Flavonoids Isolated from Heat-Processed *Epimedium koreanum* and Their Anti-HIV-1 Activities

by **Hong-Mei Li**[a]), **Cheng Zhou**[a]), **Chin-Ho Chen**[b]), **Rong-Tao Li***[a]), and **Kuo-Hsiung Lee***[c])[d])

[a]) Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, P. R. China
(phone: +86-871-65920569; fax: +86-871-65920570; e-mail: rongtaolikm@163.com)
[b]) Surgical Science, Department of Surgery, University Medical Center, Durham, North Carolina, USA
[c]) Natural Products Research Laboratories, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, North Carolina 27599, USA
(phone: +1-919-9620066; fax: +1-919-9663893; e-mail: khlee@unc.edu)
[d]) Chinese Medicine Research and Development Center, China Medical University and Hospital, Taichung, Taiwan

Systematic phytochemical investigation on heat-processed *Epimedium koreanum* led to the isolation of 13 flavonoids, including five new prenyl-flavonol glycosides, koreanosides A–E (**1**–**5**, resp.). Their structures were elucidated on the basis of detailed analysis of the 1D- and 2D-NMR spectroscopic data and chemical reactions. Apigenin (**11**) exhibited moderate anti-HIV-1 activity with an EC_{50} value of 12.8 ± 3.27 μg/ml.

Introduction. – Herba epimedii, also named Horny Goat Weed or '*Yin Yang Huo*', has been used in Traditional Chinese Medicine (TCM) as a tonic, antirheumatic, and aphrodisiac for more than 2000 years. The aerial parts of *Epimedium koreanum* (Berberidaceae), together with three other species, *E. brevicornu*, *E. sagittatum*, and *E. pubescens*, were officially adopted in the Chinese Pharmacopoeia [1]. Previous chemical and pharmacological investigations on epimedii found that the major bioactive constituents are prenyl-flavonoids, which exhibit a wide range of biological activities, such as anti-osteoporosis, androgenic, anti-inflammatory, and anticancer effects [2]. Epimedin C, icariin, and baohuoside I, three main flavonoids in the crude herb, have been used as markers for quality evaluation by HPLC/UV [1][3].

Traditionally, herba epimedii was processed by heating with sheep fat to enhance hormonal and anti-inflammatory effects [4][5]. Reportedly, HPLC analysis showed that the content of prenyl-flavonoid bisdesmosides (such as icarrin and, epimedins A–C) decreased in the heat-processed form, whereas that of monodesmosides (such as baohuoside I) increased [6]. In an *in vitro* cultured Caco-2 cell model, the absorption and metabolism of the monoglycoside baohuoside I was greater than those of bisdesmoside prenyl-flavonoids with two or more sugar moieties [7].

However, no systematic phytochemical study on heat-processed epimedii has been reported. As a continuation of our research on medicinal plants in the genus *Epimedium* [8][9], 13 flavonoids, including five new prenyl-flavonol glycosides, koreanosides A – E (**1**–**5**, resp.; *Fig. 1*), were isolated from heat-processed *E.*

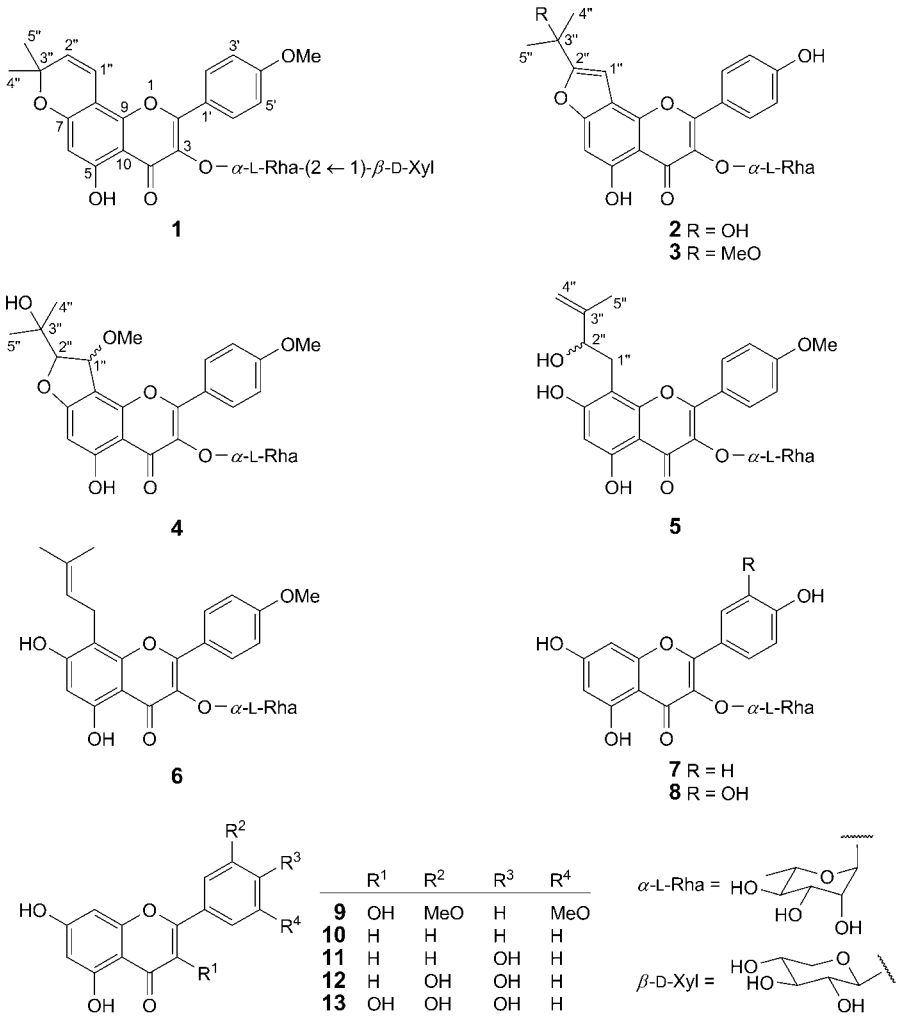

Fig. 1. *Structures of compounds* **1**–**13** *isolated from heat-processed* Epimedium koreanum

koreanum NAKAI. The known compounds were identified as icariside II (**6**) [10], afzelin (**7**) [11], quercitrin (**8**) [12], morelosin (**9**) [13], chrysin (**10**) [14], apigenin (**11**) [15], luteolin (**12**) [15], and quercetin (**13**) (*Fig. 1*) [15]. Among them, compounds **9** and **10** were isolated from the genus *Epimedium* for the first time. Compounds **2**, **7**, **8**, **11**, and **13** were evaluated for anti-HIV-1 activity, and among them, compound **11** showed moderate anti-HIV-1 activity with an EC_{50} value of 12.8 ± 3.27 μg/ml. This article reports the isolation, structural determination, and biological evaluation of these flavonoids.

Results and Discussion. – Compounds **1**–**5** were determined to be flavonol glycosides based on positive results from a HCl/Mg reaction and with *Molisch* reagent, as well as the ^{13}C-NMR data of C(2) (δ(C) 158.8, 160.1, 160.5, 158.6, and 158.8, resp., each *s*) and C(3) (δ(C) 137.2, 134.8, 135.1, 136.7, and 136.1, resp., each *s*).

Compound **1**, yellow amorphous powder, showed *quasi*-molecular-ion peaks at *m*/*z* 667 ($[M+Na]^+$) and 1311 ($[2M+Na]^+$) in positive-ion-mode ESI-MS, indicating a molecular weight of 644. The molecular formula was determined as $C_{32}H_{36}O_{14}$ by HR-EI-MS (*m*/*z* 644.2098 (M^+)) with 15 degrees of unsaturation and confirmed by ^{13}C-NMR and DEPT spectroscopic data.

In the ^{1}H-NMR spectrum of **1** (*Table 1*), an A_2B_2 coupling system at δ(H) 7.88 (*d*, *J* = 8.6, H–C(2′,6′)) and 7.11 (*d*, *J* = 8.6, H–C(3′,5′)) indicated the presence of a *para*-substituted ring *B*. HMBCs (*Fig. 2*) of H–C(2′) and H–C(6′) with C(4′) (δ(C) 164.5), of H–C(3′) and H–C(5′) with C(4′), and of MeO H-atoms (δ(H) 3.89) with C(4′) suggested that C(4′) was substituted by a MeO group. A *singlet* H-atom signal at δ(H) 6.17 showed HMBCs with C(5) (δ(C) 163.6), C(7) (160.9), C(8) (102.6), and C(10) (106.7), assigning this H-atom as H–C(6) of ring *A*. A series of H-atom signals at δ(H) 6.72 (*d*, *J* = 10.0, H–C(1″)), 5.68 (*d*, *J* = 10.0, H–C(2″)), 2.14 (*s*, Me(4″)), and 1.45 (*s*, Me(5″)) in the ^{1}H-NMR spectrum, as well as a group of C-atom signals at δ(C) 115.4 (*d*, C(1″)), 129.0 (*d*, C(2″)), 79.5 (*s*, C(3″)), 28.4 (*q*, C(4″)), and 30.6 (*q*, C(5″)) in the ^{13}C-NMR spectrum (*Table 2*), indicated the presence of a γ,γ-dimethyl-chromene ring, which was confirmed by HMBCs of Me(4″) with C(2″) and C(3″), H–C(2″) with C(3″) and C(4″), as well as H–C(1″) with C(3″). HMBCs of H–C(1″) with C(7) and C(8) and of H–C(2″) with C(8) indicated that the γ,γ-dimethyl-chromene moiety was fused to C(7) and C(8). Furthermore, the C(5) signal was present at low-field, which, in combination with the molecular formula of **1**, suggested the substitution by a OH group. Therefore, compound **1** possesses the same aglycone moiety as sutchuenmedin A [16], but differs in the composition of the oligosaccharide chain.

Acid hydrolysis of **1** with 1M HCl showed the presence of L-rhamnose (Rha) and D-xylose (Xyl) as sugar residues, as determined by GC analysis. H-Atom signals at δ(H) 5.47 (*s*, H–C(1) of Rha), 0.96 (*d*, *J* = 6.1, Me(6) of Rha), 4.28 (*d*, *J* = 6.3, H–C(1) of Xyl), 3.03 (*t*, *J* = 11.0, H_a–C(5) of Xyl), and 3.68 (overlapped, H_b–C(5) of Xyl) further confirmed the presence of the two sugar moieties. In accordance with the ^{13}C-NMR

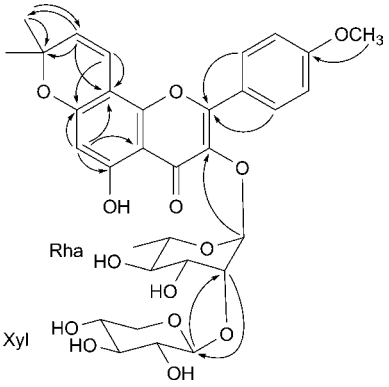

Fig. 2. *Key HMBCs of compound* **1**

Table 1. *^{1}H-NMR Data of Compounds* **1**–**5**. Arbitrary atom numbering indicated in *Fig. 1*; δ in ppm, J in Hz.

Position	**1**[a])[b])	**2**[c])[d])	**3**[c])[d])	**4**[a])[e])	**5**[a])[b])
6	6.17 (*s*)	7.04 (*s*)	7.07 (*s*)	6.31 (*s*)	6.25 (*s*)
2′, 6′	7.88 (*d*, *J* = 8.6)	7.91 (*d*, *J* = 8.8)	7.93 (*d*, *J* = 8.8)	7.88 (*d*, *J* = 8.8)	7.93 (*d*, *J* = 8.7)
3′, 5′	7.11 (*d*, *J* = 8.6)	6.96 (*d*, *J* = 8.8)	6.95 (*d*, *J* = 8.8)	7.11 (*d*, *J* = 8.8)	7.09 (*d*, *J* = 8.7)
1″	6.72 (*d*, *J* = 10.0)	6.99 (*s*)	7.25 (*s*)	5.29 (*d*, *J* = 2.6)	3.00 (*dd*, *J* = 11.4, 6.3), 3.07 (*dd*, *J* = 11.4, 5.1)
2″	5.68 (*d*, *J* = 10.0)			4.51 (*d*, *J* = 2.6)	4.36 (*t*, *J* = 5.7)
4″	2.14 (*s*)	1.53 (*s*)	1.56 (*s*)	1.29 (*s*)	4.67 (*s*) 4.73 (*s*)
5″	1.45 (*s*)	1.53 (*s*)	1.56 (*s*)	1.16 (*s*)	1.71 (*s*)
Rha					
1	5.47 (*s*)	5.36 (*d*, *J* = 1.4)	5. 37 (*d*, *J* = 1.4)	5.42 (*s*)	5.44 (*d*, *J* = 1.2)
2	4.20 (*s*)	3.16–4.02 (overlap)	3.15–4.02 (overlap)	3.31–4.22 (overlap)	3.18–4.24 (overlap)
3, 4, 5	3.15–3.81 (overlap)	3.16–4.02 (overlap)	3.15–4.02 (overlap)	3.31–4.22 (overlap)	3.18–4.24 (overlap)
6	0.96 (*d*, *J* = 6.1)	0.80 (*d*, *J* = 5.6)	0.80 (*d*, *J* = 5.5)	0.90 (*d*, *J* = 5.9)	0.85 (*d*, *J* = 6.1)
Xyl					
1	4.28 (*d*, *J* = 6.3)				
2, 3, 4	3.15–3.81 (overlap)				
5	3.03 (*t*, *J* = 11.0), 3.68 (overlap)				
4′-MeO	3.89 (*s*)			3.89 (*s*)	3.89 (*s*)
1″-MeO				3.51 (*s*)	
3″-MeO			2.98 (*s*)		
5-OH		12.64 (*s*)	12.68 (*s*)		
3″-OH		5.56 (*s*)			

[a]) Measured in CD_3OD. [b]) Measured at 500 MHz. [c]) Measured in (D_6)DMSO. [d]) Measured at 600 MHz. [e]) Measured at 400 MHz.

data, the configuration at the anomeric C-atom of the Rha moiety was determined as α [17]. Simultaneously, the β-configuration at the anomeric C-atom of the Xyl moiety was assigned on the basis of the coupling constant of its anomeric H-atom ($J = 6.3$) [18]. In the HMBC spectrum, a correlation was observed between the anomeric H-atom of Rha and C(3), indicating that the Rha moiety was attached to C(3) of the aglycone. The Xyl moiety was located at C(2) (δ(C) 82.5) of the Rha moiety, based on a HMBC of the anomeric H-atom of the Xyl moiety with C(2) of the Rha moiety. In addition, a HMBC of H–C(2) (δ(H) 4.20) of Rha with the anomeric C-atom (δ(C) 107.7) of Xyl further supported this conclusion. The above evidence supported the location and sequence of the disaccharide chain as shown in *Fig. 1*. Compound **1** was therefore determined to be 3-[(6-deoxy-2-*O*-β-D-xylopyranosyl-α-L-mannopyranosyl)oxy]-5-hydroxy-2-(4-methoxyphenyl)-8,8-dimethyl-4*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]-dipyran-4-one, and named koreanoside A.

Compound **2** was obtained as yellow amorphous powder. The IR spectrum displayed absorption bands at 3440 (OH), 1658 (C=O), and 1609 (benzene ring), which were consistent with the structure of a flavonoid. The molecular formula $C_{26}H_{26}O_{11}$ was determined on the basis of HR-EI-MS (*m*/*z* 514.1463 (M^+)), in combination with ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*), indicating 14 degrees of unsaturation.

Detailed comparison of the 1D- and 2D-NMR spectra of **2** with those of 5-hydroxy-2″-(1-hydroxy-1-methylethyl)-3-methoxyfurane-(2″,3″:7,8)-flavone (literature name) [19] revealed that the compounds had the same substitution pattern in ring *A*, but different substitution modes in rings *B* and *C*. In the ^{1}H-NMR spectrum, a broad *singlet* at δ(H) 12.64 indicated the presence of a 5-OH group. A series of signals (δ(C) 97.0 (*d*, C(1″)), 164.0 (*s*, C(2″)), 67.2 (*s*, C(3″)), 28.6 (*q*, C(4″,5″)); δ(H) 6.99 (*s*, H–C(1″)), 1.53 (*s*, Me(4″,5″))) suggested the presence of a prenyl group. In the HMBC spectrum, the cross-peaks of Me(4″) and Me(5″) with C(2″) and C(3″) and of H–C(1″) with C(2″) further confirmed the above deduction. Furthermore, the O-bearing nature of C(2″) and C(7) (δ(C) 148.0) and the HMBCs of H–C(1″) with C(8) (δ(C) 108.8), indicated that the prenyl group was incorporated into a furan ring fused to C(7) and C(8). A H-atom at δ(H) 7.04 (*s*) was assigned as H–C(6) by means of the HMBCs of this signal with C(5) (δ(C) 156.9), C(8), and C(10) (δ(C) 106.7). In addition, an A_2B_2 coupling system at δ(H) 7.91 (*d*, $J = 8.8$, H–C(2′,6′)) and 6.96 (*d*, $J = 8.8$, H–C(3′,5′)) together with the molecular formula of **2** suggested the presence of a *para*-OH substituted ring *B*. Furthermore, the H-atom signals at δ(H) 5.36 (*d*, $J = 1.4$, H–C(1) of Rha) and 0.80 (*d*, $J = 5.6$, Me(6) of Rha) indicated the presence of a Rha moiety. Acid hydrolysis of **2** afforded L-Rha as sugar residue, which was confirmed by GC analysis of its corresponding trimethylsilylated L-cysteine derivative. HMBCs of the anomeric H-atom of Rha with C(3) indicated that the sugar was linked to C(3). Thus, the structure of **2** was deduced as 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-5-hydroxy-8-(1-hydroxy-1-methylethyl)-2-(4-hydroxyphenyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one, and the compound was named koreanoside B.

Compound **3** was isolated as yellow amorphous powder. The HR-EI-MS revealed a peak at *m*/*z* 528.1647 (M^+), indicating a molecular formula of $C_{27}H_{28}O_{11}$, corresponding to 14 degrees of unsaturation. A detailed analysis of the 1D- (*Tables 1* and *2*) and 2D-NMR spectroscopic data of **3** revealed high structural similarity to **2**. The only

Table 2. *^{13}C NMR Data of Compounds* **1**–**5**. Arbitrary atom numbering indicated in *Fig. 1*; δ in ppm.

Position	**1**[a][b]	**2**[c][d]	**3**[c][d]	**4**[a][e]	**5**[a][b]
2	158.8	160.1	160.5	158.6	158.8
3	137.2	134.8	135.1	136.7	136.1
4	180.0	178.6	178.9	179.8	180.0
5	163.6	156.9	157.5	165.8	161.3
6	100.7	94.4	94.8	94.9	99.9
7	160.9	148.0	148.4	169.1	165.0
8	102.6	108.8	108.8	105.5	105.9
9	158.6	157.8	158.4	154.5	156.4
10	106.7	106.7	107.1	106.7	105.5
1′	123.8	120.0	120.2	123.7	124.3
2′, 6′	131.8	130.7	131.0	131.8	132.1
3′, 5′	115.4	115.4	115.6	115.3	115.3
4′	164.5	157.2	157.7	163.5	163.5
1″	115.4	97.0	101.6	80.2	30.3
2″	129.0	164.0	159.3	97.8	76.6
3″	79.5	67.2	72.8	71.6	148.9
4″	28.4	28.6	25.0	26.2	111.4
5″	30.6	28.6	25.0	24.4	17.8
Rha					
1	103.2	101.6	101.9	103.5	103.4
2	82.5	70.6	70.4	72.0	72.0
3	73.6	70.8	71.1	72.1	73.2
4	71.9	70.1	70.9	71.9	72.2
5	75.3	69.9	70.2	73.1	72.0
6	17.7	17.3	17.6	17.7	17.7
Xyl					
1	107.7				
2	73.6				
3	77.8				
4	71.0				
5	67.1				
4′-MeO	56.1			56.0	56.1
1″-MeO				56.5	
3″-MeO			50.4		

[a]) Measured in CD_3OD. [b]) Measured at 125 MHz. [c]) Measured in (D_6)DMSO. [d]) Measured at 150 MHz. [e]) Measured at 100 MHz.

observed difference was the replacement of the OH group at C(3″) in **2** by a MeO group (δ(C) 50.4 (*q*); δ(H) 2.98 (*s*)) in **3**, which was supported by a HMBC of the MeO H-atoms with C(3″) (δ(C) 72.8). Therefore, compound **3** is a methyl derivative of compound **2**. Its structure was elucidated to be 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-5-hydroxy-2-(4-hydroxyphenyl)-8-(1-methoxy-1-methylethyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one, and compound **3** was named koreanoside C.

Compound **4**, yellow amorphous powder, has the molecular formula $C_{28}H_{32}O_{12}$ as revealed by its HR-EI-MS (*m*/*z* 560.1896 (M^+)), with 13 degrees of unsaturation. The

IR spectrum exhibited absorption bands at 3396 (OH), 1659 (C=O), and 1596 (benzene ring) compatible with the structure of a flavonoid. Analysis of the trimethylsilylated L-cysteine derivative of the sugar residue obtained by acid hydrolysis of **4** proved the presence of L-Rha.

Careful comparison of the 1D- (*Tables 1* and *2*) and 2D-NMR data of **4** with those of **2** revealed that the two compounds were similar, except for missing one degree of unsaturation in the furan ring and the presence of two additional MeO groups in **4**. Signals for the unsaturated C=C bond (δ(C) 97.0 (*d*, C(1″)), 164.0 (*s*, C(2″)); δ(H) 6.99 (*s*, H–C(1″))) in the furan ring of **2** were replaced by the following signals in **4**, δ(C) 80.2 (*d*, C(1″)), 97.8 (*d*, C(2″)); δ(H) 5.29 (*d*, $J = 2.6$, H–C(1″)), 4.51 (*d*, $J = 2.6$, H–C(2″)), indicating a C–C bond in **4**. A series of HMBCs (*Fig. 3*) of Me(4″) (δ(H) 1.29 (*s*)) and Me(5″) (δ(H) 1.16 (*s*)) with C(2″) and C(3″) (δ(C) 71.6); H–C(2″) with C(1″), C(3″), C(4″) (δ(C) 26.2), C(5″) (δ(C) 24.4), and C(7) (δ(C) 169.1), as well as H–C(1″) with C(8) (δ(C) 105.5) and C(3″), further proved the above conclusion. The MeO H-atoms at δ(H) 3.51 showed a HMBC with C(1″), which indicated that C(1″) was substituted by a MeO group. Moreover, a HMBC cross-peak was observed between the second MeO group at δ(H) 3.89 and C(4′) at δ(C) 163.5, which suggested that the OH group on ring *B* of **2** was replaced by a MeO group in **4**.

Furthermore, the Rha sugar was assigned at C(3) on the basis of the HMBC of H–C(1) (δ(H) 5.42) of Rha with C(3) of the aglycone. Thus, the structure of **4** was unambiguously determined to be 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-8,9-dihydro-5-hydroxy-8-(1-hydroxy-1-methylethyl)-9-methoxy-2-(4-methoxyphenyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one, and compound **4** was named koreanoside D.

Compound **5** was obtained as yellow amorphous powder. The HR-EI-MS showed a *quasi*-molecular-ion peak at *m*/*z* 530.1795 (M^+), corresponding to the molecular formula $C_{27}H_{30}O_{11}$ with 13 degrees of unsaturation. In the ^{1}H-NMR spectrum (*Table 1*), a MeO group (δ(H) 3.89), an A_2B_2 coupling system (δ(H) 7.93 (*d*, $J = 8.7$, H–C(2′,6′)) and 7.09 (*d*, $J = 8.7$, H–C(3′,5′))), an aromatic H-atom *singlet* at δ(H) 6.25 (*s*, H–C(6)), a tertiary Me (δ(H) 1.71 (*s*, Me(5″))), a terminal C=C bond (δ(H) 4.67 and 4.73 (each *s*, CH_2(4″))), and an O-bearing CH (δ(H) 4.36 (*t*, $J = 5.7$, H–C(2″))) were observed. In addition, the H-atom signals at δ(H) 5.44 (*d*, $J = 1.2$, H–C(1) of Rha) and 0.85 (*d*, $J =$ 6.1, Me(6) of Rha) suggested the presence of a Rha moiety, which was further

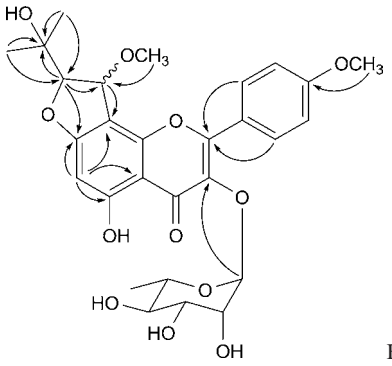

Fig. 3. *Key HMBCs of compound* **4**

confirmed by acid hydrolysis and GC analysis. In addition to signals belonging to a MeO group and the Rha residue, the ^{13}C-NMR and DEPT spectra (*Table 2*) of **5** showed resonances for 20 C-atoms: one tertiary Me (δ(C) 17.8 (*q*, C(5″)), one CH_2 (30.3 (*t*, C(1″))), one olefinic CH_2 (111.4 (*t*, C(4″))), six CH groups, including five aromatic and one O-bearing (δ(C) 76.6 (*d*, C(2″))), together with eleven sp^2 quaternary C-atoms, including one C=O (δ(C) 180.0 (*s*, C(4))) and six O-bearing ones (δ(C) 158.8 (C(2)), 136.1 (C(3)), 161.3 (C(5)), 165.0 (C(7)), 156.4 (C(9)), 163.5 (C(4′))), from which 15 C-atoms were assigned to a flavonoid skeleton, and the remaining five C-atoms were ascribed to a prenyl group. The prenyl group was located at C(8) (δ(C) 105.9) on the basis of HMBCs of CH_2(1″) (δ(H) 3.00 (*dd*, $J = 11.4$, 6.3) and 3.07 (*dd*, $J = 11.4$, 5.1)) with C(7), C(8), and C(9), as well as of H–C(2″) with C(8). A comparison of the 1D- and 2D-NMR spectroscopic data of **5** with those of 2″-hydroxy-3″-en-anhydroicaritin (literature name) [8] showed that the two compounds closely resembled each other, with the only difference being the appearance of a Rha unit in **5**. This sugar was linked to C(3), according to an HMBC of the anomeric H-atom (δ(H) 5.44) with C(3). Therefore, compound **5** was assigned as 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-5,7-dihydroxy-8-(2-hydroxy-3-methylbut-3-en-1-yl)-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one and named koreanoside E.

The anti-HIV-1 activities of compounds **2**, **7**, **8**, **11**, and **13** were evaluated using AZT as positive control, and the results are summarized in *Table 3*. Compound **11** showed moderate anti-HIV-1 activity with an EC_{50} value of 12.8 ± 3.27 μg/ml, whereas the remaining four tested compounds exhibited only weak activity. Compound **11** was toxic to MT4 cells with an IC_{50} value of 48.2 μg/ml. These results suggested that structural optimization of compound **11** is needed to improve both anti-HIV-1 potency and selectivity in the future.

Table 3. *Anti-HIV-1 Replication Activities of Compounds* **2**, **7**, **8**, **11**, *and* **13** *in HIV-1$_{NL4-3}$ Infected MT-4 Lymphocytes*

Compound	EC_{50} [μg/ml]	IC_{50} [μg/ml]
2	> 20	> 20
7	> 20	> 20
8	> 20	> 20
11	12.8 ± 3.27	48.2 ± 4.5
13	> 20	> 20
AZT	0.0092 ± 0.0025	> 1

In addition, to determine if the above compounds have anticancer potential, they were tested for cytotoxic activity against a panel of human cancer cell lines (A549, DU145, KB, and KB-VIN) with paclitaxel as positive control. However, all compounds were inactive at the highest concentration tested, 40 μg/ml.

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Experimental Part

General. TLC: Silica gel GF_{254} precoated plates (*Qingdao Marine Chemical and Industrial Factory*, P. R. China); visualization under UV light (254/365 nm) or by spraying with $FeCl_3$ soln., 10% H_2SO_4/EtOH followed by heating. Column chromatography (CC): *Sephadex LH-20* (*Amersham Biosciences AB*, Sweden), SiO_2 (200–300 mesh, *Qingdao Marine Chemical and Industrial Factory*, P. R. China), *Lichroprep RP-18* (43–63 μm, *Merck*, Germany), and *MCI* gel (*CHP 20P*, 75–150 μm, *Mitsubishi Chemical Corporation*, Tokyo, Japan). HPLC: *Agilent 1200* liquid chromatography using *Zorbax SB-C18* (5 μm, 9.4 mm × 250 mm) semi-prep. column or *Zorbax SB-C18* (4.6 mm × 250 mm) analysis column with MeOH/H_2O in gradient. GC: *Agilent HP5890* gas chromatograph with flame ionization detector (FID). IR Spectra: *Bio-Rad FtS-135* spectrophotometer in KBr pellets; $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker AM-400*, *DRX-500*, and *AVANCE III* instruments; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *API Qstar Pulsar* instrument; in *m*/*z*. HR-EI-MS (70 eV): *VG Auto Spec-3000* spectrometer; in *m*/*z*.

Plant Material. The aerial parts of *Epimedium koreanum* NAKAI were purchased in Jilin Province, P. R. China, in May 2011 and identified by Dr. *Haizhou Li*, Kunming University of Science and Technology. A voucher specimen (KMUST 20110501) has been deposited with the Laboratory of Phytochemistry, Faculty of Life Science and Technology. The raw materials were sliced and then heated at 180° in an oil bath for 10 min to give the heat-processed material.

Extraction and Isolation. The heat-processed *E. koreanum* NAKAI (10 kg) was extracted with 75% aq. acetone at r.t. and then concentrated under vacuum to yield an extract, which was successively extracted with petroleum ether, AcOEt, and BuOH, resp. The AcOEt layer (100 g) was subjected to CC (*Sephadex LH-20*, MeOH/H_2O 30, 60, 90, and 100%) to afford five fractions, *Frs. 1–5*. TLC Detection demonstrated that flavonoids were mainly concentrated in *Frs. 2–5*. *Fr. 2* (26 g) was further fractionated by CC (SiO_2; $CHCl_3$/MeOH gradient 20:1, 10:1, 8:1, and 4:1) to give four subfractions, *Frs. 2.1–2.4*. *Fr. 2.1* was separated over *Sephadex LH-20* ($CHCl_3$/MeOH 1:1), followed by semi-prep. HPLC (65% MeOH/H_2O, flow rate 3 ml/min) to yield compound **5** (6.5 mg, t_R 19.0 min). *Fr. 2.2* was successively purified through *RP-18* column (MeOH/H_2O, from 20 to 80% gradient), *Sephadex LH-20* ($CHCl_3$/MeOH 1:1), and semi-prep. HPLC (65% MeOH/H_2O, flow rate 3 ml/min) to give compounds **3** (6.0 mg, t_R 13.0 min) and **4** (4.8 mg, t_R 14.5 min). *Fr. 2.3* was repeatedly purified by *RP-18* and *Sephadex LH-20* (MeOH/H_2O 0:1 to 1:0 gradient), as well as CC (SiO_2; $CHCl_3$/MeOH/H_2O 10:1:0.05 to 8:2:0.2 gradient) to yield compounds **2** (5.5 mg) and **6** (78.2 mg). *Fr. 2.4* was successively subjected to *RP-18* (MeOH/H_2O 20 to 80% gradient) and *Sephadex LH-20* ($CHCl_3$/MeOH 1:2) to obtain compounds **7** (7.2 mg) and **8** (5.9 mg). Similarly, the purification of *Fr. 3* (3.5 g) by *RP-18* (MeOH/H_2O 20 to 80% gradient) and *Sephadex LH-20* ($CHCl_3$/MeOH 2:1) led to the isolation of compounds **9** (6.7 mg) and **10** (5.7 mg). *Fr. 4* (2.4 g) was subjected to CC (SiO_2; $CHCl_3$/MeOH 30:1 to 10:1 gradient) to give three subfractions, *Frs. 4.1–4.3*. Each subfraction was finally purified by *Sephadex LH-20* ($CHCl_3$/MeOH 2:1) to obtain compounds **11** (6.8 mg), **12** (3.8 mg), and **13** (4.3 mg). The BuOH fraction (50 g) was successively separated by *RP-18* column (MeOH/H_2O 10 to 70% gradient) and semi-prep. HPLC (80% MeOH/H_2O, flow rate 3 ml/min) to yield compound **1** (6.0 mg, t_R 8.0 min).

Koreanoside A (= *3-[(6-Deoxy-2-*O*-β-*D*-xylopyranosyl-α-*L*-mannopyranosyl)oxy]-5-hydroxy-2-(4-methoxyphenyl)-8,8-dimethyl-4*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-4-one*; **1**). Yellow amorphous powder. $[\alpha]_D^{23} = -71.2$ ($c = 0.14$, MeOH). IR (KBr): 3423, 2940, 1660, 1605, 1512, 1450. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 667 ($[M+Na]^+$), 1311 ($[2M+Na]^+$). HR-EI-MS: 644.2098 (M^+, $C_{32}H_{36}O_{14}^+$; calc. 644.2105).

Koreanoside B (= *3-[(6-Deoxy-α-*L*-mannopyranosyl)oxy]-5-hydroxy-8-(1-hydroxy-1-methylethyl)-2-(4-hydroxyphenyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one*; **2**). Yellow amorphous powder. $[\alpha]_D^{23} = -4.5$ ($c = 0.25$, MeOH). IR (KBr): 3440, 2926, 1658, 1609, 1441, 1352, 1281, 1177, 1155, 982. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (neg.): 514 (M^-), 549 ($[M+Cl]^-$). HR-EI-MS: 514.1463 (M^+, $C_{26}H_{26}O_{11}^+$; calc. 514.1475).

Koreanoside C (= *3-[(6-Deoxy-α-*L*-mannopyranosyl)oxy]-5-hydroxy-2-(4-hydroxyphenyl)-8-(1-methoxy-1-methylethyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one*; **3**). Yellow amorphous powder. $[\alpha]_D^{23} = -96.7$

($c = 0.09$, MeOH). IR (KBr): 3438, 2921, 1658, 1608, 1439, 1352, 1280, 985. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (neg.): 527 ($[M-H]^-$). HR-EI-MS: 528.1647 (M^+, $C_{27}H_{28}O_{11}^+$; calc. 528.1632).

Koreanoside D (= *3-[(6-Deoxy-α-*L*-mannopyranosyl)oxy]-8,9-dihydro-5-hydroxy-8-(1-hydroxy-1-methylethyl)-9-methoxy-2-(4-methoxyphenyl)-4*H*-furo[2,3-*h*]-1-benzopyran-4-one*; **4**). Yellow amorphous powder. $[\alpha]_D^{23} = -45.7$ ($c = 0.20$, MeOH). IR (KBr): 3396, 1659, 1596, 1511, 1482, 1396, 1363, 1255, 1181, 1155, 1131, 1054, 938. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (neg.): 596 ($[M + Cl]^-$). HR-EI-MS: 560.1896 (M^+, $C_{28}H_{32}O_{12}^+$; calc. 560.1894).

Koreanoside E (= *3-[(6-Deoxy-α-*L*-mannopyranosyl)oxy]-5,7-dihydroxy-8-(2-hydroxy-3-methylbut-3-en-1-yl)-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one*; **5**). Yellow amorphous powder. $[\alpha]_D^{23} = -96.2$ ($c = 0.15$, MeOH). IR (KBr): 3398, 3025, 2940, 1662, 1604, 1498, 890. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 554 ($[M + H + Na]^+$). HR-EI-MS: 530.1795 (M^+, $C_{27}H_{30}O_{11}^+$, calc. 530.1788).

Acid Hydrolysis and GC Analysis. Compounds **1**–**5** (1 mg, each) were hydrolyzed individually with 1M HCl (1 ml) at 90–100° in a screw-capped vial for 8 h. The mixture was filtered after being cooled to 2–4°, and the filtered liquor was evaporated to dryness under vacuum to obtain a residue. Then, the residue was dissolved in 0.5 ml of pyridine containing L-cysteine methyl ester (10 mg/ml, 1 ml) and reacted at 60° for 1 h. Then, a soln. (0.5 ml) of 1-(trimethylsilyl)-1*H*-imidazole in pyridine (10 mg/ml) was added to the above mixture, and it was heated at 60° for another 1 h. After centrifugation, the supernatant was directly analyzed by GC (*30QC2/AC-5* quartz cap. column (30 m × 0.32 mm)) with the following conditions: column temp. 180–280° at 3°/min, carrier gas N_2 (1 ml/min), injection and detector temp. 250°, injection volume 4 µl, split ratio 1:50. The standards were prepared following the same procedure. The absolute configuration of L-rhamnose in compounds **1**–**5** and D-xylose in compound **1** were determined by comparison of the retention times (14.9 and 13.3 min, resp.) of their hydrolysates with those of standard L-rhamnose and D-xylose.

HIV-1$_{NL4-3}$ Replication Inhibition Assay in MT-4 Lymphocytes. A previously described HIV-1 infectivity assay was used to assess the anti-HIV activity of the isolated flavonoids [20][21]. MT4 Cells were infected with NL4–3 at a multiplicity of infection (MOI) of 0.01 in the presence of various concentrations of the tested compounds. The compounds were dissolved in DMSO at 10 mg/ml and a series of 4-fold dilutions were prepared in cell culture medium starting at 20 and 50 µg/ml for anti-HIV-1 and cytotoxicity assays, resp. Azidothymidine (AZT, *Sigma–Aldrich*) and DMSO, diluted in the same manner as the compounds, were included in the assays as positive and negative controls, resp. Culture supernatants were collected on day 4 post infection for p24 assay using an *ELISA* kit from *ZeptoMetrix Corporation* (Buffalo, NY). The 50% inhibition concentration (EC_{50}) was defined as the concentration that inhibits HIV-1_{NL4-3} replication by 50%. A *CytoTox-Glo*™ cytotoxicity assay (Promega) was used to determine the cytotoxicity of the compounds to MT4 cells. MT4 Cells were cultured in the presence of various concentrations of the compounds for 4 d. Percent of viable cells was determined by following the protocol provided by the manufacturer. The 50% cytotoxic concentration (IC_{50}) was defined as the concentration that caused a 50% reduction of cell viability.

Cytotoxicity Analysis (*SRB Assay*). Cytotoxicity was determined by the sulforhodamine B (SRB) colorimetric assay according to our protocol [22]. Briefly, the cells (A549 (lung carcinoma), DU145 (prostate cancer), KB (epidermoid carcinoma of the nasopharynx), and KB-VIN (MDR line overexpressing P-glycoprotein), $3-5 \cdot 10^3$ cells/well) were seeded in 96-well plates filled with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) containing various concentrations of compounds, and incubated for 72 h. At the end of the exposure period, the attached cells were fixed with cold 50% CCl_3COOH for 30 min followed by staining with 0.04% SRB (*Sigma Chemical Co.*) for 30 min. The bound SRB was solubilized in 10 mM *Tris*-base, and the absorbance was measured at 515 nm on a *Microplate Reader ELx800* (*Bio-Tek Instruments*, Winnooski, VT) with a Gen5 software. All results were representative of three or more experiments.

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