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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)

and preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent; MeOH-H_2O, from 6:94 to 23:77) to afford compounds **18** (35 mg) and **19** (5 mg). Fr. B4-2 (11.3 g) was chromatographed on silica gel CC (5.6 × 38, 320 g) using $CHCl_3$-MeOH (94:6) as the eluent to give compound **15** (8.9 g). Fr. 4–3 (18.7 g) was subjected to silica gel CC (5.6 × 38, 320 g; CHCl3-MeOH, 90:10) to yield compound **16** (15.8 g). Fr. B4-4 (7.8 g) was separated by silica gel CC (4 × 45 cm, 200 g; $CHCl_3$-MeOH, 90:10), and further purified by ODS CC (3.81 × 50 cm, 300 g) eluting with MeOH-H2O (from 30:70 to 55: 45) to afford compounds **12** (300 mg), **14** (879 mg) and **16** (3.65 g). Fr. B5 (4.6 g) was chromatographed on silica gel (4 × 36 cm, 150 g; $CHCl_3$-MeOH, 80:20), purified using preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent) using MeOH-H_2O (30:70) as the eluent to give compounds **23** (18 mg), **24** (35 mg) and **25** (7 mg).

The water part (810 g, C) was subjected to silica gel CC (18 × 32 cm, 3000 g) eluting with $CHCl_3$-MeOH-H_2O (95:5:0, 90:10:1, 80:20:2, 60:40:4) to afford four subfractions (Frs. C1–4). Fr. C2 (23 g) was separated by MCI CHP-20P gel CC (4.0 × 40 cm, 500 mL) eluting with MeOH-H_2O (from 10:90 to 60:40) to yield three subfractions (Frs. C2-1–2-3). Fr. C2-1 (17 g) was recrystallized from MeOH to give compound **41** (13.7 g). Fr. C2-2 (3.2 g) was chromatographed on silica gel (4 × 36 cm, 150 g; $CHCl_3$-MeOH-H_2O, from 87:13:1 to 80:20:2) to give two subfractions Fr. C2-2-1 and C2-2-2. Fr. C2-2-1 (457 mg) was purified using preparative HPLC (SB-C18, 5 μm, 9.4 × 250 mm, Agilent) using MeOH-H_2O (from 20:80 to 50:50) as the eluent to give compounds **26** (71 mg) and **27** (13 mg). Fr. C2-2-2 (1.08 g) was separated on silica gel (1.6 × 35 cm, 30 g; $CHCl_3$-MeOH-H_2O, 85:15:1) and further purified by Sephadex LH-20 CC (1.4 × 135 cm, 53 g; $CHCl_3$-MeOH, 50:50) to give compounds **38** (27 mg), **39** (13 mg) and **45** (53 mg). Fr. C4 (4.25 g) was subjected to MCI CHP-20P gel CC (2.54 × 40 cm, 125 mL) eluting with MeOH-H_2O (from 10:90 to 80:20) to yield three subfractions (Frs. C4-1–4-3). Fr. C4-1 (457 mg) was purified using preparative HPLC (SB-C18, 5 μm, 21 × 250 mm, Agilent) using MeOH-H_2O (from 10:90 to 50:50) as the eluent to give compounds **47** (36 mg), **28** (113 mg) and **43** (241 mg). Fr. C4-2 (1.27 g) was separated on ODS CC (2.54 × 50 cm, 125 g) using MeOH-H_2O (from 10:90 to 50:50) as the eluent, and further purified by Sephadex LH-20 CC (1.4 × 135 cm, 53 g; MeOH) to give compounds **30** (16 mg), **33** (17 mg) and **34** (77 mg). Fr. C4-3 (158 mg) was purified by preparative HPLC (SB-C18, 5 μm, 21 × 250 mm, Agilent) using MeOH-H_2O (from 10:90 to 50:50) as the eluent to give compounds **35** (9 mg), **36** (4 mg) and **37** (11 mg).

(−)-swerimusic acid A (**1**): colorless oil; [α]25 D = −54.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.39) nm; IR (KBr): ν_{max} = 3432, 2970, 1767, 1677, 1640, 1433, 1388, 1301, 1277, 1176, 1022, 928 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 600/125 MHz) data, see Table 1; (+) HRESIMS *m/z* 193.0444 $[M + Na]^+$, calcd 193.0471; 171.0637 $[M + H]^+$, calcd for 171.0652.

(−)-swerimuslactone A (**2**): colorless oil; [α]20 D = −93.8 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 225 (3.84) nm; IR (KBr): = ν_{max} 2975, 2937, 2907, 1705, 1664, 1413, 1327, 1277, 1245, 1160, 1086, 1070, 982, 939, 817, 758 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 1; (+) HRESIMS *m/z* 169.0832 $[M + H]^+$, calcd for 169.0859.

(−)-swerimusic acid B (**3**): colorless oil; [α]25 D = −75.3 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 208 (3.73), 235 (3.80) nm; IR (KBr): ν_{max} = 3434, 2970, 1712, 1468, 1404, 1309, 1175, 1136, 1068, 764 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 1; (+) HRESIMS *m/z* 199.0931 $[M + H]^+$, calcd for 199.0965; (−) HRESIMS *m/z* 197.0789 $[M-H]^-$, calcd for 197.0819.

6′-*O*-formylsweroside (**4**) amorphous solids; [α]21 D = −312.4 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 244 (4.15) nm; IR (KBr): ν_{max} = 3427, 2924, 1691, 1617, 1408, 1320, 1280, 1205, 1073, 986, 900 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 2; (+) HRESIMS *m/z* 387.1326 ($[M + H]^+$, calcd for 387.1286; 197.0838, $[M + H\text{-}C_7H_{10}O_6]^+$; 431.1199 $[M + HCOO]^-$, calcd for 431.1195; 403.1244 $[M + HCOO\text{-}CO]^-$.

6′-*O*-formylgentiopicroside (**5**) amorphous solids; [α]21 D = −140.7 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.80) 253 (3.77), 272 (3.80), nm; IR (KBr): ν_{max} = 3432, 2922, 1718, 1612, 1424, 1275, 1073, 1018, 935 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 2; (+) HRESIMS *m/z* 407.0950 $[M + Na]^+$, calcd for 407.0949; 385.1152 $[M + H]^+$, calcd for 385.1125.

6′-*O*-acetylamarogentin (**6**) amorphous solids; [α]21 D = −83.9 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 202 (4.32),

Table 1
^1H-NMR and ^{13}C NMR data of compounds **1–3** in $CDOD_3$ (δ in ppm, *J* in Hz in parentheses).

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		169.0				174.9
2		131.5				
3	3.90, t, 10.0	39.5	4.37, m 4.23, m	65.0	1.94, s	13.0
4	2.44, dddd, 12.4, 10.0, 7.6, 2.6; 2.35, m	29.1		123.4		125.8
5	4.49, td, 9.0, 2.6 4.34, td, 9.0, 7.6	68.7		153.0		152.1
6		181.1	2.50, m 2.36, m	29.1	2.45. m	25.8
7	1.91, d, 7.2	14.7	4.41, m	67.4	4.30, m	67.1
8	7.13, q, 7.2	143.3	3.67, m	70.7	1.95, m 1.62, m	23.6
9			2.22, dd, 6.2, 4.2	37.3	3.61, t, 7.6	51.0
10			1.26, d, 6.2	21.3	0.92, t, 7.6	12.3
11				165.7		168.2

Table 2
^{1}H-NMR and ^{13}C NMR data of compounds **4** and **5** in $CDOD_3$ (δ in ppm, J in Hz in parentheses).

Position	4		5	
	δ_H	δ_C	δ_H	δ_C
1	5.54, m	98.2	5.49, d, 3.2	98.8
3	7.59, d, 2.4	153.9	7.46, s	150.7
4		106.1		105.0
5	3.13, m	28.4		126.9
6	1.72, m	25.9	5.63, brs	117.4
7	4.46, m	69.7	5.04, m	71.0
	4.37, td, 11.7, 2.3			
8	5.54, dt, 11.7, 10.0	133.2	5.72, m	134.8
9	2.70, dd, 8.0, 5.4	43.9	3.31, m	46.6
10	5.24, m	121.0	5.23, m	118.9
11		168.5		166.3
1′	4.70, d,	99.8	4.68, d, 8.0	100.3
2′	3.20, m	74.6	3.17	74.4
3′	3.38, m	77.8	3.37, m	77.6
4′	3.30, m	71.3	3.34, m	71.2
5′	3.55, m	75.5	3.56, m	74.4
6′	4.52, d, 10.7	63.9	4.52, d, 10.8	63.9
	4.29, dd, 12.0, 5.7		4.29, dd, 12.0, 5.6	
–HC = O	8.15	162.9	8.16, s	163.0

215 (4.32), 226 (4.32), 263 (3.96), 305 (3.67) nm; IR (KBr): ν_{max} = 3431, 2966, 1692, 1656, 1618, 1584, 1446, 1407, 1324, 1250, 1202, 1171, 1110, 1082, 1066, 1045, 986 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 3; (+) HRESIMS m/z 629.1758 $[M + H]^+$, calcd for 629.1865); (−) HR-ESIMS m/z 627.1607 $[M-H]^-$, calcd for 627.1719.

Table 3
^{1}H-NMR and ^{13}C NMR data of compounds **6** and **7** in $CDOD_3$ (δ in ppm, J in Hz in parentheses).

Position	6		7	
	δ_H	δ_C	δ_H	δ_C
1	5.24, br s	96.8	5.40, s	96.5
3	7.43, br s	153.6	7.29, s	148.2
4		104.0		104.6
5	2.72, m	28.7		126.1
6	1.67, d, 12.5; 1.55, m	25.8	5.60, brs	117.7
7	4.36, m; 4.24, m	69.6	4.82, br s	69.5
8	5.41, m	132.7	5.68, ddd, 17.1, 10.2, 6.8	134.5
9	2.59, m	43.4	3.21, d, 5.8	45.6
10	5.23, br s	121.3	5.14, m	118.1
11		167.6		165.7
1′	4.29, d, 8.0	97.3	4.18, d, 7.8	96.2
2′	4.75, m	74.3	4.72, t, 8.4	73.9
3′	2.83, m	74.6	3.00, m	75.2
4′	3.30, m	71.4	3.35, d, 7.9	70.9
5′	3.27, m	75.5	3.21, m	74.4
6′	4.35, d, 10.8	64.3	4.43, d, 11.8	63.7
	4.16, dd, 11.9, 4,6		4.18, m	
1″		148.6		148.3
2″		105.7		103.7
3″		163.8		163.0
4″	6.31, s	103.2	6.37, s	102.7
5″		166.0		165.7
6″	6.18, s	112.9	6.23, s	112.3
7″		171.4		171.0
1‴		146.5		145.8
2‴	6.71, s	116.5	6.78, s	116.2
3‴		157.4		157.2
4‴	6.79, d, 7.7	114.6	6.81, d, 7.0	114.2
5‴	7.18, t, 7.7	129.4	7.17. t, 7.0	129.0
6‴	6.73, d, 7.7	121.2	6.76, d, 7,1	120.6
$CH_3\underline{C}O$–		172.8		170.4
$\underline{CH_3}CO$–	2.04, s	20.8	2.05	20.6

6′-*O*-acetylamaronitidin (**7**) amorphous solids; [α]21 D = −99.9 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 203 (4.59), 212 (4.58), 267 (4.32) nm; IR (KBr): ν_{max} = 3427, 2924, 1705, 1657, 1613, 1584, 1452, 1371, 1327, 1251, 1208, 1167, 1138, 1112, 1077, 1042, 998 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD, 400/100 MHz) data, see Table 3; (+) HRESIMS m/z 627.1607 $[M + H]^+$, calcd for 627.1708); (−) HR-ESIMS m/z 625.1471 $[M-H]^-$, calcd for 625.1563.

The anti-HBV assay was performed according to our previous report [10]. The toxicity was assayed by a modified MTT (GIBCO Invitrogen, Carlsbad, CA, USA) method. The levels of HBsAg and HBeAg in the supernatants were measured with an ELISA method. Real-time PCR assay was used to detect the HBV DNA. Tenofovir, purchased from Jiangxi Chenyang Pharmaceutial Co. Ltd., China (purity > 97.6%) was used as a positive control.

3. Results and discussion

Compound **1** was isolated as a colorless oil and had the molecular formula $C_8H_{10}O_4$, which was deduced from positive HRESIMS (m/z 193.0444 $[M + Na]^+$, calcd for 193.0471), indicating four degrees of unsaturation (Fig. 1). The presence of OH (3432 cm^{-1}), C = O (1767 and 1677 cm^{-1}) and C = C (1640 cm^{-1}) groups was deduced from the IR spectrum. The ^{13}C NMR spectra (Table 1) displayed eight carbon resonances due to three quaternary carbons, two methines, two methylenes and one methyl group. The HSQC spectrum allowed assignments of all the protons to their bonding carbons. The ^{1}H-^{1}H COSY spectrum correlations of H-4/H-3/H-5, together with the HMBC correlation: from H-3 (δ_H 3.90) to and C-5 (δ_C 68.7), from H-4 (δ_H 2.44 and 2.35) to C-2 (δ_C 131.5), and from C-1 (δ_C 169.0) to H-5 (δ_H 4.49 and 4.34), fully supported the establishment of a lactone ring. The CH_3-CH moiety was constructed by the ^{1}H-^{1}H COSY corrections of H-7 (δ_H 1.91) and H-8 (δ_H 7.13), which was linked with C-2 from the HMBC cross-peaks of H-7 (δ_H 1.91) with C-2 (δ_C 131.5), H-8 (δ_H 7.13) with C-1 (δ_C 169.0) and C-3 (δ_C 39.5). In addition, the HMBC correlations of C-6 (δ_C 181.1) with H-3 (δ_H 3.90), H-4 (δ_H 2.44 and 2.35) revealed the carboxyl group located at C-3. The (*E*)-geometry of the C = C bond was established by the cross-peak H-7 (δ_H 1.91) with H-5 (δ_H 4.49 and 4.34) in the ROESY plot (Fig. 2). Consequently, the structure of compound **1** was elucidated as (*E*)-3-ethylidene-2-oxo- tetrahydro-2H-pyran-4-carboxylic acid and named as (−)-swerimusic acid A (**1**). (See Fig. 1.)

Compound **2**, a colorless oil, had the molecular formula $C_9H_{12}O_3$ by the positive HRESIMS (m/z 169.0832 $[M + H]^+$, calcd for 169.0859), indicating four degrees of unsaturation. Its IR spectrum suggested the presence of a carbonyl group (1705 cm^{-1}) and a double bond (1664 cm^{-1}). The ^{13}C NMR spectra (Table 1) showed nine carbon resonances due to three quaternary carbons, one methine, four methylenes and one methyl group. An obvious α, β-unsaturated δ-lactone ring was revealed from the ^{1}H NMR [δ_H 4.41 (2H, m, H-7); 2.50 (1H, m, H-6a), 2.36 (1H, m, H-6b)] and ^{13}C NMR [δ_C 165.7 (s, C-11), 153.0 (s, C-5), 123.4 (s, C-4)] data (Table 1), which was confirmed by the ^{1}H-^{1}H COSY correlations of δ_H 4.41 (H-7) with 2.50 (H-6a) and 2.36 (H-6b), and HMBC correlations of δ_C 123.4 (s, C-4) with δ_H 2.50 (H-6a) and 2.36 (H-6b), and δ_H 4.41 (H-7)

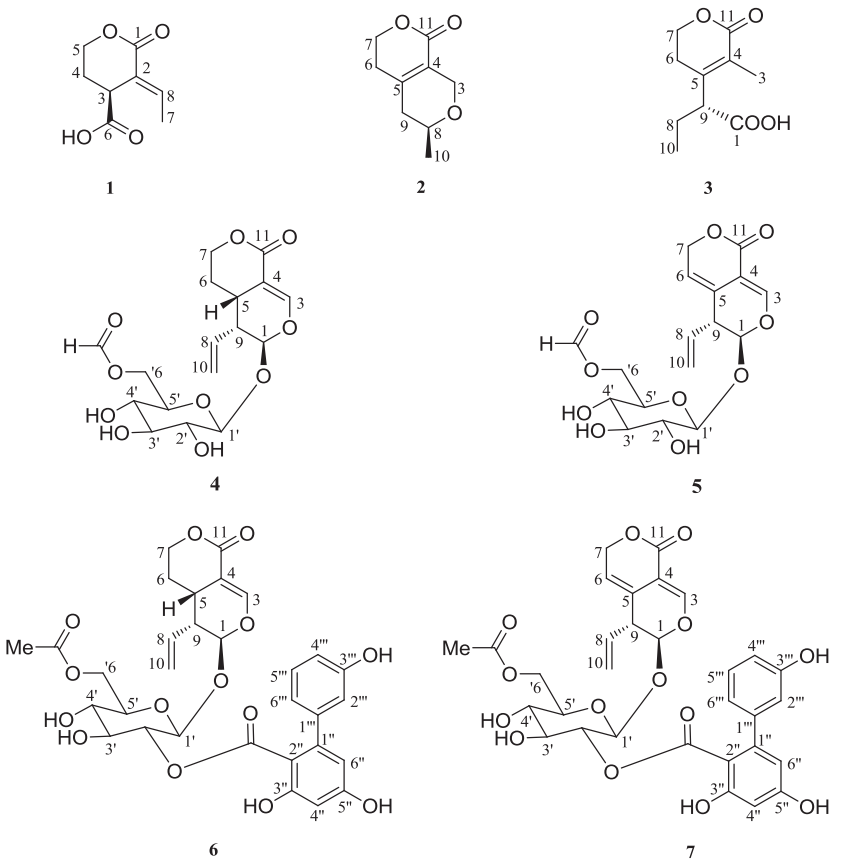

Fig. 1. Chemical structure of compounds **1–7**.

with δ_C 153.0 (C-5) and 165.7 (C-11). Besides the δ-lactone ring fragment, the four residual carbons were proposed to occupy a cyclic pattern to fulfill the unsaturation degree. In addition, a pyran ring joined with the δ-lactone ring at C-4 and C-5 positions was deduced from the ^{1}H-^{1}H COSY correlations: δ_H 3.67 (H-8) with δ_H 2.22 (H-9) and the HMBC correlations: from δ_H 4.37 and 4.23 (H-3) to δ_C 153.0 (C-5) and 165.7 (C-11); from δ_H 3.67 (H-8) to δ_C 65.0 (C-3) and 37.3 (C-9); from δ_H 2.22 (H-9) and δ_C 29.1 (C-6) and 123.4 (C-4). The residual methyl group was assigned at C-8 by the correlations of δ_H 2.22 (H-9) with 1.26 (H-10) in ^{1}H-^{1}H COSY spectrum, and δ_H 1.26 (H-10) with δ_C 37.3 (C-9) in HMBC spectrum (Fig. 2). Based on the above evidence, the structure of compound **2** was determined as 6-methyl-3,4,5,6-tetrahydropyrano[3,4-c]pyran-1(8*H*)-one and named as (−)-swerimuslactone A (**2**).

Compound **3**, a white power, possessed the molecular formula of $C_{10}H_{14}O_4$ by positive HRESIMS, which showed $[M + H]^+$ at *m/z* 199.0931 (calcd for 199.0965), with three degrees of unsaturation. The IR spectrum displayed absorption bands for OH (3434 cm^{-1}) and C = O (1712 cm^{-1}) functionalities. Ten carbons involving four quaternary carbons, one methine, three methylenes and two methyl groups were detected in the ^{13}C NMR spectrum (Table 1). The characteristic signals at δ_C 168.2 (s, C-11), 152.1 (s, C-5), 125.8 (s, C-4), 67.1 (t, C-7) and 25.8 (t, C-6) indicated an α,β-unsaturated δ-lactone fragment, which was supported by the ^{1}H-^{1}H COSY correlation of δ_H 4.30 (2H, m, H-7) with 2.45 (2H, m, H-6) and HMBC correlations of δ_H 2.45 (H-6) with δ_C 67.1 (C-7), δ_H 4.30 (H-7) with δ_C 152.1 (C-5) and 174.9 (C-1). The connectivity of CH_3(10)-CH_2(8)-CH(9)-COOH(1) was manifested by the ^{1}H-^{1}H COSY correlations of δ_H 1.95 (1H, m, H-8a) and 1.62 (1H, m, H-8b) with 3.61 (1H, t, J = 7.6 Hz, H-9) and 0.92 (3H, t, J = 7.6 Hz, H-10), and HMBC correlations (Fig. 2) of δ_H 3.61 (H-9) with δ_C 12.3 (C-10), and δ_H 1.95 (H-8a) and 1.62 (H-8b) with δ_C 174.9 (C-1), which was proposed at C-5 based on HMBC correlations of δ_H 1.95 (H-8a) and 1.62 (H-8b) with δ_C 152.1 (C-5), δ_H 3.61 (H-9) with δ_C 125.8 (C-4) and 25.8 (C-6). The residual methyl group was assigned at C-4 by the HMBC correlations of δ_H 1.94 (3H, s, H-3) with δ_C 152.1 (C-5) and 168.2 (C-11). Hence, the structure of **3** was established as 2-(5-methyl-6-oxo-3,6-dihydro-2H-pyran-4-yl)butanoic acid and named as (−)-swerimusic acid B (**3**).

Compound **4** was a white amorphous powder. The molecular formula $C_{17}H_{22}O_{10}$ was deduced from the positive HRESIMS (*m/z* 387.1326 $[M + H]^+$, calcd for 387.1286). The ^{1}H and ^{13}C NMR data of compound **4** (Table 2) showed the presence of a formyl group [δ_H 8.15 (1H, s); δ_C 163.0 (d)]. Its ^{1}H and ^{13}C NMR data were similar to sweroside with the major difference at C-5′ and C-6′ [12,13]. The HMBC spectrum (Fig. 2), correlation of δ_H 8.16 (1H, s, CHO) with δ_C 63.9 (C-6′), δ_H 4.52 (1H, d, J = 10.7 Hz, H-6′a) and 4.29 (1H, d, J = 10.7 Hz, H-6′a) with δ_C 163.0 (d, CHO) revealed the formyl group at C-6′. In the ROESY spectrum (Fig. 2), correlation of H-8 with H-5 and H-9 indicated the same configuration of compound **4** and sweroside. Based on

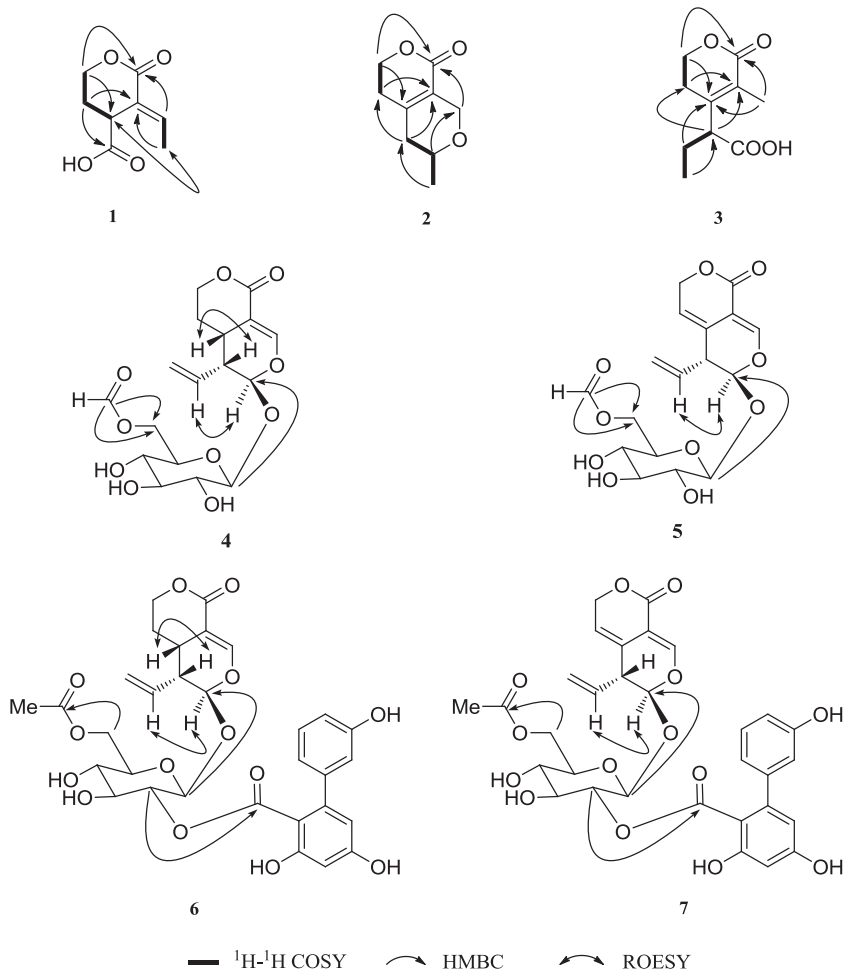

Fig. 2. Key 2D-NMR correlation of compounds **1–7**.

the above evidences, compound **4** was determined to be 6′-*O*-formylsweroside.

Compound **5** was a white amorphous powder. The molecular formula $C_{17}H_{20}O_{10}$ was deduced from the positive HRESIMS (*m/z* 407.0950 $[M + Na]^+$, calcd for 407.0949). The ^{1}H and ^{13}C NMR characteristics (Table 2) were very similar to those of 6′-*O*-acetylgentiopicroside [11]. The major difference was that the acetyl group [δ_H 2.02 (3H, s); δ_C 173.0 (s), 20.7 (q)] in 6′-*O*-acetylgentiopicroside was replaced by a formyl group [δ_H 8.16 (1H, s); δ_C 163.0 (d)] in compound **5**. In the HBMC spectrum (Fig. 2), a correlation peak from δ_H 8.16 (−C<u>H</u>O) to δ_C 63.9 (C-6′) conformed the formyl group at C-6′. The orientation of H-1 and H-9 were supported by ROESY correlation of H-1 with H-9. Based on the above evidences, compound **5** was determined to be 6′-*O*-formylgentiopicroside.

Compound **6** was obtained as a white amorphous power and had a molecular of $C_{31}H_{32}O_{14}$ by positive HRESIMS at *m/z* 629.1758 $[M + H]^+$ (calcd for 629.1865). The ^{1}H and ^{13}H NMR data (Table 3) aided by 2D NMR analyses (Fig. 2) indicated an amarogentin fragment, and the major difference was the presence of an addition acetyl group [δ_H 2.04 (3H, s); δ_C 172.8 and 20.8] in compound **6** [14]. The location of acetyl group was assigned to C-6′ from the cross-peak of HMBC between δ_H H-6′ [δ_H 4.17 (1H, dd, J = 11.9, 4.6 Hz) and 4.34 (1H, br d, J = 10.8 Hz)] and δ_C 172.8. In the ROESY spectrum, correlation of H-8 with H-5 and H-9 indicated the same configuration of compound **6** and amarogentin. Thus, the structure of compound **6** was established as 6′-*O*-acetylamarogentin.

Compound **7** was a white amorphous powder. The molecular formula $C_{31}H_{30}O_{14}$ was deduced from the negative HRESIMS (*m/z* 627.1607 $[M-H]^-$, calcd for 627.1719). The ^{1}H and ^{13}C NMR data (Table 3) revealed the presence of an OAc group [δ_H 2.04 (3H, s); δ_C 172.8 (s), 20.8 (q)]. The ^{1}H and ^{13}C NMR data were very similar to those of amaronitidin, except for the upfield shift of C-5′ and downfield shift of C-6′ [15]. Accordingly, the OAc group was located at C-6′ by the HMBC correlations from H-6′ to C = O. The α orientation of the olefinic group at C-8 were supported by the ROESY correlations of H-8 with H-1 (Fig. 2). Thus, the structure of compound **7** was established as 6′-*O*-acetylamaronitidin.

In addition to the compounds discussed above, 40 known compounds including 18 iridoids, 9 phenols and other 14 compounds were further determined by comparing their NMR data with those in previous reports or chromatographic behaviors on TLC with reference compounds. The chemical structures of 40 known compounds are shown. The eighteen

Table 4
Anti-HBV activities of selected compounds from *S. mussotii*.

Compounds	CC_{50} [mM]	HBsAg		HBeAg		HBV DNA	
		IC_{50} [mM]	SI[e]	IC_{50} [mM]	SI	IC_{50} [mM]	SI
9	1.73 ± 0.23	0.69 ± 0.07	2.5	1.14	1.5	–	–
11	1.76 ± 0.16	1.30 ± 0.10	1.4	2.84	<1.0	–	–
26	>9.42	3.76 ± 0.32	>2.5	>9.42	–	2.3 ± 0.13	>4.0
27	>10.14	5.18 ± 0.48	>2.0	>10.14	–	2.54 ± 0.18	>4.0
28	>10.48	4.55 ± 0.39	>2.3	7.46	>1.4	2.62 ± 0.14	>4.0
29	<0.67	<0.67	–	<0.67	–	<0.06	11.2
31	>5.83	0.14 ± 0.02	>41.6	5.03	>1.2	0.22 ± 0.03	>26.5
32	>7.43	0.23 ± 0.03	>32.3	3.74	>2.0	0.29 ± 0.03	>25.6
45	>3.54	0.79 ± 0.12	>4.5	1.12	>3.1	0.02 ± 0.001	>177.0
Tenofovir	>1.54	1.43 ± 0.11	>1.1	1.31	>1.2	0.00058 ± 0.0001	>2655.2

All values are the mean of two independent experiments; CC_{50} = 50% cytotoxic concentration, IC_{50} = 50% inhibition concentration, SI (selectivity index) = CC_{50}/IC_{50}; Tenofovir, an antiviral agent used as a positive control.

known iridoids are (*R*)-(−)-gentiolactone (**8**) [16], (*R*)-gentianol (**9**) [17], 5-vinyl-3,4-dihydro-isocoumarin (**10**) [18], erythrocentaurin (**11**) [19], erythrocentauric acid (**12**) [19], swertiaside A (**13**) [20], 3,4-dihydro-1*H*,6*H*,8*H*-naphtho-[1,2-*c*:4,5-*c*′,*d*′]dipyrano-1,8-dione (**14**) [21], sweroside (**15**) [12], swertiamarin (**16**) [12], gentiopicroside (**17**) [12], loganic acid (**18**) [12], 8-*epi*-loganic acid (**19**) [12], adoxosidic acid (**20**) [22], 8-hydroxy-10-hydrosweroside (**21**) [23], amarogentin (**22**) [14], 3′-*O*-β-D-glucopyranosylsweroside (**23**) [24], 4′-*O*-β-D-glucopyranosyl- gentiopicroside (**24**) [25] and 6′-*O*-β-D-glucopyranosylgentiopicroside (**25**) [25]. The nine known phenols were *m*-hydroxybenzoic acid (**26**) [26], *p*-hydroxybenzoic acid (**27**) [27], *m*-hydroxy benzenmethanol (**28**) [28], 3,4-dihydroxybenzoic acid (**29**) [28], vanillic acid (**30**) [29], ethyl 3,4-dihydroxybenzoate (**31**) [30], ethyl 2,5-dihydroxybenzoate (**32**) [31], 4-hydroxy-3,5-dimethoxybenaldehyde (**33**) [32] and 3,3′,5-trihydroxydiphenyl (**34**) [33]. The other thirteen known compounds were oleanolic acid (**35**) [34], maslinic acid (**36**) [35], erythrodiol 3-*O*-palmitate (**37**) [36], furan-2-carboxylic acid (**38**) [37], 5-hydroxymethyl-2-furan-carboxaldehyde (**39**) [38], methyl-5-hydroxy-2-pyridine-carboxylat**e** (**40**) [39], *D*-mannitol (**41**) [40], (*E*)-6-hydroxy-2,6-dimethy-locta-2,7-dienoic acid (**42**) [41], uracil (**43**) [42], uridine (**44**) [42] and isoorientin (**45**) [43]. In addition, β-sitosterol (**46**) and daucosterol (**47**) were also isolated from this plant and identified by comparing with authentic samples.

To evaluate their anti-HBV activities, namely, inhibiting the secretion of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), as well as HBV DNA replication, compounds **1**–**45** were assayed on HepG 2.2 15 cell line stably transfected with the HBV genome *in vitro*, as reported previously [10]. Tenofovir (a clinically popular anti-HBV agent) was used as the positive control. Anti-HBV activity, cytotoxicity and selectivity index (SI) are summarized in Table 4. Only two secoiridoids of compounds **9** and **11** displayed activities against the secretion of HBsAg (IC_{50} 0.69 and 1.30 mM) and HBeAg (IC_{50} 1.14 and 2.84 mM). However, other secoiridoid aglycones and glycosides showed no activity or cytotoxicity at the highest tested concentration. Six of nine known phenols exhibited significant anti-HBV activity. Compounds **26**–**28**, with one hydroxyl and one carboxyl showed anti-HBV activity with IC_{50} values of 3.76, 5.18 and 4.55 mM for inhibitory HBsAg secretion, and 2.36, 2.54 and 2.62 mM for inhibitory HBV-DNA replication, respectively. Compounds **29**, **31**, **32** with two hydroxyls and one carboxyl displayed remarkable inhibition on HBV-DNA replication with IC_{50} values of <0.06, 0.22 and 0.29 mM. Furthermore, compounds **31** and **32** showed significant inhibitory effect on the secretion of HBsAg (IC_{50} 0.14 and 0.23 mM) and HBeAg (IC_{50} 5.03 and 3.74 mM). In addition, isoorientin (**45**) displayed significant anti-HBV activity which inhibited the secretion of HBsAg and HBeAg with IC_{50} values of 0.79 and 1.12 mM, respectively, and also exhibited inhibitory activity on HBV DNA replication with IC_{50} values of 0.02 mM.

Conflict of interest

The authors indicated no potential conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.01.020.

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