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Anti-oxidant Constituents of the Roots and Stolons of Licorice (*Glycyrrhiza glabra*)

YOUNG-WON CHIN,[†] HYUN-AH JUNG,[†,‡] YUE LIU,[§] BAO-NING SU,[†,||] JOHN A. CASTORO,[||] WILLIAM J. KELLER,[⊥] MICHAEL A. PEREIRA,[§] AND A. DOUGLAS KINGHORN*[,†]

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and Division of Hematology and Oncology, Department of Internal Medicine, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210, Isolation and Structure Elucidation Group, Analytical Research and Development, Pharmaceutical Research Institute, Bristol-Myers Squibb, One Squibb Drive, New Brunswick, New Jersey 08903, and Nature's Sunshine Products, Inc., 1655 North Main Street, Spanish Fork, Utah 84660

As part of a search for new cancer chemopreventive agents, a new chalcone derivative (**1**), a novel group of neolignan lipid esters (**2**), and seven known phenolic compounds (formononetin, glabridin, hemileiocarpin, hispaglabridin B, isoliquiritigenin, 4′-*O*-methylglabridin, and paratocarpin B) (**3**−**9**) were isolated from the roots and stolons of licorice (*Glycyrrhiza glabra*). The structures of compound **1** and the individual components of isolate **2** were elucidated using various spectroscopic and chemical methods. All isolates were tested in an authentic peroxynitrite anti-oxidant assay. Of these compounds, hispaglabridin B (**6**), isoliquiritigenin (**7**), and paratocarpin B (**9**) were found to be the most potent anti-oxidant agents. Furthermore, isoliquiritigenin (**7**) was demonstrated to prevent the incidence of 1,2-dimethylhydrazine-induced colon and lung tumors in mice when administered at a dose of 300 mg/kg.

KEYWORDS: ***Glycyrrhiza glabra*; licorice; Fabaceae; chalcones; neolignan lipid ester; mixture; phenolic derivatives; peroxynitrite; anti-oxidant activity; 1,2-dimethylhydrazine; colon and lung tumors**

INTRODUCTION

Cancer chemoprevention refers to interventions such as the prevention, delay, or reversal of the process of carcinogenesis by ingestion of food, dietary supplements, or synthetic agents (*1*). Of the various processes of carcinogenesis, cell damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is known to be involved in the initiation, promotion, or progression of cancer (*2*, *3*). Anti-oxidants, which are able to eliminate ROS and RNS, may play an important role in preventing or delaying the onset of some types of cancer and subsequent cancer development. Among the well-known RNS in the human body, peroxynitrite ($ONOO^-$), a product of nitric oxide (NO) and superoxide (O_2^-), is able to trigger multiple forms of DNA damage, leading to the risk of cancer incidence and other related diseases (*4*). Thus, peroxynitrite scavengers are recognized as being potential anti-carcinogenic agents or cancer chemopreventive agents (*5*). In the present investigation, as a part of a search for new cancer chemopreventive agents, licorice was selected for study because of its peroxynitrite scavenging activity (88.3% scavenging activity at 20 μg/mL) evident on testing an initial chloroform-soluble extract of this plant.

Licorice, the dried roots and stolons (or rhizomes) of *Glycyrrhiza glabra* L. (Fabaceae), referred to in certain countries as Glycyrrhizae Radix, represents an important agricultural commodity (*6*). Extracted licorice, containing glycyrrhizin (a mixture of metallic salts of the oleanane-type triterpenoid diglucuronide, glycyrrhizic acid), has been used as an additive for flavoring and sweetening tobacco, candies, chewing gum, toothpaste, and beverages in Japan, while in the U.S., ammonium glycyrrhizinate has GRAS (generally regarded as safe) status as a flavoring and foaming agent. There is also a long-established medicinal use of licorice in both Eastern and Western countries (*7*). In recent years, licorice has been used increasingly as a botanical dietary supplement ingredient in products marketed for detoxification in the U.S. Previous phytochemical investigations on licorice have revealed that two major structural secondary metabolite types are present, namely, triterpenoids (e.g., glycyrrhizic acid) and phenolic derivatives (e.g., liquiritigenin) (*8*, *9*). Biological studies have demonstrated that the chemical constituents of licorice have a variety of

* Author to whom correspondence should be addressed. Tel.: (614) 247-8094; fax: (614) 247-8081; e-mail: kinghorn.4@osu.edu.
[†] College of Pharmacy, The Ohio State University.
[‡] Present Address: Faculty of Food Science and Biotechnology, Pukyong National University, Busan, Korea.
[§] College of Medicine and Public Health, The Ohio State University.
[||] Bristol-Myers Squibb.
[⊥] Nature's Sunshine Products, Inc.

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biological effects, such as anti-inflammatory, anti-ulcer, anti-hepatotoxic, anti-microbial, anti-oxidant, cytoprotective, and cytotoxic activities (*10*−*16*).

Previous investigations of potential cancer chemopreventive compounds from licorice to date have focused on glycyrrhizin and its aglycones obtained by acid hydrolysis, 18α-glycyrrhetinic acid and 18*β*-glycyrrhetinic acid, which were found to be active in several in vitro and in vivo cancer chemopreventive models (*6*). However, the cancer chemopreventive potential of the phenolic constituents of licorice has only partially been determined thus far (*17*).

In the present study, we describe the isolation and structure elucidation of two new isolates (**1** and **2**) and the identification of seven known phenolic compounds (**3**−**9**) from licorice (*G. glabra*). Isolate **2** was found to be an unusual mixture of neolignan long-chain lipid esters, which although unresolved chromatographically, were fully characterized structurally, using spectroscopic and chemical methods. The peroxynitrite scavenging potential of these licorice flavonoid and neolignan constituents has been determined in an in vitro test system. The cancer chemopreventive activity of the anti-oxidant isoliquiritigenin (**7**) was evaluated in an in vivo mouse model expressing tumors in the colon and lungs by the treatment of 1,2-dimethylhydrazine, when conducted according to a standard protocol (*18*).

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured with a PerkinElmer 241 automatic polarimeter. UV spectra were obtained with a PerkinElmer lambda 10 UV−vis spectrometer. Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter. IR spectra were obtained on an ATI Mattson Genesis Series FT-IR spectrophotometer. NMR spectroscopic data were recorded at room temperature on Bruker Avance DPX-300 and DRX-400 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. Additional 2-D NMR data were obtained on a Bruker DRX-600 NMR spectrometer equipped with a TXI cryoprobe. Electrospray ionization (ESI) mass spectrometric analyses were performed with a 3-Tesla Finnigan FTMS-2000 Fourier transform mass spectrometer, and electron-impact (EI) ionization data were obtained on a Kratos MS-25 mass spectrometer, using 70 eV ionization conditions. GC-MS analyses were carried out with an Agilent 6890N Network GC system equipped with a Waters Micromass GCT mass spectrometer. The accurate mass and product ion mass spectra of isolate **2** were obtained using a Waters Micromass Q-TOF premier mass spectrometer. A 150 mm × 19 mm i.d., 5 μm SunFire PrepC_{18}OBD column (Waters, Milford, MA) and a 10 mm × 19 mm i.d., 5 μm SunFire PrepC_{18} guard column (Waters) were used for preparative HPLC, along with two Waters 515 HPLC pumps and a Waters 2487 dual λ absorbance detector (Waters). Column chromatography was carried out with Diaion HP20 (Supelco, Bellefonte, PA), 230−400 mesh Purasil (Whatman, Clifton, NJ), Plus tC_{18} Sep-Pak cartridges (Waters), and Sephadex LH-20 (Sigma, St. Louis, MO). Analytical thin-layer chromatography (TLC) was performed on precoated 250 μm thick Partisil K6F (Whatman) glass plates, while preparative thin-layer chromatography was conducted on precoated 20 cm × 20 cm, 500 μm thick Partisil K6F (Whatman) glass plates.

Chemicals. DL-2-Amino-3-mercapto-3-methylbutanoic acid (DL-penicillamine), 2,4-dihydroxyacetophenone, dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid (DTPA), and 4-hydroxybenzaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 was purchased from Molecular Probes (Eugene, OR), and authentic peroxynitrite was obtained from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals and solvents utilized were of the highest grade.

Plant Material. The powdered roots and stolons of *G. glabra* L. (Fabaceae) used for the present study were provided by Nature's Sunshine Products, Inc. A representative sample (OSUADK-CCP0010) was deposited as a powder in the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University.

Extraction and Isolation. The dried and powdered roots and stolons of *G. glabra* (5 kg) were extracted with $CHCl_3$ (4 × 5 L) at room temperature, and a crude $CHCl_3$ extract (208 g) was obtained. A portion of this $CHCl_3$ crude extract (200 g) was subjected to silica gel column chromatography (2000 g), by elution with a gradient solvent system of CH_2Cl_2-acetone (100:0 to 1:1), to furnish 39 fractions (F01−F39). Fraction F03 (1.27 g) was chromatographed over a further silica gel column (40 g) with a gradient mixture of hexanes-EtOAc (100:1 to 0:100) for elution, to give 23 sub-fractions (F0301−F0323). Hemileiocarpin (**5**, 4 mg) was obtained by purification with a Sep-Pak C_{18} cartridge (60% MeCN) from sub-fraction F0306 (8 mg). Sub-fraction F0320 (36.7 mg) was chromatographed over a silica gel column (600 mg) with hexanes-EtOAc (gradient elution; 100:1 to 0:100) and gave the new chalcone **1** (3 mg).

Fraction F04 (1.16 g) was chromatographed over a silica gel column (35 g) with a hexanes-EtOAc gradient solvent system (50:1 to 0:100) to give 14 sub-fractions (F0401−F0414). Hispaglabridin B (**6**, 20 mg) was isolated from F0406 (300 mg) by repeated chromatography, using a Sep-Pak C_{18} cartridge (30% MeCN). Isolate **2** (25 mg) was precipitated from sub-fractions F0409 and F0410 in MeOH, with all attempts to separate the individual compounds in this mixture being unsuccessful. The supernatant of F0410 was chromatographed on a Sephadex LH-20 column (MeOH), which led to the isolation of paratocarpin B (**9**, 10 mg). Fraction F06 was subjected to silica gel column chromatography with hexanes-EtOAc mixtures of increasing polarity (20:1 to 0:100) to afford 11 sub-fractions. 4′-*O*-Methylglabridin (**8**, 58 mg) was purified from sub-fraction F0604. Formononetin (**3**, 60 mg) was precipitated from fractions F27 and F28 in MeOH.

Fraction F29 (6 g) was subjected to Diaion HP20 (200 g) (H_2O-acetone = 30:70 to 0:100) column chromatography to give five sub-fractions (F2901−F2905). From F2903, glabridin (**4**, 14.0 mg, t_R 32.9) was purified using HPLC (MeCN−H_2O = 50:50, 7 mL/min). Isoliquiritigenin (**7**, 2.3 mg, t_R 31.5) was isolated from F2902, through the use of HPLC (MeCN−H_2O = 35:65, 7 mL/min).

3,4-(3″,3″-Dimethylpyrano)-3′,4′-(3‴,3‴-dimethyldihydropyrano)-2′-hydroxychalcone (1,2-Dihydroparatocarpin A, 1). Amorphous yellow powder; UV (MeOH) λ_{max} (log ϵ) 210 (4.46), 289 (3.14), 376 (4.49) nm; IR (film) ν_{max} 3475, 2978, 2925, 1628, 1560, 1486, 1249, 1156 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H 7.88 (1H, d, J = 9.0 Hz, H-6′), 7.76 (1H, d, J = 15.4 Hz, H-*β*), 7.66 (1H, d, J = 15.4 Hz, H-α), 7.52 (1H, dd, J = 8.3, 1.9 Hz, H-6), 7.45 (1H, d, J = 1.9 Hz, H-2), 6.78 (1H, d, J = 8.3 Hz, H-5), 6.44 (1H, d, J = 9.8 Hz, H-1″), 6.37 (1H, d, J = 9.0 Hz, H-5′), 5.76 (1H, d, J = 9.8 Hz, H-2″), 2.69 (2H, t, J = 6.8 Hz, H-1‴), 1.83 (2H, t, J = 6.8 Hz, H-2‴), 1.44 (6H, s, H-4″, H-5″), 1.35 (6H, s, H-4‴, H-5‴); ^{13}C NMR ($CDCl_3$, 100 Hz) δ_C 193.7 (CO), 165.0 (C-2′), 162.1 (C-4′), 156.9 (C-4), 145.2 (C-*β*), 132.6 (C-2″), 131.4 (C-6), 130.2 (C-6′), 129.3 (C-1), 128.0 (C-2), 122.9 (C-3), 122.8 (C-1″), 119.3 (C-α), 117.9 (C-5), 114.1 (C-1′), 110.3 (C-5′), 110.2 (C-3′), 78.4 (C-3″), 76.8 (C-3‴), 32.8 (C-2‴), 28.5 (C-4″, C-5″), 27.0 (C-4‴, 5‴), 17.4 (C-1‴); HRESIMS *m*/*z* 413.1726 (calcd for $C_{25}H_{26}O_4Na$, 413.1723).

Mixture of Neolignan Lipid Esters (2). Amorphous white powder; IR (film) ν_{max} 3542, 3418, 3019, 2917, 2850, 1732, 1705, 1608, 1506, 1467, 1175 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.58 (1H, d, J = 15.9 Hz, H-7′), 7.15 (1H, brs, H-2′), 7.10 (1H, brd, J = 8.4 Hz, H-6′), 6.97 (1H, d, J = 8.4 Hz, H-5′), 6.95 (1H, d, J = 8.5 Hz, H-5), 6.88 (1H, brd, J = 8.5 Hz, H-6), 6.84 (1H, brs, H-2), 6.29 (1H, d, J = 15.9 Hz, H-8′), 5.73 (1H, OH-4), 4.88 (1H, d, J = 8.1 Hz, H-7), 4.33 (1H, d, J = 12.2 Hz, H-9a), 4.27 (1H, m, H-8), 4.18 (2H, t, J = 6.6 Hz, H-1‴), 3.94 (1H, overlapped, H-9b), 3.92 (3H, s, OCH_3-3), 2.32 (2H, t, J = 7.4 Hz, H-2″), 1.69 (2H, pentet, J = 6.8 Hz, H-2‴), 1.61 (2H, m, H-3″), 1.25 [$(CH_2)_n$], 0.88 (CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.3 (C-1″), 167.3 (C-9′), 147.0 (C-3), 146.6 (C-4), 145.0 (C-4′), 144.0 (C-7′), 143.8 (C-3′), 128.4 (C-1′), 127.3 (C-1), 122.3 (C-6′), 120.9 (C-6), 117.5 (C-5′), 116.7* (C-2′), 116.6* (C-8′), 114.7 (C-5), 109.3 (C-2), 76.5 (C-7), 76.1 (C-8), 64.7 (C-1‴), 62.5 (C-9), 56.0 (OCH_3-3) 34.1 (C-2″), 29.1−31.9 [$(CH_2)_n$], 28.7 (C-2‴), 26.0 (C-3‴), 24.9 (C-3″), 14.1 (CH_3) (*: signals may be interchangeable); HRESI-TOFMS *m*/*z* 983.7278 (calcd for $C_{61}H_{100}O_8Na$, 983.7316), 1011.7585 (calcd for $C_{63}H_{104}O_8Na$, 1011.7629), 1039.7892 (calcd for $C_{65}H_{108}O_8$-Na, 1039.7942).

Hydrolysis of Isolate 2. A 20 mg aliquot of isolate **2** was added in solution (3 mL $CHCl_3$, 5 mL MeOH, 5 mL H_2O) to K_2CO_3 (60 mg), and then the mixture was refluxed for 6 h. Compounds **2a** (2 mg) and **2b** (12 mg) were purified using silica gel column chromatography (hexanes-EtOAc = 10:1). Compound **2b** (10 mg) was treated with 1 N KOH in solution (3 mL $CHCl_3$, 5 mL MeOH, 5 mL H_2O) and then refluxed for 5 h, and finally compound **2c** (3 mg) was obtained.

Isolate 2a. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.67 (3H, s, OCH_3), 2.30 (2H, t, J = 7.2 Hz, H-2), 1.62 (2H, m, H-3), 1.25 [$(CH_2)_n$], 0.88 (3H, t, J = 7.2 Hz, terminal CH_3).

Isolate 2b. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.58 (1H, d, J = 15.9 Hz, H-7′), 7.17 (1H, d, J = 1.9 Hz, H-2′), 7.10 (1H, dd, J = 8.4, 1.9 Hz, H-6′), 6.98 (1H, d, J = 8.4 Hz, H-5′), 6.93−6.97 (3H, overlapped peaks, H-2, 3, 4), 6.30 (1H, d, J = 15.9 Hz, H-8′), 4.96 (1H, d, J = 8.3 Hz, H-7), 4.18 (2H, t, J = 6.8 Hz, H-1″), 4.07 (1H, m, H-8), 3.93 (3H, s, OCH_3-3), 3.82 (1H, dd, J = 12.5, 2.6 Hz, H-9a), 3.56 (1H, dd, J = 12.5, 3.8 Hz, H-9b), 1.68 (1H, pentet, J = 6.8 Hz, H-2″), 1.25 [$(CH_2)_n$], 0.88 (3H, t, J = 6.8 Hz, terminal methyl).

Isolate 2c. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.64 (2H, t, J = 6.5 Hz, H-1), 1.56 (2H, overlapped with water peak, H-2), 1.25 [$(CH_2)_n$], 0.88 (3H, t, J = 6.8 Hz, terminal methyl).

GC-MS Analysis of 2a and 2c. A 30 m × 0.25 mm i.d., 0.25 μm film DB-XLBITD column (J&W Scientific, Folsom, CA) was used. The detector and injector temperatures were 340 and 270 °C, respectively. The GC oven was programmed initially at 40 °C for 3 min, then increased by 20 °C/min to 340 °C and held for 2 min at this temperature. Helium was used as a carrier gas (1 mL/min). From compound **2a**, docosanoic acid methyl ester ($C_{23}H_{46}O_2$) and lignoceric acid methyl ester ($C_{25}H_{50}O_2$) were detected at 16.45 min (*m*/*z* 354.3514) and 17.23 min (*m*/*z* 382.3818), respectively. The GC of **2c** exhibited two peaks at 15.44 and 16.32 min, which were identified as 1-eicosanol ([M − H_2O], *m*/*z* 280.3154) and 1-docosanol ([M − H_2O], *m*/*z* 308.3472), respectively.

Synthesis of Isoliquiritigenin (7). A modified method of van Hulle et al. (*19*) was used to synthesize isoliquiritigenin (**7**). 2,4-Dihydroxyacetophenone (60.0 g) and 4-hydroxybenzaldehyde (48 g) were dissolved in MeOH (5 mL), and then a solution of 10 N KOH in water was added. The reaction mixture was refluxed for 3 h, cooled at room temperature, and acidified to precipitate isoliquiritigenin by adding a diluted HCl solution. The precipitated isoliquiritigenin was washed with water and dried in an oven. Finally, 22 g of pure isoliquiritigenin (**7**, ca. 20% yield) was obtained. The spectroscopic data were identical to those of isoliquirtigenin (**7**) isolated from licorice in the present study.

Measurement of Peroxynitrite Scavenging Activity. Peroxynitrite scavenging activity was measured by monitoring the oxidation of nonfluorescent dihydrorhodamine 123 to highly fluorescent rhodamine 123 using the modified method of Kooy et al. (*20*). Briefly, dihydrorhodamine 123 (5 mM) in EtOH, purged with nitrogen, was stored at −80 °C as a stock solution. This solution was not exposed to light, prior to the study. The rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM diethylenetriaminepentaacetic acid. The final concentration of dihydrorhodamine 123 was 5 μM. The buffer in this assay was prepared before use and placed on ice. The concentrations of compounds tested were in the range of 0.2−100 μM in 10% DMSO. The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic peroxynitrite in 0.3 N sodium hydroxide (10 μM). Dihydrorhodamine 123 was oxidized rapidly by peroxynitrite, superoxide anion ($O_2^{\bullet -}$), and nitric oxide ($NO^{\bullet}$). The fluorescence intensity of oxidized dihydrorhodamine 123 was measured with a LS55 luminescence spectrometer (PerkinElmer, Boston, MA) at the excitation and emission wavelengths of 480 and 530 nm, respectively. Values of peroxynitrite scavenging activity (50% inhibition, IC_{50}) were expressed as the mean (n = 3) for the final fluorescence intensity minus background fluorescence by the detection of oxidation of dihydrorhodamine 123. DL-Penicillamine was used as a positive control.

Animals and Administration. Cancer chemopreventive activity of isoliquiritigenin (**7**) was determined in the 1,2-dimethylhydrazine-induced colon and lung tumor model (*18*). This assay was used since it allows in a single bioassay the determination of chemopreventive activity in both organs. Female Swiss-Webster mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) at 9 weeks of age were administered by i.p. injection 10 mg/kg body weight of 1,2-dimethylhydrazine twice a week for 8 weeks. At the seventh day after the last treatment of 1,2-dimethylhydrazine, the mice were administered 0, 50, 100, or 300 mg/kg isoliquiritigenin in their AIN-76A diet (Dyets, Inc., Bethlehem, PA) until they were sacrificed by CO_2 asphyxiation at 36 weeks after the first dose of 1,2-dimethylhydrazine. Each treatment group comprised 25 mice, except for the control group with 44 mice. At necropsy, the colons and lungs were evaluated for tumors, which were then harvested. The colon tumors and lungs were then fixed in formalin overnight and transferred to 70% alcohol. The lungs were evaluated for tumors prior to embedding in paraffin. Sections of the colon tumors and the skipped sections of the lungs were stained with hematoxylin and eosin for histopathologic evaluation.

Statistical Analysis. The results of this in vivo experiment are expressed as the mean ± SEM. Statistical significances between control and sample groups were evaluated using a one-way analysis of variance (ANOVA), followed by a Bonferroni t test (p-value < 0.05).

RESULTS AND DISCUSSION

The powdered roots and stolons of licorice were extracted using $CHCl_3$, and the resultant $CHCl_3$ extract exhibited potent anti-oxidant activity (88.3% scavenging activity at 20 μg/mL) using the authentic peroxynitrite method. Therefore, this extract was fractionated by repeated chromatography, which led to the isolation of a new prenylated chalcone (**1**), and a mixture of new neolignan lipid esters (**2**), along with seven known phenolic compounds (**3**−**9**) (**Figure 1**). The seven known substances, all previously found to occur in licorice roots, namely, formononetin (**3**) (*13*, *21*), glabridin (**4**) (*13*), hemileiocarpin (**5**) (*22*), hispaglabridin B (**6**) (*13*), isoliquiritigenin (**7**) (*13*, *23*), 4′-*O*-methylglabridin (**8**) (*13*), and paratocarpin B (**9**) (*24*, *25*), were identified by comparing their physical and spectroscopic data ($[\alpha]_D$, CD, ^{1}H NMR, ^{13}C NMR, DEPT, 2D NMR, and MS) with those of published values.

The new chalcone derivative (**1**), a yellow powder, exhibited a sodiated molecular ion peak consistent with the molecular formula $C_{25}H_{26}O_4Na$. The ^{1}H NMR spectrum of **1** showed signals at δ_H 7.66 (1H, d, J = 15.4 Hz, H-α) and 7.76 (1H, d, J = 15.4 Hz, H-β) for two olefinic protons, signals at δ_H 6.78 (1H, d, J = 8.3 Hz, H-5), 7.45 (1H, d, J = 1.9 Hz, H-2), and 7.52 (1H, dd, J = 8.3, 1.9 Hz, H-6) for a trisubstituted aromatic ring, and signals at δ_H 6.37 (1H, d, J = 9.0 Hz, H-5′) and 7.88 (1H, d, J = 9.0 Hz, H-6′) for a tetrasubstituted aromatic ring. These signals were assignable to a chalcone derivative similar in structure to paratocarpin A (*24*). The presence of 2,2-dimethylchroman and 2,2-dimethyl-2*H*-chromene structural units in **1** was suggested by interpretation of the chemical shifts, splitting patterns, and 2D correlations of the remaining ^{1}H and ^{13}C NMR resonances. The occurrence of these two additional rings was also required by the unsaturation value of the determined molecular formula. Their locations were determined by the HMBC NMR spectroscopic correlations from H-1‴ (δ_H 2.69) to C-2′ (δ_C 165.0), C-3′ (δ_C 110.2), and C-4′ (δ_C 162.1) and from H-1″ to C-3, C-4, and C-3″, as shown in **Figure 2**. Thus, the new chalcone (**1**), 1,2-dihydroparatocarpin A, was assigned as 3,4-(3″,3″-dimethylpyrano)-3′,4′-(3‴,3‴-dimethyldihydropyrano)-2′-hydroxychalcone.

The ^{1}H NMR spectrum of **2** disclosed that this isolate consisted of a lignan nucleus and long-chain fatty acid and alkanol moieties. All attempts to resolve this mixture into its component esters were unsuccessful, as was experienced by others for a group of lipo norditerpenoid alkaloids isolated from the root tubers of *Acontium ferox* and for two series of *N*-fatty

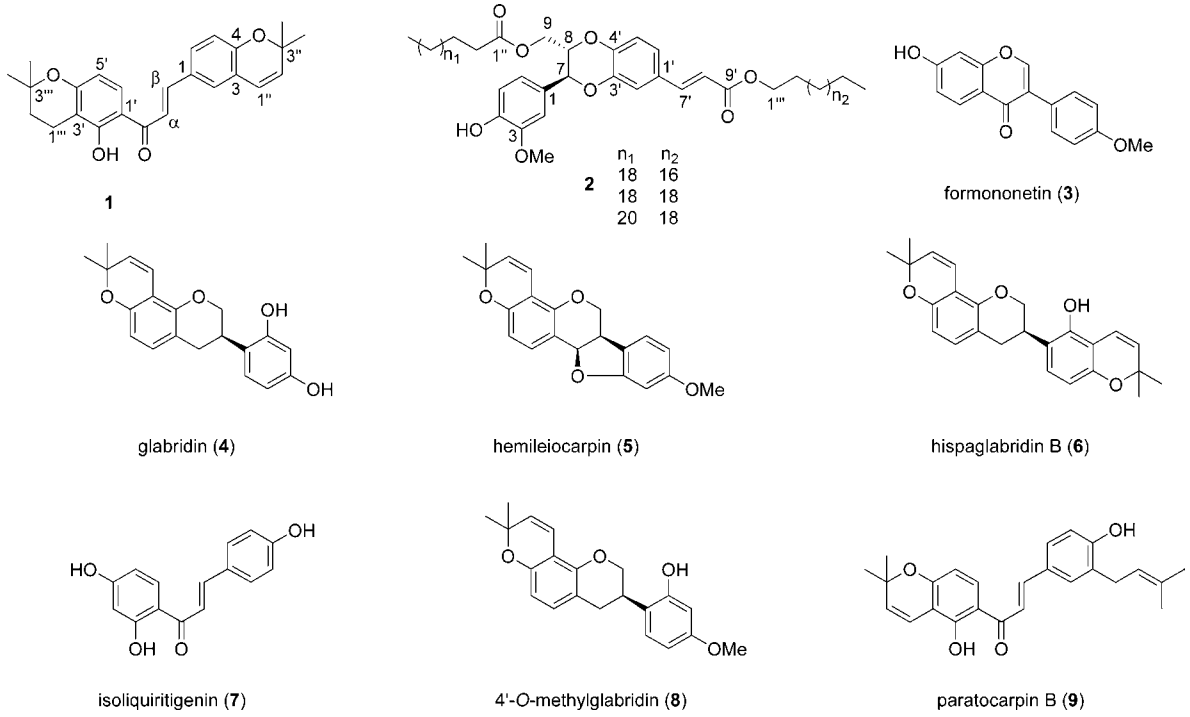

Figure 1. Structures of compounds isolated from the roots and stolons of *G. glabra*.

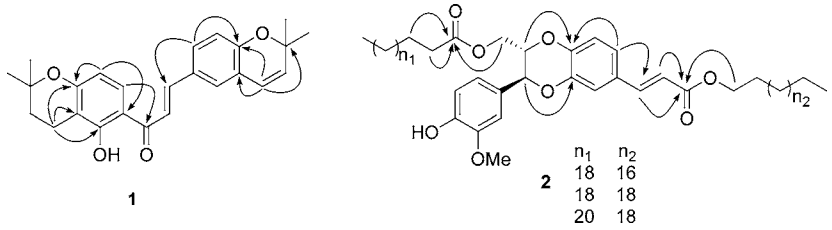

Figure 2. Selected HMBC correlations for compound **1** and isolate **2**.

acid acyl tryptamines from the seeds of *Annona atemoya* (*26*, *27*). The resonances at δ_H 4.88 (1H, d, J = 8.1 Hz, H-7), 4.27 (1H, m, H-8), 4.33 (1H, d, J = 12.2 Hz, H-9a), and 3.94 (1H, m, H-9b) suggested the occurrence in **1** of a 1,4-benzodioxane-type neolignan moiety with a trans configuration ($J_{7,8}$ = 8.1 Hz) at C-7 and C-8. A pair of *trans*-olefinic proton peaks at δ_H 7.58 (1H, d, J = 15.9 Hz, H-7′) and 6.29 (1H, d, J = 15.9 Hz, H-8′) was apparent. The remaining aromatic signals at δ_H 7.15−6.84 (6H) were assigned as two 1,3,4-trisubstituted aromatic rings (*28*). The presence of an alkanol moiety was inferred from the ^{1}H NMR signals at δ_H 4.18 (2H, t, J = 6.6 Hz, H-1‴) and 1.69 (2H, pentet, J = 6.8 Hz, H-2‴) and the occurrence of a fatty acid group from signals at δ_H 2.32 (2H, t, J = 7.4 Hz, H-2″) and 1.61 (2H, m, H-3″). HMBC correlations were used to place the fatty acid group at C-9 via an ester linkage (H-9 to δ_C 173.3), and an alkanol moiety was proposed at C-9′ through an ether linkage (H-1‴ to δ_C 167.3). Since the long-range coupling constants from H-8 to C-4′ and from H-7 to C-3′ of benzodioxane-type neolignans are sometimes very small (*29*), the three-bond HMBC correlations from H-8 to C-4′ and from H-7 to C-3′ would have been difficult to observe using the generally used BBO (broadband observe) probe with a standard relaxation delay of about 70 ms. In the present study, the HMBC spectrum of compound **2** was acquired using a cryoprobe at 600 MHz with a relaxation delay value of 90 ms. Thus, the observed correlations of H-6′ (δ_H 7.10) and H-8 (δ_H 4.27) to C-4′ (δ_C 145.0) and H-7 (δ_H 4.88) to C-3′ (δ_C 143.8) in the HMBC spectrum were used to finalize the structure of the lignan moiety (3-methoxy-4,9-dihydroxy-7,3′-epoxy-8,4′-oxyneolign-7′-en-9′-oic acid) in this isolate, as shown in **Figure 2**. The HRESI-TOF mass spectrum of isolate **2** exhibited three sodiated molecular ion peaks consistent with $C_{61}H_{100}O_8Na$, $C_{63}H_{104}O_8Na$, and $C_{65}H_{108}O_8Na$, showing that this isolate represented a mixture of three compounds, with the ester units having different chain lengths. To confirm the chain lengths of the fatty acid and alkanol units, isolate **2** was hydrolyzed in a stepwise reaction (**Figure 3**), and the component fatty acid methyl esters and alkanols were obtained. GC-MS analysis of the fatty acid methyl esters (**2a**) permitted the detection of docosanoic acid (C_{22}) and lignoceric acid (C_{24}). On the GC-MS analysis of the alkanols (**2c**), 1-eicosanol (C_{20}) and 1-docosanol (C_{22}) were identified as ester components. The product ion mass spectra of the sodiated molecular ion of the two most abundant components of isolate **2** were in agreement with the GC-MS data of the hydrolysis products mentioned previously. The sodiated molecular ion at *m*/*z* 983.7278 [fragments at *m*/*z* 703.4208, $C_{41}H_{60}O_8Na$ (+3.1 ppm error) and *m*/*z* 481.3285, $C_{29}H_{46}O_4Na$ (−1.8 ppm error)] and *m*/*z* 1011.7585 [fragments *m*/*z* 703.4189, $C_{41}H_{60}O_8Na$ (+0.4 ppm error) and *m*/*z* 509.3577, $C_{31}H_{50}O_4Na$ (−5.9 ppm error)] produced a common fragment ion at *m*/*z* 703 with the same elemental composition. The elemental composition of this ion corresponds to the loss of the aliphatic group from the enolate portion of the isolate **2**. Thus, these fragments have the same aliphatic chain length, n = 18. The two other

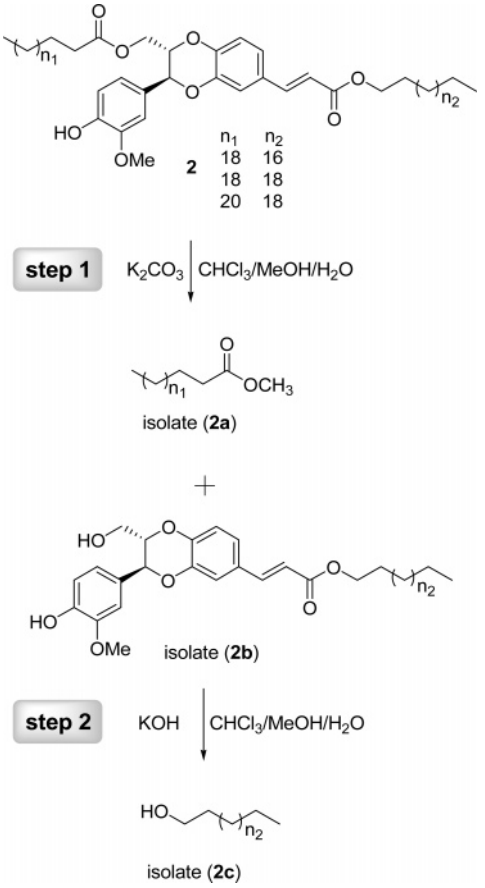

Figure 3. Hydrolysis of isolate **2**.

fragments generated at *m*/*z* 481 and 509 differed by C_2H_4 and were assigned to the different aliphatic chain lengths on the enolate portion of the molecule, $n = 16$ and 18, respectively. Careful analysis of the molecular ions of this compound complex and the GC-MS data of the fatty acid methyl esters and the alkanols led to the conclusion that this mixture (**2**) was comprised of the neolignan nucleus with a C_{22} fatty acid and a C_{20} alkanol, a C_{22} fatty acid and a C_{22} alkanol, and a C_{24} fatty acid and a C_{22} alkanol, in a ratio of 11:33:6, respectively (**Figure 1**). To the best of our knowledge, this neolignan−lipid ester isolate (**2**) represents the first report of the identification of a lignan from licorice. Moreover, neolignan lipid esters of this type do not appear to be known previously as natural products.

All isolates (**1**−**9**) obtained in the present investigation were also evaluated for anti-oxidant activity using the authentic peroxynitrite in vitro method (**Table 1**) (*20*). Of these compounds, **6**, **7**, and **9** exhibited the most potent peroxynitrite scavenging activities, with IC_{50} values of 3.2, 9.3, and 2.3 μM, respectively, which were comparable to that of the positive control used, DL-penicillamine (IC_{50} 3.1 μM). An extract of licorice roots has been reported recently to possess scavenging effects against peroxynitrite as well as nitric oxide, but the active principles were not individually established (*30*). The phenolic compounds isolated and investigated in the present study may be responsible, at least in part, for such peroxynitrite scavenging activity.

Of the three most active constituents found in the present study, a previous biological evaluation of hispaglabridin B (**6**) in a low-density lipoprotein (LDL) oxidation system has demonstrated that this compound possesses anti-oxidant activity (*13*), which is consistent with the results reported herein. There is now considerable prior literature on the biological activities

Table 1. Anti-oxidant Activities of Compounds Isolated from Licorice[a]

compound	IC_{50} (μM)
1, 2-dihydroparatocarpin A (**1**)	19.8
neolignan lipid esters (**2**)	>20
formononetin (**3**)	12.4
glabridin (**4**)	12.9
hemileiocarpin (**5**)	19.7
hispaglabridin B (**6**)	3.2
isoliquiritigenin (**7**)	9.3
4′-*O*-methylglabridin (**8**)	18.5
paratocarpin B (**9**)	2.3
DL-penicillamine[b]	3.1

[a] A compound with an IC_{50} value less than 20 μM is considered to be active in this anti-oxidant assay. [b] Positive control used.

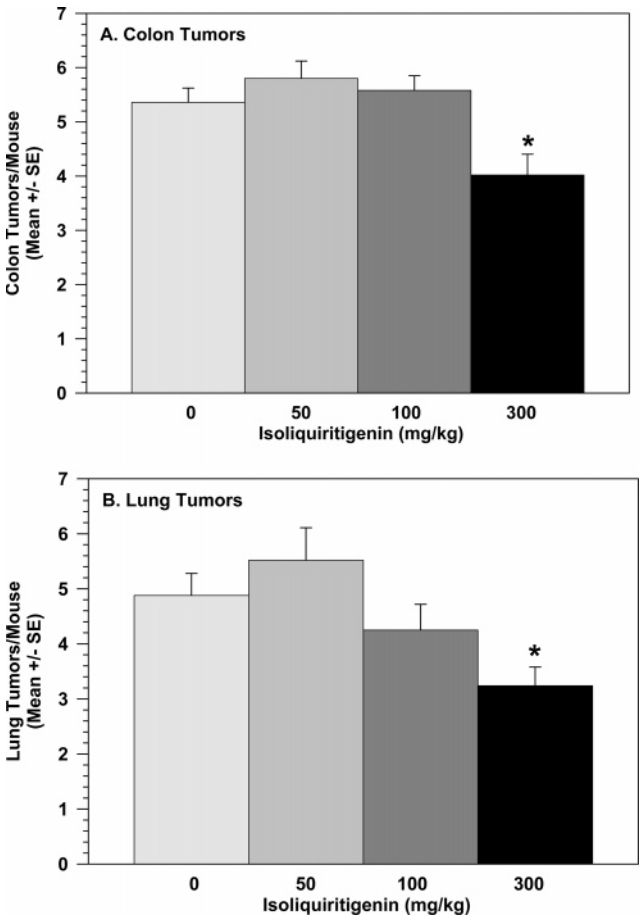

Figure 4. Isoliquiritigenin (**7**) prevention of colon (**A**) and lung (**B**) tumors. Results are expressed as means ± SE for each treatment group of 25 mice each, except for the control group with 44 mice. The asterisk indicates a significant difference from the control group, *p*-value < 0.05.

of isoliquiritigenin (**7**), in particular in regard to cancer. Thus, it has been found that isoliquiritigenin inhibits the production of nitric oxide (NO) and prostaglandin E_2 (*31*) and causes the induction of phase II enzymes (glutathione-*S*-transferase and quinone reductase) (*32*). Isoliquiritigenin (**7**) acts as a monofunctional inducer of phase II enzymes through the activation of the anti-oxidant response element (ARE) (*17*). This compound exhibited cytotoxic activity against several cancer cell lines (*33*−*38*), and inhibitory effects of isoliquiritigenin in test systems related to metastasis have been reported (*39*). Moreover, isoliquiritigenin (**7**) was reported to inhibit 7,12-dimethylbenz-[*a*]anthracene (DMBA) initiated and 12-*O*-tetradecanoylphorbol-

13-acetate (TPA) promoted skin papillomas, as well as azoxymethane-induced murine colon aberrant crypt foci formation in mice (*40*, *41*). In addition, isoliquiritigenin (**7**) was found to inhibit alveolar duct formation produced by DMBA in a mouse mammary organ culture assay (*42*). There have been no earlier reports published on the biological evaluation of paratocarpin B (**9**), other than this compound being shown to be inactive in an anti-microbial assay (*43*). The cancer chemopreventive potential of additional naturally occurring and synthetic chalcones has been reported by others (*44*, *45*).

Isoliquiritigenin (**7**), available in a large amount through a synthetic method, was further examined to assess its cancer chemopreventive activity in the 1,2-dimethylhydrazine-induced colon and lung tumor model (*18*). Three dose levels of isoliquiritigenin (50, 100, and 300 mg/kg diet) were evaluated, with each treatment containing 25 mice. There were 44 mice in the vehicle, the control diet group. Only one mouse (100 mg/kg isoliquiritigenin) died prior to the terminal sacrifice. None of the three dose levels of isoliquiritigenin (**7**) was toxic to the mice, nor did it alter the body weight of these test animals. With respect to lung tumors, all of the mice had at least one tumor except for one mouse each in the 50 mg/kg isoliquiritigenin (**7**) and the vehicle control groups. Over 97.1% of the lung tumors in the four different treatment groups were adenomas with the rest being adenocarcinomas. The percentage of the tumors found to be adenocarcinomas did not vary among the treatment groups, ranging from 1.46−2.91% of the tumors. With respect to the colon tumors, all the mice had at least one tumor. The highest dose level of isoliquiritigenin used decreased the multiplicity of both the colon and the lung tumors (**Figure 4**). These results, consistent with the additional activities mentioned previously, suggest that isoliquiritigenin (**7**) is a potential cancer chemopreventive agent. Thus, isoliquiritigenin (**7**) and other phenolic constituents of licorice seem to be worthy of more detailed investigation in this regard.

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