Steroids 78 (2013) 670–682

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Steroidal glycosides from the bulbs of *Fritillaria meleagris* and their cytotoxic activities

Yukiko Matsuo *, Daisuke Shinoda, Aina Nakamaru, Yoshihiro Mimaki *

Tokyo University of Pharmacy and Life Sciences, School of Pharmacy, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan

ARTICLE INFO

Article history:
Received 3 August 2012
Received in revised form 29 January 2013
Accepted 27 February 2013
Available online 13 March 2013

Keywords:
Fritillaria meleagris
Liliaceae
Steroidal glycosides
HL-60 cells
A549 cells
Apoptosis

ABSTRACT

Steroidal glycosides (**1**–**18**), including 10 new compounds (**1**–**10**), were isolated from the bulbs of *Fritillaria meleagris* (Liliaceae). The structures of the new compounds were determined by two-dimensional (2D) NMR analysis, and by hydrolytic cleavage followed by spectroscopic and chromatographic analysis. The isolated compounds and their aglycones were evaluated for cytotoxic activity against HL-60 human promyelocytic leukemia cells and A549 human lung adenocarcinoma cells. Morphological observation and flow cytometry analysis showed that 5β-spirostanol glycoside (**2**) and a cholestane derivative (**17a**) induced apoptotic cell death in HL-60 cells through different mechanisms of action. Furthermore, the (22*R*)-spirosolanol glycoside (**11**) selectively induced apoptosis in A549 cells without affecting the caspase-3 activity level.

1. Introduction

Plants of the genus *Fritillaria* belong to the family Liliaceae, and their bulbs contain steroidal alkaloids with various biological activities such as anticholinergic, antitussive, expectorant, and anti-inflammatory effects [1–3]. *Fritillaria meleagris* L., commonly called snake's head fritillary, is a perennial plant distributed widely throughout Asia and northwestern Europe. The plant grows to a height of 30–40 cm and has flowers with a checkered pattern [4]. Only one systematic chemical characterization of *F. meleagris* has been conducted; a publication in 1958 reported the putative presence of two alkaloids by paper chromatography [5]. As part of our continuing chemical investigation of the saponin constituents of Liliaceae plants [6], a phytochemical screen of the bulbs of *F. meleagris* was performed. We report the discovery of a new steroidal alkaloid (**1**), two new spirostanol glycosides (**2**, **3**), five new furostanol glycosides (**4**–**8**), two new cholestane-type glycosides (**9**, **10**), together with eight known compounds (**11**–**18**). Extensive spectroscopic studies were conducted to determine the structures of the new steroidal glycosides, including two-dimensional (2D) NMR, and hydrolytic cleavage followed by spectroscopic and chromatographic analysis. The cytotoxicity of the isolated compounds and their aglycones was evaluated against HL-60 human promyelocytic leukemia cells and A549 human lung adenocarcinoma cells.

2. Experimental

2.1. General methods

Optical rotations were obtained using a P-1030 (Jasco, Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded with a FT-IR 620 spectrophotometer (Jasco). NMR spectra (500 MHz for ^{1}H NMR) were recorded with a DRX-500 spectrometer (Bruker, Karlsruhe, Germany), using standard Bruker pulse programs. Chemical shifts are given as δ values referenced to tetramethylsilane (TMS) as an internal standard. HRESITOFMS data were obtained with an LCT mass spectrometer (Waters-Micromass, Manchester, U.K.). 5 ppm error in HRESITOFMS data has achieved the level of accuracy for formula confirmation and established the molecular formula of isolated compound. Porous-polymer polystyrene resin Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), BW-300 silica gel (Fuji-Silysia Chemical, Aichi, Japan), and octadecylsilanized (ODS) silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography (CC). TLC was carried out on precoated Silica gel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP_{18} $F_{254}S$ plates (0.25 mm thick, Merck), and the spots were visualized by spraying the plates with 10% H_2SO_4 in H_2O and then heating. HPLC was performed with a system composed of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh), and a Rheodyne injection port. A TSK gel ODS-100Z column (10 mm i.d. × 250 mm, 5 μm, Tosoh) was employed for the preparative HPLC. Purities of all isolated compounds were confirmed by NMR, optical rotation,

* Corresponding authors. Tel.: +81 42 676 4577; fax: +81 42 676 4579.
E-mail addresses: matsuoy@toyaku.ac.jp (Y. Matsuo), mimakyi@toyaku.ac.jp (Y. Mimaki).

0039-128X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2013.02.012

TLC, and mass spectrometry, respectively. The following materials and reagents were used for the cell cultures and the assay of cytotoxic activities: Spectra Classic microplate reader (Tecan, Salzburg, Austria); 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan); JCRB 0085 HL-60 cells and JCRB 0076 A549 cells (Human Science Research Resources Bank, Osaka, Japan); fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, U.S.A.); 0.25% Trypsin-EDTA solution, RPMI 1640 medium, minimum essential medium (MEM), phosphate buffered saline (PBS) (Wako Pure Chemical Industries, Osaka, Japan), etoposide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, U.S.A.); penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). Paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan), Triton X-100 (Sigma), propidium iodide (PI) (Molecular Probes, Eugene, OK, U.S.A.), and ribonuclease A (RNase) (Wako Pure Chemical Industries, Osaka, Japan). All other chemicals used were of biochemical reagent grade.

2.2. Plant material

The bulbs of *F. meleagris*, which were collected in India in 2007, were obtained from Sakata-no-Tane (Kanagawa, Japan) and identified by Dr. Yutaka Sashida, professor emeritus at Tokyo University of Pharmacy and Life Sciences. A voucher specimen was deposited in our laboratory (voucher no. 07-004-FM, Department of Medicinal Pharmacognosy).

2.3. Extraction and isolation

The *F. meleagris* bulbs (fresh weight, 6.0 kg) were extracted with hot MeOH (20 L) for 12 h. After removing the solvent, the MeOH extract (300 g) was resuspended in MeOH/H_2O (3:7) to pass through a Diaion HP-20 column (50 mesh, 2000 g, 8.5 × 60 cm) and then successively eluted with MeOH/H_2O (3:7), MeOH, EtOH, and EtOAc (each 9 L). CC of the MeOH-eluted fraction (80.0 g) on silica gel (200–300 mesh, 1500 g, 8.5 × 30 cm), eluted with a stepwise gradient mixture of $CHCl_3$/MeOH/H_2O (40:10:1; 20:10:1 and 7:4:1) and finally with MeOH alone, provided five fractions (A–E). Fraction B was chromatographed on ODS silica gel (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN/H_2O (1:4; 1:3; 1:2 and 1:1) to afford **3** (19.1 mg) and **10** (8.7 mg). Fraction C was separated by an ODS silica gel column (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN/H_2O (1:4, 1:3, 1:2 and 1:1) to give **2** (14.6 mg), **13** (3.3 mg), **14** (2.1 mg), **15** (13.0 mg), **16** (3.9 mg), and **17** (37.0 mg). Fraction D was chromatographed on silica gel (200–300 mesh, 1800 g, 8.5 × 35 cm) eluted with $CHCl_3$/MeOH/H_2O (30:10:1; 20:10:1 and 7:4:1) and on ODS silica gel (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN-H_2O (1:3; 1:2 and 1:1) to afford **1** (91.8 mg), **4** (158 mg), **5** (10.1 mg), **6** (32.0 mg), **7** (9.2 mg), **8** (39.4 mg), **9** (3.5 mg), **11** (1.4 mg), **12** (8.0 mg), and **18** (1.3 mg). Isolated compounds have been attempted to crystallize but all attempts were unsuccessful. Steroidal glycosides were described as "amorphous solids" and melting point determinations should not be needed for compounds described as "amorphous solids".

2.3.1. Compound **1**

(22*S*,25*S*)-spirosol-5-en-3β-yl*O* -β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**1**): amorphous solid; $[\alpha]_D^{25}$ −64.8 (*c* 0.05, MeOH); IR ν_{max} (film) cm^{-1}: 3445 (OH), 2932 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS m/z: 884.5045 $[M + H]^+$ (calcd for $C_{45}H_{74}O_{16}N$: 884.5008).

2.3.2. Acid hydrolysis of **1**

A solution of **1** (10.0 mg) in 1 M HCl in dioxane/H_2O (1:1; 3.0 mL) was heated at 95 °C for 1 h under an Ar atmosphere. The reaction mixture was cooled, neturalized with aqueous 1 M NaOH (2.0 mL), diluted with H_2O (20 mL), and extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ extract (7.8 mg) was purified by preparative TLC ($CHCl_3$/MeOH, 30:1) to give tomatidenol (**1a**, 2.4 mg). Steroid aglycones which were stable towards acid were obtained by the above procedures. The purity of aglycone was confirmed by NMR, optical rotation, TLC, and mass spectrometry. The aqueous residue (2.9 mg) was directly analyzed by HPLC under the following conditions that β-D-glycoside and α-D-glycoside were detected as one peak: Capcell Pak NH2 UG80 column (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan); MeCN/H_2O (85:15); detection by refractive index (RI) and optical rotation (OR); flow rate of 1.0 mL/min. D-Glucose and L-rhamnose in the aqueous residue were identified by comparing their retention times (t_R [min]) and signs of optical rotation with those of authentic samples [7,8]: L-rhamnose (7.89, negative optical rotation), and D-glucose (13.99, positive optical rotation).

2.3.3. Compound **1a**

An amorphous solid; $[\alpha]_D^{25}$ −57.2 (*c* 0.05, MeOH) (Lit. −23.5 [9]); ^{13}C NMR (125 MHz, $CDCl_3$): δ 36.7(C-1), 31.6 (C-2), 71.7 (C-3), 42.3 (C-4), 140.8 (C-5), 121.4 (C-6), 32.1 (C-7), 31.4 (C-8), 50.1 (C-9), 37.2 (C-10), 20.9 (C-11), 39.9 (C-12), 40.7 (C-13), 56.0 (C-14), 32.7 (C-15), 78.8 (C-16), 62.0 (C-17), 16.8 (C-18), 19.4 (C-19), 42.8 (C-20), 15.8 (C-21), 99.2 (C-22), 26.6 (C-23), 28.4 (C-24), 29.7 (C-25), 50.0 (C-26), 19.4 (C-27); HRESITOFMS *m/z*: 414.3374 $[M + H]^+$ (calcd for $C_{27}H_{44}NO_2$: 414.3372).

2.3.4. Compound **2**

(25*R*)-5β-spirostan-3β-yl*O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**): an amorphous solid; $[\alpha]_D^{25}$ −60.4 (*c* 0.10, MeOH); IR ν_{max} (film) cm^{-1}: 3397 (OH), 2927 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 887.4977 $[M + H]^+$ (calcd for $C_{45}H_{75}O_{17}$: 887.5004).

2.3.5. Enzymatic hydrolysis of **2**

Compound **2** (5.0 mg) was treated with naringinase (EC 232-96-4, Sigma; 554 mg) in a HOAc/KOAc buffer (pH 4.3, 5.0 mL) at room temperature for 432 h. The reaction mixture was purified by CC on silica gel ($CHCl_3$/MeOH/H_2O; 10:1:0, 7:4:1) (200–300 mesh, 100 g, 2 × 30 cm) to give smilagenin (**2a**, 0.3 mg), and a sugar fraction (2.3 mg). Acid hydrolysis of **2** with 1 M HCl have resulted in giving only D-glucose and L-rhamnose, whereas the labile aglycone decomposed under acidic conditions [10]. The preparation of the aglycone **2a** has been finally completed by enzymatic hydrolysis under the above conditions. HPLC analysis of the sugar fraction under the same conditions as those for **1** showed the presence of D-glucose (14.22, positive optical rotation) and L-rhamnose (7.98, negative optical rotation).

2.3.6. Compound **2a**

An amorphous solid; $[\alpha]_D^{25}$ −53.6 (*c* 0.05, MeOH) (Lit. −61 [11]); ^{13}C NMR (125 MHz, C_5D_5N): δ 30.6(C-1), 28.6 (C-2), 66.1 (C-3), 34.4 (C-4), 37.0 (C-5), 27.2 (C-6), 26.9 (C-7), 35.6 (C-8), 41.0 (C-9), 35.6 (C-10), 21.2 (C-11), 40.1 (C-12), 40.4 (C-13), 56.6 (C-14), 32.2 (C-15), 81.3 (C-16), 63.2 (C-17), 16.6 (C-18), 24.3 (C-19), 42.0 (C-20), 15.1 (C-21), 109.3 (C-22), 31.8 (C-23), 29.3 (C-24), 30.0 (C-25), 66.9 (C-26), 17.3 (C-27); HRESITOFMS *m/z*: 417.3382 $[M + H]^+$ (calcd for $C_{27}H_{45}O_3$: 417.3369).