Anti-human Immunodeficiency Virus-1 Constituents of the Bark of *Poncirus trifoliata*

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A total of 36 compounds (1—36) were obtained from the stem bark of *Poncirus trifoliata* including three new prenylated flavonoids, (−)-5,4′-dihydroxy-7,8-[(3″,4″-*cis*-dihydroxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (1), (−)-5,4′-dihydroxy-7,8-[(3″-hydroxy-4″-one)-2″,2″-dimethylpyrano]-flavone (2), and (−)-5,4′-dihydroxy-7,8-[(*cis*-3″-hydroxy-4″-ethoxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (3). The new structures were elucidated by means of spectroscopic methods. Compounds 1—20 were evaluated for their anti-human immunodeficiency virus-1 (HIV-1) activity, in which 2 showed significant anti-HIV-1 activity with high therapeutic index (TI) of 143.65.

Key words *Poncirus trifoliata*; chemical constituent; prenylated flavonoid; anti-human immunodeficiency virus-1 activity

Poncirus trifoliata (L.) Raf., a 1—5 m tree in the family Rutaceae, is widely distributed in P.R. China.[1)] Its dried immature fruit has been traditionally used for uterine contraction and relaxation, and treating gastrointestinal and cardiovascular diseases.[2)] The crude extract also has reported for anti-inflammatory, anti-bacterial and anti-mucin releasing activities.[3—5)] Previous study on the roots and fruits of this plant reported lots of coumarins,[6—12)] flavonoids,[2)] and nortriterpenoids.[13,14)] However, the chemical constituent of the stem bark of this plant was seldom reported. As part of searching for novel and bioactive compounds, now we report the result of a phytochemical investigation on the stem bark of *P. trifoliata*.

Results and Discussions

The chemical investigation on a MeOH extact of the stem bark of *P. trfoliata* led to the isolation of 36 compounds (**1**—**36**) including flavonoids, coumarins, triterpenoids, steroids, and lignans. Compounds **1**—**3** are new prenylated flavonoids which were elucidated as (−)-5,4′-dihydroxy-7,8-[(3″,4″-*cis*-dihydroxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (**1**), (−)-5,4′-dihydroxy-7,8-[(3″-hydroxy-4″-one)-2″,2″-dimethylpyrano]-flavone (**2**), and (−)-5,4′-dihydroxy-7,8-[(*cis*-3″-hydroxy-4″-ethoxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (**3**) by means of spectroscopic methods. Compounds **4**—**20** were identified as atalantoflavone (**4**),[15)] citflavanone (**5**),[16)] 4′,5,7-trihydroxyflavanone (**6**),[17)] 5-methyl-tovoxanthone (**7**),[18)] auraptene (**8**),[19)] anisocoumarin B (**9**),[20)] seselin (**10**),[21)] xanthyletin (**11**),[22)] isoangenomalin (**12**),[23)] 3-(1,1-dimethylallyl)-8-hydroxy-7-methoxycoumarin (**13**),[24)] poncitrin (**14**),[25)] clausrin (**15**),[26)] 7,8-dihydrofurocoumarin (**16**),[27)] 5,7-dimethoxycoumarin (**17**),[28)] 6,8-dimethoxycoumarin (**18**),[28)] limonin (**19**),[29)] syringaresinol (**20**),[30)] by comparison with data in the literature. Compounds **21**—**36** were common triterpenoids and steroids which were identified as oleanan-3β-ol (**21**), 3-acetyl-β-amyrin (**22**), oleanolic acid (**23**), α-amyrin (**24**), ursolic acid (**25**), neoilexonol (**26**), 12-lupen-3-ol (**27**), betulinic acid (**28**), 3-acetyl-betulinic acid (**29**), 3-hydroxy-20(30)-lupen-29-al (**30**), wallichenol (**31**), β-sitosterol (**32**), stigmasta-5,22-dien-3-ol (**33**), stigmast-5-ene-3,4-diol (**34**), stigmast-5-ene-3,7-diol (**35**), β-sitosterol palmitate (**36**) by comparison 1D-NMR spectra and *Rf* values in TLC plates with standard compounds deposited in the laboratory.

Compound **1** was obtained as a yellow powder. The UV absorption bands at 333, 271, 216, and 202 nm suggested that **1** had a flavonoid skeleton with hydroxy substitutes at C-4′ and C-5,[15)] while the IR absorption bands at 3426 and 1656 cm^{-1} also suggested the existence of hydroxy and carbonyl groups. The molecular formula $C_{20}H_{18}O_7$ was established by the negative HR-electrospray ionization (ESI)-MS at *m/z*: 369.0973 $[M-H]^+$ (Calcd 369.0974). The ^{13}C-NMR spectrum displayed 20 carbon resonances ascribable to two methyls (δ_C 21.3, 26.4), eight methines (δ_C 60.5, 71.1, 98.8, 103.5, 115.9×2, 128.8×2), and ten quaternary carbons (δ_C 79.4, 102.8, 104.7, 121.2, 155.8, 158.7, 160.3, 161.2, 163.8, 181.9) (Table 1). Of them, five carbon signals at δ_C 21.3 (q), 26.4 (q), 60.5 (d), 71.1 (d), and 79.4 (s) constructed a prenyl group on the basis of heteronuclear multiple bond correlation (HMBC) correlations (Fig. 1). Subsequently, a 2″,2″-dimethyldihydropyran moiety with hydroxy substitutes at C-3″ and C-4″ was established according to the 2D-NMR data and MS analysis (Fig. 1). In the ^{1}H-NMR spectrum (Table 1), two singlets at δ_H 1.35 (3H) and 1.37 (3H) were ascribed to protons of two methyls. Two doublets at δ_H 6.89 (2H, d, J=8.5 Hz) and 8.02 (2H, d, J=8.5 Hz) were ascribed to aromatic protons of ring B. A downfield singlet at δ_H 12.95 (1H) was ascribed to the proton of OH-5 due to the hydrogen bond formed between OH-5 and the carbonyl group at C-4. A singlet at δ_H 10.38 (1H) was assigned to OH-4′. The HMBC correlation of δ_H 12.95 (1H, s, OH-5) with δ_C 160.3 (s), 98.8 (d), and 104.7 (s) ascribed these three carbon resonances to C-5, C-6, and C-4a, respectively, while the HMBC correlation of δ_H 10.38 (1H, s, OH-4′) with δ_C 161.2 (s) ascribed this carbon resonance to C-4′. According to heteronuclear single quantum coherence (HSQC) spectrum, signals at δ_H 6.11 (1H, s, H-6) and 4.95 (1H, dd, J=7.0, 5.0 Hz, H-4″) were ascribed to H-6 and H-4″. In the HMBC spectrum, the correlations of δ_H 6.11 (1H, s, H-6)/δ_C 158.7 (s, C-7), δ_H

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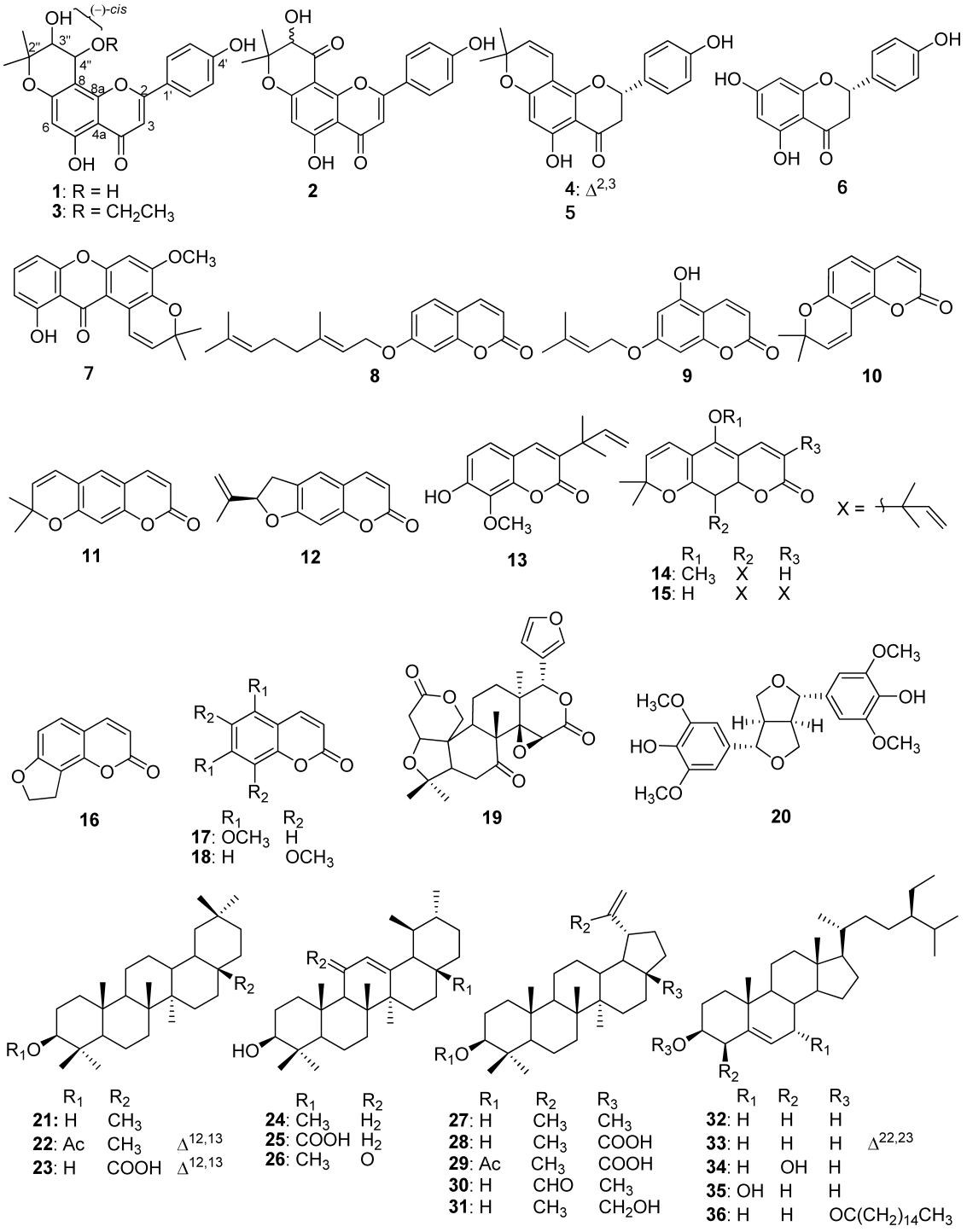

4.95 (1H, dd, J=7.0, 5.0 Hz, H-4″)/δ_C 158.7 (s, C-7), and δ_H 4.95 (1H, dd, J=7.0, 5.0 Hz, H-4″)/δ_C 155.8 (s, C-8a) suggested the ^{13}C-NMR signals at δ_C 158.7 and 155.8 should be assigned to C-7 and C-8a, respectively. A singlet at δ_H 6.86 (1H) were assigned to the protons of H-3 which showed key HMBC correlations with δ_C 163.8 (s, C-2) and 181.9 (s, C-4) (Fig. 1). These evidence suggested that **1** was similar to that of atalantoflavone (**4**)[15] except for the change at C-3″ and C-4″, as elucidated above. A known flavonoid 5,4′-dihydroxy-(3″,4″-dihydro-3″,4″-dihydroxy)-2″,2″-dimethylpyrano-(5″,6″:7,8)-flavone with the same planar structure was previously isolated from *Retama raetam* and *Rodgersia sambucifolia*[31,32] but without establishment of configuration at stereogenic centers. In addition, some significant differences of ^{1}H- and ^{13}C-NMR data (in particular with data of CH-3″ and CH-4″) suggested the different configuration of stereogenic centers between **1** and 5,4′-dihydroxy-(3″,4″-dihydro-3″,4″-dihydroxy)-2″,2″-dimethylpyrano-(5″,6″:7,8)-flavone. The rotating frame Overhauser enhancement spectroscopy (ROESY) correlation between H-3″ and H-4″, as well as the coupling constant of $J_{3'',4''}$=5.0 Hz,[33] suggested both H-3″ and H-4″ to be the same side. The absolute configuration was not elucidated due to the limited amount available. Detailed analysis of 2D-NMR (HMBC, ROESY) and MS data established the

Table 1. ^{1}H- and ^{13}C-NMR Data of **1**—**3** (δ in ppm, J in Hz)

No.	**1**[a]		**2**[b]		**3**[c]	
	H, J (Hz)	C	H, J (Hz)	C	H, J (Hz)	C
2	—	163.8 s	—	165.9 s	—	165.3 s
3	6.86 s	103.5 d	7.11 s	104.0 d	6.67 s	104.7 d
4	—	181.9 s	—	182.5 s	—	183.1 s
4a	—	104.7 s	—	106.0 s	—	106.0 s
5	—	160.3 s	—	157.0 s	—	157.3 s
6	6.11 s	98.8 d	6.48 s	100.2 d	6.11 s	100.0 d
7	—	158.7 s	—	167.4 s	—	160.3 s
8	—	102.8 s	—	102.1 s	—	102.5 s
8a	—	155.8 s	—	165.3 s	—	162.4 s
1′	—	121.2 s	—	121.7 s	—	123.4 s
2′	8.02 d (8.5)	128.8 d	8.48 d (8.5)	129.8 d	7.98 d (8.5)	129.4 d
3′	6.89 d (8.5)	115.9 d	7.32 d (8.5)	117.0 d	7.04 d (8.5)	116.8 d
4′	—	161.2 s	—	163.4 s	—	161.9 s
5′	6.89 d (8.5)	115.9 d	7.32 d (8.5)	117.0 d	7.04 d (8.5)	116.8 d
6′	8.02 d (8.5)	128.8 d	8.48 d (8.5)	129.8 d	7.98 d (8.5)	129.4 d
2″	—	79.4 s	—	84.4 s	—	80.2 s
3″	3.61 dd (6.0, 5.0)	71.1 d	4.65 s	77.2 d	3.96 dd (5.8, 4.5)	72.0 d
4″	4.95 dd (7.0, 5.0)	60.5 d	—	190.2 s	4.98 d (4.5)	70.7 d
Me-2″a	1.35 s	26.4 q	1.69 s	26.1 q	1.49 s	26.4 q
Me-2″b	1.37 s	21.3 q	1.53 s	19.1 q	1.40 s	22.4 q
OH-3″	5.20 d (6.0)	—	—	—	4.15 d (5.8)	—
OH-4″	5.24 d (7.0)	—	—	—		—
OH-5	12.95 s	—	—	—	12.99 s	—
OH-4′	10.38 br s	—	—	—	9.37 br s	—
O$\underline{CH_2}CH_3$	—	—	—	—	3.88 q (5.9)	67.6 t
					4.04 q (5.9)	
$OCH_2\underline{CH_3}$	—	—	—	—	1.10 t (5.9)	16.0 q

a) At 500 and 125 MHz, resp., in DMSO-d_6. *b*) At 500 and 125 MHz, resp., in pyridine-d_5. *c*) At 500 and 125 MHz, resp., in Me_2CO-d_6.

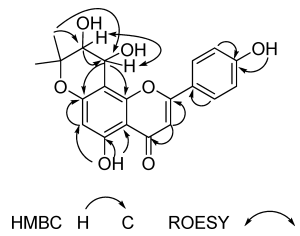

Fig. 1. Key 2D NMR Correlations of **1**

structure of **1** to be (−)-5,4′-dihydroxy-7,8-[(3″,4″-*cis*-dihydroxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone, as shown.

Compound **2** was deduced to have a molecular formula of $C_{20}H_{16}O_7$ by the HR-ESI-MS at m/z: 369.0982 $[M+H]^+$ (Calcd 369.0974). The UV absorption bands at 321, 284, 269, 226 and 211 nm suggested a flavonoid skeleton with hydroxyl substitutes at C-5 and C-4′.[15] The ^{13}C-NMR displayed 20 carbon resonances (Table 1), two methyls (δ_C 19.1, 26.1), seven methines (δ_C 77.2, 100.2, 104.0, 117.0, 117.0, 129.8, 129.8), and eleven quaternary carbons (δ_C 84.4, 102.1, 106.0, 121.7, 157.0, 163.4, 165.3, 165.9, 167.4, 182.5, 190.2). In the ^{1}H-NMR spectrum (Table 1), two doublets at δ_H 8.48 (2H, d, J=8.5 Hz) and 7.33 (2H, d, J=8.5 Hz) could be assigned to protons at 2′,6′ and 3′,5′, respectively. Two singlets at δ_H 7.11 and 6.48 were assigned to the protons of H-3 and H-6, respectively, as revealed by HMBC correlations of H-3 with δ_C 165.9 (s, C-2) and 182.5 (s, C-4) and of H-6 with δ_C 157.0 (s, C-5) and 167.4 (s, C-7). The above data suggested that compound **2** was similar to **1** except C-4″ was oxidated to be a carbonyl (δ_C 190.2) in **2**, as revealed by the key HMBC correlation of δ_H 1.69 (Me-2″a) with δ_C 77.2 and the weak HMBC correlation of δ_H 1.69 (Me-2″a) with δ_C 190.2. The absolute configuration was not elucidated due to the limited amount available. Detailed analysis of HMBC spectrum (Fig. 1) established the structure of **2** to be (−)-5,4′-dihydroxy-7,8-[(3″-hydroxy-4″-one)-2″,2″-dimethylpyrano]-flavone (**2**), as shown.

Compound **3** possessed a molecular formula $C_{22}H_{22}O_7$ as revealed by the HR-ESI-MS (m/z: 399.1440 $[M+H]^+$ (Calcd 399.1443). The UV spectrum at 329, 303, 285, 274 and 217 nm suggested a flavonoid skeletion. The ^{1}H-NMR spectrum (Table 1) revealed a pattern characteristic of a substituted 2″,2″-dimethyldihydropyran unit by two singlets at δ_H 1.40 (3H, s) and 1.49 (3H, s) assignable to C-2″ and two downfield shift at δ_H 3.97 (1H, t, J=4.6 Hz) and 4.99 (1H, d, J=4.2 Hz) assignable to H-3″ and H-4″, respectively. The ^{1}H- and ^{13}C-NMR data (Table 1) revealed that **3** was closely related to **1**, except –OH at C-4″ in **1** was substituted by $-OCH_2CH_3$ [δ_H 3.87 and 4.04 (each 1H, q, J=7.0 Hz), δ_C 67.6 (t); δ_H 1.10 (3H, t, J=6.0 Hz), δ_C 16.0 (q)] in **3**, as revealed by HMBC correlations of δ_H 3.87 and 4.04 with δ_C 70.7 (d, C-4″) (Fig. 1). The ROESY correlation between H-3″ and H-4″, as well as the coupling constant of $J_{3'',4''}$=4.5 Hz, suggested the stereoconfiguration was the same to that of **1**. The absolute configuration was not elucidated due the limited amount available. Detailed analysis of 2D-NMR data established the structure of **3** to be (−)-5,4′-dihydroxy-7,8-[(*cis*-3″-hydroxy-4″-ethoxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (**3**), as shown.

Compounds **1**—**20** were evaluated for their anti-human immunodeficiency virus-1 (HIV-1) activities (Table 2). Compounds **1**—**3**, **9**—**11**, **13**, and **17** showed anti-HIV-1 activities

Table 2. Anti-HIV-1_{IIIB} Activity of **1**—**20**

Compound	Cytotoxicity CC_{50} (μM)	Anti-HIV-1_{IIIB} activity EC_{50} (μM)	Therapeutic index (TI) CC_{50}/EC_{50}
1	387.1	8.6	45.19
2	136.6	0.9	143.65
3	149.5	6.7	22.11
4	246.8	27.9	8.83
5	210.8	30.1	7.0
6	378.2	203.3	1.86
7	>493.8	183.4	>2.69
8	217.8	59.7	3.62
9	813.0	18.3	>44.44
10	329.0	25.5	12.91
11	732.0	41.2	17.76
12	636.1	145.8	4.36
13	330.2	10.7	31.00
14	68.4	9.5	7.24
15	10.0	18.4	6.48
16	>1063.8	336.3	>3.16
17	417.8	33.8	12.36
18	>970.9	102.9	>7.36
19	>340.4	>340.4	Inactive
20	346.9	79.5	4.36
AZT	>749.1	0.0045	>163934

with therapeutic index (TI) of >10. It is noteworthy that compound **2** (EC_{50}, 0.35 μg/ml; CC_{50}, 50.28 μg/ml) showed significant anti-HIV-1 activity with high therapeutic index (TI) of 143.65. In addition, the result of anti-HIV-1 assay suggested that these prenylated flavonoids and coumarins could be considered as candidates of anti-HIV-1 agents.

Experimental

General Methods Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy using KBr pellets. 1D and 2D spectra were run on Bruker DRX-500 and AM-400 spectrometers with tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. HR-ESI-MS were performed on an API-Qstar-Pulsar-1 spectrometer. Column chromatography was performed on Silica gel (200—300 mesh, Qingdao Haiyang Chemical Co., Ltd., P. R. China) and RP-18 (20—45 μm, Fuji Silysia Chemical Ltd., Japan). Fractions were monitored by TLC (GF 254, Qingdao Haiyang Chemical Co., Ltd., P. R. China), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4.

Plant Material The stem bark of *P. trifoliata* was collected in Jingshan, Hubei Province, P. R. China, and identified by Dr. Chun-Xia Zeng. A voucher specimen (NO. FT20070810) has been deposited at Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

Extraction and Isolation The air-dried stem bark of *P. trifoliata* (10 kg) was powdered and extacted with MeOH at room temperature (24 h×3). After removal of the MeOH under reduced pressure, the viscous concentration was partitioned with EtOAc (4 l×4) to afford EtOAc and H_2O extract. The EtOAc fraction (208 g) was subjected to column chromatography (silica gel, 2.0 kg) eluted with a $CHCl_3$–Me_2CO gradient (1 : 0, 15 : 1, 10 : 1, 7 : 1, 5 : 1, 3 : 1, 1 : 1) to give seven fractions (1—7). Fraction 1 (29 g) was subjected to column chromatography (silica gel, 800 g; petroleum ether–Me_2CO, 20 : 1—8 : 1) to give **21** (800 mg), **22** (1.1 g), **24** (1.3 g), **27** (110 mg), **32** (2.1 g), and **33** (9 mg). Fraction 2 (40 g) was separated by column chromatography (silica gel, 1000 g; petroleum ether–Me_2CO, 10 : 1—2 : 1) to afford **8** (9 mg), **10** (17 mg), **11** (23 mg), **17** (4 mg), **18** (8 mg), **26** (110 mg), **30** (12 mg), **36** (120 mg). Fraction 3 (20 g) was subjected to column chromatography (silica gel, 450 g; $CHCl_3$–Me_2CO, 13 : 1) and further separated by column chromatography (RP-18, 180 g; MeOH–H_2O, 3 : 7—8 : 2) to afford **9** (4 mg), **13** (21 mg), **14** (18 mg), and **15** (27 mg). Fraction 4 (27 g) was separated by column chromatography (silica gel, 700 g) to obtain four subfractions (4a—4d). Fraction 4a (5 g) was further subjected to column chromatography (silica gel, 180 g; $CHCl_3$–Me_2CO, 10 : 1) to afford **16** (3 mg) and **23** (118 mg). Fraction 4b (3.5 g) was subjected to column chromatography (RP-18, 100 g; MeOH–H_2O, 3 : 7—8 : 2) to afford **25** (7 mg) and **28** (18 mg). Fraction 4d (7 g) was repeatedly separated by column chromatography (silica gel, 220 g; $CHCl_3$–Me_2CO, 8 : 1—4 : 1) to afford **20** (128 mg), **29** (88 mg), and a mixture (300 mg). The mixture was further purified by column chromatography (Sephadex LH-20, 100 g; MeOH) to afford **31** (28 mg). Fraction 5 (17 g) was subjected to column chromatography (silica gel, 550 g; $CHCl_3$–Me_2CO, 8 : 1—4 : 1) to afford **34** (11 mg), **35** (21 mg), and a mixture (240 mg). The mixture was purified by column chromatography (Sephadex LH-20, 100 g; MeOH) to afford **12** (6 mg). Fraction 6 (20 g) was subjected to column chromatography (RP-18, 400 g; MeOH–H_2O, 2 : 8—9 : 1) to afford **7** (228 mg), **19** (200 mg), **5** (38 mg), and a mixture (7 g). Compound **4** (1.8 g) precipitated from the mixture. Fraction 7 (24 g) was subjected to column chromatography (silica gel, 700 g; $CHCl_3$–MeOH, 12 : 1—5 : 1) and separated repeatedly by column chromatography (RP-18, 300 g; MeOH–H_2O, 2 : 8—4 : 6) to afford **2** (3 mg), **3** (4 mg), **1** (6 mg), and **6** (6 mg).

(−)-5,4′-Dihydroxy-7,8-[(3″,4″-*cis*-dihydroxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (**1**): Yellow powder. $[\alpha]_D^{22}$ −13.8° (c=0.12, MeOH). UV λ_{max} (MeOH) nm (log ε): 333 (4.14), 271 (4.09), 216 (4.37), 202 (4.38). IR (KBr) cm^{-1}: 3426, 2929, 1656, 1607, 1364, 1174, 1109, 837. ^{1}H- (500 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6) see Table 1. Negative HR-ESI-MS m/z: 369.0973 $[M-H]^+$ (Calcd for $C_{20}H_{18}O_7$−H: 369.0974).

(−)-5,4′-Dihydroxy-7,8-[(3″-hydroxy-4″-one)-2″,2″-dimethylpyrano]-flavone (**2**): Yellow powder. $[\alpha]_D^{22}$ −8.1° (c=0.12, MeOH). UV λ_{max} (MeOH) nm (log ε): 321 (4.04), 284 (3.98), 269 (3.97), 226 (4.05), 211 (4.02). IR (KBr) cm^{-1}: 3369, 3260, 1686, 1645, 1590, 1567, 1513, 1475, 1432, 1358, 1242, 1172, 1101, 840. ^{1}H- (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Table 1. Positive HR-ESI-MS m/z: 369.0982 $[M+H]^+$ (Calcd for $C_{20}H_{16}O_7$+H: 369.0974).

(−)-5,4′-Dihydroxy-7,8-[(*cis*-3″-hydroxy-4″-ethoxy-3″,4″-dihydro)-2″,2″-dimethylpyrano]-flavone (**3**): Yellow powder. $[\alpha]_D^{22}$ −24.3° (c=0.12, MeOH). UV λ_{max} (MeOH) nm (log ε): 329 (3.97), 303 (3.94), 285 (3.93), 274 (3.94), 217 (4.21). IR (KBr) cm^{-1}: 3434, 3152, 1659, 1602, 1555, 1492, 1357, 1284, 1241, 1174, 1120, 836. ^{1}H- (500 MHz, Me_2CO-d_6) and ^{13}C-NMR (125 MHz, Me_2CO-d_6) see Table 1. Positive HR-ESI-MS m/z: 399.1440 $[M+H]^+$ (Calcd for $C_{22}H_{22}O_7$+H: 399.1443).

***In Vitro* Anti-HIV-1 Assays** Compounds **1**—**20** were evaluated for their anti-HIV-1 activity by microtiter syncytium formation infectivity assay, using the method previously described, with 3′-azido-3′-deoxythymidine (AZT) as a positive control.[34,35] The assays included cytotoxicity (CC_{50}) in C8166 cell and inhibition (EC_{50}) of syncytium formation in HIV-1_{IIIB}-infected C8166 cell *in vitro*. The cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The absorbance at 595 nm/630 nm ($A_{595/630}$) was read in an enzyme-linked immunosorbent assay (ELISA) reader (Elx800, Bio-Tek Instrument Inc., U.S.A.). The minimum cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was determined from dose response curve. In the presence or absence of various concentrations of test compounds, 3×104 C8166 cells were exposed to HIV-1_{IIIB} at a multiplicity of infection (M.O.I.) of 0.01. The cells were incubated in 96-well plates or 24-well plates at 37 °C in 5% CO_2 for 3 d. AZT (3′-azido-3′-deoxythymidine, Sigma) was used as a positive control. At 3 d post-infection, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cells) in each well of 96-well plates under an inverted microscope, and HIV-1 p24 antigen in the culture supernatants was determined by an ELISA. The virus particles in the culture supernatant of each well of 96-well plates were precipitated by 30% PEG (Mw 8000), lysed with lysis buffer and the activity of reverse transcriptase (RT) was measured by a commercial RT kit (Roche Molecular Biochemicals). The minimum inhibitory concentrations that reduced CPE, HIV-1 p24 antigen and RT production by 50% (EC_{50}) were interpolated from plots generated from the data. The therapeutic index (TI) was calculated from the ratio of CC_{50}/EC_{50}.

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