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Chemical constituents of *Swertia mussotii* and their anti-hepatitis B virus activity

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ABSTRACT

Three new secoiridoid aglycones of (−)-swermusic acid A (**1**) and B (**3**), and (−)-swerimuslatone A (**2**), and four new secoiridoid glycosides of 6′-*O*-formylsweroside (**4**), 6′-*O*-formylgentiopicroside (**5**), 6′-*O*-acetylamarogentin (**6**) and 6′-*O*-acetylamaronitidin (**7**), along with 40 known compounds (**8**–**47**) were isolated from *Swertia mussotii*. Their structures were elucidated on the basis of extensive spectroscopic analyses including MS, IR, UV, 1D- and 2D-NMR. Forty-five compounds from *S. mussotii* were evaluated for their anti-HBV activity on the HepG 2.2.15 cell line *in vitro* inhibiting the secretions of HBsAg and HBeAg, as well as HBV DNA replication. Six of the nine phenols **26**–**29**, **31** and **32** exhibited activities inhibiting HBsAg and HBeAg secretion with IC_{50} values from 0.23 to 5.18 mM, and HBV DNA replication with IC_{50} values from <0.06 to 2.62 mM. Moreover, isooriention (**45**) displayed significant anti-HBV activities against secretions of HBsAg and HBeAg with IC_{50} value of 0.79 and 1.12 mM, as well as HBV DNA replication with IC_{50} value of 0.02 mM.

1. Introduction

Hepatitis B virus (HBV) infection is a major global health problem due to its worldwide distribution and potential adverse sequel; however, the present therapeutic strategies for HBV infection are far from satisfactory. Therefore, novel anti-HBV drugs with new mechanisms are further needed [1–3]. Traditional Chinese medicines (TCMs) with multiple components and diverse activities are fascinating sources for drug discovery. *Swertia mussotii*, well known as "Zang-Yin-Chen" in Tibet, China, belongs to the *Swertia* genus of the family Gentianaceae, which has long been used to treat virus hepatitis in Tibet and has been documented in many monographs [4–6]. Presently, many anti-hepatitis agents developed from *S. mussotii* have been widely applied in the clinic [7,8]. In order to clarify its active components, our preliminary bioassay-guided fractionation has led to the isolation of twenty xanthones from *S. mussotii*, some of which exhibited significant anti-HBV activity, and the detailed structure–activity relationships were discussed [9]. As part of our ongoing search for anti-HBV active compounds from natural sources, further investigation on this plant resulted in 47 compounds, including seven new ones. Herein, the isolation, structural elucidation and anti-HBV activity of these compounds were reported.

2. Materials and methods

2.1. General experimental procedures

1D (^1H and ^{13}C) and 2D (HSQC, ^1H-^1H COSY, HMBC and ROESY) NMR experiments were acquired on Bruker AM-400, DRX-500 or AVANCE III-600 spectrometers (Bruker, Bremerhaven, Germany). The chemical shifts were given in δ (ppm) scale and referenced to the deuterated solvent signals.

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Mass spectra were obtained on LCMS-IT-TOF spectrometer (Shimadzu, Kyoto, Japan). IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets (Bio-Rad, Hercules, California, USA). UV spectra were taken with a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were obtained on a Jasco model 1020 digital polarimeter (Horiba, Tokyo, Japan) at room temperature. Column chromatography was performed on silica gel (200–300 mesh; Qingdao Makall Chemical Company, Qingdao, P.R. China). Semi-preparative HPLC was carried out on Waters Alliance 2695 liquid chromatography with an ZORBAX SB-C_{18} (5 μm, 9.4 × 250 mm) column (Agilent, USA) at a flow rate of 3.0 mL/min. Sephadex LH-20 (20–150 μm) for chromatography was purchased from Pharmacia Fine Chemical Co. Ltd. (Pharmacia, Uppsala, Sweden) and Rp-18 (40–63 μm) was from Fuji Silysia Chemical Ltd. (Fuji, Japan). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

Microplate reader (Model 680) was purchased from Bio-Rad Inc. (Hercules, CA, USA). ELISA reader was purchased from AutoBio diagnostics Co. Ltd. (Beijing, P.R. China). The amplification and detection of HBV DNA were performed in a Mastercycler Ep Realplex System (German). Total DNA was isolated by using TIANamp Gemomic DNA Kits (TIANGEN Biotech Co. Ltd., China).

2.2. Plant material

The whole plants of *S. mussotii* Franch. were collected in Yushu, Qinghai Province, P.R. China, in November 2008 and authenticated by Prof. Dr. Yan-Duo Tao, Northwest Institute of Plateau Biology, Chinese Academy of Sciences. A voucher specimen (No. 20101128) was kept in the Laboratory of Antivirus and Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried and powdered whole plants of *S. mussotii* (6 kg) were extracted 2 times with 90% EtOH (50 L) at room temperature, for 24 h each. The combined EtOH extracts were concentrated *in vacuo* to yield a brown-yellow gum (1.47 kg). The residue was suspended in H_2O (6 L), and partitioned with petroleum ether (PE), EtOAc (5 L × 4) and *n*-butanol (4 L × 3), successively.

The EtOAc part (A, 300 g) was subjected to silica gel column chromatograph (CC, 18 × 28 cm, 2500 g) eluted with $CHCl_3$-MeOH-H_2O (100:0:0, 95:5:0, 90:10:1, 80:20:2, 60:40:4, *v/v/v*, each 30 L). The collected fractions were combined based on their TLC characteristics to yield 6 fractions (Frs. A1–A6). Fr. A1 (93 g) was further divided into 4 subfractions (Frs. A1-1–A1-4), by chromatograph over silica gel CC (9 × 40 cm, 1000 g) using PE-EtOAc (90:10, 80:20, 70:30, each 12 L) as the eluent. Fr. A1-2 (7.8 g) was applied to silica gel CC (4 × 36 cm, 150 g, PE-Me_2CO, 90:10, 2.5 L), to give four subfractions (Frs. A1-2-1–A1-2-4). Fr. A1-2-2 (3.17) was chromatographed over a silica gel column (4 × 36 cm, 150 g) using PE-Me_2CO (96:4) as the eluent to yield compounds **1** (13 mg), **3** (24 mg) and **9** (592 mg). Compounds **2** (11 mg) and **32** (42 mg) were obtained from Fr. A1-2-3 by preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent) using MeOH-H_2O (50:50) as the eluent. Fr. A1-2-2 (3.17) was further separated by ODS CC (1.90 × 40 cm, 40 g) and eluted with a MeOH-H_2O gradient (from 10:90 to 80:20) to afford compounds **10** (413 mg) and **13** (21 mg). Fr. A2 (68 g) was chromatographed on silica gel column (9 × 30 cm, 750 g) eluted with PE-Me_2CO (90:10, 80:20, 70:30) to provide 3 subfractions (Frs. A2-1–A2-3). Fr. A2-1 (18.4 g) was subjected to silica gel CC (9 × 30 cm, 750 g) eluted with $CHCl_3$-Me_2CO (from 95:5 to 80:20) to give Fr. A2-1-2 (1.18 g), which was purified using silica gel CC (2.5 × 36 cm, 50 g) to generate compounds **4** (67 mg) and **5** (48 mg). Compound **16** (38 g) was obtained from Fr. A2-2 (43 g) by silica gel CC (9 × 30 cm, 750 g) using PE-EtOAc (90:10) as the eluent. Fr. A2-3 (5.4 g) was further separated by CC over silica gel (4 × 25 cm, 120 g) eluting with $CHCl_3$-Me_2CO (80:20, 70:30) to give four subfractions (Frs. A2-3-1–A2-3-4). Fr. A2-3-1 was submitted to Sephadex LH-20 CC (1.4 × 135 cm, 53 g) and eluted with $CHCl_3$-MeOH (50:50) to yield compound **11** (5 mg). Fr. A2-3-2 (2.76 g) was recrystallized from $CHCl_3$-MeOH (50:50) to afford compound **8** (2.48 g). Fr. A6 (33 g) was chromatographed on silica gel column (9 × 30 cm, 750 g) using $CHCl_3$-MeOH-H_2O (80:20:2) as the eluent and further purified by silica gel CC (1.90 × 40 cm, 40 g) eluted with $CHCl_3$-MeOH (90:10) to give compound **16** (23.8 g).

The *n*-butanol part (230 g, B) was chromatographed on a silica gel column (18 × 23 cm, 2000 g), successively eluted with $CHCl_3$-MeOH-H_2O (95:5:0, 90:10:1, 80:20:2, 60:40:4), to yield five subfractions (Frs. B1–B5). Fr B1 (3.28 g) was separated by ODS CC (3.81 × 50 cm, 300 g) and eluted with MeOH-H_2O (from 10:90 to 100:0) to give four subfractions (Frs. B1-1–B1-4). Fr. B1-1 (89 mg) and Fr. B1-2 were purified using preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent) using MeOH-H_2O (47:53) as the eluent to give compounds **40** (29 mg) and **44** (24 mg), respectively. Fr. B1-3 (243 mg) was purified by Sephadex LH-20 CC (1.32 × 135 cm, 53 g; $CHCl_3$-MeOH, 50:50) to yield compounds **6** (53 mg) and **20** (82 mg). Fr. B2 (12.1 g) was separated by ODS column chromatography (3.81 × 50 cm, 300 g) and eluted with MeOH-H_2O (from 10:90 to 100:0) to afford three subfractions (Frs. B2-1–B2-3). Fr. B2-1 (46 mg) was purified using preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent) using MeOH-H_2O (43:57) as the eluent to give compound **7** (20 mg). Fr. B2-2 (1.76 g) was chromatographed on silica gel CC (1.6 × 35 cm, 30 g) using $CHCl_3$-MeOH (95:5, 90:10) as the eluent and further purified by Sephadex LH-20 CC (1.32 × 135 cm, 53 g; $CHCl_3$-MeOH, 50:50) to yield compounds **17** (21 mg) and **21** (547 mg). Fr. B2-3 (5.16 g) was separated on ODS CC (3.81 × 50 cm, 300 g) using MeOH-H_2O (from 20:90 to 70:30) as the eluent, and further purified by Sephadex LH-20 CC (1.4 × 135 cm, 53 g; $CHCl_3$-MeOH, 50:50) and preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent; MeOH-H_2O, 43:57) to yield compounds **31** (73 mg), **32** (12 mg) and **42** (461 mg). Fr. B3 (20.8 g) was separated by silica gel CC (8 × 29 cm, 500 g; AcOEt-MeOH-H_2O, 90:10:1) to give 4 subfractions (Frs. B3-1–3-4). Fr. B3-1 (8.32) was further purified by silica gel CC (4 × 50 cm, 200 g; $CHCl_3$-MeOH-H_2O, 85:15:1) to afford compound **22** (4.72 g). Fr. B4 (42 g) was subjected to MCI CHP-20P gel CC (4.0 × 40 cm, 500 mL) eluting with MeOH-H_2O (from 10:90 to 100:0) to yield four subfractions (Frs. B4-1–4-4). Fr. B4-1 (103 mg) was separated by Sephadex LH-20 CC (1.4 × 135 cm, 53 g; MeOH)