

## Anti-Coxsackie Virus B3 Norsesquiterpenoids from the Roots of *Phyllanthus emblica*

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Three new norsesquiterpenoid glycosides, 4'-hydroxyphyllaemblicin B (**1**) and phyllaemblicins E (**2**) and F (**3**), were isolated from the roots of *Phyllanthus emblica*, together with three known compounds, phyllaemblic acid (**4**), phyllaemblicin B (**5**), and phyllaemblicin C (**6**). Of these, **3** is a new norsesquiterpenoid dimer. The structures of **1–3** were established by spectroscopic data information and by acidic hydrolysis. The isolated compounds, together with two other known analogues, phyllaemblic acid methyl ester (**7**) and phyllaemblicin A (**8**), were evaluated for their antiviral activity toward coxsackie virus B3 (CVB3) by an in vitro cytopathic effect inhibitory assay. Compounds **5–7** exhibited strong anti-CVB3 activity.

*Phyllanthus emblica* L. (Euphorbiaceae) is a shrub or tree with a greenish-gray bark and feathery leaves, growing in tropical and subtropical areas of mainland China, India, Indonesia, and the Malay Peninsula. It has been used extensively in many traditional medicinal systems, such as Ayurvedic medicine, Chinese herbal medicine, and those used by the Tibetan, Mongolian, Dai, and Uigur people.<sup>1</sup> The roots, bark, and leaves have been used for treating eczema, warts, diarrhea, and headache after a fever in the People's Republic of China, and the roots have been used as an astringent and hemostatic agent in Nepal.<sup>1</sup> The fruit and its juice have been used widely due to their purported biological activities, including antibacterial,<sup>2</sup> antioxidant,<sup>3</sup> anti-inflammatory,<sup>4</sup> antitumor,<sup>1</sup> and hepatoprotective effects.<sup>5</sup> The methanolic and water extracts of the fruits were found to markedly inhibit the reverse transcription of HIV.<sup>6</sup>

Our previous collaborative chemical studies of *P. emblica* have led to the isolation of several norsesquiterpenoids from the roots,<sup>7,8</sup> organic acid gallates and polyphenols from the fruit juice,<sup>9,10</sup> and ellagitannins and flavonoids from the branches and leaves.<sup>10,11</sup> Of these, phyllaemblic acid and its glycosides phyllaemblicins A–C were found to be the major norsesquiterpenoids from the roots. In the course of our study on antiviral compounds from natural sources, the norsesquiterpenoid glycoside phyllaemblicin B (**5**) exhibited stronger anti-CVB3 activity in vitro than a positive control, ribovirin. In order to evaluate its anti-CVB3 activity in vivo, a large-scale preparation of **5** was carried out. This led to the isolation of three new analogues, namely, 4'-hydroxyphyllaemblicin B (**1**) and phyllaemblicins E (**2**) and F (**3**), together with two known compounds, phyllaemblic acid (**4**) and phyllaemblicin C (**6**), in addition to **5**. The isolated compounds **1–6**, together with two previously isolated analogues, phyllaemblic acid methyl ester (**7**) and phyllaemblicin A (**8**), were evaluated for their anti-CVB3 activity in vitro.

The methanolic extract of the fresh roots of *P. emblica* was subjected to column chromatography to afford compounds **1–6**. Of these, compounds **4–6** were identified as the previously reported norsesquiterpenoid phyllaemblic acid (**4**)<sup>7</sup> and the glycosides phyllaemblicins B (**5**) and C (**6**),<sup>8</sup> respectively, by comparison with authentic samples and of their spectroscopic and physical data with previously reported values.

Compound **1** was obtained as a white, amorphous powder. Its molecular formula, C<sub>33</sub>H<sub>44</sub>O<sub>20</sub>, was determined on the basis of the HRESIMS ([M – H]<sup>–</sup>, *m/z* 759.2333) and <sup>13</sup>C and DEPT NMR data. The IR spectrum showed the presence of hydroxyl (3421 cm<sup>–1</sup>) and carbonyl (1692 cm<sup>–1</sup>) groups in **1**. The <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data of **1** were closely related to those of **5**, which showed two sets of signals for two hexoses [anomeric centers at δ<sub>H</sub> 5.60 (d, *J* = 8.0 Hz) and 4.20 (d, *J* = 7.7 Hz); δ<sub>C</sub> 93.7 and 106.2, respectively] and 14 carbon resonances, including one ketone (δ 213.8), one carbonyl (δ 175.9), and six oxygen-bearing carbons (δ 100.6, 76.2, 75.9, 71.5, 70.4, and 63.5). However, instead of the benzoyl group in **5**, the downfield shift of C-4' (δ 163.2) and the appearance of two doublet aromatic proton signals at δ 8.03 and 6.91 (each 2H, d, *J* = 8.7 Hz) revealed the presence of a *p*-hydroxybenzoyl group in **1**. Acidic hydrolysis of **1** gave D-glucose as the sole sugar moiety, which was determined to have a β configuration on the basis of the large coupling constants of the anomeric protons [δ<sub>H</sub> 5.60 (d, *J* = 8.0 Hz) and 4.20 (d, *J* = 7.7 Hz)]. The locations of the sugar units and the *p*-hydroxybenzoyl group in **1** were confirmed by a HMBC experiment, in which correlations of H-1''' (δ 4.20, terminal glucosyl unit) with C-2'' (δ 83.2, inner glucosyl unit), H-1'' (δ 5.60, inner glucosyl unit) with C-13 (δ 175.9), and H-10 (δ 5.29) and H-2',6' (δ 8.03) with C-7' (δ 168.0) were observed. Therefore, the structure of **1** was determined as 4'-hydroxyphyllaemblicin B.

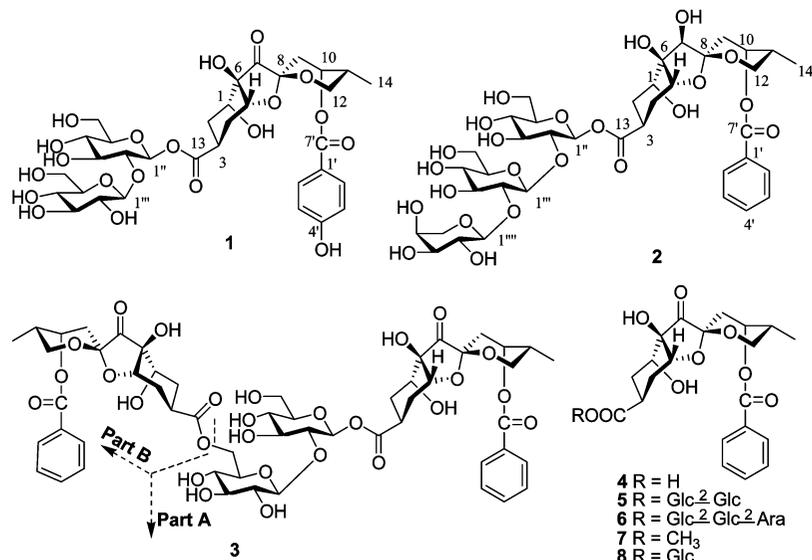
Compound **2** was assigned the molecular formula C<sub>38</sub>H<sub>54</sub>O<sub>23</sub> from the positive HRESIMS ([M + Na]<sup>+</sup>, *m/z* 901.2929), which was 2 mass units larger than that of **6**. This difference corresponded to the presence of two additional protons. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of **2** were very similar to those of **6**, except for the loss of the ketone signal at δ 213 and the appearance of an additional oxygen-bearing methine carbon signal at δ 84.2. This observation suggested that the C-7 ketone in **6** was reduced to a secondary alcohol in **2**. Acidic hydrolysis of **2** gave D-glucose and L-arabinose as sugar residues. The coupling constants (*J* = 8.1, 7.8 Hz) of anomeric protons of the two glucose moieties indicated these to be in the β configuration, and the coupling constants (*J*<sub>1,2</sub> = 7.1 Hz, *J*<sub>2,3</sub> = 9.0 Hz, *J*<sub>3,4</sub> = 9.0 Hz, *J*<sub>4,5</sub> = 12.6 and 2.6 Hz) of the L-arabinose moiety suggested it to be in the α-pyranose form. Connectivities of the sugar and benzoyl moieties were further confirmed by the HMBC correlations of H-1'' of the inner glucose (δ 5.53), H-1''' of the middle glucose (δ 4.19), and H-1'''' of the arabinose (δ 4.50) with C-13 (δ 175.8), C-2'' of inner glucose (δ 84.1), and C-2''' of middle glucose (δ 85.0), respectively. In the ROESY spectrum of **2**, H-7 was only correlated with H-9, but not with H-5, suggesting that

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H-7 was  $\alpha$ -oriented. Accordingly, the structure of phyllaemblicin E was established as shown in 2.

Compound **3** was obtained as a white, amorphous powder. The molecular formula C<sub>54</sub>H<sub>66</sub>O<sub>27</sub> was deduced from the positive HRESIMS ([M + Na]<sup>+</sup>, *m/z* 1169.3658) data. Acidic hydrolysis of **3** gave D-glucose as the sole sugar residue. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of **3** were closely related to those of **5**, which exhibited signals due to two  $\beta$ -glucopyranosyl units [ $\delta$  5.67 (d, *J* = 8.1) and 4.25 (d, *J* = 6.5)] together with two sets of signals almost superimposable on those of **4**, suggesting that there was another phyllaemblic acid (**4**) moiety attached to the phyllaemblicin B (**5**) unit in **3**. On comparison with those of **5** ( $\delta_{\text{H}}$  3.52 and 3.56,  $\delta_{\text{C}}$  62.0), the H-6''' and C-6''' signals of the second glucose moiety in **3** were shifted downfield to  $\delta_{\text{H}}$  4.53 and 4.31 and  $\delta_{\text{C}}$  63.5, respectively, suggesting that the C-6''' of this glucose unit is acylated with another phyllaemblic acid (**4**) unit, in which the C-13 signal was shifted upfield to  $\delta$  175.6 relative to that of the free phyllaemblic acid (**4**,  $\delta$  179.0). The connectivities of each unit in **3** were further determined by 2D NMR experiments, including <sup>1</sup>H-<sup>1</sup>H COSY, HMQC, and HMBC. In the HMBC spectrum of **3**, H-1'' ( $\delta$  5.67) of the first glucose moiety and H-6''' ( $\delta$  4.53, 4.31) of the second glucose moiety were correlated with C-13 ( $\delta$  174.8, 175.6) of the two carboxyls. Moreover, HMBC correlations of H-1''' ( $\delta$  4.25) of the second glucose moiety with C-2'' ( $\delta$  83.8) of the first glucose moiety and of both H-10 ( $\delta$  5.33) and H-2',6' ( $\delta$  8.18, 8.10) with C-7' ( $\delta$  166.6) of the benzoyl group were observed. Accordingly, the structure of **3** was determined to be a dimer of **4** and **5** through an ester linkage between C-6''' of **5** and C-13 of **4** and was named phyllaemblicin F.

The isolated compounds **1–6** were tested for their *in vitro* anti-CVB3 activities, together with two previously isolated analogues, phyllaemblic acid methyl ester (**7**) and phyllaemblicin A (**8**), and their CC<sub>50</sub> (concentration that reduced the viability of HeLa cell cultures by 50%), IC<sub>50</sub> [concentration required to reduce 50% of cytopathic effect (CPE)], and TI (therapeutic index = CC<sub>50</sub>/IC<sub>50</sub>) values are shown in Table 3. Compounds **5–7** exhibited significant anti-CVB3 activity, while other compounds (**1–4**, **8**) showed no antiviral activity. This suggests that the C-7 ketone, the benzoyl group at C-10, and esterification at C-13 are essential for anti-CVB3 activity. Once the ketone at the C-7 position was reduced to be a secondary alcohol or the benzene ring of a benzoyl group was substituted with a hydroxyl group, the anti-CVB3 activity of the molecule (**1** and **2**) was lost. In addition, at least two sugar units attached at the C-13 position are necessary for anti-CVB3 activity. Compounds **4** and **8**, with no or only one sugar unit, showed a lack of anti-CVB3 activity.

## Experimental Section

**General Experimental Procedures.** [ $\alpha$ ]<sub>D</sub> and UV data were measured on a JASCO-20 polarimeter and Shimadzu UV-2401PC spectrometer, respectively. IR spectra were measured on a Bruker Tensor 27 spectrometer with KBr pellets. 1D- and 2D-NMR spectra were run on Bruker AM-400 and DRX-500 instruments operating at 400 and 500 MHz for <sup>1</sup>H and 100 and 125 MHz for <sup>13</sup>C, respectively. Coupling constants are expressed in hertz, and chemical shifts are given on a ppm scale with tetramethylsilane as internal standard. The MS data were recorded on a VG Auto Spec-3000 spectrometer (VG, Manchester, U.K.) with glycerol as the matrix. HRESIMS were recorded on an API Qstar Pulsar LC/TOF spectrometer. GC analysis was run on a Shimadzu GC-14C gas chromatograph. Evaluations of cytotoxicity and antiviral effects were performed on an Emax precision microplate reader (Bio-Rad Company, Hercules, CA).

Column chromatography was performed with Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd. Uppsala, Sweden), Diaion HP20SS (Mitsubishi Chemical Co., Tokyo, Japan), silica gel (200–300 mesh, Qingdao Makall Group Co., Ltd. Qingdao, People's Republic of China), Chromatorex ODS (100–200 mesh, Fuji Silysia Chemical Co., Ltd. Tokyo, Japan), and MCI gel CHP 20P (Mitsubishi Chemical Co.). Thin-layer chromatography (TLC) was carried out on silica gel H-precoated plates (Qingdao Makall Group Co., Ltd.). Dulbecco's modified Eagle's medium (DMEM) and ribovirin were purchased from HyClone Company (Logan, UT) and Guangzhou Shiqiao Pharmaceutical Co., Ltd. (Guangzhou, People's Republic of China), respectively.

**Plant Material.** Roots of *P. emblica* were collected from Simao city, Yunnan Province, People's Republic of China, in May 2007. The plant was identified by Mr. Jiang Li from Yunnan Academy of Forestry. A voucher specimen (KUN No. 0619422) has been deposited in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and Isolation.** The fresh roots of *P. emblica* (21.7 kg) were extracted with methanol three times (2 h  $\times$  3) under reflux. The extract (490 g) was divided into two portions, and each portion was subjected to column chromatography over Sephadex LH-20 (6.4  $\times$  50 cm), eluted with MeOH-H<sub>2</sub>O (3:7-1:0), to afford four fractions in all. Fraction 1 (152 g) was applied to a Diaion HP20SS column (6.8  $\times$  60 cm) and eluted with MeOH-H<sub>2</sub>O (0:1-1:0) to give 11 subfractions. Subfraction 8 (26.3 g) was chromatographed sequentially over silica gel (4.8  $\times$  50 cm, CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O, 8:2:0.2 and 7:3:0.5), Chromatorex ODS (MeOH-H<sub>2</sub>O, 1:9-6:4), and MCI gel CHP 20P (MeOH-H<sub>2</sub>O, 7:3-9:1) to afford **1** (358 mg), **2** (38 mg), **5** (9.1 g), and **6** (6.2 g). Subfraction 9 (2.5 g) was subjected to passage over Sephadex LH-20 (MeOH-H<sub>2</sub>O, 4:6-7:3), silica gel (CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O, 8:2:0.2), and MCI gel CHP 20P (MeOH-H<sub>2</sub>O, 7:3-9:1) to afford **3** (50 mg) and **4** (63 mg).

**4'-Hydroxyphyllaemblicin B (1):** white, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> 0 (c 0.23, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 258 (3.68), 203 (3.68), 195 (3.41), 191 (3.42) nm; IR (KBr)  $\nu_{\text{max}}$  3421, 2934, 1778, 1744, 1692, 1609, 1281, 1169, 1097, 851, 773, 617 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data,

**Table 1.** <sup>1</sup>H and <sup>13</sup>C NMR Spectroscopic Data of **1** and **2**<sup>a</sup>

position	<b>1</b>		<b>2</b>	
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C
1	3.91 brs	71.5	3.84 brs	72.1
2	2.04 brd (13.6)	32.2	2.19–2.22 m	28.7
	1.75 dt (13.6, 2.5)		1.57–1.66 m	
3	2.90 tt (12.9, 2.5)	32.1	2.63–2.72 m	34.5
4	2.35 brd (14.0)	29.4	2.08 dt (13.9, 3.8)	28.4
	1.86 dt (14.0, 3.6)		1.90 dt (13.9, 3.8)	
5	4.28 brs	76.2	3.83 brs	76.0
6		75.9		76.7
7		213.8	4.15 t (3.2)	82.4
8		100.6		102.6
9	2.25 dd (14.8, 3.1)	32.8	2.18–2.23 m	36.1
	1.96 dd (14.8, 3.1)		2.12 dd (14.6, 2.6)	
10	5.29 brd (2.8)	70.4	5.32 d (2.5)	71.9
11	2.15 m	34.4	2.16–2.24 m	34.1
12	4.02 t (11.7)	63.5	4.07 t (11.3)	63.1
	3.54 dd (11.7, 2.8)		3.55–3.61 m	
13		175.9		175.8
14	0.87 d (6.8)	13.1	0.89 d (6.8)	13.1
1'		123.1		132.2
2', 6'	8.03 d (8.7)	133.2	8.14 d (7.2)	130.9
3', 5'	6.91 d (8.7)	116.5	7.55 t (7.7)	129.9
4'		163.2	7.67 t (7.4)	134.5
7'		168.0		167.9
Glc-1''	5.60 d (8.0)	93.7	5.53 d (8.1)	93.8
2''	3.49 brd (9.2)	83.2	3.23 brd (8.1)	84.1
3''	3.60 brd (9.2)	77.9	3.45 brd (8.8)	77.5
4''	3.36–3.40 m	70.5	3.44 brd (8.8)	70.3
5''	3.40–3.43 m	79.0	3.35–3.38 m	78.8
6''	3.92 dd (12.0, 2.4)	62.3	3.85 brd (11.6)	62.3
	3.78 dd (12.0, 5.6)		3.64–3.68 m	
Glc-1'''	4.20 d (7.7)	106.2	4.19 d (7.8)	104.4
2'''	3.11 dt (7.7, 2.5)	75.5	3.27 brd (7.8)	85.0
3'''	3.25 dd (7.0, 2.5)	77.8	3.54–3.59 m	77.3
4'''	3.22–3.29 m	70.6	3.83 brs	70.1
5'''	2.72–2.76 m	77.6	3.28–3.33 m	77.7
6'''	3.69 dd (12.1, 5.0)	61.8	3.45–3.50 m	61.9
	3.59 dd (12.1, 2.0)		3.54–3.60 m	
Ara-1''''			4.50 d (7.1)	107.0
2''''			3.63–3.68 m	73.6
3''''			3.56 dt (9.0, 3.0)	74.2
4''''			3.83 brs	69.6
5''''			3.56 m	67.6
			3.99 dd (12.6, 2.6)	

<sup>a</sup> Measured in CD<sub>3</sub>OD, 400 and 100 MHz for <sup>1</sup>H and <sup>13</sup>C NMR, respectively, δ in ppm, *J* values in Hz.

see Table 1; FABMS *m/z* 759 [M – H]<sup>–</sup> (100), 597 [M – H – 162 (glucosyl)]<sup>–</sup> (2), 435 [M – H – 324 (2 × glucosyl)]<sup>–</sup> (25); HRESIMS *m/z* 759.2333 [M – H]<sup>–</sup> (calcd for C<sub>33</sub>H<sub>43</sub>O<sub>20</sub>, 759.2347).

**Phyllaemblicin E (2):** white, amorphous powder; [α]<sub>D</sub><sup>25</sup> +11.0 (c 0.24, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 272 (2.56), 228 (3.56), 202 (3.55), 194 (3.31) nm; IR (KBr) ν<sub>max</sub> 3405, 2932, 1747, 1705, 1283, 1078, 781, 720 cm<sup>–1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; FABMS *m/z* 902 [M + Na]<sup>+</sup> (7), 879 [M + H]<sup>+</sup> (12); HRESIMS *m/z* 901.2929 [M + Na]<sup>+</sup> (calcd for C<sub>38</sub>H<sub>54</sub>O<sub>23</sub>Na, 901.2953).

**Phyllaemblicin F (3):** white, amorphous powder; [α]<sub>D</sub><sup>23</sup> +11.6 (c 0.26, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 280 (2.49), 273 (2.64), 229 (3.98), 202 (3.91) nm; IR (KBr) ν<sub>max</sub> 3435, 3422, 2935, 1779, 1716, 1283, 1176, 1081, 1005, 952, 719 cm<sup>–1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 2; FABMS *m/z* 1169 [M + Na]<sup>+</sup> (30), 1147 [M + H]<sup>+</sup> (20), 727 [M + H – 420 (phyllaemblicin acid)]<sup>+</sup> (15), 413 [M + H – 420 – 324 (2 × glucosyl)]<sup>+</sup> (17); HRESIMS *m/z* 1169.3658 [M + Na]<sup>+</sup> (calcd for C<sub>54</sub>H<sub>66</sub>O<sub>27</sub>Na, 1169.3689).

**Acidic Hydrolysis of Compounds 1–3.** Compounds **1–3** (each 8–10 mg) were hydrolyzed with 2 M HCl–dioxane (1:1, 4 mL) under reflux for 6 h. The reaction mixture was extracted with CHCl<sub>3</sub> five times (2 mL × 5). The aqueous layer was neutralized with 2 M NaOH and then dried to give a monosaccharide mixture. Then, a solution of the sugar mixture in pyridine (2 mL) was added to L-cysteine methyl ester hydrochloride (about 1.5 mg) and kept at 60 °C for 1 h. Next, trimethylsilylimidazole (about 1.5 mL) was added to the reaction mixture in ice water and kept at 60 °C for 30 min. The mixture was subjected to GC analysis, run on a Shimadzu GC-14C gas chromatograph equipped with a 30 m × 0.32 mm i.d. 30QC2/AC-5 quartz capillary column and an H<sub>2</sub> flame ionization detector with the following conditions: column temperature, 180–280 °C; programmed increase,

**Table 2.** <sup>1</sup>H and <sup>13</sup>C NMR Spectroscopic Data of **3**<sup>a</sup>

position	part A		part B	
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C
1	3.99 d (2.5)	71.6	4.04 d (3.5)	71.4
2	2.11–2.16 m	32.1	1.98–2.03 m	32.2
	1.74 dt (16.7, 3.0)		1.83 dt (16.0, 3.0)	
3	2.92 brs	31.5	3.00 dt (16.0, 3.0)	31.6
4	2.27 brd (18.5)	28.8	2.54 brd (18.5)	28.8
	1.94 dt (18.5, 4.5)		2.02–2.04 m	
5	4.23 brs	74.9	4.32 brs	75.2
6		75.9		75.7
7		212.2		212.3
8		99.8		99.8
9	1.94 dt (14.9, 2.7)	32.1	2.20 dt (18.5, 2.7)	32.7
10	5.33 q (2.7)	70.1	5.33 q (2.7)	69.9
11	2.10–2.16 m	33.4	2.10–2.16 m	33.6
12	3.48 dd (14.0, 5.5)	62.6	3.85 t (14.3)	62.6
	3.38–3.42 m		3.72–3.75 m	
13		174.8		175.6
14	0.77 d (6.8)	13.0	0.81 d (6.9)	13.0
1'		131.9		132.0
2', 6'	8.10 d (7.5)	130.5	8.18 d (7.4)	130.5
3', 5'	7.57–7.68 m	129.3	7.57–7.68 m	129.5
4'	7.57–7.68 m	133.7	7.57–7.68 m	134.0
7'		166.6		166.6
Glc-1''	5.67 d (8.1)	92.8		
2''	3.41 dd (11.0, 8.1)	83.8		
3''	3.60–3.64 m	77.4		
4''	3.57–3.60 m	70.1		
5''	3.54 t (6.8)	75.6		
6''	3.58–3.62 m	62.0		
	3.47 dd (14.0, 5.5)			
Glc-1'''	4.25 d (6.5)	106.1		
2'''	3.20–3.25 m	75.5		
3'''	3.30 dt (10.8, 6.5)	77.3		
4'''	3.20–3.25 m	70.5		
5'''	3.69–3.71 m	77.8		
6'''	4.53 dd (15.0, 5.0)	63.5		
	4.31 dd (15.0, 2.0)			

<sup>a</sup> Measured in CD<sub>3</sub>COCD<sub>3</sub>, 500 and 125 MHz for <sup>1</sup>H and <sup>13</sup>C NMR, respectively, δ in ppm, *J* values in Hz.

**Table 3.** Anti-Coxsackie Virus B3 Effects of Compounds **5–7**<sup>a</sup>

compound	CC <sub>50</sub> (μg/mL) <sup>b</sup>	IC <sub>50</sub> (μg/mL) <sup>c</sup>	TI <sup>d</sup>
<b>5</b>	50.2	7.8	6.4
<b>6</b>	67.7	11.0	6.2
<b>7</b>	213.6	21.8	9.8
ribovirin	426.0	20.3	21.0

<sup>a</sup> All data represent mean values for three independent experiments. <sup>b</sup> CC<sub>50</sub> = concentration that reduced the viability of HeLa cell cultures by 50%. <sup>c</sup> IC<sub>50</sub> = concentration required to reduce 50% of cytopathic effect. <sup>d</sup> TI = CC<sub>50</sub>/IC<sub>50</sub>.

3 °C/min; carrier gas, N<sub>2</sub> (1 mL/min); injector and detector temperature, 250 °C; injection volume, 4 μL; and split ratio, 1/50. The configuration of the sugar moiety was determined by comparing the retention time with the derivatives of authentic samples. The retention times of D-/L-glucose were 19.21/14.94 min, and those of D-/L-arabinose were 14.60/13.65 min, respectively.

**Virus and Cell Cultures.** Coxsackie virus B3 Nacy strain, which was generously provided by the Wuhan Institute of Virology, Chinese Academy of Sciences, was propagated in HeLa cell monolayers and stored at –80 °C until used. Viral titers were determined by tissue culture infectious dose (TCID<sub>50</sub>) assays, and the concentration of the CVB3 titer used for the infections was 100 TCID<sub>50</sub>. HeLa cells were obtained from the American Type Culture Collection and grown routinely in complete medium (DMEM medium supplemented with 10% heat-inactivated newborn calf serum, 0.1% L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) at 37 °C in an atmosphere of 5% CO<sub>2</sub>. The test medium used for the cytotoxic assay as well as the antiviral assay contained 2% of the appropriate serum.

**Cytotoxicity.** Cytotoxicity of compounds **1–8** was determined by a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method,<sup>12</sup> as previously reported. Briefly, HeLa cells were seeded in 96-well plates and grown to monolayers. After removal of the growth medium (DMEM maintenance with 10% newborn calf serum), the test compound solutions at series of concentrations (200–6.25 μg/mL) in

the test medium were added (100  $\mu$ L each well). Then, the plates were incubated at 37 °C for 48 h, and 20  $\mu$ L of MTT solution (0.5 mg/mL, in phosphate-buffered saline) was added to each well followed by reacting for 4 h. Subsequently, the supernatant was removed and 100  $\mu$ L of DMSO was added. After incubation at room temperature for 30 min, the optical density (OD) values were read on a microplate spectrophotometer at dual wavelengths of 570 and 630 nm. The  $CC_{50}$  values (the concentration of test compounds that reduced the viability of HeLa cell cultures by 50%) were calculated from each mean dose–response curve.

**Anti-CVB3 Activity Assay.** Anti-CVB3 activities of the test compounds were evaluated by the cytopathic effect (CPE) inhibitory assay described previously.<sup>13,14</sup> In brief, HeLa cells were grown in 96-well plates and were allowed to form monolayers. Then, 50  $\mu$ L of 100 TCID<sub>50</sub> viral suspensions and an equal volume of serial 2-fold dilutions of the test compounds at doses below  $CC_{50}$  were added to each well. Noninfected and infected cells without the test compounds served as cell and virus control, respectively. The plates were incubated at 37 °C in a humidified CO<sub>2</sub> atmosphere. When virus control showed a maximum CPE, the virucidal effects were determined by the MTT assay as described above, and the  $IC_{50}$  values (the concentration of test compounds required to reduce 50% of CPE) were calculated. Thus, the therapeutic index (TI) was calculated from the ratio  $CC_{50}/IC_{50}$ .

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