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Swerilactones L−O, Secoiridoids with C_{12} and C_{13} Skeletons from *Swertia mileensis*

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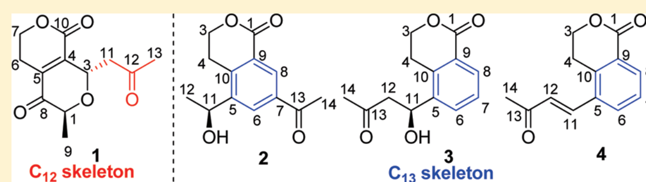
S Supporting Information

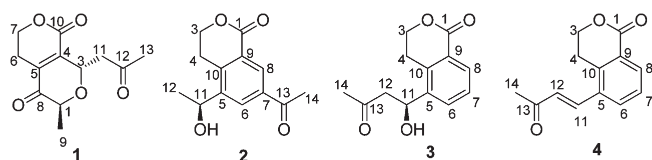
ABSTRACT: Swerilactones L−O (**1**−**4**), four unusual secoiridoids with unprecedented C_{12} and C_{13} skeletons, were isolated from the traditional Chinese herb *Swertia mileensis*. Compounds **1** and **2** had moderate inhibitory activities against the secretion of hepatitis B virus surface antigen (IC_{50} = 1.47 and 1.20 mM, with SI < 1 and 1.53, respectively) and hepatitis B virus e antigen (IC_{50} = 0.88 and >2.69 mM, with SI 1.62 and <1, respectively) in an antihepatitis B virus assay on the Hep G 2.2.15 cell line in vitro.



Iridoids are a group of monoterpenes with a methylcyclopentane skeleton, mainly distributed in plants of the families Scrophulariaceae, Rubiaceae, Labiatae, Gentianaceae, Verbenaceae, and Oleaceae.[1] Most of the iridoids in the Gentianaceae are secoiridoid glycosides and are biogenetically derived from the cleavage of the cyclopentane ring.[2] To date, more than 1000 iridoids have been isolated as natural products[3] and can be classified into three types, according to the number of carbons in the aglycone parts: C_8, C_9, and C_{10} skeletons.[4] From a biosynthetic point of view, iridoids with C_8 and C_9 frameworks are derived by removing carbons from the C_{10} architecture, and skeletons other than C_8, C_9, and C_{10} are rarely reported. Therefore, iridoids with different architectures, especially skeletons of more than 10 carbons, are interesting from phytochemical and biosynthetic perspectives.

Swertia mileensis (Gentianaceae) is famous in China under the name "Qing-Ye-Dan" and has been documented in the *Chinese Pharmacopoeia* (1977−2010 editions) for the treatment of hepatitis.[5] In our ongoing search for active antihepatitis B virus (HBV) compounds from natural sources,[6] our previous study of *S. mileensis* produced a series of new active anti-HBV lactones: swerilactones A−K. Of these, swerilactones A, B,[7a] C, D,[7b] and H−K[7c] are considered secoiridoid dimers and trimers from a biosynthetic point of view; swerilactones E−G may also be regarded as secoiridoid dimers, whose biosynthetic pathway is unclear;[7d] and swerilactosides A−C are three unusual secoiridoid glycoside dimers.[7e] This promising outcome prompted us to continue our investigation, which produced four new secoiridoid monomers, swerilactones L−O (**1**−**4**), with unprecedented C_{12} and C_{13} skeletons, from the active part (50% and 90% aqueous ethanol extracts) of *S. mileensis*.

Swerilactones L−O (**1**−**4**) are the first examples of secoiridoids with C_{12} and C_{13} skeletons. Structurally, swerilactone L (**1**) has an additional C_3 side chain attached to C-3, and swerilactones M−O (**2**−**4**) contain an unusual phenyl framework rarely observed in secoiridoids. Based on an anti-HBV assay on the Hep G 2.2.15 cell line in vitro, compounds **1** and **2** have moderate activities that inhibit the secretion of hepatitis B virus surface antigen (HBsAg) (IC_{50} = 1.47 and 1.20 mM, respectively) and hepatitis B virus e antigen (HBeAg) (IC_{50} = 0.88 and >2.69 mM, respectively). Therefore, the isolation, structural determination, and anti-HBV properties of swerilactones L−O (**1**−**4**) not only enrich the skeletal types of the secoiridoids, but also provide information for the comprehensive identification of the active constituents of *S. mileensis*.



Swerilactone L (**1**) was isolated as a white powder and had a molecular formula of $C_{12}H_{14}O_5$ on the basis of positive ESIMS (*m/z* 261 $[M + Na]^+$) and (+) HR-ESIMS (*m/z* 261.0751 $[M + Na]^+$, calcd for $C_{12}H_{14}O_5Na$, 261.0738), indicating six degrees of unsaturation. The IR spectrum displayed absorptions of C=O (1716 cm^{-1}) and C=C (1655 cm^{-1}) groups. The ^{13}C NMR (DEPT) spectrum exhibited 12 carbon resonances attributed to five quaternary carbons (including three carbonyls and two olefinic carbons), two oxygenated methines, three methylenes (one oxygenated), and two methyls.

In the ^1H and ^{13}C NMR spectra, the carbons observed at δ_C 163.2 (s, C-10), 140.4 (s, C-4), 138.7 (s, C-5), 66.6 (t, C-7), and 20.3 (t, C-6), in combination with proton resonances at δ_H 4.50

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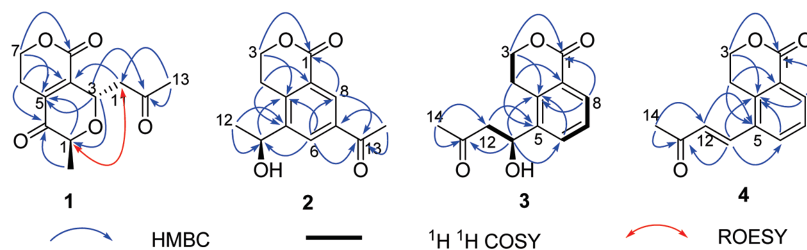

Figure 1. Selected 2D NMR correlations for compounds **1**−**4**.

(2H, dd, J = 7.3, 5.1 Hz, H-7) and 2.63 (2H, m, H-6), indicated an α,β-unsaturated δ-lactone fragment,[7d] which was further confirmed by ^{1}H−^{1}H COSY (H-6/H-7) and HMBC (H-7/C-10, C-5 and H-6/C-4) experiments. One 2-oxygenated propionyl group (C_9−C_1−C_8(O)−) attached to C-5 was deduced on the basis of ^{1}H−^{1}H COSY (H-9/H-1) and HMBC (H-6, H-9/C-8 and H-1/C-5) analyses, together with the downfield shifts of δ_C 72.5 (d, C-1) and δ_H 4.38 (1H, q, J = 6.9 Hz, H-1) in the ^{1}H and ^{13}C NMR spectra. Similarly, the connectivity of C_{13}−C_{12}(O)−C_{11}−C_3−C_4 was determined from the cross-peak of H-3/H-11 in the ^{1}H−^{1}H COSY spectrum and correlated signals in the HMBC spectrum, i.e., from H-13 to C-12, C-11; H-3 to C-12, C-5; and H-11 to C-4. With the HMBC correlations of H-1/C-3 and H-3/C-1, and the structural features deduced above, the ether bond between C-1 and C-3 was determined, completing the structure of swerilactone L (**1**).

In the ROESY spectrum, the correlation between H-1 and H-11 suggested that H-1 and the (C_{11}−C_{12}(O)−C_{13}) fragment were cofacial. Therefore, the relative configuration of swerilactone L (**1**) was determined to be 1S* and 3S*, as shown in Figure 1.

Swerilactone M (**2**) has a molecular formula of $C_{13}H_{14}O_4$, deduced by positive HR-ESIMS (m/z 257.0787 [M + Na]$^+$; calcd for $C_{13}H_{14}O_4Na$, 257.0789), indicating seven degrees of unsaturation. The IR spectrum showed the presence of OH (3497 cm^{-1}), C═O (1716, 1680 cm^{-1}), and a phenyl group (1605, 1581, 1475 cm^{-1}). The ^{13}C NMR (DEPT) spectrum displayed 13 carbons, ascribed as six quaternary carbons, three methines, two methylenes, and two methyls.

In the ^{1}H and ^{13}C NMR spectra, the resonances for one ester carbonyl (δ_C 164.6, s, C-1), six aromatic carbons (between δ_C 143.2 and 126.1), and two methylenes at δ_C 66.4 (t, C-3) and 24.8 (t, C-4), as well as protons at δ_H 8.58 (1H, d, J = 0.7 Hz, H-8), 8.34 (1H, bs, H-6), 4.55 (2H, m, H-3), and 3.20 (2H, m, H-4), indicated a disubstituted 1-isochromanone moiety,[7d] which was confirmed by HMBC correlations from H-3 to C-1 and C-10; H-4 to C-9 and C-5; H-8 to C-1, C-6, and C-10; and H-6 to C-10 and C-8. An additional acetyl group [δ_C 196.9 (s, C-13), 26.7 (q, C-14); δ_H 2.65 (3H, s, H-14)] was proposed at C-7 on the basis of the HMBC correlation of H-14 with C-13 and C-7, and H-6 and H-8 with C-13. Accordingly, the residual 1-hydroxyethyl group [(δ_H 1.55 (3H, d, J = 6.4 Hz, H-12), 5.14 (1H, q, J = 6.4 Hz, H-11)] was located at C-5 by the HMBC correlations from H-12 to C-11, C-5; from H-11 to C-10, C-6; and from H-6 to C-11.

Swerilactone M (**2**) has one stereogenic center (C-11); therefore, a different configuration of C-11 will result in a reverse optical rotation. On the basis of the optical rotation measurements in three different solvents (MeOH, acetone, and $CHCl_3$), compound **2** exhibited a negative optical rotation consistent with those of 1S-phenylethanol analogues. Thus, the absolute configuration of C-11 was determined as S (Supporting Information, S47).

Swerilactone N (**3**) had a molecular formula of $C_{13}H_{14}O_4$ on the basis of positive HR-ESIMS (m/z 257.0785, [M + Na]$^+$; calcd for $C_{13}H_{14}O_4Na$, 257.0789), suggesting seven degrees of unsaturation. The presence of hydroxy (3433 cm^{-1}), carbonyl (1712 cm^{-1}), and phenyl (1599 and 1475 cm^{-1}) groups was deduced from the IR spectrum. The ^{13}C NMR (DEPT) spectrum showed 13 carbon resonances, assigned to five quaternary carbons, four methines, three methylenes, and one methyl group.

Similar to compound **2**, a 1-isochromanone fragment was characterized from protons at δ_H 7.95 (1H, dd, J = 7.7, 1.0 Hz, H-8), 7.43 (1H, t, J = 7.7 Hz, H-7), 7.78 (1H, dd, J = 7.7, 1.0 Hz, H-6), 4.51 (2H, m, H-3), and 3.15 (2H, m, H-4), as well as nine carbons attributed to one ester carbonyl (167.6, s, C-1), six aromatic carbons (δ_C 142.8−126.5), and two methylenes [δ_C 68.3 (t, C-3), 25.2 (t, C-4)], which was also supported by ^{1}H−^{1}H COSY correlations of H-3/H-4 and H-6/H-7/H-8, and HMBC from H-3 to C-1, C-10; from H-4 to C-9, C-5; from H-6 to C-10; and from H-8 to C-1, C-10 analyses.

Besides the 1-isochromanone part, a $C_4H_7O_2$ fragment was required to fulfill the requirements of the molecular formula and was deduced as a 1-hydroxy-3-butanoyl group located at C-5 on the basis of the HMBC correlation of H-14 to C-13, C-12; H-12 to C-5; and H-11 to C-13, C-10, C-6, in combination with an ^{1}H−^{1}H COSY correlation of H-11 to H-12. Similarly, the absolute configuration of compound **3** was proposed as 11S by comparing its optical rotation in three solvents (MeOH, acetone, and $CHCl_3$) with those of (S)-4-hydroxy-4-phenyl-2-butanone analogues (Supporting Information, S48).

Swerilactone O (**4**) had a molecular formula of $C_{13}H_{12}O_3$ deduced from positive HR-ESIMS (m/z 217.0865, [M + H]$^+$; calcd for $C_{13}H_{13}O_3$, 217.0864), with eight degrees of unsaturation. The IR spectrum showed absorptions for carbonyl (1710, 1670 cm^{-1}), double bond (1622 cm^{-1}), and phenyl ring (1579, 1472, 1448 cm^{-1}) functionalities. The ^{13}C NMR (DEPT) spectrum showed 13 carbon resonances, including five quaternary carbons, five methines, two methylenes, and one methyl group.

The ^{1}H and ^{13}C NMR data were similar to those for swerilactone N (**3**), except for the downfield shifts of C-11 and C-12 (see Table 1). In conjunction with an 18 lower mass, it permitted swerilactone O (**4**) to be identified as a C-11(12)-dehydrated derivative of swerilactone N (**3**). This deduction was confirmed by HMBC correlations from H-14 to C-12, H-12 to C-5, and H-11 to C-6, C-10, and C-13.

The C-11−C-12 double-bond configuration was identified as E on the basis of the $J_{11,12}$ coupling constant of 16.0 Hz.

Table 1. **^{1}H and ^{13}C NMR Data for Swerilactones L−O (1−4), δ in ppm, *J* in Hz**

	1[a]		2[a]		3[b]		4[a]	
no.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4.38, q, 6.9	72.5, d		164.6, s		167.6, s		164.5, s
3	5.15, t, 6.1	66.4, d	4.55, m	66.4, t	4.51, m	68.3, t	4.55, m	66.4, t
4		140.4, s	3.20, m	24.8, t	3.15, m	25.2, t	3.18, m	24.9, t
5		138.7, s		143.2, s		142.8, s		132.4, s
6	2.63, m	20.3, t	8.34, bs	129.4, d	7.78, dd, 7.7, 1.0	132.6, d	7.82, d, 7.8	131.5, d
7	4.50, dd, 7.3, 5.1	66.6, t		136.4, s	7.43, t, 7.7	128.4, d	7.46, t, 7.8	127.7, d
8		195.6, s	8.58, d, 0.7	129.9, d	7.95, dd, 7.7, 1.0	130.3, d	8.18, d, 7.8	132.3, d
9	1.38, d, 6.9	15.2, q		126.1, s		126.5, s		126.3, s
10		163.2, s		141.6, s		138.6, s		138.8, s
11	3.06, d, 6.1	45.0, t	5.14, q, 6.4	66.8, d	5.37, dd, 8.8, 4.2	66.9, d	7.72, d, 16.0	137.7, d
12		205.2, s	1.55, d, 6.4	24.0, q	a: 2.93, dd, 16.6, 8.8	51.9, t	6.71, d, 16.0	129.8, d
					b: 2.79, dd, 16.6, 4.2			
13	2.23, s	30.4, q		196.9, s		209.2, s		197.3, s
14			2.65, s	26.7, q	2.18, s	30.7, q	2.40, s	28.4, q

[a] Data were recorded in $CDCl_3$. [b] Data were recorded in methanol-d_4.

Table 2. **Anti-HBV Activities of Swerilactones L−O (1−4) (mM)**

		HBsAg		HBeAg	
no.	CC_{50}	IC_{50}	SI	IC_{50}	SI
1	1.43	1.47	<1	0.88	1.62
2	1.84	1.20	1.53	>2.69	<1
3	>0.51	>0.51	−[a]	>0.51	−
4	>1.44	>1.44	−	>1.44	−
3TC	33.25	25.94	1.28	30.26	1.10

[a] IC_{50} and CC_{50} values were not reached at the (highest) tested concentrations.

To evaluate their anti-HBV activities, swerilactones L−O (**1**−**4**) were assayed in the Hep G 2.2.15 cell line in vitro, and 3TC (lamivudine, a frequently used clinical anti-HBV agent) was used as the positive control.[6a] Swerilactone L (**1**) exhibited moderate inhibitory activity against the secretion of HBsAg (IC_{50} = 1.47 mM) and HBeAg (IC_{50} = 0.88 mM). Swerilactone M (**2**) also showed inhibition of HBsAg secretion, with an IC_{50} value of 1.20 mM. However, compounds **1** and **2** had low selectivity indices of <2.00 because of their cytotoxicity (CC_{50} 1.43 and 1.84 mM, respectively). Compounds **3** and **4** showed neither anti-HBV activity nor cytotoxicity at the highest tested concentrations of 0.51 and 1.44 mM (Table 2).

EXPERIMENTAL SECTION

General Experimental Procedures. 1D and 2D NMR spectra were recorded on a Bruker AM-400 NMR or DRX-500 spectrometer with TMS as the internal standard (Bruker, Bremerhaven, Germany). MS data were collected on a VG Auto Spec-3000 spectrometer (VG, Manchester, UK). IR (KBr) spectra were recorded on a Bio-Rad FTS-135 spectrometer (Bio-Rad, Hercules, CA, USA). UV data were collected on a Shimadzu UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were collected on a Jasco model 1020 polarimeter (Horiba, Tokyo, Japan). Silica gel (200−300 mesh and H) for column chromatography was obtained from Qingdao Meigao Chemical Company (Makall, Qingdao, China). Sephadex LH-20 (20−150 μm) was purchased from Pharmacia Fine Chemicals Co. Ltd. (Pharmacia, Uppsala, Sweden).

Plant Material. Whole plants of *S. mileensis* were collected in Mile County, Yunnan Province, China, on November 6, 2008, and were identified by Prof. Li-Gong Lei (Kunming Institute of Botany, CAS). A voucher specimen (no. 2008-11-01) was deposited in the Laboratory of Antivirus and Natural Medicine Chemistry, Kunming Institute of Botany, CAS.

Extraction and Isolation. Fraction A4[7b] (10.2 g) was partitioned with silica gel column chromatography (150.0 g, 4.0 × 25.0 cm) with $CHCl_3/Me_2CO$ gradient elution (90:10 → 30:70, v/v) to afford five subfractions, A4-1 to A4-5. Fraction A4-1 (800 mg) was further purified on a silica gel column (25.0 g, 1.5 × 30.0 cm) and eluted with petroleum ether/EtOAc (80:20 → 50:50, v/v) to give three fractions, A4-1-1 to A4-1-3. Fraction A4-1-1 (50 mg) was purified with Sephadex LH-20 (50.0 g, 1.4 × 145.0 cm, $CHCl_3$/MeOH, 1:1, v/v) to afford swerilactone L (**1**, 25 mg). Fraction A4-1-2 (100 mg) was loaded onto a silica gel column (30 g, 1.7 × 25.0 cm) and eluted with $CHCl_3/Me_2CO$ (90:10, v/v) and onto Sephadex LH-20 (50.0 g, 1.4 × 145.0 cm, $CHCl_3$/MeOH, 1:1, v/v) to yield swerilactone O (**4**, 10 mg). Fraction A4-1-3 (150 mg) was purified with silica gel column chromatography (30 g, 1.7 × 25.0 cm, petroleum ether/Me_2CO, 65:35, v/v) to yield swerilactones M (**2**, 20 mg) and N (**3**, 9 mg).

*Swerilactone L (**1**):* colorless powder; $[\alpha]_D^{24.0}$ −4.1 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 193 (3.74), 196 (3.75), 227 (3.87) nm; IR (KBr) ν_{max} 1716, 1655, 1414, 1377, 1265, 1209, 1138, 1099, 772 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$, 400/100 MHz), see Table 1; (+) ESIMS *m/z* 261 $[M + Na]^+$; (+) HRESIMS *m/z* 261.0751 $[M + Na]^+$ (calcd for $C_{12}H_{14}O_5Na$, 261.0738).

*Swerilactone M (**2**):* colorless gum; $[\alpha]_D^{27.0}$ −4.6 (*c* 0.3, MeOH); $[\alpha]_D^{24.5}$ −12.0 (*c* 0.1, acetone); $[\alpha]_D^{24.4}$ −10.7 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 196 (4.17), 225 (4.41), 293 (3.29) nm; IR (KBr) ν_{max} 3497, 1716, 1680, 1605, 1581, 1475, 1427, 1357, 1274, 1223, 780 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$, 500/100 MHz), see Table 1; EIMS *m/z* 234 ($[M]^+$, 5), 219 (100), 216 (81), 201 (60), 188 (28), 173 (35), 145 (10), 115 (18); (+) HRESIMS *m/z* 257.0787 $[M + Na]^+$ (calcd for $C_{13}H_{14}O_4Na$, 257.0789).

*Swerilactone N (**3**):* colorless gum; $[\alpha]_D^{26.0}$ −1.2 (*c* 0.2, MeOH); $[\alpha]_D^{24.5}$ −8.9 (*c* 0.1, acetone); $[\alpha]_D^{24.7}$ −20.1 (*c* 0.1, $CHCl_3$); UV

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(MeOH) λ_{max} (log ε): 238 (3.94), 284 (3.37) nm; IR (KBr) ν_{max} 3433, 1712, 1599, 1475, 1399, 1363, 1299, 1124, 759 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD, 500/100 MHz), see Table 1; EIMS m/z 234 (M^+, 16), 216 (100), 177 (85), 174 (97), 159 (25), 156 (32), 148 (45), 129 (43), 105 (47), 91 (45); (+) HRESIMS m/z 257.0785 $[M + Na]^+$ (calcd for $C_{13}H_{14}O_4Na$, 257.0789).

*Swerilactone O (**4**):* colorless gum; UV (MeOH) λ_{max} (log ε) 193 (4.12), 196 (4.12), 233 (4.21), 279 (4.10) nm; IR (KBr) ν_{max} 1710, 1670, 1622, 1579, 1472, 1448, 1401, 1361, 1326, 1292, 1118, 1038, 980, 760 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$, 500/125 MHz), see Table 1; EIMS m/z 216 (M^+, 46), 201 (75), 186 (31), 171 (72), 157 (27), 145 (36), 128 (54), 115 (100), 89 (12); (+) HRESIMS m/z 217.0865 $[M + H]^+$ (calcd for $C_{13}H_{13}O_3$, 217.0864).

***In Vitro* Anti-HBV Assay.** The anti-HBV procedure was performed according to our previous report.[6a] The anti-HBV activities and cytotoxicity of compounds **1**−**4** were evaluated on the Hep G 2.2.15 cell line, which was stably transfected with the HBV genome using Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA, USA). The anti-HBV antigen secretion activities were assayed by the enzyme-linked immunosorbent assay (ELISA; Autobio Diagnostics Co., Ltd., China). Cytotoxicity was assayed with a modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Gibco Invitrogen, Carlsbad, CA, USA). All the compounds evaluated were dissolved in DMSO (Gibco; solvent control) for the anti-HBV activity and cytotoxicity assays. The concentration of DMSO in the culture was kept below 2.5 μL/mL to ensure that the growth of the cells was not affected.

Assay for HBV Antigen Secretion. Hep G 2.2.15 cells were seeded in a 48-well microplate at a density of 1×10^4 cells/well and cultured for 72 h at 37 °C under 5% CO_2. The culture medium with (or without) the tested compounds was refreshed, and the cells were cultured for an additional 72 h. The culture media were collected and tested for HBsAg and HBeAg levels using ELISAs (Autobio Diagnostics Co., Ltd.). The absorbance (A) of each well was measured at 490 nm with a microplate reader (model 680; Bio-Rad, Inc., USA). The inhibition ratio (η) and IC_{50} value were calculated as follows (lg^{-1} is the inverse function of lg, and lg is log_{10}):

$$\eta = (A_{contrasted} - A_{tested})/(A_{contrasted} - A_{blank}) \times 100$$

$$IC_{50} = lg^{-1}[(50 - \eta_{<50\%})/(\eta_{>50\%} - \eta_{<50\%}) \times lg(C_{>50\%}/C_{<50\%}) + lg(C_{<50\%})]$$

Cytotoxicity Assay. After the culture medium used for the HBV antigen secretion assay was removed, the plates were air-dried and MTT (400 μg/mL, 200 μL/well) was added. Four hours later, the MTT was removed and replaced with DMSO (300 μL/well), and the samples were incubated for 10 min. The supernatants (100 μL) were transferred to 96-well plates, and the absorbance (A) was measured at 490 nm with an automatic plate reader (model 680; Bio-Rad Inc.). The damage ratio (η) and CC_{50} value were calculated as follows:

$$\eta = (A_{contrasted} - A_{tested})/(A_{contrasted} - A_{blank}) \times 100$$

$$CC_{50} = lg^{-1}[(50 - \eta_{<50\%})/(\eta_{>50\%} - \eta_{<50\%}) \times lg(C_{>50\%}/C_{<50\%}) + lg(C_{<50\%})]$$

Cell Line and Cell Culture. Hep G 2.2.15 cells are used widely to assay anti-HBV activities and are derived from the Hep G 2 hepatoma cell line (American Type Culture Collection, Manassas, VA, USA). In this study, Hep G 2.2.15 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 μg/mL G148 (Gibco), 100 IU/mL penicillin (Gibco), and 100 IU/mL streptomycin (Gibco) and maintained at 37 °C in a moist atmosphere containing 5% CO_2.

ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR, HSQC, HMBC, $^{1}H-^{1}H$ COSY, and MS spectra for compounds **1**−**4** are supplied in the supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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