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BIOACTIVE METABOLITES FROM *Penicillium* sp. P-1, A FUNGAL ENDOPHYTE IN *Huperzia serrata*

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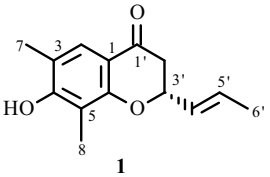
A chemical study of metabolites of the strain Penicillium *sp. P-1, an endophyte from the stems of* Huperzia serrata*, furnished a new chromone derivative, (2*S*)-2,3-dihydro-7-hydroxy-6,8-dimethyl-2-[(*E*)-prop-1-enyl]-chroman-4-one (**1**), an enantiomer of a known compound, and seven known compounds **2–8**. The structure and absolute configuration of **1** were established using spectroscopic methods, including extensive 2D NMR and CD analyses. Cytotoxic activity of compounds **1–3** against HeLa and HepG2 cell lines were evaluated, in which compounds **2** and **3** exhibited marked cytotoxic activity against HeLa cells.*

Keywords: endophytes, *Penicillium* sp., *Huperzia serrata*, cytotoxic.

An endophyte refers to a bacterial (including actinomycete) or fungal microorganism that spends the whole or part of its life cycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease [1, 2]. Fungal endophytes have been a hotspot of intense research since the realization of their ecological relevance and the potential of yielding metabolites with diverse structural and biological functions. As a result, many fungal endophytes have been cultured and subjected to detailed investigations during the past two decades, which led to the chemical characterization and biological evaluation of a large number of natural products with novel structures and interesting biological activities [3].

In the process of our research on the endophytes, the strain *Penicillium* sp. P-1 was obtained from the stems of *Huperzia serrata* and exhibited marked cytotoxic activity in the preliminary experiments. Chemical investigation of the metabolites of the fungus led to the isolation of a new chromone derivative **1** and seven known compounds **2–8**. The structures of all eight compounds were established on the basis of in-depth MS and NMR analyses. Compounds **1–3** were subjected to *in vitro* cytotoxic activitiy assay against HeLa and HepG2 cell lines.



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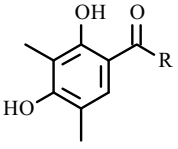

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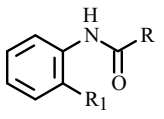

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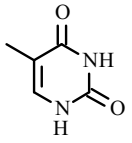

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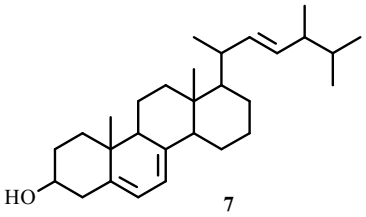
2: R = -CH=CH-CH=CH-CH_3
3: R = -CH_2-CH_2-CH=CH-CH_3

4: R_1 = H, R_2 = -CHCl-CH_3
5: R_1 = OH, R_2 = CH_3



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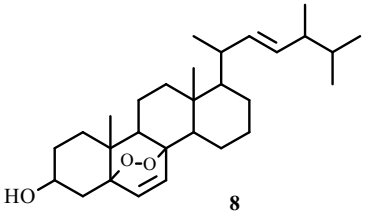

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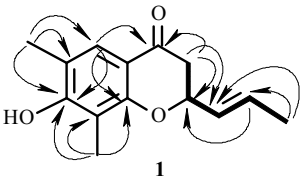
TABLE 1. NMR Data of **1** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_H	δ_C	C atom	δ_H	δ_C
1	–	114.4 (C)	1′	–	191.9 (C)
2	7.56 (1H, s)	125.8 (CH)	2′	2.70 (2H, m)	42.7 (CH_2)
3	–	117.3 (C)	3′	4.85 (1H, m)	78.2 (CH)
4	–	159.0 (C)	4′	5.72 (1H, dd, J = 15.5, 6.5)	128.9 (CH)
5	–	110.7 (C)	5′	5.87 (1H, dq, J = 15.5, 6.5)	129.8 (CH)
6	–	159.5 (C)	6′	1.77 (3H, d, J = 6.5)	17.8 (CH_3)
7	2.21 (3H, s)	15.6 (CH_3)			
8	2.14 (3H, s)	8.1 (CH_3)			



1

Fig. 1. ^1H–^1H COSY (━) and key HMBC (→) correlations of **1**.

Compound **1** was obtained as a colorless needle crystal. It was assigned the molecular formula $C_{14}H_{16}O_3$ from HR-ESI-MS measurement that showed a pseudomolecular ion peak at *m/z* 233.1173 $[M + 1]^+$ (calcd for $C_{14}H_{17}O_3$, 233.1178). The IR spectrum exhibited bands at 3379 and 1651 cm^{-1} due to hydroxyl and carbonyl groups, respectively. The ^1H NMR spectrum of **1** revealed the presence of three methyl groups (δ 2.21, 2.14, and 1.77) and a disubstituted double bond signal at δ 5.72 (1H, dd, J = 15.5, 6.5 Hz) and 5.87 (1H, dq J = 15.5, 6.5 Hz). It also showed signals due to an oxygen-bearing methine at δ 4.85 (1H, m). The ^{13}C NMR and DEPT spectra of **1** exhibited the presence of 14 C atoms comprising six quaternary, four tertiary, and one secondary C atom, as well as three methyl groups. Among them, except for the carbon signals assigned the carbonyl group (δ 191.9), the eight relatively downfield carbon signals were ascribed to the benzene ring and one double-bond group. Thus, the remaining one out of the total of seven degrees of unsaturation had to account for another ring in the structure. The data mentioned above, combined with the ^{13}C NMR data, showed that **1** was a chromane derivative [4, 5].

The proton and corresponding carbon signals in the NMR spectra of **1** were unequivocally assigned by HMQC experiments. The ^1H–^1H COSY and HMBC correlations (Fig. 1) showed the existence of a propenyl that was connected to the C-3′ deduced from the HMBC correlations of H-5′/C-3′ and H-4′/C-3′; the C-7 was attached to C-3 by the strong HMBC correlations of H_3-7/C-2, H_3-7/C-4, and H_3-7/C-3; the methyl signal at δ 2.14 showed correlation with C-4, C-5, and C-6, indicating the linkage of this methyl to the C-5. The correlation of H-2/C-1′ offered evidence that the C-2 of the benzene ring was not substituted. Hence, the hydroxyl carrying the active proton was allocated to C-4. The planar structure of **1** was thus outlined.

The NMR and IR data of **1** showed good agreement with that of the known compound (2*R*)-2,3-dihydro-7-hydroxy-6,8-dimethyl-2-[(*E*)-prop-1-enyl]chroman-4-one (KC for short) [6]. However, the optical rotation of **1** showed the opposite result against KC, with $[\alpha]_D^{20}$ −2.67° (*c* 1.1, MeOH). A discrepancy was also observed in the data of CD spectra between **1** and KC. Compound **1** exhibited a positive Cotton effect at 286 nm and 223 nm, while KC exhibited a negative Cotton effect at the corresponding position. Based on the optical rotation and CD spectra, the conclusion can be drawn that **1** was an enantiomer of KC, and the absolute configuration in the C-3′ can be identified as S. Thus, **1** was named (2*S*)-2,3-dihydro-7-hydroxy-6,8-dimethyl-2-[(*E*)-prop-1-enyl]-chroman-4-one.

Compound **4** was obtained as a white powder whose structure was tentatively elucidated as 2-chloro-*N*-phenylpropanamide by interpreting and comparing the NMR and MS data with the literature [7]. Results from an optical rotatory dispersion experiment aiming at determining the absolute configuration of **4** indicated that **4** was a racemic compound consisting of equal amounts of enatiomers and that it was not optically active. Based on the results above, we speculated that compound **4** was not naturally occurring and the chloroform used as extractant may contribute to its formation during the extracting process.

Furthermore, another six known compounds were identified as sorbicillin (**2**) [8], 2′,3′-dihydrosorbicillin (**3**) [8, 9], *N*-(2-hydroxyphenyl)-acetamide (**5**) [10], thymine (**6**) [11], (3β,22*E*)-ergosta-5,7,22-trien-3-ol (**7**) [12] and 5α,8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol (8) [13] by comparison of their spectroscopic data with literature values.

The cytotoxic activity of **1–3** was evaluated *in vitro* using HeLa and HepG2 cell lines as targets. The results showed that compound **2** exhibited potent cytotoxic activity against HeLa cells and weak activity against HepG2 cells with IC_{50} of 1.6 and 27.2 μM, respectively. Compound **3** showed moderate activity against HeLa cells and weak activity against HepG2 cells with IC_{50} of 7.4 and 44.4 μM.

EXPERIMENTAL

General Experiment Procedures. Melting points were measured on a Chinese X-4 melting point apparatus (uncorrected). Optical rotation was measured on a Perkin–Elmer 341 polarimeter. CD spectrum was acquired on a Jasco J-815 spectrometer. NMR data were recorded on a Bruker AM-500 NMR spectrometers using TMS as internal standard. The ESI-MS were recorded on an Agilent 6210-LC/TOF mass spectrometer, and the EI-MS were recorded on a Finnigan LCQoacw mass spectrometer. All solvents used were of analytical grade (Hangzhou Gaojing Fine Chemical Plant, Hangzhou, P. R. China). Silica gel (200–300 mesh), Toyopearl HW-40C gel (50–100 μm; Tosoh), and Lichroprep RP-18 (40–63 μm; Merck) were used for column chromatography, and a pre-coated silica gel GF_{254} plate (Qingdao Marine Chemical Plant, Qingdao, P. R. China) was used for TLC.

Fungal Material and Culture Conditions. The fungus P-1 was isolated from the healthy stems of *Huperzia serrata* using the same method we previously reported [14]. The plant samples were collected in Xishuangbanna Tropical Plant Garden, Chinese Academy of Science, Yunnan Province, P. R. China. The fungus was identified on the morphology level as *Penicillum* sp. by Prof. Wen-Hong Liu of Zhejiang Chinese Medical University. The original culture (ZJUT HS-P1) was deposited at Zhejiang University of Technology, People′s Republic of China. The fungus was cultured on PDA (potato 200 g/L, glucose 20 g/L, agar 18 g/L) slant at 28°C for 4 days. The fermentation was carried out in liquid potato-dextrose medium (potato 200 g/L, glucose 20 g/L) in Erlenmeyer flasks to a total of 100 L (250 mL × 400) at 28°C. The flasks were first inoculated on rotary shakers for 6 days at 185 r.p.m. and then cultivated for another 20 days without agitation.

Extraction and Isolation of the Metabolites. At the end of fermentation, the culture was filtered through cheesecloth. The mycelium was air-dried (dried weight 1.65 kg) and extracted three times in 95% EtOH by refluxing. The combined extract was evaporated to dryness under reduced pressure to afford the residue (456 g). The residue was suspended in water (2 L) and then partitioned successively with chloroform (6 × 0.5 L) and *n*-butanol (6 × 0.5 L). The chloroform extract (180 g) was first subjected to column chromatography over silica gel eluted with $CHCl_3$–MeOH (20:1→1:1) to afford five chemically distinct fractions (Fr. 1–Fr. 5). Fraction 1 (2.7 g) was separated over Lichroprep RP-18 eluting with MeOH–H_2O (3:2→5:1) to give three refined fractions that were further purified over size-exclusion chromatography on Toyopearl HW−40C eluting with methanol to afford **1** (35 mg), **2** (27 mg), and **3** (14.6 mg). Fraction 2 (6.3 g) was subjected to a silica gel column eluting with $CHCl_3$–MeOH (15:1), followed by repeated size-exclusion chromatography on Toyopearl HW-40C to give **4** (21.5 mg) and **5** (18 mg). Repeated chromatography of Fr. 3 (4.0 g) over silica gel afforded **7** (1.1 g) and **8** (75 mg). The *n*-butanol extracts (15 g) was subjected to silica gel eluting with $CHCl_3$–MeOH (5:1) to give **6** (15 mg).

(2*S*)-2,3-Dihydro-7-hydroxy-6,8-dimethyl-2-[(*E*)-prop-1-enyl]-chroman-4-one (1). Colorless needle crystal, mp 184–186°C; $[\alpha]_D^{20}$ – 2.7° (*c* 1.1, MeOH). UV spectrum (MeOH, λ_{max}, nm) (log ε): 216 (4.26), 283 (4.04). IR spectrum (KBr, ν, cm^{-1}): 3379, 2919, 1651, 1584, 1352, 1296, 1192, 958. CD spectrum (MeOH, λ_{max}, nm) (De): 286 (+0.56), 223 (+0.40), ESI-MS *m/z* 233 $[M + 1]^+$; HR-ESI-MS *m/z* 233.1173 $[M + 1]^+$ (calcd for $C_{14}H_{17}O_3$, 233.1178). ^1H and ^{13}C NMR, see Table 1.

Sorbicillin (2). Pale yellow powder, mp 129–131°C. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 13.58 (1H, s), 7.46 (1H, dd, J = 14.5, 10.5), 7.45 (1H, s), 6.94 (1H, d, J = 15), 6.38–6.25 (2H, m), 2.22 (3H, s), 2.15 (3H, s), 2.22 (3H, d, J = 6.5). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 192.6 (C), 162.6 (C), 158.8 (C), 144.5 (CH), 140.9 (CH), 130.7 (CH), 128.8 (CH), 122.0 (CH), 114.4 (C), 113.7 (C), 110.5 (C), 18.9 (CH_3), 15.6 (CH_3), 7.5 (CH_3). ESI-MS *m/z:* 232 $[M]^+$, 231 $[M – 1]^+$.

2′,3′-Dihydrosorbicillin (3). Pale yellow powder, mp 68–70°C. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.98 (1H, s), 7.39 (1H, s), 5.52–5.49 (2H, m), 5.42 (1H, s), 2.96 (2H, t, J = 7.5), 2.42–2.38 (2H, m), 2.20 (3H, s), 2.13 (3H, s), 1.65 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 204.5 (C), 161.4 (C), 158.7 (C), 129.6 (CH), 129.1 (CH), 126.1 (CH), 114.5 (C), 113.0 (C), 110.4 (C), 37.9 (CH_2), 27.6 (CH_2), 17.9 (CH_3), 15.6 (CH_3), 7.5 (CH_3). ESI-MS *m/z:* 234 $[M]^+$, 233 $[M – 1]^+$.

2-Chloro-*N*-phenylpropanamide (4). White powder, mp 91–93°C. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 8.28 (1H, br.s), 7.55 (2H, d, J = 7.5), 7.36 (2H, t, J = 7.5), 7.16 (1H, t, J = 7.5), 4.55 (1H, dd, J = 14.0, 7.0), 1.83 (3H, d, J = 7.0). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 167.5 (C-7), 136.9 (C-1), 129.1 (C-3, C-5), 125.1 (C-4), 120.1 (C-2, C-6), 56.2 (C-8), 22.6 (C-9). EI-MS *m/z* (I_{rel}, %): 183 (19, M^+), 148 (3), 120 (100).

***N*-(2-Hydroxyphenyl)-acetamide (5)**. White powder, mp 210–212°C. ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.58 (1H, dd, J = 8.0, 1.0), 7.02 (1H, dt, J = 1.5, 8.0), 6.88 (1H, dd, J = 8.0, 1.0), 6.82 (1H, dt, J = 1.5, 8.0), 2.19 (3H, s). ^{13}C NMR (125 MHz, CD_3OD, δ): 172.3 (C), 149.8 (C), 127.0 (C), 126.9 (CH), 124.0 (CH), 120.6 (CH), 117.4 (CH), 23.5 (CH_3). EI-MS *m/z* (I_{rel}, %): 151 (20, M^+), 109 (100), 80 (21).

Thymine (6). Bright yellow needle crystal, mp 314–316°C. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 10.86 (2H, br.s), 7.25 (1H, s), 1.73 (3H, s). ^{13}C NMR (125 MHz, DMSO-d_6, δ): 164.9 (C), 151.5 (C), 137.7 (CH), 107.6 (C), 11.8 (CH_3). EI-MS *m/z* (I_{rel}, %): 126 (100, M^+), 55 (81), 28 (35).

(3β,22*E*)-Ergosta-5,7,22-trien-3-ol (7). White powder, mp 153–155°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 5.57 (1H, m), 5.38 (1H, m), 5.15–5.25 (2H, m), 3.64 (1H, m), 1.04 (3H, d, J = 6.5), 0.95 (3H, s), 0.92 (3H, d, J = 6.5), 0.84 (3H, d, J = 7.5), 0.82 (3H, d, J = 7.0), 0.63 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 141.4 (C), 139.8 (C), 135.6 (CH), 132.0 (CH), 119.6 (CH), 116.3 (CH), 70.5 (CH), 55.7 (CH), 54.6 (CH), 46.3 (CH), 42.9 (C), 42.8 (CH), 40.8 (CH_2), 40.4 (CH), 39.1 (CH_2), 38.4 (CH_2), 37.0 (C), 33.1 (CH), 32.0 (CH_2), 28.3 (CH_2), 23.0 (CH_2), 21.2 (CH_2), 21.1 (CH_3), 20.0 (CH_3), 19.7 (CH_3), 17.6 (CH_3), 16.3 (CH_3), 12.0 (CH_3).

5α,8α-Epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol (8). Colorless needle crystal, mp 182–184°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.50 (1H, d, J = 8.5), 6.24 (1H, d, J = 8.5), 5.22 (1H, dd, J = 15.0, 7.5), 5.14 (1H, dd, J = 15.0, 7.5), 3.97 (1H, m), 1.00 (3H, d, J = 6.5), 0.90 (3H, d, J = 6.5), 0.88 (3H, s), 0.83 (3H, d, J = 5.0), 0.82 (3H, d, J = 3.0), 0.80 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 135.4 (CH), 135.2 (CH), 132.3 (CH), 130.7 (CH), 82.1 (C), 79.4 (C), 66.4 (CH), 56.2 (CH), 51.6 (CH), 51.0 (CH_2), 44.5 (C), 42.7 (CH), 39.7 (CH), 39.3 (CH_2), 36.9 (CH), 34.7 (C), 33.0 (CH), 30.1 (CH_2), 28.6 (CH_2), 23.4 (CH_2), 20.8 (CH_2), 20.6 (CH_3), 19.9 (CH_3), 19.6 (CH_3), 18.1 (CH_3), 17.5 (CH_3), 12.8 (CH_3).

Bioassays. The cytotoxic assays were performed using the MTT assay method [15]. Compound **2** exhibited potent cytotoxic activity against HeLa cells and weak activity against HepG2 cells with IC_{50} of 1.6 and 27.2 μM, respectively. Compound **3** showed moderate activity against HeLa cells and weak activity against HepG2 cells with IC_{50} of 7.4 and 44.4 μM.

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