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Chalcone derivatives from the fern *Cyclosorus parasiticus* and their anti-proliferative activity

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ABSTRACT

Three new chalcone derivatives, named parasiticins A–C (**1**–**3**), were isolated from the leaves of *Cyclosorus parasiticus*, together with four known chalcones, 5,7-dihydroxy-4-phenyl-8-(3-phenyl-trans-acryloyl)-3,4-dihydro-1-benzopyran-2-one (**4**), 2′-hydroxy-4′,6′-dimethoxychalcone (**5**), 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**6**), 2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone (**7**). The chemical structures of the new isolated compounds were elucidated unambiguously by spectroscopic data analysis. The cytotoxic activities of compounds **1**–**7** were evaluated against six human cancer cell lines in vitro. Compounds **3** and **6** exhibited substantial cytotoxicity against all six cell lines, especially toward HepG2 with the IC_{50} values of 1.60 and 2.82 μM, respectively. Furthermore, we demonstrated that compounds **3** and **6** could induce apoptosis in the HepG2 cell line, which may contribute significantly to their cytotoxicity.

1. Introduction

The fern *Cyclosorus parasiticus* (L.) Farwell (Thelypteridaceae) is distributed throughout southern China, the Indo-China peninsula and Philippines. This plant is used in Chinese folk medicines for the treatment of rheumatic pain, cold, dysentery and fever. Since 2006, our research on the Thelypteridaceae has led to discovery of a variety of bioactive flavanoids with novel structures (Fang et al., 2011, 2006; Tang et al., 2010; Wei et al., 2011; Zhao et al., 2011, 2010, 2007, 2006).

Chalcone and its derivatives are widely distributed in nature from ferns to higher plants. They display a broad spectrum of biological activities and contribute to the medicinal value of herbs (Ni et al., 2004). Due to numerous potential pharmacological activities, such as anticancer, antioxidant, antimalarial and anti-inflammatory, chalcone and its derivatives have attracted continuously growing interest amongst the scientists (Batovska and Todorova, 2010). Many researches have shown that a number of chalcone derivatives exhibit cytotoxic and antitumor activities (Go et al., 2005; Amor et al., 2007; Simirgiotis et al., 2008).

Our researches on Thelypteridaceae are devoted to finding novel promising compounds. As part of this programme, in this paper, the leaves of *C. parasiticus* were collected for phytochemical and biological study, which led to the isolation of three new chalcone derivatives, named parasiticins A–C (**1–3**), and four known chalcones, 5,7-dihydroxy-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-3,4-dihydro-1-benzopyran-2-one (**4**), 2′-hydroxy-4′,6′-dimethoxychalcone (**5**), 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**6**), and 2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone (**7**). In this paper, we report the isolation and structure determination of compounds **1–7**, as well as their preferential cytotoxic activity against six human cancer cell lines (lung cancer A549, hepatocellular carcinoma HepG2, breast cancer MCF-7 and MDA-MB-231, leukemia ALL-SIL, and pancreatic cancer SW1990).

2. Materials and methods

2.1. General experimental procedures

Optical rotations were determined in $CHCl_3$ on a Perkin–Elmer 341 polarimeter. UV data were obtained using a Cary 50 UV–Visible spectrophotometer. 1D NMR and 2D NMR spectra were recorded on a Bruker-AM-400 spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants are given in Hz. HRESIMS was performed on a Thermo Scientific

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LTQ-Orbitrap XL mass spectrometer and ESIMS on a Agilent LC/MSD Trap XCT ion trap mass spectrometer. Column chromatography (CC) was carried out on silica gel (100–200 mesh and 200–300 mesh, Qingdao Haiyang Chemical Industry Co., Ltd., People's Republic of China) and Sephadex LH-20 (Pharmacia, USA). Fractions were monitored with TLC, and spots were visualized by spraying with 5% H_2SO_4 in EtOH, followed by heating.

2.2. Samples and reagents

The leaves of *C. parasiticus* were collected at Shenzhen, Guangdong province, People's Republic of China, in June 2012. The plant material was identified by Dr. Jianping Wang of the School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology. A voucher specimen (CP201206) has been deposited at School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology.

2.3. Anti-proliferation activity assay

Six human cancer cell lines were used, namely, A549 lung cancer, HepG2 hepatocellular carcinoma, MCF-7 and MDA-MB-231 breast cancer, ALL-SIL human leukemia, and SW1990 pancreatic cancer. Cells were cultured in RPMI1640 or in DMEM medium (Hyclone), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere, 5% CO_2. To measure in vitro cell sensitivity to test compounds, a sulforhodamine B (SRB) assay was performed (Ihsan-ul-Haq et al., 2013). Doxorubicin was used as a positive control. Briefly, cells were harvested from exponential phase cultures, counted and plated in the 96-well plates. After treatment, cells were fixed with trichloroacetic acid (final concentration 10%) at 4 °C for 1 h. Then, the cells were washed five times with deionized water and stained with 0.4% SRB (dissolved in 1% acetic acid) for 15 min. Subsequently, each well was washed four times with 1% acetic acid to remove unbound stain and left to dry at room temperature. The bound protein stain was solubilized in a tris-base (tris(hydroxymethyl)aminomethane) solution. The optical density was read at 540 nm in a microplate reader (BioTek Instruments Inc., USA). The IC_{50} values were determined as the concentrations that inhibited cell viability by 50%.

2.4. Fluorescent staining of nuclei for HepG2 cells

HepG2 cells from exponentially growing cultures were seeded within 24-well culture plates and treated with compound **3** or **6**. After treatment, cells were washed with phosphate-buffered saline (PBS), and fixed in MeOH-HAc (3:1, v/v) for 10 min at 4 °C. Cells were stained with Hoechst 33258 (5 μg/mL in PBS) for 5 min at room temperature and then examined in a Nikon Eclipse fluorescence microscope at 356 nm.

2.5. Flow cytometry assay for HepG2 cells

Flow cytometry analysis was performed to detect apoptosis by AnnexinV-FITC Apoptosis Detection Kits (Key GEN BioTECH, China). Briefly, HepG2 cells were treated with compound **3** or **6**, then collected and washed twice with PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in binding buffer. Then, the cells were incubated with 5 μL FITC-conjugated annexin V and 5 μL propidium iodide (PI) for 15 min at room temperature in the dark. Finally, the cells were analyzed with a FACSort flow-cytometer (Becton–Dickinson, USA).

2.6. Clonogenicity assay

To determine long-term effects of compound **3**, HepG2 cells were seeded at low density (10^3 per well) into six-well plates for 12 h. After complete adhesion, cells were treated with compound **3** for 24 h. The medium was discarded, and fresh medium was added to the wells. Cells were allowed to grow until visible colonies formed. The cell colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

2.7. Statistical analysis

Data are expressed as means ± S.D. of at least three independent experiments. Statistical comparisons between groups were analyzed by one-way ANOVA with Tukey's posthoc tests. Significance of difference was indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$.

3. Results and discussion

3.1. Structural elucidation of the isolated compounds

Parasiticin A (**1**) was obtained as yellow needles. Its molecular formula was assigned on the basis of HRESIMS as $C_{24}H_{16}O_5$ (*m/z* 383.0921 $[M-H]^-$). The ^1H NMR spectrum (in acetone-d_6, Table 1) exhibited three single protons at δ 14.22 (1H, s, 7-OH), δ 6.32 (1H, s, 6-H) and δ 6.03 (1H, s, 3-H), while the resonances of a *trans*-configured double bond group were recorded at δ 7.92 (1H, dd, J = 15.5, 7.4 Hz, 3′-H) and δ 8.35 (1H, dd, J = 15.5, 3.7 Hz, 2′-H). The spectrum also showed aromatic signals of 10H at δ 7.42–7.86. Inspection of the ^{13}C NMR and DEPT spectra (Table 1) indicated 20 signals belonging to 24 sp^2 carbons including 14 methine and 10 quaternary carbons. The ^1H NMR and correlations of ^1H—^{13}C by HMBC (Fig. 1) experiments indicated the presence of two single-substituted aromatic rings. By comparing the ^1H and ^{13}C NMR data with the corresponding literature, we found that structure of **1** was very similar with 5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-1-benzopyran-2-one (interruptin B, Quadri-Spinelli et al., 2000), which was isolated from the congeneric plant, *C. interruptus*, and it exhibited the skeletons of a chalcone and a neoflavone. The HBMC correlation of 3-H at δ 6.03 with 2-C (δ 158.3), 4a-C (δ 101.9) and 1″-C (δ 139.8) revealed the existence of the neoflavone skeleton. The HMBC relationship of the double bond group (δ 8.35 and 7.92) suggested a fragment of O═C—C═C—C, exhibiting the skeleton of a chalcone. However, the connection position of 3-phenyl-*trans*-acryloyl substituent with the neoflavone (6-C or 8-C) cannot be determined by HMBC experiment. According to previous study, the 8a-C has lower chemical shifts (δ 150–158) than 5-C or 7-C (δ 160–168) in compounds with similar structures (Asai et al., 1991). The proton at δ 6.32 showed HMBC correlation with four carbon signals at δ 101.9, 104.3, 161.9, and 168.7, indicating that the proton had no ^1H—^{13}C relationship with 8a-C. Therefore, the 3-phenyl-*trans*-acryloyl substituent must be attached at 8-C. In conclusion, **1** could be proposed structurally as 5,7-dihydroxy-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-1-benzopyran-2-one, named parasiticin A.

Parasiticin B (**2**) was obtained as a yellow powder. Its molecular formula was assigned on the basis of HRESIMS as $C_{35}H_{36}O_6$ (*m/z* 551.2430 $[M-H]^-$). The ^{13}C NMR data of **2** (Table 1) was similar to **1** and interruptin B (Quadri-Spinelli et al., 2000), except the presence of a long-chain saturated fatty acid. The ESIMS result showed ions at *m/z* 550.9 $[M-H]^-$ and 396.9 $[M-155]^-$, indicating that **2** was a decanoic acid ester of interruptin B, which had a molecular weight of 398. The correlations of ^1H—^{13}C of **2** were showed in Fig. 1. Based on the ^1H NMR and DEPT spectrums of **2** (Table 1) and the structure of interruptin B, the esterification position must be at 5-OH of interruptin B. Therefore, the structure of **2** was elucidated as 7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-1-benzopyran-2-one-5-yl decanoate, named parasiticin B. It is the first example of long-chain fatty acid ester of this class of compounds.

Parasiticin C (**3**) was obtained as yellow needles. Its molecular formula was assigned on the basis of HRESIMS as $C_{25}H_{20}O_5$ (*m/z* 399.1315 $[M-H]^-$). It can be suggested that **3** has a very similar structure with **1** and interruptin B by comparing their ^{13}C and DEPT NMR data (Table 1), except the presence of two aliphatic carbon signals (δ 34.5, CH; δ 37.0, CH_2) and absence of the 3-C and 4-C of interruptin B. The HMQC and HMBC (Fig. 1) experiments indicated that the signal at δ 37.0 should be assigned to 3-C and the signal at δ 34.5 to be 4-C. Therefore, the structure of **3** was confirmed as 5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-3,4-dihydro-1-benzopyran-2-one.

The known compounds were identified as 5,7-dihydroxy-4-phenyl-8-(3-phenyl-*trans*-acryloyl)-3,4-dihydro-1-benzopyran-2-one (**4**, Dietz et al., 1980), 2′-hydroxy-4′,6′-dimethoxychalcone (**5**, Ahmed et al., 1988), 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**6**, Salem and Werbovetz, 2005), 2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone (**7**, Resurreccion-Magno et al., 2005), respectively, on the basis of NMR and mass spectra and comparison with the reported data. We also first report the ^{13}C NMR data