Seven New Secoiridoids with Anti-Hepatitis B Virus Activity from *Swertia angustifolia*

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- *Swertia angustifolia*
- Gentianaceae
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Abstract

Seven new secoiridoids, swertianglide (**1**) and swertianosides A–F (**2**–**7**), together with fifteen known compounds, were isolated from the whole plants of *Swertia angustifolia*. Their structures were elucidated on the basis of extensive spectroscopic analyses ($[\alpha]_D$, UV, IR, MS, 1D- and 2D-NMR). Fourteen compounds were evaluated for their anti-hepatitis B virus (HBV) activities on the Hep G 2.2.15 cell line *in vitro*. Compound **2**, an unusual secoiridoid glycoside dimer, showed significant activities inhibiting the secretion of HBsAg (IC_{50} 0.18 mM, SI 3.11) and HBeAg (IC_{50} 0.12 mM, SI 4.67).

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

The plant genus *Swertia* (Gentianaceae) comprises about 170 species, 79 of which are present in China. Many *Swertia* plants are widely used as folk herbs to treat hepatitis in both TCM and Tibetan medicine systems [1,2]. For example, *S. mileensis*, known as "*Qing-Ye-Dan*", has been documented in the Chinese Pharmacopoeia (1977–2010 editions) to cure viral hepatitis clinically [3]. Our previous investigation on *S. mileensis* led to the isolation of a series of novel anti-HBV active lactones, swerilactones A–K [4–8], as well as three new secoiridoid glycoside dimers, swerilactosides A–C [9].

S. angustifolia, the congener plant of *S. mileensis*, is also used for treating hepatitis and cholecystitis in the folk of Yunnan province [10]. Previous studies on this plant revealed the presence of xanthones, secoiridoids, and steroids [11–14]. However, none was concerned with its anti-HBV property. As part of a continuous search for anti-HBV constituents from natural resources, our focused investigation on *S. angustifolia* resulted in the isolation of seven new compounds, including one new secoiridoid, swertianglide (**1**), and six new secoiridoid glycosides, swertianosides A–F (**2**–**7**) (**Fig. 1**), together with fifteen known ones. This paper describes the isolation and structural elucidation of compounds **1**–**7** by extensive spectroscopic analyses, as well as their anti-HBV activities.

Materials and Methods

General experimental procedures

Optical rotations were obtained on a JASCO model 1020 polarimeter (Horiba). UV spectra were taken on a Shimadzu UV-2401PC spectrophotometer (Shimadzu). IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets (Bio-Rad). 1D- and 2D-NMR spectra were recorded on Bruker AM-400, DRX-500, or AVANCE III-600 spectrometers with TMS as an internal standard (Bruker). Mass spectra were run on a VG Auto Spec-3000 mass spectrometer (VG). Column chromatography was performed on silica gel (200–300 mesh; Qingdao Makall Chemical Company). Semipreparative HPLC was carried out on a Waters Alliance 2695 liquid chromatograph with an Eclipse XDB-C_{18} (9.4 × 250 mm) column (Waters). Sephadex LH-20 (20–150 µm) for chromatography was purchased from Pharmacia Fine Chemical Co. Ltd. (Pharmacia). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

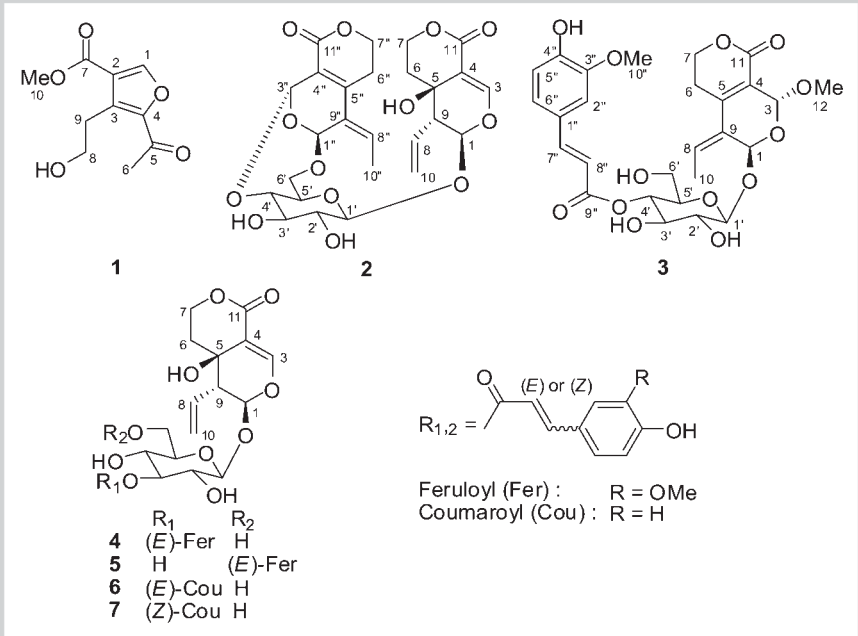

Fig. 1 Structures of compounds **1**–**7**.

Plant material

The whole plants of *S. angustifolia* were collected in Luquan, Yunnan province, PR China, in November 2008 and identified by Prof. Dr. Li-Gong Lei, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 20081127) was deposited at the Laboratory of Antivirus and Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The air-dried and powdered whole plants of *S. angustifolia* (5 kg) were extracted with 90% (50 L) EtOH under reflux two times, 2 h for each time. The combined extracts (1.2 kg) were concentrated, and suspended in H_2O (10 L). The suspension was partitioned with petroleum ether, EtOAc, and *n*-butanol, successively. The EtOAc layer provided 210 g of residue which was subjected to silica gel column (9 × 40 cm, 1000 g) eluted with $CHCl_3$-MeOH-H_2O (100 : 0 : 0, 95 : 5 : 0, 8 : 2 : 0.2, 7 : 3 : 0.3, 6 : 4 : 0.4, 0 : 100 : 0, *v*/*v*, each 10 L) to yield 6 fractions (Frs. A–F). Fr. B (24.6 g) was further divided into 5 subfractions (Frs. B1–B5) by chromatography over a silica gel column (4 × 19 cm, 100 g) using $CHCl_3$-Me_2CO (9 : 1, 8 : 2, 7 : 3, 6 : 4, 1 : 1, *v*/*v*, each 1.5 L) as the eluent. Fr. B2 (240 mg) was applied to silica gel column (1.5 × 26 cm, 12 g, petroleum ether-Me_2CO, 8 : 2, *v*/*v*, 800 mL), yielding compounds **1** (4 mg), **12** (21 mg), and **13** (18 mg). Fr. B3 (360 mg) and Fr. B4 (280 mg) were further isolated by column chromatography over silica (1.5 × 30 cm, 25 g) with petroleum ether-EtOAc (7 : 3) (60 mL × 20) and petroleum ether-Me_2CO (8 : 2) (60 mL × 25), respectively, to give compounds **8** (17 mg), **9** (23 mg), **10** (20 mg), and **11** (19 mg). Fr. D (18.6 g) was treated with the same procedures as Fr. B to obtain 5 subfractions (Frs. D1–D5). Compound **2** (16 mg) was purified from Fr. D2 (120 mg) by repeated column chromatography over silica gel (1.5 × 26 cm, 12 g, $CHCl_3$-MeOH, 95 : 5, 9 : 1, *v*/*v*, each 280 mL). Fr. D3 (3.2 g) was performed on silica gel column (4 × 19 cm, 100 g) with a gradient eluent of EtOAc-MeOH (100 : 1, 95 : 5, 9 : 1, 85 : 15, 8 : 2, *v*/*v*, each 1.5 L) to produce 5 subfractions (Frs. D3-1–D3–5). Compounds **4** (100 mg) and **5** (150 mg) were obtained from Fr. D3-1 (550 mg) by silica gel column chromatography (2 × 35 cm, 50 g) using $CHCl_3$-MeOH (100 : 1, *v*/*v*, 1.5 L) as the eluent. Fr. D3-2 (120 mg) was purified by column chromatography over silica gel (1.5 × 26 cm, 12 g, $CHCl_3$-MeCO, 1 : 1, *v*/*v*, 800 mL), and followed by semipreparative HPLC (MeOH-H_2O, *v*/*v* = 3 : 7) to provide compounds **18** (4 mg, t_R = 18.96 min), **19** (6 mg, t_R = 20.32 min), and **20** (4 mg, t_R = 21.61 min). After silica gel column chromatography (2 × 35 cm, 50 g, $CHCl_3$-Me_2CO, 9 : 1, 8 : 2, 7 : 3, 1 : 1, *v*/*v*, each 1 L), Fr. D3-3 (740 mg) was divided into four fractions. Fr. D3-3–1 (520 mg) was first loaded on silica gel column (2 × 35 cm, 50 g, 12 g, $CHCl_3$-MeOH, 9 : 1, 8 : 2, *v*/*v*, each 300 mL), and then purified on Sephadex LH-20 (1.4 × 150 cm, 48 g) yielding compounds **3** (5 mg), **15** (180 mg), **17** (120 mg), and **16** (12 mg). Fr. D3-3–2 (60 mg) was purified by semipreparative HPLC (MeOH-H_2O, *v*/*v* = 3 : 7) to obtain compounds **6** (19 mg, t_R = 19.16 min) and **7** (5 mg, t_R = 20.86 min). Compounds **14** (30 mg), **21** (4 mg), and **22** (5 mg) were isolated from Fr. D4 (170 mg) by column chromatography over silica gel (1.5 × 26 cm, 12 g) with $CHCl_3$-MeOH (9 : 1, *v*/*v*, 850 L) as the eluent. All purified compounds had a degree of purity > 90%, based on the TLC method in three different solvent systems (all compounds exhibited one spot both under UV radiation and when sprayed with H_2SO_4) and NMR spectra (the baseline was smooth without impurity peaks).

Swertianglide (**1**): white powder; $[\alpha]_D^{18}$: − 11.0 (*c* 0.14, MeOH); UV (MeOH): λ_{max} (log ε) = 265 (4.0), 215 (3.7), 195 (3.5) nm; IR (KBr): ν_{max} = 3435, 3136, 2959, 2931, 1730, 1680, 1583, 1439, 1408, 1268, 1234, 1163, 1102, 1045 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see ▶ **Table 1**; negative ESI-MS: *m*/*z* = 211 [M − H]$^-$; HR-ESI-MS: *m*/*z* = 211.0603 [M − H]$^-$ (calcd. for $C_{10}H_{11}O_5$, 211.0606).

Swertianoside A (**2**): white powder; $[\alpha]_D^{15}$: − 79.1 (*c* 0.25, MeOH); UV (MeOH): λ_{max} (log ε) = 269 (4.0), 241 (4.0) nm; IR (KBr) ν_{max} = 3428, 2927, 1707, 1620, 1433, 1271, 1248, 1208, 1156, 1125, 1114, 1060, 1024, 949, 931, 904, 847, 760 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see ▶ **Tables 2** and **3**; negative ESI-MS: *m*/*z* = 549 [M − H]$^-$; HR-ESI-MS: *m*/*z* = 549.1609 [M − H]$^-$ (calcd. for $C_{26}H_{29}O_{13}$, 549.1608).

Swertianoside B (**3**): white powder; $[\alpha]_D^{16}$: − 70.1 (*c* 0.12, MeOH); UV (MeOH): λ_{max} (log ε) = 326 (4.2), 277 (4.3) nm; IR (KBr): ν_{max} = 3432, 2958, 2927, 1712, 1632, 1601, 1516, 1431, 1273,

Table 1 ^{1}H-NMR and ^{13}C-NMR data for **1** (600 MHz, in CD_3COCD_3) and angelone (400 MHz, in $CDCl_3$), *J* in Hz.

Position	1		Angelone	
	δ_H	δ_C	δ_H	δ_C
1	8.30 (s)	151.1 (d)	8.15 (s)	148.2 (d)
2	–	121.4 (s)	–	117.5 (s)
3	–	130.7 (s)	–	128.2 (s)
4	–	150.9 (s)	–	147.4 (s)
5	–	189.3 (s)	–	188.4 (s)
6	2.46 (s)	27.5 (q)	2.53 (s)	26.8 (q)
7	–	163.6 (s)	–	160.8 (s)
8	3.68 (m)	62.2 (t)	4.53 (t, 6.0)	68.4 (t)
9	3.31 (t, 6.5)	28.2 (t)	3.22 (t, 6.0)	21.3 (t)
10	3.82 (s)	51.0 (q)	–	–

1126, 1069, 1030, 985 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see **Tables 2** and **3**; negative ESI-MS: *m/z* = 563 [M – H]$^-$; HR-ESI-MS: *m/z* = 563.1774 [M – H]$^-$ (calcd. for $C_{27}H_{31}O_{13}$, 563.1764).

Swertianoside C (**4**): white powder; $[\alpha]_D^{16}$: – 116.2 (*c* 0.27, MeOH); UV (MeOH): λ_{max} (log ε) = 326 (4.3), 236 (4.3), 220 (4.2) nm; IR (KBr): ν_{max} = 3426, 2974, 2935, 1696, 1619, 1516, 1428, 1270, 1157, 1069, 1029 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see **Tables 2** and **3**; negative ESI-MS: *m/z* = 549 [M – H]$^-$; HR-ESI-MS: *m/z* = 549.1610 [M – H]$^-$ (calcd. for $C_{26}H_{29}O_{13}$, 549.1608).

Swertianoside D (**5**): white powder; $[\alpha]_D^{16}$: – 83.1 (*c* 0.25, MeOH); UV (MeOH): λ_{max} (log ε) = 327 (4.3), 237 (4.3) nm; IR (KBr): ν_{max} = 3429, 2962, 2920, 1707, 1620, 1515, 1429, 1270, 1159, 1071 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see **Tables 2** and **3**; negative ESI-MS: *m/z* = 549 [M – H]$^-$; HR-ESI-MS: *m/z* = 549.1601 [M – H]$^-$ (calcd. for $C_{26}H_{29}O_{13}$, 549.1608).

Swertianoside E (**6**): white powder; $[\alpha]_D^{16}$: – 131.3 (*c* 0.14, MeOH); UV (MeOH): λ_{max} (log ε) = 313 (4.3), 230 (4.3) nm; IR (KBr): ν_{max} = 3431, 2924, 1694, 1619, 1514, 1410, 1270, 1169, 1069 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see **Tables 2** and **3**; negative ESI-MS: *m/z* = 519 [M – H]$^-$; HR-ESI-MS: *m/z* = 519.1494 [M – H]$^-$ (calcd. for $C_{25}H_{27}O_{12}$, 519.1502).

Swertianoside F (**7**): white powder; $[\alpha]_D^{17}$: – 150.9 (*c* 0.13, MeOH); UV (MeOH): λ_{max} (log ε) = 312 (4.3), 230 (4.3) nm; IR (KBr): ν_{max} = 3431, 2924, 1694, 1609, 1515, 1410, 1269, 1170, 1069 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see **Tables 2** and **3**; negative ESI-MS: *m/z* = 519 [M – H]$^-$; HR-ESI-MS: *m/z* = 519.1493 [M – H]$^-$ (calcd. for $C_{25}H_{27}O_{12}$, 519.1502).

Acid hydrolysis of compounds 2–7

A solution of compound **2** (3 mg) in a mixture of MeOH (1.0 mL) and 10% HCl (1.0 mL) was refluxed for 2 h. The hydrolysate was allowed to cool, diluted with 2 mL of H_2O, and extracted with EtOAc (4 mL) 3 times. The aqueous layer was neutralized with aqueous $Ba(OH)_2$ and concentrated *in vacuum* to give a residue, in which glucose was identified by comparison with the authentic sample (BuOH-EtOAc-H_2O, 4 : 1 : 5, upper layer, R_f 0.45; PhOH-H_2O, 4 : 1, R_f 0.50) on PC by spraying with phthalic acid-aniline reagent (1.66 g phthalic acid and 0.93 g aniline dissolved in 100 mL H_2O/sat. BuOH), followed by heating. The residue was purified by column chromatography over silica gel (EtOAc-MeOH, 3 : 2) and identified to be D-glucose based on its $[\alpha]_D$ value ($[\alpha]_D^{17}$: + 44.0, *c* 0.03, H_2O). The hydrolysis processes of compounds **3**–**7** were the same as for **2**.

Anti-HBV activities on the HBV transfected Hep G 2.2.15 cell line *in vitro*

The anti-HBV assay was performed according to our previous report [6]. Tenofovir, purchased from Jiangxi Chenyang Pharmaceutical Co. Ltd., China (purity > 97.6%) was used as the positive control.

Supporting information

1D- and 2D-NMR, HR-ESI-MS, IR, and UV spectra of **1**–**4**, 1D-NMR spectroscopic and HR-ESI-MS data of compounds **5**–**7**, as well as the procedure for anti-HBV assay and the anti-HBV data of compounds **2, 4**–**6**, and **8**–**17** from two independent experiments are available as Supporting Information.

Results and Discussion

Swertianglide (**1**) had a molecular formula of $C_{10}H_{12}O_5$ by negative HR-ESI-MS (*m/z* 211.0603 [M – H]$^-$, calcd. 211.0606), indicating five degrees of unsaturation. The IR spectrum suggested the existence of a hydroxy group (3435 cm^{-1}), a carbonyl group (1730 cm^{-1}), and a double bond (1680 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra (**Table 1**) displayed ten carbon resonances due to five quaternary carbons, one methine, two methylenes, and two methyl groups, including one keto and one ester carbonyl groups, and two double bonds. One acetyl, one hydroxyethyl, and one methoxycarbonyl groups were inferred from the ^{1}H-NMR [δ_H 2.46 (3H, s, H-6), 3.82 (3H, s, H-10), 3.68 (2H, m, H-8), 3.31 (2H, t, *J* = 6.5, H-9)] and ^{13}C-NMR [δ_C 189.3 (C-5, s), 163.6 (C-7, s)] data, combined with the correlation of H-8/H-9 in the ^{1}H-^{1}H COSY spectrum (**Fig. 2**) and the correlations of H-6/C-5 and H-10/C-7 in the HMBC spectrum. The residual two oxygenated double bonds [δ_C 151.1 (C-1, d), 121.4 (C-2, s), 130.7 (C-3, s), 150.9 (C-4, s)], together with the correlations of H-1/C-2, H-1/C-3, and H-1/C-4 in the HMBC spectrum suggested a tetrahydrofuran moiety. The acetyl, hydroxyethyl, and methoxycarbonyl groups were proposed at C-4, C-3, and C-2, respectively, based on the HMBC (**Fig. 2**) correlation of H-6/C-4, H-8/C-3, H-9/C-2, and H-1/C-7. Thus, the structure of **1** was determined and resembled angelone [15]. Comparison of their NMR spectra revealed that the lactone ring in angelone was opened to give a methyl ester connected to C-7, and a hydroxy group connected to C-8 in **1**.

Swertianoside A (**2**) was isolated as white powder, with a molecular formula of $C_{26}H_{30}O_{13}$, deduced by negative HR-ESI-MS (*m/z* 549.1609 [M – H]$^-$). The IR spectrum showed the presence of hydroxy group (3428 cm^{-1}), carbonyl group (1707 cm^{-1}), double bond (1620 cm^{-1}), and ether bond (1114, 1060, 1024 cm^{-1}) functionalities. Acid hydrolysis of compound **2** with 10% HCl yielded D-glucose, which was identified by comparison with an authentic sample on PC in combination with an $[\alpha]_D$ experiment ($[\alpha]_D^{17}$: + 44.0, *c* 0.03, H_2O). The coupling constant (*J* = 8.0 Hz) of the anomeric protons (δ_H 4.65) indicated a β-glucosyl moiety. The ^{1}H- and ^{13}C-NMR spectra (**Tables 2** and **3**) exhibited 26 carbon resonances ascribed to seven quaternary carbons, twelve methines, six methylenes, and one methyl group. Compound **2** possessed the same molecular formula as that of swerilactoside C [9], as well as the similar UV, IR, and 1D-NMR spectral data, which suggested that they possessed a similar skeleton. Based on the extensive 2D-NMR analyses, **2** was proposed to have the same partial fragments **2a** (swertiamarin fragment) and **2b** with swerilactoside C [9], but a different connective mode. The glycosidic linkage between C (1″) and C (6′) was deduced by the HMBC

Table 2 ^{1}H-NMR data for **2**–**7** (in CD_3OD, *J* in Hz).

Pos.	2[a]	3[b]	4[a]	5[a]	6[a]	7[a]
1	5.56 (d, 1.2)	6.27 (s)	5.71 (d, 1.2)	5.58 (d, 1.2)	5.71 (d, 1.2)	5.70 (d, 1.2)
3	7.62 (s)	5.41 (s)	7.61 (s)	7.64 (s)	7.60 (s)	7.61 (s)
6a	1.88 (ddd, 13.8, 13.0, 5.0)	2.75 (m)	1.87 (ddd, 13.8, 13.6, 5.0)	1.86 (ddd, 13.9, 13.5, 5.0)	1.87 (ddd, 14.1, 13.5, 5.0)	1.87 (ddd, 14.1, 13.5, 5.0)
6b	1.74 (br d, 13.8)	2.68 (m)	1.70 (br d, 13.8)	1.71 (br d, 13.9)	1.71 (br d, 14.1)	1.70 (br d, 14.1)
7a	4.75 (m)	4.49 (m)	4.70 (m)	4.74 (m)	4.72 (m)	4.72 (m)
7b	4.35 (m)	4.37 (m)	4.30 (m)	4.32 (m)	4.30 (m)	4.31 (m)
8	5.42 (m)	6.45 (m)	5.39 (m)	5.35 (m)	5.40 (m)	5.39 (m)
9	2.89 (dd, 1.2, 9.2)	–	2.90 (dd, 1.2, 9.2)	2.90 (dd, 1.2, 9.2)	2.89 (dd, 1.2, 9.2)	2.88 (dd, 1.2, 9.2)
10a	5.36 (dd, 2.5, 17.0)	2.00 (d, 7.2)	5.35 (dd, 2.5, 9.4)	5.33 (dd, 2.5, 9.4)	5.37 (dd, 2.5, 9.4)	5.34 (dd, 2.5, 9.4)
10b	5.28 (dd, 2.5, 9.5)	–	5.26 (dd, 2.5, 17.0)	5.18 (dd, 2.5, 17.0)	5.27 (dd, 2.5, 9.4)	5.26 (dd, 2.5, 9.4)
12	–	3.60 (s)	–	–	–	–
1′	4.65 (d, 8.0)	4.94 (d, 7.9)	4.75 (d, 7.9)	4.68 (d, 7.9)	4.74 (d, 7.9)	4.73 (d, 7.9)
2′	3.25 (dd, 8.0, 9.2)	3.34 (m)	3.39 (dd, 7.9, 9.3)	3.26 (dd, 7.9, 9.1)	3.38 (dd, 7.9, 9.3)	3.40 (dd, 7.9, 9.1)
3′	3.44 (m)	3.75 (t, 9.2)	5.04 (t, 9.3)	3.43 (m)	5.03 (t, 9.3)	5.02 (t, 9.1)
4′	3.76 (t, 9.2)	4.86 (t, 9.2)	3.52 (t, 9.3)	3.42 (m)	3.51 (t, 9.3)	3.43 (t, 9.1)
5′	3.39 (m)	3.66 (m)	3.44 (m)	3.62 (m)	3.44 (m)	3.41 (m)
6′a	4.03 (dd, 4.7, 11.5)	3.67 (m)	3.86 (m)	4.50 (br d, 12.0)	3.85 (br d, 12.0)	3.84 (br d, 12.0)
6′b	3.96 (br d, 11.5)	3.58 (m)	3.68 (dd, 5.6, 12.0)	4.41 (dd, 5.6, 12.0)	3.68 (dd, 5.6, 12.0)	3.67 (dd, 5.6, 12.0)
1″	5.75 (s)	–	–	–	–	–
2″	–	7.22 (d, 1.4)	7.15 (d, 1.8)	7.17 (d, 1.3)	7.44 (d, 8.6)	7.62 (d, 8.6)
3″	5.69 (s)	–	–	–	6.76 (d, 8.6)	6.69 (d, 8.6)
5″	–	6.82 (d, 8.2)	6.76 (d, 8.2)	6.81 (d, 8.2)	6.76 (d, 8.6)	6.69 (d, 8.6)
6″	2.71 (m)	7.10 (dd, 1.4, 8.2)	7.04 (dd, 1.8, 8.2)	7.05 (dd, 1.3, 8.2)	7.44 (d, 8.6)	7.62 (d, 8.6)
7″a	4.46 (m)	7.68 (d, 15.9)	7.61 (d, 15.9)	7.62 (d, 15.9)	7.63 (d, 15.9)	6.83 (d, 12.8)
7″b	4.39 (m)	–	–	–	–	–
8″	6.51 (q, 7.3)	6.42 (d, 15.9)	6.38 (d, 15.9)	6.38 (d, 15.9)	6.35 (d, 15.9)	5.80 (d, 12.8)
10″	1.94 (d, 7.3)	3.90 (s)	3.84 (s)	3.89 (s)	–	–

[a] Recorded at 400 MHz; [b] recorded at 600 MHz

Table 3 ^{13}C-NMR data for **2**–**7** (In CD_3OD, *J* in Hz).

Pos.	2[a]	3[b]	4[a]	5[a]	6[c]	7[c]
1	99.1 (d)	91.4 (d)	99.1 (d)	97.9 (d)	99.2 (d)	99.1 (d)
3	154.6 (d)	95.9 (d)	154.8 (d)	153.4 (d)	154.7 (d)	154.8 (d)
4	108.9 (s)	119.7 (s)	108.9 (s)	107.4 (s)	109.1 (s)	108.9 (s)
5	64.2 (s)	147.5 (s)	64.3 (s)	62.9 (s)	64.4 (s)	64.3 (s)
6	33.7 (t)	23.9 (t)	33.7 (t)	32.2 (t)	33.7 (t)	33.7 (t)
7	65.9 (t)	66.9 (t)	66.0 (t)	64.5 (t)	65.9 (t)	65.9 (t)
8	133.8 (d)	137.3 (d)	133.8 (d)	132.3 (d)	133.8 (d)	133.8 (d)
9	52.0 (d)	130.6 (s)	51.9 (d)	50.5 (d)	52.0 (d)	51.9 (d)
10	121.2 (t)	14.8 (q)	121.3 (t)	119.9 (t)	121.3 (t)	121.2 (t)
11	167.9 (s)	165.8 (s)	168.0 (s)	166.5 (s)	167.9 (s)	167.9 (s)
12	–	58.3 (q)	–	–	–	–
1′	100.4 (d)	99.3 (d)	100.1 (d)	99.0 (d)	100.1 (d)	100.2 (d)
2′	74.6 (d)	75.3 (d)	72.9 (d)	72.9 (d)	73.0 (d)	72.8 (d)
3′	75.5 (d)	75.8 (d)	78.6 (d)	76.1 (d)	78.4 (d)	78.1 (d)
4′	79.2 (d)	72.6 (d)	69.6 (d)	70.0 (d)	69.7 (d)	69.6 (d)
5′	72.3 (d)	76.8 (d)	78.4 (d)	74.4 (d)	78.7 (d)	78.4 (d)
6′	68.4 (t)	62.8 (t)	62.2 (t)	62.8 (t)	62.3 (t)	62.2 (t)
1″	94.3 (d)	127.8 (s)	127.8 (s)	126.2 (s)	127.3 (s)	127.6 (s)
2″	–	111.8 (d)	111.7 (d)	110.2 (d)	131.2 (d)	133.7 (d)
3″	92.7 (d)	149.5 (s)	149.3 (s)	147.9 (s)	116.9 (d)	115.8 (d)
4″	119.8 (s)	150.9 (s)	150.6 (s)	149.2 (s)	161.3 (s)	160.0 (s)
5″	147.8 (s)	116.6 (d)	116.5 (d)	115.0 (d)	116.9 (d)	115.8 (d)
6″	23.5 (t)	124.4 (d)	124.1 (d)	122.9 (d)	131.2 (d)	133.7 (d)
7″	66.9 (t)	147.8 (d)	147.0 (d)	145.7 (d)	146.7 (d)	144.8 (d)
8″	137.9 (d)	115.1 (d)	115.6 (d)	113.7 (d)	115.4 (d)	116.8 (d)
9″	130.8 (s)	168.7 (s)	168.9 (s)	167.6 (s)	169.0 (s)	167.9 (s)
10″	14.5 (q)	56.5 (q)	56.4 (q)	55.0 (q)	–	–
11″	165.9 (s)	–	–	–	–	–

[a] Recorded at 100 MHz; [b] recorded at 150 MHz; [c] recorded at 125 MHz

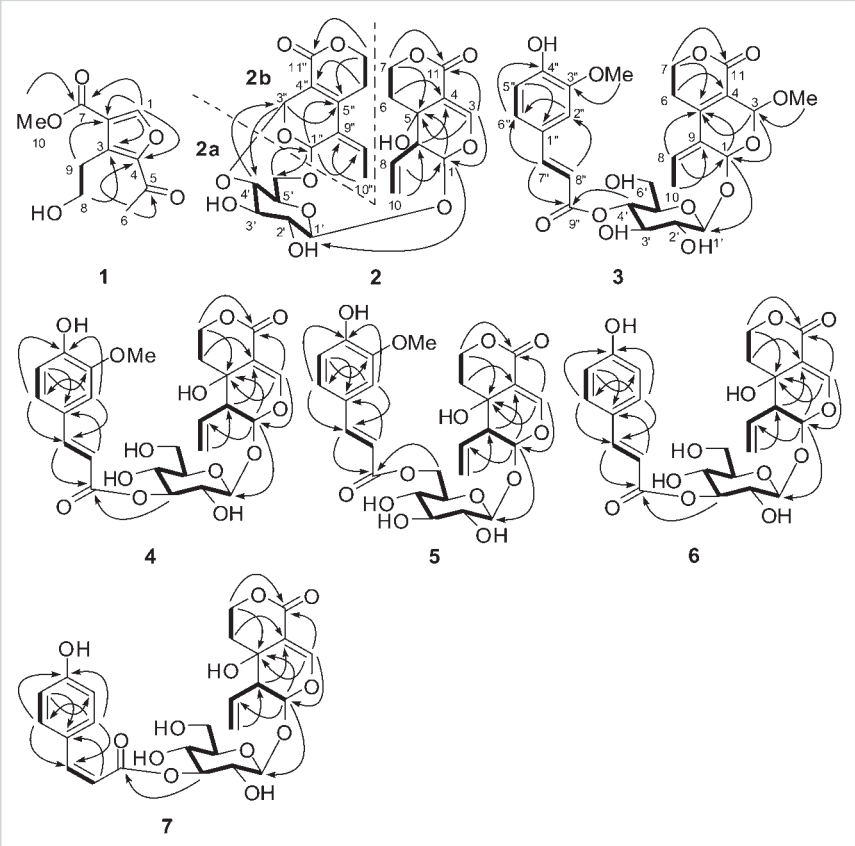

Fig. 2 Selected COSY (–) and HMBC (→) correlations of compounds **1**–**7**.

correlations of H-1″/C-6′ and H-6′/C-1″. Similarly, the connection of C-3″ and C-4′ by a glycosidic bond was detected by the HMBC correlations of H-3″/C-4′ and H-4′/C-3″. The correlations of H-1″/H-6′, H-3″/H-4′, and H-1′/H-1 in the ROESY spectrum (**Fig. 3**) suggested that H-1 and H-1″ were α-oriented and H-3″ was β-oriented. The *Z*-configuration of the double bond (C-8″ = C-9″) was deduced by the ROESY correlation of H-1″/H-10″ (δ_H 1.94).

Swertianoside B (**3**) was obtained as white powder. The negative HR-ESI-MS revealed its molecular formula to be $C_{27}H_{32}O_{13}$ (*m/z* 563.1774 [M – H]$^-$). The IR spectrum showed the absorption bands of a hydroxy group (3432 cm^{-1}), carbonyl group (1712 cm^{-1}), double bond (1632 cm^{-1}), and aromatic ring (1601, 1516, 1431 cm^{-1}), as well as ether bond (1126, 1069, 1030 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of compound **3** (**Tables 2** and **3**) displayed 27 carbon signals due to eight quaternary carbons, thirteen methines, three methylenes, and three methyls, of which two carbonyl carbons, one benzene ring, three double carbons, and one glucosyl group were characterized. Acid hydrolysis of compound **3** with 10% HCl yielded glucose. Detailed analyses of its NMR spectra exhibited the presence of a 3-*epi*-swertiajaposide C [16] fragment, as confirmed by the ^{1}H-^{1}H COSY, HMBC, and ROESY spectra. In addition to the 3-*epi*-swertiajaposide C fragment, the ^{1}H-NMR spectrum of **3** indicated a *trans*-feruloyl moiety, which was evident from three aromatic protons at δ_H 6.82 (1H, d, *J* = 8.2 Hz, H-5″), 7.10 (1H, dd, *J* = 1.4, 8.2 Hz, H-6″), 7.22 (1H, d, *J* = 1.4 Hz, H-2″), an (*E*)-configured C=C bond at δ_H 6.42 (1H, d, *J* = 15.9 Hz, H-8″), 7.68 (1H, d, *J* = 15.9 Hz, H-7″), and a methoxy group at δ_H 3.90 (3H, s, H-10″). In the HMBC spectrum (**Fig. 1**), the correlation of H-4′ (δ_H 4.86) with C-9″ (δ_C 168.7) proposed that the feruloyl moiety was attached to C-4′ of the glucosyl group. Consequently, the structure of compound **3** was elucidated.

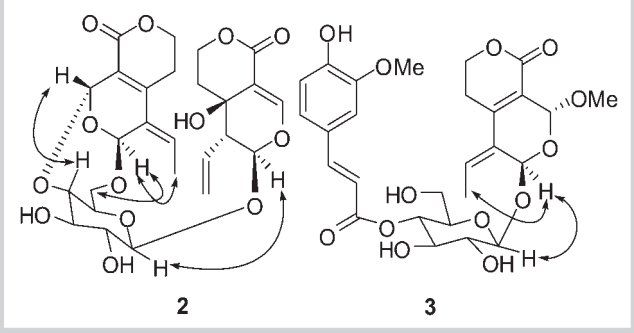

Fig. 3 Selected ROESY (↔) correlations of compounds **2** and **3**.

Swertianoside C (**4**) possessed a molecular formula of $C_{26}H_{30}O_{13}$, as determined by HR-ESI-MS ([M – H]$^-$ *m/z* 549.1610, calcd. 549.1608). The IR spectrum suggested the presence of a hydroxy group (3426 cm^{-1}), carbonyl group (1696 cm^{-1}), aromatic ring (1619, 1516, 1428 cm^{-1}), and an ether bond (1157, 1069, 1029 cm^{-1}). Acid hydrolysis of compound **4** with 10% HCl yielded glucose. The ^{1}H- and ^{13}C- NMR (DEPT) data (**Tables 2** and **3**) aided by 2D analyses indicated a swertiamarin fragment [17]. Besides the swertiamarin fragment, the signals for three aromatic protons at δ_H 6.76 (1H, d, *J* = 8.2 Hz, H-5″), 7.04 (1H, dd, *J* = 1.8, 8.2 Hz, H-6″), 7.15 (1H, d, *J* = 1.8 Hz, H-2″), an (*E*)-configured C=C bond at δ_H 6.38 (1H, d, *J* = 15.9 Hz, H-8″), 7.61 (1H, d, *J* = 15.9 Hz, H-7″), and a methoxy group at δ_H 3.84 (3H, s, H-10″) in the

Table 4 Anti-HBV activities of compounds **2, 4–6**, and **8–17**[a].

Compounds	CC_{50} [mM]	HBsAg[b]		HBeAg[c]		DNA[d]	
		IC_{50} [mM]	SI[e]	IC_{50} [mM]	SI	IC_{50} [mM]	SI
2	0.56 (0.48 ~ 0.64)	0.18 (0.12 ~ 0.24)	3.11	0.12 (0.05 ~ 0.19)	4.67	0.22 (0.17 ~ 0.27)	2.54
4	> 1.85[f]	> 1.85	–	> 1.85	–	> 0.46	–
5	> 1.87	> 1.87	–	> 1.87	–	> 0.47	–
6	> 1.77	> 1.77	–	> 1.77	–	> 0.44	–
8	1.97 (1.85 ~ 2.09)	1.39 (1.24 ~ 1.54)	1.42	2.84 (2.71 ~ 2.97)	< 1	0.86 (0.78 ~ 0.94)	2.29
9	> 1.02	> 1.02	–	> 1.02	–	–	–
10	3.47 (3.34 ~ 3.60)	3.32 (3.18 ~ 3.46)	1.04	2.29 (2.12 ~ 2.46)	1.52	–	–
11	> 0.71	> 0.71	–	> 0.71	–	–	–
12	> 4.58	> 4.58	–	> 4.58	–	> 1.14	–
13	> 6.59	> 6.59	–	> 6.59	–	> 1.65	–
14	> 3.19	> 3.19	–	> 3.19	–	> 0.80	–
15	> 2.49	> 2.49	–	> 2.49	–	> 0.62	–
16	> 1.40	> 1.40	–	> 1.40	–	> 0.35	–
17	> 2.30	> 2.30	–	> 2.30	–	> 0.75	–
Tenofovir[g]	> 1.63	1.31 (1.22 ~ 1.40)	> 1.24	1.15 (1.04 ~ 1.26)	> 1.42	0.00065 (0.00051 ~ 0.00079)	> 2507.7

[a] All values are the mean of two independent experiments; [b] HBsAg: HBV surface antigen; [c] HBeAg: HBV e antigen; [d] DNA: HBV DNA replication; [e] CC_{50} = 50% cytotoxic concentration, IC_{50} = 50% inhibition concentration, SI (selectivity index) = CC_{50}/IC_{50}; [f] CC_{50} or IC_{50} values were not reached at the highest tested concentration; [g] Tenofovir, an antiviral agent used as a positive control

^{1}H-NMR spectrum showed a *trans*-feruloyl moiety. The correlation of H-3′ (δ_H 5.04) with C-9″ (δ_C 168.9) in the HMBC spectrum (**Fig. 1**) proposed that the feruloyl moiety was attached to C-3′ of the glucosyl group.

The molecular formula of swertianoside D (**5**) was established as $C_{26}H_{30}O_{13}$ according to the quasimolecular ion peak at *m/z* 549.1601 [M – H]$^-$ in the HR-ESI-MS. Its IR and NMR data (**Tables 2** and **3**) were close to those of compound **4**, suggesting a similar skeleton. The ^{1}H-^{1}H COSY and HMBC analyses indicated that compound **5** contained the identical fragments of the *trans*-feruloyl and swertiamarin moiety. The HMBC correlations (**Fig. 1**) of H-6′ (δ_H 4.50 and 4.41) with C-9″ (δ_C 167.6) proved that the feruloyl moiety was located at C-6′ of glucosyl group. Based on the above evidence, the structure of compound **5** was determined as shown.

Swertianoside E (**6**) had a molecular formula of $C_{25}H_{28}O_{12}$ by the negative HR-ESI-MS (*m/z* = 519.1494 [M – H]$^-$). The IR and NMR data (**Tables 2** and **3**) were similar to those of **4**, except for the absence of a methoxy group and the presence of an AA′BB′ benzene ring system in the ^{1}H-NMR spectrum, which demonstrated that the *trans*-feruloyl at C-3′ in **4** was replaced by a *trans*-coumaroyl in **6**. The above deduction was confirmed by 2D-NMR analyses.

Swertianoside F (**7**) possessed the same molecular formula of $C_{25}H_{28}O_{12}$ as that of compound **6**. The UV, IR, and NMR spectra (**Tables 2** and **3**) of compound **7** were very similar to those of compound **6**; the only difference was that the *trans*-coumaroyl at C-3′ in **6** was replaced by the *cis*-coumaroyl in **7**, as confirmed by the coupling constant ($J_{H\text{-}7''/H\text{-}8''}$ = 12.8 Hz). Thus, the structure of compound **7** was established as shown.

The other fifteen known compounds were determined as erythrocentaurin (**8**) [18], gentiolactone (**9**) [19], gentiogenal (**10**) [20], (*R*)-gentianol (**11**) [21], 4-carboxy-boonein (**12**) [22], angelone (**13**) [15], *epi*-eustomoside (**14**) [23], amarogentin (**15**) [24], angustioside (**16**) [23], angustiamarin (**17**) [23], 6′-O-[(*Z*)-coumaroyl]swertiamarin (**18**) [25], 6′-O-[(*E*)-coumaroyl]swertiamarin (**19**) [25], 4′-O-[(*E*)-coumaroyl]swertiamarin (**20**) [26], sweroside (**21**) [27], and swertiamarin (**22**) [17], by comparison of their spectroscopic data with those reported.

Some compounds (**2, 4–6**, and **8–17**) were tested for 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 in Hep G 2.2.15 cells, as reported previously (Tenofovir was used as the positive control) [6]. The results of their activities and cytotoxicities are listed in **Table 4**. The most active compound **2**, an unusual secoiridoid glycoside dimer with two molecules of secoiridoids connected by the glucosyl group, possessed significant activities inhibiting the secretion of HBsAg (IC_{50} 0.18 mM, SI 3.11) and HBeAg (IC_{50} 0.12 mM, SI 4.67). However, other secoiridoid glycosides displayed no activity or cytotoxicity at the tested (highest) concentration. This result proposed that the introduction of an additional secoiridoid aglycone forming an eight-member ring system could significantly enhance their anti-HBV activity. Of the secoiridoid aglycones, only compounds **8** and **10** with a formyl group showed activities against the secretion of HBsAg (IC_{50} 1.39 and 3.32 mM) and HBeAg (IC_{50} 2.84 and 2.29 mM), respectively, which indicated that the formyl group played an important role in their anti-HBV activity. However, none of the assayed compounds showed equivalent activity compared to Tenofovir against HBV DNA replication. This may be due to little structural similarity between the isolates and nucleoside analogues, which led to different mechanisms of action.

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Conflict of Interest

There are no conflicts of interest among all authors of this manuscript.

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