The Isolation of Two New Chromone Derivatives from the New Zealand Fungus *Tolypocladium extinguens*

Yunjiang Feng,[†] John W. Blunt,[†] Anthony L. J. Cole,[‡] and Murray H. G. Munro*[,†]

Department of Chemistry and Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

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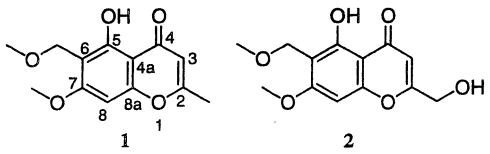
Two new chromone derivatives, 6-methoxymethyleugenin (**1**) and 2-hydroxymethyl-6-methoxymethyleugenin (**2**), and the previously reported 6-hydroxymethyleugenin (**3**) were isolated from the New Zealand fungus *Tolypocladium extinguens*. The structures of these compounds were elucidated on the basis of spectroscopic data. 6-Methoxymethyleugenin (**1**) showed moderate cytotoxicity against P388 leukemia cells.

The genus *Tolypocladium* has attracted a great deal of attention since the discovery of immunosuppressive agents, the cyclosporines from *Tolypocladium inflatum*.[1] Extensive studies on the different species suggested that in addition to the cyclosporins a variety of bioactive metabolites are produced.[2] In our continuing studies of bioactive natural products from New Zealand fungi, we became interested in one isolate, *Tolypocladium extinguens*. The crude fungal extract showed moderate cytotoxicity against murine P388 leukemia cells, and further investigation led to the isolation of two new chromone derivatives, 6-methoxymethyleugenin (**1**) and 2-hydroxymethyl-6-methoxymethyleugenin (**2**), along with the previously reported metabolite 6-hydroxymethyleugenin (**3**).[3−5]



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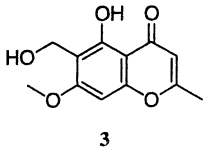

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The entomopathogenic fungus *T. extinguens* (CANU-T880) was isolated from the New Zealand glow-worm *Arachnocampa luminosa*. After 4 weeks fermentation in half-strength Sabouraud dextrose yeast broth (SDY) under static conditions at 26 °C, an ethyl acetate extract of the mycelium and culture filtrate was separated using flash reverse-phase (rp) chromatography. Repeated Diol chromatography on selected fractions from the rp column yielded **1**−**3**.

The molecular formula of the major metabolite **1**, a white crystalline solid, was deduced as $C_{13}H_{14}O_5$ (seven double-bond equivalents) by HREIMS analysis in combination with ^{1}H and ^{13}C NMR data. **1** had a very simple ^{1}H NMR spectrum (seven singlets) which suggested seven uncoupled protons. The sharp singlet at 13.03 ppm was interpreted as a hydrogen-bonded phenol hydroxy group. The APT sequence experiment on **1** confirmed 13 carbon signals including three methyl, one methylene, two methine, and seven quaternary carbon signals. The presence of an oxygen-substituted aromatic system was deduced on the basis of carbon chemical shifts and supported by the corresponding UV spectrum (maxima at 231, 250, 256, and 290 nm). The quaternary carbon signal at 182.4 ppm was assigned to a ketone carbonyl group and confirmed by the IR absorption at 1664 cm^{-1}.

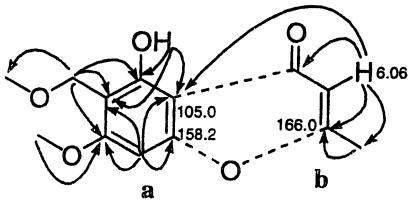

Figure 1. Substructures **a** and **b** and CIGAR correlations for **1**.

With the aid of this information, the structure of **1** was elucidated by a combination of 2D NMR experiments. A detailed analysis of the HSQC and CIGAR[6] experiments defined two subunits: a substituted aromatic ring **a** and a β-methyl-α,β-unsaturated ketone **b** (Figure 1). Further correlations from the proton at 6.06 ppm in **b** to the carbon at 105.0 ppm in **a** linked the two subunits. However, no other correlations were observed under a wide range of experimental conditions that would confirm the formation of the benzopyranone ring system between **a** and **b**. The final definition of the structure **1** was therefore based on consideration of the molecular formula, double bond equivalents, and the downfield shifts of the carbon signals at C-2 and C-8a (158.2 and 166.0 ppm) (Figure 1).

Compound **2**, also a white crystalline solid, possessed very similar ^{1}H and APT NMR data to those of **1**. The structure of **2** differed from **1** only in the replacement of a CH_3 by a CH_2OH at the C-2 position, where a methyl singlet (2.36 ppm) was replaced by a methylene singlet (4.47 ppm) in the ^{1}H NMR spectrum. The ^{13}C NMR spectral data (20.4 ppm replaced by 60.6 ppm) were consistent with this change. Further analysis of the 2D experimental data and the molecular formula $C_{13}H_{14}O_6$ from HREIMS corroborated the structure of **2**.

The minor metabolite **3** had previously been isolated from a number of different fungi and lichen, but only limited spectral data have been reported in the literature.[3−5] Full ^{1}H and ^{13}C NMR assignments were accomplished by comparison with those for compounds **1** and **2** and confirmed by the analysis of the 2D NMR spectral data.

* To whom correspondence should be addressed. Tel: 64-3-3642434. Fax: 64-3-3642429. E-mail: m.munro@chem.canterbury.ac.nz.
[†] Department of Chemistry.
[‡] Department of Plant and Microbial Sciences.

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Chromone derivatives have been isolated from a wide variety of plants and fungi.[7] Previous studies suggested they possess a spectrum of activities including anticancer[8,9] and the inhibition of gray hair by promotion of melanin formation.[10] The compounds isolated in this study are the first chromone derivatives reported from the fungus *T. extinguens*. The bioactivity evaluation showed that compound **1** had a moderate cytotoxicity against P388 cultured cells[11−13] with an ID_{50} value of 6 μg/mL. None of the compounds isolated showed activity in the antimicrobial assay[12−14] against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Trichophyton mentagrophytes*, and *Cladosporium resinae*.

Experimental Section

General Experimental Procedures. UV and IR spectra were measured with a GBC UV/vis 920 spectrometer and a Shimadzu FTIR-8201 PC spectrometer, respectively. ^{1}H, ^{13}C-APT, and 2D NMR (^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C CIGAR) spectra were recorded on a Varian INOVA 500 MHz spectrometer. EI mass spectra were acquired using a Kratos MS80RFA mass spectrometer. Column chromatography used 40 μM Prep LC Bakerbond Octadecyl (C_{18}) and 40 μM Prep LC Bakerbond Diol (COHCOH), and TLC was performed with Merck Diol TLC plates. Solvents for extraction and chromatography were distilled prior to use. Culture media SDY broth included Gibco yeast extract and peptone 100, Oxoid soya peptone and tryptose, and BDH glucose.

Fungal Material. *Tolypocladium extinguens* was isolated from an infected glow-worm, *Arachnocampa luminosa*, collected at Waitomo Caves, New Zealand. A voucher of the fungus has been deposited in the University of Canterbury fungal herbarium and assigned as CANU-T880.

Fermentation and Isolation. *T. extinguens* (CANU-T880) was fermented in half-strength SDY broth (2.5 g of yeast extract, 1 g of soya peptone, 2 g of peptone 100, 2 g of tryptose, and 10 g of glucose per liter of distilled water) under static conditions at 26 °C for 4 weeks. The culture broth (500 mL) was homogenized and filtered through Celite. The mycelium was extracted by stirring with ethyl acetate overnight (3 × 200 mL), as was the culture filtrate (3 × 500 mL). The combined ethyl acetate extracts were concentrated under vacuum, yielding a dark brown residue (275 mg). The residue was chromatographed on C_{18} using a steep, stepped solvent gradient from 10% MeOH/H_2O to MeOH to CH_2Cl_2. The fractions eluted with 40%, 50%, and 60% aqueous MeOH were repeatedly chromatographed on Diol with the gradient petroleum ether/EtOAc (4:1 to 3:2 to 1:1) to yield 6-methoxymethyleugenin (**1**) (3.3 mg), 2-hydroxymethyl-6-methoxymethyleugenin (**2**) (2.8 mg), and 6-hydroxymethyleugenin (**3**) (0.7 mg).

Compound 1: white crystalline solid; UV (MeOH) λ_{max} (log ϵ) 207 (4.47), 232 (4.38), 250 (4.38), 256 (4.39), 290 (3.94), 316 (sh) (3.67); IR (chloroform) ν_{max} 3693 (br), 2957, 2842, 1664, 1605, 1495, 1344, 1132, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 13.03 (1H, s, OH), 6.37 (1H, s, H-8), 6.06 (1H, s, H-3), 4.56 (2H, s, C*H*$_2$OCH$_3$), 3.91 (3H, s, OCH$_3$), 3.41 (3H, s, CH$_2$OC*H*$_3$), 2.36 (3H, s, CH$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 182.4 (C-4), 166.0 (C-2), 164.2 (C-7), 160.5 (C-5), 158.2 (C-8a), 109.1 (C-3), 108.9 (C-6), 105.0 (C-4a), 89.6 (C-8), 61.6 (*C*H$_2$OCH$_3$), 58.2 (CH$_2$O*C*H$_3$), 56.2 (OCH$_3$), 20.4 (CH$_3$); HREIMS *m*/*z* 250.08338 (calcd for $C_{13}H_{14}O_5$, 250.08412).

Compound 2: white crystalline solid; UV (MeOH) λ_{max} (log ϵ) 206 (4.56), 231 (4.42), 251 (4.40), 257 (4.40), 290 (3.99), 322 (sh) (3.67); IR (chloroform) ν_{max} 3693 (br), 2948, 2848, 1666, 1601, 1494, 1470, 1325, 1130, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 13.03 (1H, s, OH), 6.35 (1H, s, H-8), 6.12 (1H, s, H-3), 4.53 (2H, s, C*H*$_2$OCH$_3$), 4.47 (2H, s, C*H*$_2$OH), 3.91 (3H, s, OCH$_3$), 3.47 (3H, s, CH$_2$OC*H*$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 182.4 (C-4), 168.9 (C-2), 164.4 (C-7), 160.6 (C-5), 157.9 (C-8a), 108.8 (C-6), 106.1 (C-3), 105.2 (C-4a), 89.8 (C-8), 61.7 (*C*H$_2$-OCH$_3$), 60.6 (CH$_2$OH), 58.3 (CH$_2$O*C*H$_3$), 56.1 (OCH$_3$); HREIMS *m*/*z* 266.07868 (calcd for $C_{13}H_{14}O_5$, 266.07904).

Compound 3: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 13.03 (1H, s, OH), 6.37 (1H, s, H-8), 6.06 (1H, s, H-3), 4.81 (2H, s, CH$_2$OH), 3.91 (3H, s, OCH$_3$), 2.36 (3H, s, CH$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.4 (C-2), 163.0 (C-7), 159.4 (C-5), 157.8 (C-8a), 111.7 (C-6), 109.0 (C-3), 105.1 (C-4a), 89.5 (C-8), 55.9 (OCH$_3$), 53.6 (CH$_2$OH), 20.3 (CH$_3$).

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