Cytotoxic Allyl Retrochalcone from the Roots of *Glycyrrhiza inflata*

Goo YOON,[a] Young Do JUNG,[b] and Seung Hoon CHEON*,[a]

[a] *College of Pharmacy & Drug Development Research Institute, Chonnam National University; Gwangju 500–757, Korea: and* [b] *Department of Biochemistry, Chonnam National University Medical School; Gwangju 501–190, Korea.*
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Two known retrochalcones, licochalcone A (1) and licochalcone C (2), and one new retrochalcone, licochalcone E (4) were isolated by cytotoxicity-guided fractionation from the roots of *Glycyrrhiza inflata* along with an ordinary chalcone, isoliquiritigenin (3). The structure of the new retrochalcone was elucidated through a spectroscopic analysis.

Key words *Glycyrrhiza inflata*; licochalcone E; retrochalcone; cytotoxicity

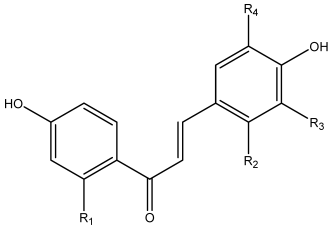
Licorice is the roots and stolons of several *Glycyrrhiza* species (Leguminosae) that has been used worldwide since ancient times as a medicine and a sweetening agent. Traditionally they have been used in the Northeast Asia for the treatment of gastric and duodenal ulcers, bronchial asthma, inflammation, and other diseases.[1)] Much of the recent research on the constituents of licorice has resulted in finding the pharmacological importance of various phenolic compounds.[2)]

Researchers identified the unusual phenolic compounds known as retrochalcones from *Glycyrrhiza inflata*, which are structurally distinguished from the normal chalcones by the lack of oxygen functionalities at C-2′ and C-6′.[3,4)] Until now five retrochalcones, licochalcones A—D and echinatin, have been isolated and characterized from the roots of *G. inflata*.[2,3)] Licochalcones A—D were shown to have various biological properties, *e.g.*, antitumor,[5—8)] antiparasitic,[9)] antileishmanial,[10)] antibacterial[11)] activities. They also displayed antioxidative and superoxide scavenging effects.[12)] This paper describes the cytotoxicity-guided isolation and structure elucidation of a new retrochalcone **4**.

The water extract of the roots of *G. inflata* was defatted with hexane and partitioned with methylene chloride, ethyl acetate, and *n*-butanol successively. Cytotoxicity-guided fractionation of the methylene chloride fraction led to an isolation of four compounds. Compounds **1**—**3** were identified as licochalcone A (**1**), licochalcone C (**2**), and isoliquiritigenin (**3**) by analysis of MS, ^{1}H-, ^{13}C-NMR, and IR spectra data of each compound and by comparison with the literature reports.[3,13)]

Licochalcone E (**4**), amorphous powder, with $[\alpha]_D$ −10.0° (c=0.2, acetone), showed quite similar IR, MS, ^{1}H-, and ^{13}C-NMR spectra to those of licochalcone A (**1**) and licochalcone C (**2**). The high resolution (HR)-EI-MS spectrum of licochalcone E (**4**) showed the peak for $[M]^+$ at m/z 338.1510 (Calcd 338.1518), indicating a molecular formula of $C_{21}H_{22}O_4$. The unique fragment peak at m/z 307 ($[M-31]^+$) indicated that methoxyl group is located at C-2 position.[3,4)] IR spectrum showed the presence of a hydroxyl (3447 cm^{-1}) and a conjugated carbonyl (1700, 1637 cm^{-1}) group, and an aromatic (1585 cm^{-1}) moiety. The ^{1}H-NMR spectrum showed the presence of a methoxyl group [δ 3.83 (3H, s)], *trans*-olefinic protons [δ 7.62 (1H, d, J=15.5 Hz, H-α), δ 8.00 (1H, d, J=15.5 Hz, H-β)], and an α,β-dimethylallyl group [δ 1.34 (3H, d, J=7 Hz, H-4″), δ 1.67 (3H, s, H-5″), δ 3.84 (1H, m, H-1″), δ 4.89 (2H, d, J=16 Hz, H-3″)]. The spectrum also showed the signals of a tetra-substituted benzene ring [δ 6.64 (1H, s, H-3), δ 7.47 (1H, s, H-6), *vide infra*], and a di-substituted benzene ring [δ 6.93 (2H, d, J=8.7 Hz, H-3′, 5′), δ 7.96 (2H, d, J=8.7 Hz, H-2′, 6′)]. ^{13}C-NMR spectrum of licochalcone E (**4**) showed a ketone carbon signal at δ 187.5, and the signal of an α,β-dimethylallyl group [δ 18.8 (C-4″), 21.5 (C-5″), 37.7 (C-1″) 109.1 (C-3″), and 149.2 (C-2″)], a methoxyl group [δ 55.0], and twelve carbons of the two aromatic rings. The complete assignment of an α,β-dimethylallyl group signal was based on the results of heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) 2D NMR experiments. According to the HMBC, the H-4″ signal was correlated with the C-2″, C-5, and C-1″ resonances indicating that C-4″ is linked to C-1″. The C-5″ methyl was confirmed to be connected to C-2″ carbon on the basis of a HMBC correlation between the H-3″ signal resonated at δ 4.89 and C-5″ at δ 21.5 (Fig. 2).

In a cytotoxicity assay with the HT1080 cell line, the IC_{50}



1: R_1 = H, R_2 = OMe, R_3 = H, R_4 = $C(Me)_2$-CH=CH_2
2: R_1 = H, R_2 = OMe, R_3 = CH_2-CH=$C(Me)_2$, R_4 = H
3: R_1 = OH, R_2 = H, R_3 = H, R_4 = H
4: R_1 = H, R_2 = OMe, R_3 = H, R_4 = CH(Me)-C(Me)=CH_2

Fig. 1. Structures of Compounds **1**—**4**

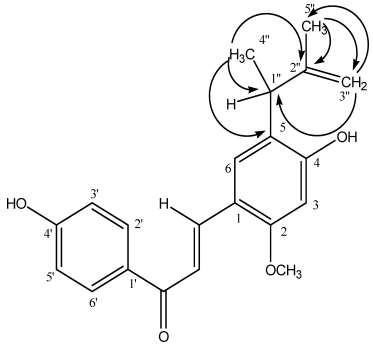


Fig. 2. Selected HMBC Correlations for **4**

* To whom correspondence should be addressed. e-mail: shcheon@chonnam.ac.kr
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values of compounds **1**—**4** were 57.0, 72.8, 96.8, and 45.2 μM, respectively. Licochalcone E (**4**) exhibited the most potent cytotoxic effect compared to the known antitumor agents, licochalcone A (**1**)[5,6] and isoliquiritigenin (**3**).[14,15]

The total synthesis of licochalcone E (**4**) and the structure–activity relationship studies of allyl retrochalcones on cytotoxicity are currently undergoing and the results will be published in due course.

Experimental

General Procedure Optical rotation was measured on a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Nicolet 520P spectrometer (Polaris/ICON). EI-MS spectra were obtained on a GC (HP6890N)-MS (Agilent5973N) spectrometer. HR-EI-MS spectrum was obtained on a JEOL JMS-700 Mstation spectrometer. UV spectrum was determined with a JASCO V-530 spectrophotometer. Nuclear magnetic resonance (NMR) spectra for ^{1}H, and ^{13}C were taken on Varian UNITY plus 300 spectrometer and Varian Unity INOVA 500 spectrometer. Chemical shifts were expressed in parts per million (ppm) relative to TMS as the internal standard and coupling constants (J) were given in Hz. TLC was carried out on Merck silica gel F_{254} precoated aluminum sheets and RP-18F_{254s} precoated glass plates. MPLC was carried out with silica gel 60 (230—400 mesh). Column chromatography was performed using Merck 60, 70—230 mesh silica gel.

Plant Matrial The roots of *Glycyrrhiza inflata* were purchased from Chonnam Herb Association. A voucher specimen (CSH-005) was deposited in the College of Pharmacy, Chonnam National University, Gwangju, Korea.

Extraction and Isolation Small scale isolation. The air-dried powdered *Glycyrrhiza inflata* roots (400 g) were extracted twice with boiling distilled water (1 l) for 2.5 h and the combined water extracts were defatted with *n*-hexane (300 ml). The aqueous layer was partitioned with methylene chloride (300 ml×2), ethyl acetate (300 ml×2), and *n*-butanol (300 ml×2) successively. The methylene chloride fraction was subjected to silica gel chromatography using a *n*-hexane–ethyl acetate–methanol gradient system (2 : 1 : 0.1 to 100% MeOH) to provide 3 fractions (fractions 1 to 3). Fraction 1 was separated to 3 subfractions (fractions 1-1 to 1-3) by preparative TLC using a *n*-hexane–ethyl acetate–methanol (2 : 1 : 0.1) solvent system. Subfraction 1-2 was separated to 3 subfractions (fractions 1-2-1 to 1-2-3) by preparative TLC using a *n*-hexane–ethyl acetate–methanol (1 : 1 : 0.1) solvent system followed by a chloroform–methanol (9 : 1) solvent system. Subfraction 1-2-2 was separated to 3 subfractions (fractions 1-2-2-1 to 1-2-2-3) by preparative TLC using a chloroform–ethyl acetate–methanol (9 : 1 : 0.1) solvent system. Subfraction 1-2-2-3 was separated to 3 subfractions (fractions 1-2-2-3-1 to 1-2-2-3-3) by preparative TLC using a *n*-hexane–ethyl acetate–methanol (1 : 1 : 0.1) solvent system. Subfractions 1-2-2-3-1 and 1-2-2-3-2 showed good cytotoxicities.

Large Scale Isolation: Powdered *Glycyrrhiza inflata* roots (50 kg) were extracted twice with boiling distilled water (50 l) for 2.5 h. The combined water extracts were defatted with *n*-hexane (5 l×3) and the aqueous phase was partitioned with methylene chloride (10 l×5). The methylene chloride extract (1.5 g) was subjected to silica gel MPLC using a *n*-hexane–ethyl acetate–methanol gradient system (2 : 1 : 0.1 to 1 : 1 : 0.1). The combined fractions (0.1 g) having similar *Rf* values to those of subfractions 1-2-2-3-1 and 1-2-2-3-2 in the small scale isolation procedure were subjected to RP18 column chromatography using a methanol–water (4 : 1) solvent system to provide 2 fractions (fractions A, B). Fraction A was purified by preparative TLC to give compound **1** (20 mg), **2** (3 mg) and **3** (5 mg) using a *n*-hexane–ethyl acetate–methanol (1 : 1 : 0.1) eluent system followed by a chloroform–methanol (10 : 1) eluent system. Fraction B was purified by preparative TLC to afford compound **4** (10 mg) using a chloroform–methanol (10 : 1) eluent system. Each compound was further purified by column chromatography using RP18 to an analytically acceptable purity.

Compound **4**: Amorphous powder. $[\alpha]_D$ −10.0° (c=0.2, acetone). IR (neat) cm^{-1}: 3447, 1700, 1637, 1602, 1585, 1560, 1287, 1260, 1211, 1166. HR-EI-MS *m/z*: 338.1510 $[M]^+$ (Calcd for $C_{21}H_{22}O_4$: 338.1518) EI-MS *m/z*: 338 ($[M]^+$), 323, 307 ($[M-OCH_3]^+$), 121. UV λ_{max} (EtOH) nm (log ε): 222 (4.58), 271 (3.47), 286 (3.70), 364 (3.57), 377 (3.62). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 1.34 (3H, d, J=7, H-4″), 1.67 (3H, s, H-5″), 3.83 (3H, s, OMe), 3.84 (1H, m, H-1″), 4.89 (2H, d, J=16, H-3″), 6.64 (1H, s, H-3), 6.93 (2H, d, J=8.7, H-3′, 5′), 7.47 (1H, s, H-6), 7.62 (1H, d, J=15.5, H-α), 7.96 (2H, d, J=8.7, H-2′, 6′), 8.00 (1H, d, J=15.5, H-β). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 187.5 (C=O), 162.9 (C-4′), 159.7 (C-4), 158.8 (C-2), 149.2 (C-2″), 139.0 (C-β), 130.6 (C-2′, C-6′), 130.0 (C-1′), 128.3 (C-6), 124.3 (C-5), 118.1 (C-α), 115.5 (C-3′, C-5′), 114.9 (C-1), 109.1 (C-3″), 99.1 (C-3), 55.0 (OMe), 37.7 (C-1″), 21.5 (C-5″), 18.8 (C-4″).

MTT Assay HT1080 cells (2×10^4) were seeded in 96-wells and allowed to attach for 8—12 h. The media were changed to 0.5% FBS containing DMEM before reagents were added to the medium. Twenty-four hours after the addition of reagents, MTT was added to the cell culture medium with a final concentration of 0.5 mg/ml. After incubating for 4—6 h, the media were discarded. The remained cells were lysed by addition of 100 μl dimethylsulfoxide (DMSO). Absorption was measured in a 96-well spectrometer using a 540 nm filter.

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