7-*O*-Methylkaempferol and -quercetin Glycosides from the Whole Plant of *Nervilia fordii*

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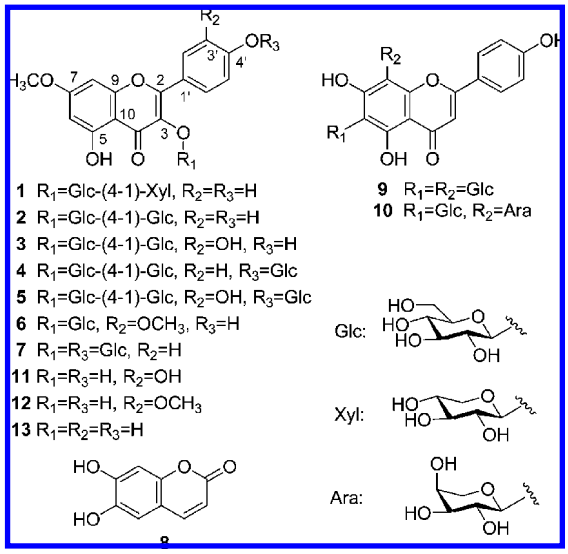
Five new 7-*O*-methylkaempferol and -quercetin glycosides, namely, nervilifordins A−E (**1**−**5**), were isolated from the whole plant of *Nervilia fordii*, together with seven known flavonoids (**6**, **7**, and **9**−**13**) and one known coumarin (**8**). Their structures were elucidated on the basis of extensive spectroscopic analyses, including HSQC, HMBC, ROESY, and chemical methods. Compounds **1**−**3** and **6**−**13** were evaluated for their anti-herpes simplex virus 1 (HSV-1) activity and cytotoxicity on African green monkey kidney cells (Vero cells) in vitro. Of the tested compounds, only esculetin (**8**) exhibited antiviral activity against HSV-1, while the aglycones (**11**−**13**) showed stronger cytotoxicity on Vero cells than their glycosides (**1**−**3**, **6**, and **7**).

Nervilia fordii (Hance) Schltr. is an orchidaceous plant endemic to the south of China. It has been used for the treatment of cough and throat swelling in Chinese folk medicine.[1] The decoction of the plant was reported to have antiviral activity against influenza in vitro.[2] Several triterpenes, sterols, and flavonoids were reported from the petroleum ether and EtOAc extracts of this plant.[3−5] Our preliminary experiment showed that the MeOH extract of *N. fordii* exhibited antiviral activity on herpes simplex virus type 1 (HSV-1). Thus, we have carried out a chemical investigation of the MeOH extract, leading to the isolation of five new 7-*O*-methylkaempferol and -quercetin glycosides (**1**−**5**), together with eight known compounds (**6**−**13**). Their structures were determined by extensive spectroscopic analyses, including HSQC, HMBC, ROESY, and chemical methods. In addition, the in vitro anti-HSV-1 activity of the isolated compounds is also described.



Results and Discussion

The MeOH extract of the whole plant of *N. fordii* was defatted with petroleum ether and then subjected to column chromatography (CC) over Diaion HP-20SS, MCI-gel CHP-20P, Sephadex LH-20, and silica gel to afford five new 7-*O*-methylkaempferol and -quercetin glycosides (**1**−**5**) and eight known compounds (**6**−**13**). The known compounds were identified as rhamnazin-3-*O*-*β*-D-glucopyranoside (**6**),[6] complanatuside (**7**),[7] esculetin (**8**),[8] vicenin-2 (**9**),[9] schaftoside (**10**),[10] rhamnetin (**11**),[11] rhamnazin (**12**),[12] and rhamnocitrin (**13**)[13] on the basis of detailed spectroscopic analyses. The known compounds **6**−**11** were reported from *N. fordii* extract for the first time.

Compound **1**, a yellow amorphous powder, had a molecular formula of $C_{27}H_{30}O_{15}$ on the basis of its negative ion mode HRESIMS ([M − H]$^-$, *m*/*z* 593.1505) and the ^{13}C NMR data (Tables 1 and 2). The ^{1}H and ^{13}C NMR spectra were closely related to those of rhamnocitrin (**13**),[13] except for the appearance of two sets of signals arising from one hexosyl and one pentosyl unit. Acid hydrolysis of **1** produced D-xylose and D-glucose as sugar residues, which were determined by GC analysis of their corresponding trimethylsilylated L-cysteine adducts. The coupling constants of the anomeric protons [*δ* 4.23 (d, *J* = 7.6 Hz) and 5.50 (1H, d, *J* = 7.5 Hz)] indicated that both the D-xylose and D-glucose moieties had *β*-pyranosyl configurations. Connectivities of the methoxy and sugar moieties were confirmed by 2D NMR experiments. In the NOESY spectra of **1**, correlations of the methoxy protons at *δ* 3.84 with H-6 (*δ* 6.31) and H-8 (*δ* 6.67) indicated its substitution at C-7. The HMBC correlations of H-1″ (*δ* 4.23) with C-3 (*δ* 133.4) and of H-1‴ with C-4″ (*δ* 78.7) revealed that the xylosyl unit was attached at C-4″ of the glucosyl unit, which was linked to C-3 of the aglycone. On the basis of the above evidence, compound **1** was determined to be rhamnocitrin-3-*O*-*β*-D-xylopyranosyl-(1−4)-*β*-D-glucopyranoside and named nervilifordin A.

Compound **2** was obtained as a yellow amorphous powder possessing a molecular formula of $C_{28}H_{32}O_{16}$ deduced from HRESIMS ([M − H]$^-$, *m*/*z* 623.1621) and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, except that instead of a xylosyl unit in **1**, a set of signals due to a glucosyl moiety appeared in **2**. Acid hydrolysis of **2** produced only D-glucose as sugar residue. The *J* values of the anomeric protons [*δ* 5.49 (d, *J* = 8.3 Hz, H-1″) and 4.24 (d, *J* = 8.0 Hz, H-1‴)] of the two glucose moieties assigned their *β*-configured anomeric configurations. The location of the C-7 methoxy group in **2** was determined by the NOESY correlations of *δ* 3.84 with H-6 (*δ* 6.36) and H-8 (*δ* 6.72). The HMBC correlations of H-1″ (*δ* 5.49) of the inner glucose with C-3 (*δ* 133.4), and H-1‴ (*δ* 4.24) of the terminal glucose with C-4″ (*δ* 80.1), confirmed the sugar location and connectivity in **2**. Therefore, the structure of nervilifordin B (**2**) was characterized as rhamnocitrin-3-*O*-*β*-D-glucopyranosyl-(1−4)-*β*-D-glucopyranoside.

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Table 1. ^{1}H NMR Data of Compounds **1**−**5** in DMSO-d_6 (δ values; J in Hz, in parentheses)

position	1	2	3	4	5
6	6.31 d (2.1)	6.36 s	6.31 s	6.38 s	6.37 s
8	6.67 d (2.1)	6.72 s	6.67 s	6.78 s	6.76 s
2′	8.05 d (8.6)	8.05 d (8.7)	7.60 s	8.13 d (8.7)	7.64 s
3′	6.88 d (8.6)	6.88 d (8.7)		7.15 d (8.7)	
5′	6.88 d (8.6)	6.88 d (8.7)	6.84 d (8.4)	6.75 d (8.7)	7.19 d (8.4)
6′	8.05 d (8.6)	8.05 d (8.7)	7.58 d (8.4)	8.13 d (8.7)	7.61 d (8.8)
7-OMe	3.84 s	3.84 s	3.84 s	3.84 s	3.84 s
1″	5.50 d (7.5)	5.49 d (8.3)	5.50 d (7.6)	5.50 d (6.3)	5.50 d (7.3)
2″	3.11 m	3.24 m	3.33[a]	3.26 m	3.27 m
3″	3.31[a]	3.36[a]	3.38[a]	3.40[a]	3.38[a]
4″	3.32[a]	3.35[a]	3.38[a]	3.37[a]	3.36[a]
5″	3.19 m	3.24 m	3.26 m	3.20 m	3.26 m
6″	3.51 m	3.58 m	3.62 d (11)	3.69 m	3.63 m
	3.43	3.47[a]	3.47[a]	3.41[a]	3.48[a]
1‴	4.23 d (7.7)	4.24 d (8.0)	4.24 d (7.8)	4.25 d (7.8)	4.25 d (6.4)
2‴	2.90 m	2.95 m	2.98 t (8.5)	2.87 m	2.97 m
3‴	3.00 m	3.16 t (7.0)	3.13 t (8.0)	3.30[a]	3.14 m
4‴	3.21 m	3.02 t (9.0)	3.04 t (9.0)	3.04 m	3.03 t (9.0)
5‴	3.67 dd (5.5, 11)	3.12 t (7.0)	3.19 t (8.0)	3.18 m	3.18 m
	3.07 m				
6‴		3.68 d (11)	3.69 d (10)	3.61 m	3.70 m
		3.39[a]	3.40[a]	3.41[a]	3.40[a]
4′-O-glc-1⁗				5.02 d (7.5)	4.86 d (6.4)
2⁗				3.27 m	3.12 m
3⁗				3.30[a]	3.31[a]
4⁗				3.19 m	3.18 m
5⁗				3.40[a]	3.38[a]
6⁗				3.68 m	3.69 m
				3.48[a]	3.42[a]

[a] Overlapped with the signals of H_2O for each column.

Table 2. ^{13}C NMR Data (δ values) of Compounds **1**−**5** in DMSO-d_6

position	1	2	3	4	5
2	156.7	156.8	156.7	156.5	156.0
3	133.4	133.4	133.5	134.0	134.1
4	177.5	177.6	177.6	177.7	177.6
5	160.9	161.0	161.0	161.0	161.0
6	97.9	98.0	98.0	98.1	98.0
7	165.2	165.2	165.2	165.3	165.3
8	92.3	92.4	92.2	92.5	92.3
9	156.3	156.4	156.3	156.1	156.4
10	105.0	105.0	105.0	105.2	105.1
1′	120.7	120.8	121.0	123.6	124.4
2′	131.0	131.1	116.4	130.7	116.7
3′	115.2	115.3	145.0	115.9	146.6
4′	160.1	160.2	148.8	159.4	147.8
5′	115.2	115.3	115.3	115.9	115.5
6′	131.0	131.1	121.7	130.7	120.8
7-OMe	56.1	56.2	56.2	56.3	56.2
1″	100.6	100.6	100.5	100.6	100.5
2″	74.1	74.0	73.9	74.0	73.9
3″	74.3	74.8	74.9	74.8	74.9
4″	78.7	80.1	80.4	80.1	80.3
5″	75.5	75.4	75.5	75.5	75.5
6″	59.8	60.2	60.3	60.2	60.3
1‴	103.5	103.2	103.3	103.2	103.2
2‴	73.3	73.3	73.3	73.3	73.3
3‴	76.5	76.5	76.5	76.6	76.5
4‴	69.4	70.1	70.1	70.1	70.1
5‴	65.8	76.9	76.9	76.9	76.8
6‴		61.1	61.1	61.1	61.1
4′-O-glc-1⁗				99.9	101.5
2⁗				73.3	73.3
3⁗				76.5	75.9
4⁗				69.7	69.8
5⁗				77.1	77.3
6⁗				60.7	60.8

Compound **3** had a molecular formula of $C_{28}H_{32}O_{17}$, determined by HRESIMS ([M − H]$^-$, m/z 639.1568), which was 16 mass units larger than that of **2**. This difference corresponded to the mass of an additional oxygen atom. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **11**,[11] except for the appearance of two sets of signals assignable to two glucosyl units. The sugar moieties were both determined to be D-glucose by acidic hydrolysis, and the anomeric configurations were assigned as β on the basis of the J values of their anomeric protons [δ 5.50 (d, J = 7.6 Hz, H-1″) and 4.24 (d, J = 7.8 Hz, H-1‴)]. The NOESY and HMBC experiments further confirmed that the connectivities of the methoxy and sugar moieties in **3** were the same as those of **2**. Thus, the structure of nervilifordin C (**3**) was elucidated as rhamnetin-3-*O*-β-D-glucopyranosyl-(1−4)-β-D-glucopyranoside.

Compound **4** was obtained as a yellow amorphous powder. The HRESIMS exhibited a quasi-molecular ion peak at m/z 821.1911 [M + Cl]$^-$, corresponding to a molecular formula of $C_{34}H_{42}O_{21}Cl$. The FABMS of **4** showed a quasi-molecular ion peak at 786 [M]$^-$, which was 162 mass units larger than that of **2**, corresponding to the mass of an additional hexosyl moiety. The ^{1}H and ^{13}C NMR spectra of **4** resembled those of **2**, but exhibited signals due to an additional hexosyl moiety. The sugar moieties were determined to be β-glucopyranosyl units based on their NMR spectroscopic data and the J values of their anomeric protons [δ 5.50 (d, J = 6.3 Hz, H-1″), 4.25 (d, J = 7.8 Hz, H-1‴), and 5.02 (d, J = 7.5 Hz, H-1⁗)]. The additional glucosyl moiety was determined to be attached at C-4′ on the basis of the HMBC correlation between H-1⁗ (δ 5.02) and C-4′ (δ 159.4). Other HMBC and NOESY correlations confirmed the structure of **4**. Therefore, compound **4** was assigned as 4′-*O*-β-D-glucopyranosylrhamnocitrin-3-*O*-β-D-glucopyranosyl-(4−1)- β-D-glucopyranoside and named nervilifordin D.

Compound **5** was obtained as a yellow amorphous powder. Its molecular formula, $C_{34}H_{42}O_{22}$, was deduced from the HRESIMS ([M − H]$^-$, m/z 801.2089) and ^{13}C NMR data. Comparison of the ^{1}H and ^{13}C NMR data of **5** with those of **3** revealed that **5** had an additional glucopyranosyl moiety linked at C-4′, which was identified by the NOESY and HMBC experiments. In the NOESY spectra of **5**, the anomeric proton [δ 4.86 (d, J = 6.4 Hz, H-1⁗)] correlated with H-5′ (δ 7.19). The correlation of the anomeric proton [δ 4.86 (d, J = 6.4 Hz, H-1⁗)] with C-4′ (δ 147.8) was also observed in the HMBC spectrum. Thus, the structure of nervilifordin

Table 3. Cytotoxicity and Anti-HSV-1 Activity of Compounds **1**−**3** and **6**−**13**

compound	MNCC (mM)[a]	TIC (mM)[b]
1	0.17	−[c]
2	0.16	−
3	0.16	−
6	0.20	−
7	0.32	−
8	0.14	0.14
9	0.17	−
10	0.35	−
11	0.08	−
12	0.04	−
13	0.08	−
acyclovir	1.11	0.0043[d]

[a] MNCC: maximal noncytotoxic concentration against Vero cells. [b] TIC: total inhibitory concentration against HSV-1. [c] No activity. [d] IC_{50} value (concentration required to reduce 50% of cytopathic effect).

E (**5**) was determined as 4′-*O*-*β*-D-glucopyranosylrhamnetin-3-*O*-*β*-D-glucopyranosyl-(4−1)-*β*-D-glucopyranoside.

Compounds **1**−**3** and **6**−**13** were evaluated for their in vitro anti-herpes simplex virus type 1 (HSV-1) activity using a cytopathic effect (CPE) assay and cytotoxicity on African green monkey kidney cells (Vero cells) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The total inhibitory concentrations (TIC) against HSV-1 and maximal noncytotoxic concentrations (MNCC) against Vero cells of the tested compounds are shown in Table 3. Of the tested compounds, only the coumarin esculetin (**8**) could totally inhibit HSV-1 at a concentration of 0.14 mM. The MNCC of the flavonol glycosides (**1**−**3**, **6**−**7**) are more than 0.16 mM, while the aglycones (**11**−**13**) showed stronger cytotoxicity against Vero cells with MNCC values of 0.08, 0.04, and 0.04 mM, respectively.

Experimental Section

General Experimental Procedures. Melting points were obtained on an XRC-1 apparatus and are uncorrected. Optical rotations were measured with a HORIBA SEPA-300 high-sensitive polarimeter. IR spectra were measured on a Bio-Rad FTS-135 spectrophotometer. NMR spectra were measured in DMSO-d_6 solution and recorded on a Bruker DRX-500 instrument (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) at 25 °C, using TMS as an internal standard. FABMS was recorded on an VG Auto Sepc-3000 mass spectrometer using glycerol as matrix, and HRESIMS was recorded on an API QSTAR Pular-1 mass spectrometer (for compound **4**, one drop of 0.01% aqueous NaCl was added while measuring the HRESIMS). The GC was performed on an HP5890 gas chromatograph (Agilent Technologies) with a quartz capillary column (30 mm × 0.32 mm × 0.25 μm); detection, FID. Column chromatography was performed on Diaion HP20SS (Mitsubishi Chemical Co.), MCI-gel CHP-20P (75−150 μm, Mitsubishi Chemical Co.), and Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemical Co.). Precoated silica gel plates (Qingdao Haiyang Chemical Co.) were used for TLC. Detection was done by spraying the plates with 10% sulfuric acid followed by heating. Sustainable medium used for assays was Dulbecco's modified Eagle's medium (DMEM) with 2% fetal bovine serum (FBS), whose pH value was adjusted to 7.2 by 0.75% $NaHCO_3$ and Hepes buffer (47.6 g of Hepes was dissolved into H_2O (200 mL) and its pH value adjusted to 7.5−8.0 by 1 N NaOH).

Plant Material. The whole plants of *N. fordii* were purchased from Drugs Co. Ltd. of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China), in June 2006. The sample was identified by one of the authors (Y.-F.W.). A voucher specimen has been deposited in the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The dried whole plants of *N. fordii* (2.9 kg) were extracted with MeOH under reflux (3 × 2 L, each 1 h). The extracts were combined and concentrated under reduced pressure to about 2 L in volume and then partitioned with petroleum ether (6 × 2 L). After concentration to a small volume (80 mL), the MeOH portion was subjected to Diaion HP-20SS column (8.0 × 55 cm) chromatography (CC), eluting with MeOH−H_2O (0:1−1:0), to afford 11 fractions. Fraction 8 (3.4 g) was subjected to silica gel CC, eluting with $CHCl_3$−MeOH−H_2O (8:2:0.2), to yield **1** (30 mg) and **6** (800 mg). Fraction 6 (3.1 g) was successively chromatographed over MCI-gel CHP-20P and silica gel eluted with MeOH−H_2O (0:1−1:0, each gradient 200 mL) and $CHCl_3$−MeOH−H_2O (8:2:0.2) to give **2** (500 mg). Compounds **3** (392 mg) and **7** (1.36 g) precipitated out from the MeOH solute of fractions 5 (3.9 g) and 4 (13.2 g), respectively. The filtrate of fraction 4 was chromatographed over silica gel ($CHCl_3$−MeOH−H_2O, 7:3:0.5) and MCI-gel CHP-20P (MeOH−H_2O, 4:6−7:3) CC to afford **4** (6 mg) and **5** (12 mg). CC of fraction 3 (3.6 g) over Sephadex LH-20 eluting with MeOH−H_2O (0:1−6:4) afforded **8** (70 mg). Fraction 2 (4.6 g) was chromatographed over Sephadex LH-20, silica gel, and MCI-gel CHP-20P, eluting with MeOH−H_2O (0:1−1:0), $CHCl_3$−MeOH−H_2O (8:2:0.2−6.5:3.5:0.5), and MeOH−H_2O (4:6−7:3), respectively, to give **9** (31 mg) and **10** (21 mg). A portion (200 mg) of fraction 9 (6 g) was subjected to MCI-gel CHP-20P CC, eluting with MeOH−H_2O (5:5−1:0), to afford **11** (35 mg). Fraction 10 (0.63 g) was chromatographed over silica gel ($CHCl_3$−MeOH, 100:1) to give **12** (283 mg) and **13** (17 mg).

Nervilifordin A (1): yellow, amorphous powder; $[\alpha]^{24}_D$ −9.8 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 266 (4.16), 350 (4.10) nm; IR (KBr) ν_{max} 3374, 2895, 2768, 1665, 1593, 1494, 1445, 1355, 830, 804 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; FABMS (negative ion mode) *m*/*z* 593 [M − H]$^-$; HRESIMS *m*/*z* 593.1505 [M − H]$^-$ (calcd for $C_{27}H_{29}O_{15}$, 593.1506).

Nervilifordin B (2): yellow, amorphous powder; $[\alpha]^{24}_D$ −16.1 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 266.5 (4.35), 349.5 (3.86) nm; IR (KBr) ν_{max} 3396, 2882, 1658, 1596, 1497, 1345, 1074, 829, 800 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; FABMS (negative ion mode) *m*/*z* 623 [M − H]$^-$, 461 [M − H − 162]$^-$, 299 [M − H − 162 × 2]$^-$; HRESIMS *m*/*z* 623.1621 [M − H]$^-$ (calcd for $C_{28}H_{31}O_{16}$, 623.2612).

Nervilifordin C (3): yellow, amorphous powder; $[\alpha]^{24}_D$ −13.2 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 267 (4.56), 357.5 (4.19) nm; IR (KBr) ν_{max} 3407, 2904, 1658, 1590, 1495, 1291, 1067, 844, 793, 589 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; FABMS (negative ion mode) *m*/*z* 639 [M − H]$^-$, 477 [M − H − 162]$^-$, 315 [M − H −162 × 2]$^-$; HRESIMS *m*/*z* 639.1568 [M − H]$^-$ (calcd for $C_{28}H_{31}O_{17}$, 639.1561).

Nervilifordin D (4): yellow, amorphous powder; $[\alpha]^{24}_D$ +25.0 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 264 (4.90), 347 (3.91) nm; IR (KBr) ν_{max} 3402, 2924, 1656, 1600, 1073, 1023, 635, 598 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; FABMS (negative ion mode) *m*/*z* 786 [M]$^-$; HRESIMS *m*/*z* 821.1911 [M + Cl]$^-$ (calcd for $C_{34}H_{42}O_{21}Cl$, 821.1907).

Nervilifordin E (5): yellow, amorphous powder; $[\alpha]^{24}_D$ −93.3 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 268 (4.28), 344.5 (4.15) nm; IR (KBr) ν_{max} 3419, 2919, 1597, 1072, 803, 642, 579 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; FABMS (negative ion mode) *m*/*z* 801 [M − H]$^-$, 477 [M − H − 162 × 2]$^-$; HRESIMS *m*/*z* 801.2089 [M − H]$^-$ (calcd for $C_{34}H_{41}O_{22}$, 801.2089).

Acid Hydrolysis of Compounds 1−3 and 5. Compounds **1**−**3** and **5** (each 6 mg) were hydrolyzed with 1.5 N HCl/1,4-dioxane (1:1, 4 mL) at 80 °C for 3 h. The mixture was extracted with $CHCl_3$ (3 × 4 mL). The aqueous layer was neutralized with NaOH (1 N) and was evaporated to dryness. The dry powders were dissolved in pyridine (2 mL), L-cysteine methyl ester hydrochloride (1.5 mg) was added, and the mixture was heated at 60 °C for 1 h. Trimethylsilyl imidazole (1.5 mL) was added, and the mixture was heated at 60 °C for another 30 min. An aliquot (4 μL) of the supernatant was removed and directly subjected to GC analysis under the following conditions: column temp 180−280 °C at 3 deg/min, carrier gas N_2 (1 mL/min), injector and detector temp 250 °C, split ratio 1:50. The configurations of D-glucose and D-xylose for compounds **1**−**3** and **5** were determined by comparison of the retentions times of the corresponding derivatives with those of standard D-glucose and D-xylose, giving a single peak at 19.208 and 13.674 min, respectively.

HSV-1 Inhibition Activity. HSV-1 inhibition activity was assayed with the plaque reduction assay,[14] with acyclovir as positive control. The Vero cells were seeded into 24-well culture plates. After 24 h of incubation, the cells were infected with 30 PFU HSV-1 in the presence of samples of different concentrations (samples were diluted with cell

sustainable medium), while the dilution medium without samples was used as the control. Then each well was overlaid with medium containing 1% of methylcellulose, and the plate was incubated for 3 days. Thereafter, the cell monolayer was fixed and stained with formalin and crystal violet, respectively. The viral plaques were counted under a binocular microscope. The concentration reducing plaque formation by 100% relative to control was estimated from graphic plots and defined as 100% inhibitory concentration.

Cytotoxicity Assays. Cytotoxic activity was performed using the MTT reduction assay.[15] Vero cells were seeded into a 96-well plate. Different concentrations of samples (100 μL), diluted with cell sustainable medium, were applied to the wells of a 96-well plate containing a confluent cell monolayer in triplicate, while dilution medium without sample was used as the control. After 3 days of incubation, 12 μL of the MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well. The plate was further incubated for 4 h to allow for MTT formazan formation. After removing the medium, 100 μL of DMSO was added to dissolve the formazan crystals. The content in the wells was homogenized on a microplate shaker 30 min later. The OD (optical density) was then read on a microplate spectrophotometer at double wavelengths of 540 and 630 nm. The maximal noncytotoxic concentration was defined as the maximal concentration of the sample that did not exert a cytotoxic effect evaluated from the OD values of nonviable cells.

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