-5′), 73.7 (C-2′), 60.6 (C-6′), 60.1 (4′-OMe), 19.3 (C-9); HRMS (ESITOF) *m/z* 391.0998 $[M + Na]^+$ (calcd for $C_{17}H_{20}O_9Na$, 391.1000).

Isocoumarin (3): off-white solid; mp 243.4−244.7 °C; $[\alpha]^{26}_{D}$ −32.37 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 237 (4.67), 244 (4.73), 258 sh (4.14), 277 (3.90), 289 (3.83), 328 (3.94) nm; IR (KBr) ν_{max} 3377, 1692, 1644, 1633, 1578, 1499, 1394, 1369, 1305, 1263, 1241, 1184, 1149, 1118, 1091, 1056, 1033 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 10.95 (1H, br s, 8-OH), 6.79 (1H, s, H-4), 6.41 (1H, d, J = 2.1 Hz, H-5), 6.36 (1H, d, J = 2.1 Hz, H-7), 5.30 (1H, d, J = 5.2 Hz, 2′-OH), 5.18 (1H, d, J = 5.4 Hz, 3′-OH), 4.70, (1H, t, J = 5.6 Hz, 6′-OH), 4.56 (1H, d, J = 14.0 Hz, H-9a), 4.39 (1H, d, J = 14.0 Hz, H-9b), 4.28 (1H, d, J = 8.1 Hz, H-1′), 3.61 (1H, dd, J = 8.1, 5.6 Hz, H-6a′), 3.48 (1H, dd, J = 8.1, 5.6 Hz, H-6b′), 3.42 (3H, s, 4′-OMe), 3.29 (1H, ddd, J = 9.1, 8.7, 5.4 Hz, H-3′), 3.15 (1H, m, H-5′), 3.04 (1H, ddd, J = 8.7, 8.1, 5.2 Hz, H-2′), 2.95 (1H, dd, J = 9.6, 9.1 Hz, H-4′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 166.2 (C-6), 165.4 (C-1), 163.2 (C-8), 153.3 (C-4a), 139.3 (C-3), 105.9(C-4), 104.0 (C-5), 102.7 (C-7), 102.6 (C-1′), 99.0 (C-8a), 79.9 (C-4′), 76.9 (C-3′), 76.1 (C-5′), 74.1 (C-2′), 66.3 (C-9), 61.1 (C-6′), 60.1 (4′-OMe); HRMS (ESITOF) *m/z* 407.0973 $[M + Na]^+$ (calcd for $C_{17}H_{20}O_{10}Na$: 407.0949).

Isocoumarin (4): pale brown solid; mp 242.3−242.8 °C; $[\alpha]^{26}_{D}$ −11.05 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ε) 235 (5.32), 241 (5.34), 256 sh (4.88), 275 (4.70), 287 (4.57), 327 (4.68) nm; IR (KBr) ν_{max} 1685, 1647, 1625, 1572, 1384, 1245, 1092, 1048, 992 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 10.94 (1H, br s, 8-OH), 6.73 (1H, d, J = 2.2 Hz, H-5), 6.69 (1H, s, H-4), 6.59 (1H, d, J = 2.2 Hz, H-7), 5.69 (1H, t, J = 5.7 Hz, 9-OH), 5.51 (1H, d, J = 5.3 Hz, 2′-OH), 5.33 (1H, d, J = 5.6 Hz, 3′-OH), 5.07 (1H, d, J = 8.1 Hz, H-1′), 4.75 (1H, t, J = 5.6 Hz, 6′-OH), 4.28 (2H, d, J = 5.7 Hz, H-9), 3.63 (1H, m, H-6a′),

3.51 (1H, m, H-6b′), 3.48 (1H, m, H-5′), 3.45 (3H, s, 4′-OMe), 3.40 (1H, m, H-3′), 3.25 (1H, ddd, J = 8.4, 8.1, 5.3 Hz, H-2′), 3.05 (1H, dd, J = 9.3, 9.3 Hz, H-4′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 165.4 (C-1), 164.7 (C-6), 162.9 (C-8), 157.8 (C-4a), 139.5 (C-3), 104.1 (C-5), 103.4 (C-4), 102.8 (C-7), 100.8 (C-8a), 99.8 (C-1′), 79.3 (C-4′), 76.6 (C-3′), 76.2 (C-5′), 73.7 (C-2′), 60.6 (C-6′), 59.9 (C-9); HRMS (ESITOF) m/z 407.0970 $[M + Na]^+$ (calcd for $C_{17}H_{20}O_{10}Na$: 407.0949).

Hydrolysis of Isocoumarin 1. Compound **1** (32 mg) was hydrolyzed with 10% aqueous HCl (1 mL) at 90 °C for 12 h. The reaction mixture was then diluted with H_2O (2 mL) and extracted with EtOAc (2 × 3 mL). The aqueous layer was concentrated under vacuum to yield 4-*O*-methyl-D-glycopyranose (15.2 mg, $[\alpha]^{25}_D$ +67.7, *c* 0.19, MeOH). The organic layer was evaporated to dryness under reduced pressure to obtain the aglycone (13.7 mg) whose ^{1}H NMR data were identical to those of 6,8-dihydroxy-3-methylisocoumarin (**2**).[11]

Hydrolysis of Isocoumarin 3. Compound **3** (12 mg) was hydrolyzed similarly to **1**. The aqueous layer was concentrated under vacuum to yield 4-*O*-methyl-D-glycopyranose (8 mg, $[\alpha]^{26}_D$ +61.3, *c* 0.40, MeOH). The organic layer was evaporated to dryness under reduced pressure to obtain the aglycone, 6,8-dihydroxy-3-hydroxymethylisocoumarin (5 mg).[13]

Biological Assays. Antimalarial activity against *Plasmodium falciparum* K1 was evaluated by using the microculture radioisotope technique.[18] The IC_{50} value of the standard antimalarial compound, dihydroartemisinin, was 0.0011 μg/mL. Assays for anti-Herpes simplex virus type 1 (HSV-1), anti-*Mycobacterium tuberculosis* H_{37}Ra, and cytotoxicity against Vero cells (Afican green monkey kidney fibroblast) were carried out by using the green fluorescent protein (GFP)-based method.[19] The IC_{50} value of the standard anti-HSV-1, acyclovir, was 6.86 μg/mL. The standard antitubercular drug, isoniazid, showed MIC values of 0.023−0.046 μg/mL. The IC_{50} value of a standard cytotoxic compound, ellipticine, against Vero cells was 1.16 μg/mL. Anticancer activities against KB cells (oral human epidermoid carcinoma), MCF-7 cells (human breast cancer), and NCI-H187 cells (human small-cell lung cancer) were evaluated using the resazurin microplate assay.[20] Doxorubicin, an anticancer agent, exhibited cytotoxic activities against KB, MCF-7, and NCI-H187 cell lines with IC_{50} values of 0.144, 0.899, and 0.058 μg/mL, respectively.

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Supporting Information Available: NMR spectra of compounds **1**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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