Flavanone and Flavonol Glycosides from the Leaves of *Thevetia peruviana* and Their HIV-1 Reverse Transcriptase and HIV-1 Integrase Inhibitory Activities

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Two new flavanone glucosides, (2*R*)- and (2*S*)-5-*O*-β-D-glucopyranosyl-7,4′-dihydroxy-3′,5′-dimethoxyflavanone[pervianoside I (3), peruvianoside II (4)] and a new flavonol glycoside, quercetin 3-*O*-{β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside} (peruvianoside III, 13) were isolated from the leaves of *Thevetia peruviana* SCHUM., together with nine known flavonol glycosides and two known iridoid glucosides. The structures of all compounds were determined on the basis of chemical and spectroscopic methods. Their inhibitory effects against HIV-1 reverse transcriptase and HIV-1 integrase were also investigated.

Key words HIV-1; integrase; reverse transcriptase; *Thevetia peruviana*; flavanone glucoside; flavonol glycoside

The acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus and remains one of the leading causes a crisis worldwide in public health. HIV-1 encodes three enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN). HIV-1 PR processes viral proteins into functional enzymes and structural proteins. HIV-1 RT is the multifunctional enzyme that transcripts viral RNA into viral DNA which is important for viral replication, whereas IN is responsible for the integration of double strand DNA transcribed from viral RNA into the host chromosome. For HIV-1 PR, many inhibitors have been prepared chemically and used intensively for AIDS treatments. However, their use is limited due to the emergence of drug resistance and toxicity.[1] Thus, screening of natural products provides an opportunity for the discovery of HIV-1 IN and HIV-1 RT inhibitors.

Thevetia peruviana SCHUM. is an evergreen flowering shrub belonging to the Dogbane family, Apocynaceae. It grows widely throughout tropical and subtropical regions. Its generic name is yellow oleander.[2] The latex of the oleander is poisonous to man, animals and certain insects. Despite its toxicity, oleander has been used as an abortifacient, to treat congestive heart failure, malaria, leprosy, indigestion, ringworm, venereal disease and even as a suicide instrument.[3] The leaves have been reported to contain iridoid glycosides,[4] flavonoids,[5] triterpenes,[6] monoterpenes[7] and cardiac glycosides.[8]

From the screening of Thai medicinal plants against HIV-1, an ethanol (EtOH) extract of *T. peruviana* showed high anti HIV-1 (IC_{100}=1.56 μg/ml) and HIV-1 IN inhibitory activity (IC_{50}=12.0 μg/ml).[9] Therefore, the EtOH extract of this plant was fractionated further into *n*-hexane, chloroform ($CHCl_3$), and water (H_2O) soluble fractions. In this paper, we describe the structure elucidation of new compounds from the water fraction and the inhibitory activities against HIV-1 IN and HIV-1 RT-associated DNA polymerase [RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP)] of the isolated compounds.

Results and Discussion

An EtOH extract of the leaves of *T. peruviana* was concentrated and partitioned with *n*-hexane, $CHCl_3$ and H_2O. The H_2O soluble fraction was repeatedly chromatographed over normal and reversed-phase silica gel to afford two iridoid glucosides (**1**, **2**), two flavanone glucosides (**3**, **4**) and ten flavonol glycosides (**5**—**14**) (Chart 1). The known compounds were identified as theviridoside (**1**),[4] theveside (**2**),[4] quercetin 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**5**),[5] kaempferol 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**6**),[5] quercetin 3-*O*-[(6-*O*-sinapoyl)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**7**),[5] kaempferol 3-*O*-[(6-*O*-sinapoyl)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**8**),[5] quercetin 3-*O*-[(6-*O*-feruloyl)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**9**),[10] kaempferol 3-*O*-[(6-*O*-feruloyl)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**10**),[10] quercetin 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside] (**11**),[11] kaempferol 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside] (**12**),[12] and kaempferol 3-*O*-{β-D-glucopyranosyl-(1→2)-[α-L-rhamonopyranosyl-(1→6)]-β-D-galactopyranoside} (**14**).[13] Of these flavonol glycosides, five compounds (**9**, **10**, **11**, **12** and **14**) were isolated for the first time from this plant species. The structures of the new compounds were determined as follows:

Compound **3** (peruvianoside I), a white amorphous powder, showed quasi-molecular ion peaks $[M-H]^-$ at *m/z* 493 in the negative ion fast atom bombardment mass spectrum (FAB-MS) and $[M+H]^+$ at *m/z* 495 in the positive ion FAB-MS spectrum, suggesting the molecular formula of $C_{23}H_{25}O_{12}$ [high resolution (HR)-FAB-MS, *m/z*: 493.1317 $[M-H]^-$ (Calcd for $C_{23}H_{25}O_{12}$: 493.1346)]. Moreover, a fragment ion peak at *m/z* 331 $[M+H-glc]^+$ was also observed in both positive ion FAB-MS and electron impact (EI)-MS. The infrared (IR) spectrum showed the presence of a carbonyl group at 1652 cm^{-1}, and hydroxyl groups at 3448 cm^{-1}. Ultraviolet (UV) absorption bands at 210 (ε 35000), 283 (ε 15000) and 325 (sh) were similar to those of flavanones.[14] The ^{1}H-NMR spectrum of **3** exhibited signals

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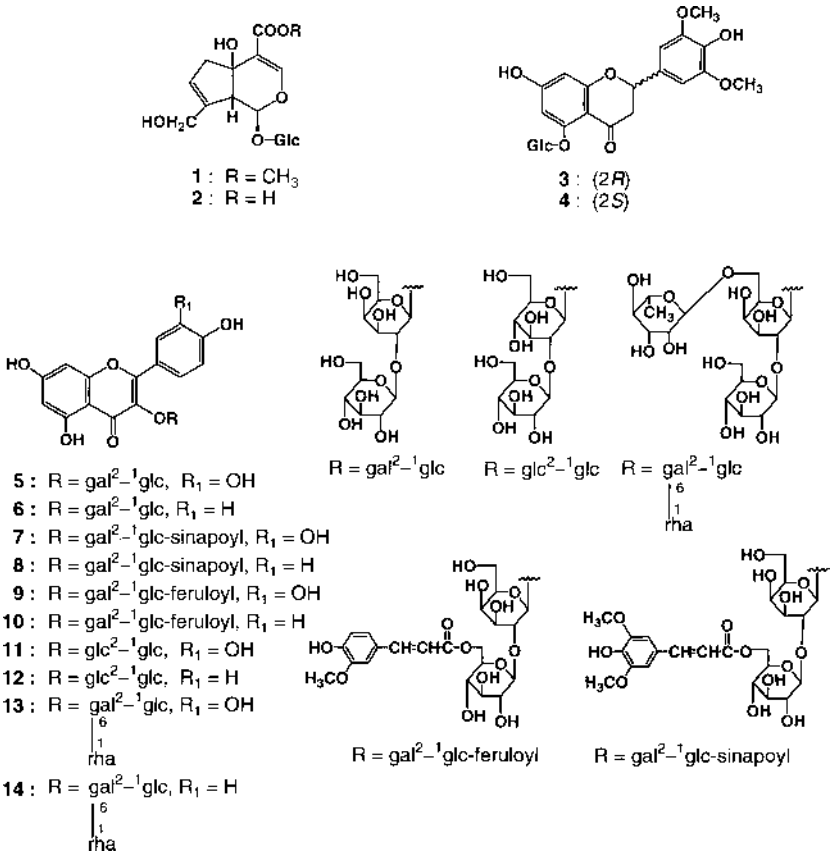

Chart 1. Structures of Compounds Isolated from *T. peruviana* (Leaves)

at δ 7.13 (d, J=2.2 Hz) and δ 6.62 (d, J=2.2 Hz), indicating the meta substitutions on the A-ring, whereas a singlet signal of two protons at δ 6.98 was assigned to H-2′ and H-6′ in the 3′,4′,5′-trisubstituted phenyl ring. Signals at δ 5.42 (dd, J=2.7, 13.2 Hz), δ 3.27 (dd, J=13.2, 17.6 Hz) and δ 2.84 (dd, J=2.7, 17.6 Hz) suggested a dihydropyrone skeleton of flavanone. A singlet signal of six protons at δ 3.81 indicated the presence of two methoxyl groups substituted on an aromatic ring. For the sugar moiety, a signal of an anomeric proton was observed at δ 5.47 (d, J=7.6 Hz), while other sugar protons exhibited multiplet signals ranging from δ 4.00 to 4.40.[15] On acid hydrolysis, **3** afforded D-glucose which was identified by co-thin layer chromatography (TLC) and gas chromatography (GC)-MS comparing with those of an authentic sample.[16] In the ^{13}C-NMR spectrum, signals at δ 80.2 and 46.3 were assigned to C-2 and C-3 of the dihydropyrone moiety,[14] whereas that at δ 56.5 was assigned to methoxyl groups (Table 1). The sugar linkage was determined by the heteronuclear multiple bond coherence (HMBC) experiment; a long-range coupling observed between a carbon signal at δ 165.0 (C-5) and a proton signal at δ 5.47 (glc-1) revealed glycosylation at C-5 with a glucose unit. This was also supported by the upfield shift of C-4 to δ 190.5, indicating the absence of intramolecular hydrogen bonding between a carbonyl group at C-4 and a hydroxyl group at C-5, thereby implying the linkage of glucose at C-5.[14] A carbon signal at δ 166.4 (C-7) was correlated with both signals at δ 6.62 (H-6) and δ 7.13 (H-8), whereas signals at δ 149.3 (C-3′ and C-5′) revealed the correlation with protons of methoxyl groups (6H, s, δ 3.80). The circular dichroism (CD) data ($[\theta]_{335}$ -10.61×10^3 and $[\theta]_{291}$ $+47.04\times10^3$) revealed the structure of *R*-configuration by comparison with a previous report based on the negative Cotton effect around 335 nm and the positive Cotton effect around 290 nm in the CD spectra.[17] From these findings, compound **3** was

Table 1. ^{13}C-NMR Spectral Data of Compounds **3** and **4** [δ (ppm) in Pyridine-d_5 (100 MHz)]

C	Compound	
	3	**4**
2	80.2	80.0
3	46.3	46.2
4	190.5	190.8
5	165.0	165.5
6	99.4	99.3
7	166.4	166.9
8	100.7	100.5
9	161.7	162.4
10	106.7	106.8
1′	138.2	138.2
2′,6′	105.3	105.3
3′,5′	149.3	149.2
4′	129.5	129.3
–OCH_3	56.5	56.4
Glc		
1	104.9	105.8
2	75.1	75.2
3	77.8	77.4
4	71.1	71.1
5	79.2	79.4
6	62.4	62.4

identified as (2*R*)-5-*O*-β-D-glucopyranosyl-7,4′-dihydroxy-3′,5′-dimethoxyflavanone.

Compound **4** (peruvianoside II) was obtained as a white amorphous powder. The spectroscopic data of **4** were similar to those of **3**. A quasi-molecular ion peak was observed at *m/z* 493 $[M-H]^-$ in the negative ion FAB-MS and $[M+H]^+$ at *m/z* 495 in the positive ion FAB-MS, consistent with the molecular formula of $C_{23}H_{25}O_{12}$ [HR-FAB-MS, *m/z*: 493.1310 $[M-H]^-$ (Calcd for $C_{23}H_{25}O_{12}$: 493.1347)]. A fragment ion peak at *m/z* 331 $[M+H-glc]^+$ was also observed in both positive ion FAB-MS and EI-MS. The IR spectrum showed the presence of a carbonyl group at 1654 cm^{-1} and hydroxy groups at 3448 cm^{-1}. The UV spectra showed absorption bands at 210 (ε 40000), 283 (ε 17000) and 325 (sh). Signals at δ 7.16 (d, J=2.2 Hz) and δ 6.62 (d, J=2.2 Hz) were assigned to H-8 and H-6, whereas a singlet signal at δ 6.97 (2H) was assignable to H-2′ and H-6′. Chemical shifts at δ 5.38 (dd, J=2.7, 13.4 Hz), δ 3.13 (dd, J=13.4, 17.6 Hz) and δ 2.88 (dd, J=2.7, 17.6 Hz) were responsible for H-2, H_{ax}-3 and H_{eq}-3, respectively. A singlet signal of two methoxyl protons at δ 3.82 (6H) was also observed. For the sugar moiety, a signal of an anomeric proton was observed at δ 5.39 (d, J=7.6 Hz), while other sugar protons appeared as multiplet signals at δ 4.01—4.41. The ^{13}C-NMR spectrum showed signals at δ 190.8, assignable to C-4 and at δ 80.0 and δ 46.2, due to the dihydropyrone moiety (C-2 and C-3). A singlet signal at δ 56.4 indicated the presence of methoxyl groups (C-3′ and C-5′). The sugar linkage was determined with the HMBC spectrum; significant cross peaks observed between a carbon signal at δ 165.5 (C-5) and a proton signal at δ 5.39 (glc-1) revealed 5-glycosylation. Moreover, a carbon signal at δ 166.9 (C-7) was also correlated with both H-6 (δ 6.62) and H-8 signals (δ 7.16), whereas signals of C-3′ and C-5′ (δ 149.2) indicated the correlation with a proton signal due to methoxyl groups (6H, s, δ 3.82). On acid hydrolysis, the sugar of **4** was identified as D-glucose. The CD data ($[\theta]_{335}$ $+22.02\times10^3$ and $[\theta]_{291}$ -34.98×10^3) indicated the structure of *S*-configuration at C-2 by comparison with the previous report.[17] From the above evidence, compound **4** was determined to be (2*S*)-5-*O*-β-D-glucopyranosyl-7,4′-dihydroxy-3′,5′-dimethoxyflavanone.

Compound **13** (peruvianoside III), a yellow amorphous powder, gave a quasi-molecular ion peak $[M-H]^-$ at *m/z* 771 in the negative ion FAB-MS and was assigned the formula $C_{33}H_{40}O_{21}$ [HR-FAB-MS *m/z*: 771.1982 $(M-H)^-$ (Calcd for $C_{33}H_{39}O_21$: 771.1984). Moreover, significant fragment ion peaks at *m/z* 593 $[M+H-glc]^+$ and 429 $[M+H-glc-rha]^+$ in the positive ion FAB-MS were also observed. The acid hydrolysis of **13** afforded D-galactose, D-glucose and L-rhamnose, which were identified by co-TLC and GC-MS comparing with authentic samples. The absolute configurations of sugars were determined to be D-galactose, D-glucose and L-rhamnose, respectively. The IR spectrum exhibited the presence of a carbonyl group at 1652 cm^{-1}, aromatic groups at 2922 cm^{-1} and hydroxyl groups at 3422 cm^{-1}. The UV absorption bands at 210 (ε 29000), 255 (ε 18000) and 355 (ε 14000) suggested it to be a flavonol analogue.[14] The ^{1}H-NMR spectrum of **13** showed characteristics of a quercetin moiety.[5] Doublet signals at δ 6.69 and 6.60 (J=1.9 Hz) were assigned to H-6 and H-8 on the A-ring. Signals at δ 8.36 (d, J=2.2 Hz) were referred to H-2′, whereas signals at δ 7.29 (d, J=8.6 Hz) and δ 8.06 (dd, J=2.2, 8.6 Hz) were assigned to H-5′ and H-6′, respectively. Three anomeric protons of the sugars were observed at δ 6.34 (1H, d, J=7.8 Hz), δ 5.41 (1H, d, J=7.3 Hz) and δ 5.11 (1H, br s), which were originated from those of β-galactose, β-glucose and α-rhamnose.[13] Signals of other sugar protons appeared at δ 3.75—4.89, while a signal at δ 1.42 (3H, d, J=5.8 Hz) was assignable to a methyl group of rhamnose. As shown in Table 2, a signal which shifted downfield of δ 82.6 was assigned to C-2 of galactose, while that of the downfield shift of δ 66.0 was assigned to C-6 of galactose. In the HMBC spectrum, significant cross peaks were observed between an anomeric proton signal of glucose (δ 5.41) and a C-2 signal of galactose (δ 82.6), along with a cross peak between an anomeric proton signal of rhamnose (δ 5.11) and a C-6 signal of galactose (δ 66.0). Thus, the structure of **13** was determined to be quercetin 3-*O*-{β-D-glucopyranosyl-(1→2)-[α-L-rhamonopyranosyl-(1→6)]-β-D-galactopyranoside}.

Table 2. ^{13}C-NMR Spectral Data of Compounds **13** and **14** [δ (ppm) in Pyridine-d_5 (100 MHz)]

C	Compound	
	13	**14**
2	157.3	157.1
3	134.6	134.3
4	178.9	178.8
5	162.9	162.8
6	99.7	99.5
7	165.6	165.7
8	94.4	94.3
9	157.5	157.5
10	105.2	105.5
1′	122.4	122.0
2′	116.3	131.9
3′	146.5	116.1
4′	150.5	161.6
5′	117.9	116.1
6′	122.7	131.9
Gal		
1	100.7	100.8
2	82.6	82.0
3	75.2	75.1
4	69.0	69.1
5	75.0	75.0
6	66.0	66.1
Glc		
1	106.2	106.8
2	76.0	75.8
3	78.3	78.6
4	71.1	71.2
5	78.3	78.3
6	62.2	62.2
Rha		
1	101.7	101.7
2	72.6	72.6
3	72.0	72.0
4	73.8	73.8
5	69.6	69.6
6	18.4	18.4

Of the isolated compounds from *T. peruviana*, compounds **7**, **9**, **11**, **14** and quercetin showed appreciable HIV-1 RDDP inhibitory activity with IC_{50} values of 33, 20, 41, 38 and 43 μM, respectively, whereas the others showed moderate or no activity. As to the structure-activity relationships, it was

Table 3. HIV-1 RT (RDDP and DDDP) and HIV-1 IN Inhibitory Activity of Compounds Isolated from *T. peruviana*

Compound	IC_{50} (μM)		
	RDDP	DDDP	IN
1	>100	>100	>100
2	>100	>100	>100
3	>100	>100	>100
4	>100	>100	>100
5	>100	>100	>100
6	>100	>100	59
7	33	69	7
8	>100	>100	30
9	20	42	5
10	52	>100	31
11	41	>100	45
12	75	>100	>100
13	>100	>100	>100
14	38	>100	43
15 (Quercetin)	43	>100	15
16 (Kaempferol)	>100	>100	40
Adriamycin (positive control)	27	6	
Suramin (positive control)			2.4

found that quercetin derivatives seemed to have relatively higher activity than kaempferol derivatives, as observed from the comparison of **7** [(IC_{50}=33, 69 μM) *vs.* **8** (IC_{50}>100 μM); **9** (IC_{50}=20, 42 μM) *vs.* **10** (IC_{50}=52, >100 μM)] for RDDP and DDDP inhibitory activities, respectively. Moreover, the compounds bearing a feruloyl moiety exhibited higher activity than those bearing a sinapoyl moiety [**7** (IC_{50}=33 μM) *vs.* **9** (IC_{50}=20 μM); **8** (IC_{50}>100 μM) *vs.* **10** (IC_{50}=52 μM)] (see Table 3).

In the case of HIV-1 IN inhibitory activity, compound **9** again showed the highest activity (IC_{50}=5 μM) among the isolated compounds, followed by **7**, **8**, **10**, **14**, **11** and **6** with IC_{50} values of 7, 30, 31, 43, 45 and 59 μM, respectively. Compounds possessing a feruloyl or sinapoyl group in the terminal glucose moiety showed more potent inhibitory activity than unsubstituted ones, when the IC_{50} values of **7** and **8** (or **9** and **10**) were compared with those of **5** and **6**. These compounds also showed higher inhibitory activity than their aglycones quercetin and kaempferol. Moreover, compounds that inhibited HIV-1 IN activity also possessed HIV-1 RDDP inhibitory activity, although they did not appreciably affect DDDP activity. Flavanone glucosides **3**, **4** and iridoid glucosides **1**, **2**, showed no activity at concentrations below 100 μM against either HIV-1 RT or HIV-1 IN (Table 3).

As regards the bioactivities of flavonol glycosides, quercetin and kaempferol 3-*O*-[(6-feruloyl)-glucopyranosyl-galactopyranoside] were reported to show neuroprotective activity in primary cultures of rat cortical cells damaged by L-glutamate,[18] whereas quercetin 3-*O*-[(6-feruloyl)-galactopyranoside] exhibited anti-superoxide production,[19] and anti-complement effects.[20] Feruloyl and sinapoyl glycosides showed radical scavenging activities against a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibitory activity against the oxidation of linoleic acid.[21] In 1998, Kim *et al.* reported that quercetin 3-*O*-(2,6-digalloyl)-galactopyranoside and quercetin 3-*O*-(2-galloyl)-arabinoside showed significant HIV-1 IN inhibitory activity with IC_{50} values of 24 and 18 μg/ml, respectively.[22] Naringenin and related compounds were previously reported to show antiherpetic activity by inhibition of the plaque formation of HSV-1 and HSV-2,[23] and also possessed the hepatoprotective activity.[24] In 1997, Min *et al.* reported that the dimethyl ethers of flavanones exhibited significant cytotoxic activity against a human leukemia cell line and a human lung carcinoma cell line.[25]

Experimental

General Melting points were measured on a Yanagimoto micro hot-stage melting point apparatus. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan). UV spectra were measured with a SHIMADZU UV-2200 (Shimadzu Co., Kyoto, Japan). CD spectra were measured with a JASCO J 805 spectropolarimeter (Jasco Co.) and IR spectra were measured with a JASCO FT/IR-230 infrared spectrometer. ^{1}H- and ^{13}C-NMR were measured with a JEOL-LA 400 Lambda (^{1}H, 400 MHz; ^{13}C, 100 MHz, JEOL Co., Tokyo) or a Varian UNITY 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz, Varian Co., California, U.S.A.) spectrometer. EI-MS were measured with a JEOL JMS-GC mate mass spectrometer at an ionization voltage of 70 eV (JEOL Co.). FAB-MS were obtained with a JEOL JMS-DX 300L spectrometer using glycerol as a matrix. HR-FAB-MS were measured with a JEOL JMS-700T mass spectrometer with a resolution of 5000 and glycerol containing a faint amount of NaCl. GC-MS was performed on a SHIMADZU GC-17A instrument and a JEOL automass system II. BIORAD, model 1250 immunowash (Biorad Co., California, U.S.A.); KUBOTA 6800, rotator (Kubota Co., Osaka, Japan); and ADVANTEC, CI-410 incubator (Advantech Co., Tokyo) were used for the multiplate integration assay.

Column chromatography was carried out on silica-gel BW-820 MH, ODS (Fuji Silysia Chemical Co., Nagoya, Japan), Diaion HP-20 (Mitsubishi Chemical Co., Tokyo), Sephadex LH-20 (Pharmacia Co., Tokyo, Japan) and Amberlite MB-3 (Organo Co., Tokyo). Preparative HPLC was carried out on a TOSOH CCPM-II system (Tosoh Co., Tokyo) equipped with a UV 8020 detector and a TSK gel ODS-80Ts column (21.5×300 mm, Tosoh Co., Tokyo). Analytical TLC was carried out on pre-coated silica-gel 60 F_{254} plates (0.25 mm, Merck Co., Darmstadt, Germany) and RP-18 F_{254} S plates (0.25 mm) and spots were detected under UV light or after spraying with anisaldehyde–5% H_2SO_4.

Plant Material The leaves of *T. peruviana* were collected at the Botanical Garden of the Department of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand in August, 2000. The voucher specimen (SKP 0132016) is deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Isolation of Compounds 1—14 from the Leaves of *T. peruviana* The dried powdered leaves (5.0 kg) were extracted with EtOH 6 times at room temperature. An EtOH extract was then concentrated and partitioned between MeOH and *n*-hexane, and the MeOH-soluble fraction was subsequently partitioned with $CHCl_3$ and H_2O. The respective fractions were evaporated to dryness *in vacuo* to give residues of 87.7, 51.5 and 98.6 g as hexane, $CHCl_3$ and H_2O soluble fractions. The H_2O fraction was chromatographed on Diaion HP-20 and eluted consecutively with water, 50% MeOH I, 50% MeOH II and 100% MeOH to give the respective fractions of 55.8, 2.2, 29.9 and 6.6 g after evaporation of solvent.

The fraction eluted with 50% MeOH I (1.1 g) was chromatographed on ODS (2.5×50 cm.) with MeOH–H_2O (1 : 5) to give 4 fractions (fr. I-1 to fr. I-4). Fraction I-2 (0.2 g) was further chromatographed on silica gel (2.0×50 cm) with a solvent system of $CHCl_3$–MeOH (2 : 1) to afford compound **2** (134 mg), whereas fraction I-4 gave compound **1** (164 mg).

The fraction eluted with 50% MeOH II (10.0 g) was passed through a silica gel column (5.0×60 cm) with $CHCl_3$–MeOH (4 : 1→1 : 1) to give 3 fractions (fr. II-1 to fr. II-3). Fr. II-2 yielded compound **1** (5.41 g), while fr. II-3 (2.4 g) was chromatographed further using a Sephadex LH-20 column (3.0×50 cm) with MeOH–$CHCl_3$ (9 : 1) to afford four fractions (fr. II-3a to fr. II-3d). Fraction II-3c (0.53 g) was subjected to prep. HPLC (40% MeOH in 0.1% TFA/H_2O, 5 ml/min, monitored at 254 nm) to give compounds **13** (10.8 mg), **14** (4.2 mg), mixture A (78.0 mg) and mixture B (39.1 mg). Mixture A was further chromatographed by prep. HPLC (13% CH_3CN in 0.1% TFA/H_2O, 5 ml/min, monitored at 254 nm) to obtain compounds **5** (9.7 mg) and **11** (11.4 mg). Meanwhile, mixture B (39.1 mg) was subjected to prep. HPLC using 13% CH_3CN in 0.1% TFA/H_2O as a mobile phase, to give compounds **6** (6.2 mg) and **12** (6.4 mg).

The fraction eluted with 100% MeOH (3.0 g) was passed through a Sephadex LH-20 column (4.0×50 cm) with MeOH–$CHCl_3$ (9 : 1) to give

four fractions (fr. I-IV). Fraction III (1.0 g) was further concentrated to give a yellowish liquid fraction (0.9 g) and white precipitates (50 mg). The precipitates were separated by prep. HPLC (20% CH_3CN in 0.1% TFA/H_2O) to afford compounds **3** (9.0 mg) and **4** (9.2 mg). On the other hand, the yellow solution (0.9 g) was applied to a column of RP-18 (MeOH–H_2O, 1 : 1, 2.5×50 cm) to give two fractions (fr. IIIa and fr. IIIb). Fraction IIIb (0.58 g) was subjected to prep. HPLC (40% MeOH in 0.1% TFA/H_2O) to obtain compounds **6** (20.8 mg), **7** (41.8 mg), **8** (61.6 mg), **9** (51.2 mg), **10** (57.0 mg) and **15** (5.0 mg).

(2*R*)-5-*O*-*β*-D-Glucopyranosyl-7,4′-dihydroxy-3′,5′-dimethoxyflavanone (**3**, peruvianoside I): White amorphous powder; mp: 194—196 °C; $[\alpha]_D^{25}$ −93° (*c*=0.02, MeOH); UV λ_{max}^{MeOH} nm (*ε*): 210 (35000), 283 (15000), 325 sh. IR (KBr) cm^{-1}: 3448, 2846, 2372, 1654, 1542, 1508, 1458 and 1115. Positive ion FAB-MS *m/z*: 495 $[M+H]^+$, 331 $[M+H-glc]^+$. Negative ion FAB-MS *m/z*: 493 $[M-H]^-$. HR-FAB-MS *m/z*: 493.1317 $[M-H]^-$ (Calcd for $C_{23}H_{25}O_{12}$: 493.1346). ^{1}H-NMR (400 MHz, pyridine-d_5) *δ*: 7.13 (1H, d, *J*=2.2 Hz, H-8), 6.98 (2H, s, H-2′, H-6′), 6.62 (1H, d, *J*=2.2 Hz, H-6), 5.47 (1H, d, *J*=7.6 Hz, anomeric proton of glucose), 5.42 (1H, dd, *J*=13.2, 2.7 Hz, H-2), 4.32—4.40 (5H, m, sugar protons), 4.00 (1H, m, H-5 of sugar), 3.81 (6H, s, –OCH_3), 3.27 (1H, dd, *J*=13.2, 17.6 Hz, H_{ax}-3), 2.88 (1H, dd, *J*=2.7, 17.6 Hz, H_{eq}-3). ^{13}C-NMR: Table 1. CD (*c*=0.02, MeOH): $[\theta]_{335}$ -10.61×10^3, $[\theta]_{291}$ $+47.04\times10^3$.

(2*S*)-5-*O*-*β*-D-Glucopyranosyl-7,4′-dihydroxy-3′,5′-dimethoxyflavanone (**4**, peruvianoside II): White amorphous powder; mp: 190—192 °C; $[\alpha]_D^{25}$ −127° (*c*=0.02, MeOH); UV λ_{max}^{MeOH} nm (*ε*): 210 (40000), 283 (17000), 325 sh. IR (KBr) cm^{-1}: 3448, 2920, 2846, 2344, 1654, 1560, 1508, 1458 and 1118. Positive ion FAB-MS *m/z*: 495 $[M+H]^+$, 331 $[M+H-glc]^+$. Negative ion FAB-MS *m/z*: 493 $[M-H]^-$. HR-FAB-MS *m/z*: 493.1310 $[M-H]^-$ (Calcd for $C_{23}H_{25}O_{12}$: 493.1347). CD (*c*=0.02, MeOH): $[\theta]_{335}$ $+22.02\times10^3$, $[\theta]_{291}$ -34.98×10^3. ^{1}H-NMR (400 MHz, pyridine-d_5) *δ*: 7.16 (1H, d, *J*=2.2 Hz, H-8), 6.97 (2H, s, H-2′, H-6′), 6.62 (1H, d, *J*=2.2 Hz, H-6), 5.39 (1H, d, *J*=7.6 Hz, anomeric proton of glucose), 5.38 (1H, dd, *J*=13.4, 2.7 Hz, H-2), 4.30—4.41 (5H, m, sugar protons), 4.01 (1H, m, H-5 of sugar), 3.82 (6H, s, –OCH_3), 3.13 (1H, dd, *J*=13.4, 17.6 Hz, H_{ax}-3), 2.88 (1H, dd, *J*=2.7, 17.6 Hz, H_{eq}-3). ^{13}C-NMR: Table 1.

Acid Hydrolysis of 3 and 4 Compounds **3** and **4** (1 mg each) were refluxed with 7% HCl in H_2O–EtOH (1 : 1, 1 ml) for 3 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The water layer was concentrated and checked by TLC [1: silica-gel, $CHCl_3$–MeOH–H_2O (65 : 40 : 10), *Rf*: D-glucose, 0.25; 2: silica-gel, EtOAc–MeOH–H_2O–AcOH (65 : 20 : 15 : 15), *Rf*: D-glucose, 0.40]. The residual water layer was desalted with Amberlite MB-3 and dried, then dissolved in pyridine (0.1 ml), and treated with 0.1 M L-cysteine methyl ester hydrochloride in pyridine (0.2 ml). The mixture was kept at 60 °C for 1.5 h. After the solvent was evaporated under reduced pressure, the residue was trimethylsilylated with hexamethyl disilazane-trimethyl chlorosilane (HMDS-TMCS) (0.1 ml) at 60 °C for 1 h. The mixture was partitioned between hexane and H_2O (0.3 ml each) and the hexane layer was analyzed by GC-MS (column, DB-1, J & W Scientific, 0.25 mm i.d×30 m; column temperature, 50—230 °C, 15 °C/min then 230 °C, 18 min; carrier gas, He). The sugar derivatives thus obtained showed a retention time of 22.26 min, identical with that of authentic D-glucose. Under the same conditions, a derivative of L-glucose showed a retention time of 23.26 min.

Quercetin 3-*O*-{*β*-D-glucopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-Galactopyranoside} (**13**, peruvianoside III): Yellow amorphous powder; mp: 205—207 °C; $[\alpha]_D^{25}$ +106° (*c*=0.05, MeOH); UV λ_{max}^{MeOH} nm (*ε*): 210 (29000), 255 (18000) and 355 (14000). IR (KBr) cm^{-1}: 3422, 2922, 1654, 1542, 1509, 1457 and 1205. Positive ion FAB-MS *m/z*: 593 $[M+H-glc]^+$, 429 $[M+H-glc-rha]^+$. Negative ion FAB-MS *m/z*: 771 $[M-H]^-$. HR-FAB-MS *m/z*: 771.1982 $[M-H]^-$ (Calcd for $C_{33}H_{39}O_{21}$: 771.1984). ^{1}H-NMR (400 MHz, pyridine-d_5) *δ*: 8.36 (1H, d, *J*=2.2 Hz, H-2′), 8.06 (1H, dd, *J*=2.2, 8.6 Hz, H-6′), 7.29 (1H, d, *J*=8.6 Hz, H-5′), 6.69 (1H, d, *J*=1.9 Hz, H-6), 6.60 (1H, d, *J*=1.9 Hz, H-8), 6.34 (1H, d, *J*=7.8 Hz, anomeric proton of galactose), 5.41 (1H, d, *J*=7.3 Hz, anomeric proton of glucose), 5.11 (1H, br s, anomeric proton of rhamnose), 3.75—4.89 (m, sugar protons), 1.42 (3H, d, *J*=5.8 Hz, methyl of rhamnose). ^{13}C-NMR data: see Table 2.

Acid Hydrolysis of 13 Compound **13** (1 mg) was refluxed with 7% HCl in H_2O–EtOH (1 : 1, 1 ml) for 3 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The water layer was concentrated and checked by TLC [system 1: silica-gel, $CHCl_3$–MeOH–H_2O (65 : 40 : 10), *Rf*: D-galactose, 0.20; D-glucose, 0.25; L-rhamnose, 0.45; system 2: silica-gel, EtOAc–MeOH–H_2O–AcOH (65 : 20 : 15 : 15), *Rf*: D-galactose, 0.35; D-glucose, 0.40; L-rhamnose, 0.56]. The residual water layer was desalted with Amberlite MB-3, treated with L-cysteine methyl ester hydrochloride and trimethylsilylated with HMDS-TMCS, then analyzed by GC-MS under the same conditions as described for **3** and **4**. The sugar derivatives were identified as D-galactose, D-glucose and L-rhamnose with the retention times of 23.02, 22.26 and 20.18 min, respectively, which were identical to the respective retention times of authentic samples. Under the same conditions, derivatives of L-galactose and L-glucose showed the retention times of 24.12 and 23.26 min, respectively. Although a derivative of D-rhamnose was not compared, Hara and his co-workers reported that L- and D-rhamnose derivatives showed significantly different retention times under the same conditions.[16]

RDDP Inhibitory Activity Assay For the assay of RDDP inhibitory activity,[26,27] HIV-1 RT was adjusted to 0.01 U/*μ*l with a solution of 0.2 M phosphate buffer (pH 7.2), 50% glycerol, 2 mM dithiothreitol (DTT) and 0.02% of Triton X-100. A reaction mixture (20 *μ*l) containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.25 *μ*g/ml (*ca.* 16 nM) poly(rA).oligo$(dT)_{12-18}$ as a template primer, 250 nM dTTP, 100 nM [methyl-^{3}H]dTTP (18.5 MBq/ml), 0.01 U/*μ*l of RT, and 1.0 *μ*l of a test compound dissolved in dimethyl sulfoxide (DMSO) (final concentration of 5%) was incubated at 37 °C for 1 h. A control reaction was done under the same conditions without adding the test compound. The reaction was terminated by the addition of 20 *μ*l of 0.02 M EDTA. The resulting mixture was applied to a Whatman DE 81 paper disc and washed batchwise with 3 ml of 5% Na_2HPO_4, distilled water 3 times, ethanol once and ether once. The paper disc was then dried and immersed in 3 ml of scintillation fluid. The amount of polymer fraction, including ^{3}H-labeled residues, was determined by counting the radioactivity on the paper disc. The calculation of the inhibitory potency for the tested compound was done as follows:

$$\text{inhibition (\%)} = [1-(\text{dpm}_{\text{comp.}}/\text{dpm}_{\text{contl.}})]\times100$$

Adriamycin (Sigma-Aldrich Co., Tokyo) was used as a positive control, and inhibited RDDP activity with an IC_{50} value of 27 *μ*M under the above conditions.

DDDP Inhibitory Activity Assay For the assay of DDDP inhibitory activity, HIV-1 RT was adjusted to 0.1 U/*μ*l with a solution of 0.2 M phosphate buffer (pH 7.2), 50% glycerol, 2 mM DTT and 0.02% of Triton X-100. A reaction mixture (20 *μ*l) containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.25 *μ*g/ml (*ca.* 16 nM) poly(dA).oligo$(dT)_{12-18}$ as a template primer, 250 nM dTTP, 200 nM [methyl-^{3}H]dTTP (18.5 MBq/ml), 0.1 U/*μ*l of RT, and 1.0 *μ*l of a test compound dissolved in DMSO (final concentration of 5%) was incubated at 37 °C for 1 h. A control reaction was done under the same conditions without adding the test compound. The reaction was terminated by the addition of 20 *μ*l of 0.02 M EDTA. The resulting mixture was applied to a Whatman DE 81 paper disc and washed in a similar manner as described for RDDP. The amount of a polymer fraction, including ^{3}H-labeled residues, was determined by counting the radioactivity on the paper disc. Calculation of the inhibitory potency for the tested compound was done as follows:

$$\text{inhibition (\%)} = [1-(\text{dpm}_{\text{comp.}}/\text{dpm}_{\text{contl.}})]\times100$$

Adriamycin was used as a positive control, and inhibited DDDP activity with an IC_{50} value of 6 *μ*M under the above conditions.

HIV-1 IN Inhibitory Activity Assay. a) HIV-1 IN HIV-1 IN protein was expressed in *Escherichia coli* and purified, and was stored at −80 °C before use.

b) Oligonucleotide Substrates Oligonucleotides of long terminal repeat (LTR) donor DNA and substrate DNA were purchased from Japan Bioservice (JBios) (Asaka city, Japan) and stored at −25 °C before use. The sequences of the biotinylated LTR donor DNA were 5′-biotin-ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG T-3′ and its unlabelled complement 3′-GAA AAT CAG TCA CAC CTT TTA GAG ATC GTC A-5′, and those of the substrate DNA (digoxigenin-labelled target DNA) were 5′-TGA CCA AGG GCT AAT TCA CT-digoxigenin and its 3′-labelled complement, digoxigenin-ACT GGT TCC CGA TTA AGT GA-5′.

c) Multiplate Integration Assay (MIA) Procedure This assay using a 96-well plate was basically similar to that reported by Hazuda *et al.*[28] First, a 96-well plate was coated with 50 *μ*l of a streptavidin solution containing 40 *μ*g/ml streptavidin, 90 mM Na_2CO_3 and 10 mM KCl. Fifty microliters of a biotinylated LTR donor DNA solution containing 10 mM Tris–HCl (pH 8.0), 1 mM NaCl and 40 fmol/*μ*l of LTR donor DNA were added to each well, and the plate was shaken gently at room temperature for 30 min, then washed with phosphate buffer saline (PBS) (pH 7.3), 4 times. A mixture (45 *μ*l) composed of 12 *μ*l of 5×IN buffer [containing 150 mM 3-(*N*-morpholino)-propane sulfonic acid (MOPS) (pH 7.2), 75 mM $MnCl_2$, 5 mM DTT, 25% glycerol and 500 *μ*g/ml bovine serum albumin], 1 *μ*l of 5 pmol/*μ*l digoxigenin-labelled target DNA and 32 *μ*l of sterilized water was added to each well. Subsequently, 6 *μ*l of a sample solution and 9 *μ*l of a 1/10 dilution of

integrase enzyme were added to the plate and the mixture was incubated at 37 °C for 80 min. The wells were washed with PBS 4 times, then 100 μl of 500 mU/ml alkaline phosphatase (AP) labelled anti-digoxigenin antibody was added and incubation followed at 37 °C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS 4 times and with PBS 4 times. Then, AP buffer (150 μl) containing 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM *p*-nitrophenyl phosphate was added to each well and they were incubated at 37 °C for 1 h. Finally, the visible absorbance of each well was measured with a microplate reader (BIO-RAD, model 3550 UV) at a wavelength of 405 nm. The positive control was composed of the reaction mixture, 50% DMSO and an integrase enzyme, while the negative control was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA.2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin was used as a positive control, which inhibited the IN activity with an IC_{50} value of 2.4 μM under the above conditions.

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