Kaempferol Acetylrhamnosides from the Rhizome of *Dryopteris crassirhizoma* and Their Inhibitory Effects on Three Different Activities of Human Immunodeficiency Virus-1 Reverse Transcriptase

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Three new kaempferol glycosides, called crassirhizomosides A (1), B (2) and C (3), were isolated from the rhizome of *Dryopteris crassirhizoma* (Aspidiaceae), together with the known kaempferol glycoside, sutchuenoside A (4). The structures of 1—3 were determined as kaempferol 3-α-L-(2,4-di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside, kaempferol 3-α-L-(3,4-di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside, and kaempferol 3-α-L-(2,3-di-*O*-acetyl)rhamnopyranosside-7-α-L-rhamnopyranoside, respectively, by chemical and spectroscopic means. Inhibitory effects of 1—4 and kaempferol on human immunodeficiency virus reverse transcriptase-associated DNA polymerase (RNA-dependent DNA polymerase and DNA-dependent DNA polymerase) and RNase H activities were investigated.

Key words crassirhizomoside A—C; kaempferol glycoside; *Dryopteris crassirhizoma*; Aspidiaceae; human immunodeficiency virus; reverse transcriptase

The development of potential drugs for the control of human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), is nowadays a cardinal goal, because HIV-infected people and AIDS patients continue to increase in population, especially in the developing countries. Reverse transcriptase (RT) of HIV has been demonstrated to be important for the viral replication. The crucial role of RT in the early stages of the HIV life cycle has made it one of the most reliable targets for potential anti-AIDS chemotheraphy.[1—3] This enzyme is a multifunctional enzyme exhibiting not only RT (RNA-dependent DNA polymerase, RDDP) activity but also DNA-dependent DNA polymerase (DDDP) and inherent ribonuclease H (RNase H) activities. The DNA polymerizing functions, together with an RNase H function, are responsible for converting the viral genomic RNA into proviral double-stranded DNA. Inhibition of each catalytic function of RT interferes with the virus production.[4,5] To date, two classes of drugs, nucleoside-analogue and non-nucleoside inhibitors, with different inhibitory mechanisms, have been developed. However, their use for treatment of patients with AIDS is limited due to the emergence of drug-resistant viruses and their toxicity.[6] The need for the development of effective and selective inhibitors of HIV RT with new mechanisms still remains. Although a great number of researches have been conducted on the development of inhibitors of RT (RDDP) activity, there are only a few reports on the selective inhibitors of RNase H activity, such as herparin,[7] illimaquinone,[8] novenamines (U-34445, U-35122, U-35401)[9] and cephalosporine degradation product (HP 0.35).[10] Therefore, we examined a conventional assay method for anti-RNase H activity associated with HIV-1 RT to find new inhibitory substances from natural sources.[11]

During *in vitro* screening of 50 Korean and 30 Chinese medicinal plants, we found that the rhizome of *Dryopteris crassirhizoma* Nakai (Aspidiaceae) appreciably inhibited RNase H activity of HIV-1 RT. The methanol extract of the rhizome inhibited RNase H activity with a 50% inhibitory concentration (IC_{50}) of 25 μg/ml, while it more potently inhibited RT activity (IC_{50}, 4.0 μg/ml). The rhizome of *D. crarrirhizoma* is a well-known Chinese herbal medicine used as a taeniacide.[12] Species of the genus *Dryopteris* are generally characterized by the presence of phloroglucinol derivatives, such as flavaspidic acids, triflavaspidic acids, dryocrassins, albaspidins and filixic acids.[13—18] Some of them showed anti-tumor promoting activity[19] and anti-bacterial activity.[20] Recently, kaempferol glycosides have been isolated from the *Dryopteris* species.[21] In this paper, we describe the structural elucidation of new kaempferol acetylrhamnosides (**1**—**3**), as well as inhibitory potencies against RDDP, DDDP and RNase H activities.

Results and Discussion

An EtOAc-soluble fraction of the MeOH extract of the rhizome of *D. crassirhizoma* was chromatographed on columns of silica gel, Sephadex LH-20 and octadecyl silane (ODS), followed by preparative high performance liquid chromatography (HPLC) to give four kaempferol glycosides (**1**—**4**) (Chart 1). Compound **4** was identified as kaempferol 3-α-L-(4-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside

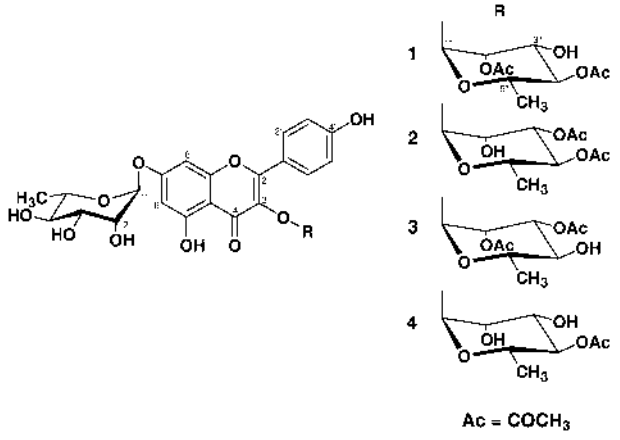

Chart 1. Structures of Compounds Isolated from the Rhizome of *Dryopteris crassirhizoma*

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Table 1. ^{1}H-NMR Spectral Data of Compounds **1**—**4** in CD_3OD (400 MHz)

	1	**2**	**3**	**4**
6	6.45 br s	6.46 br s	6.47 br s	6.36 d (2.2)
8	6.72 br s	6.73 br s	6.74 br s	6.62 d (2.2)
2′	7.76 d (8.5)	7.79 d (8.5)	7.84 d (8.7)	7.65 d (8.9)
3′	6.95 d (8.5)	6.96 d (8.5)	6.95 d (8.7)	6.85 d (8.9)
5′	6.95 d (8.5)	6.96 d (8.5)	6.95 d (8.7)	6.85 d (8.9)
6′	7.76 d (8.5)	7.79 d (8.5)	7.84 d (8.7)	7.65 d (8.9)
1″	5.53 d (1.9)	5.61 d (1.7)	5.52 d (1.7)	5.42 d (1.7)
2″	5.43 dd (1.9, 3.4)	4.34 dd (1.7, 3.1)	5.61 dd (1.7, 3.4)	4.12 dd (1.7, 3.4)
3″	4.03 dd (3.4, 10.0)	5.15 dd (3.1, 10.0)	5.08 dd (3.4, 9.4)	3.70 dd (3.4, 9.9)
4″	4.75 t (10.0)	4.99 t (10.0)	3.45 t (9.4)	4.73 m
5″	3.32 m	3.29 m	3.43 m	3.16 dd (6.3, 9.9)
6″	0.81 d (6.3)	0.81 d (6.3)	0.98 d (5.6)	0.69 d (6.3)
1‴	5.55 br s	5.55 br s	5.55 br s	5.46 br s
2‴	4.01 dd (1.9, 2.9)	4.01 dd (1.7, 3.4)	4.01 dd (1.7, 3.1)	3.92 dd (1.7, 3.4)
3‴	3.82 dd (2.9, 9.5)	3.82 dd (3.4, 9.4)	3.82 dd (3.1, 9.4)	3.72 dd (3.4, 9.4)
4‴	3.47 t (9.5)	3.47 t (9.4)	3.47 t (9.4)	3.38 t (9.4)
5‴	3.59 dd (6.0, 9.5)	3.60 dd (6.3, 9.4)	3.59 dd (6.0, 9.4)	3.51 dd (6.3, 9.4)
6‴	1.25 d (6.0)	1.26 d (6.0)	1.26 d (6.0)	1.17 d (6.3)
2″-$COCH_3$	2.03 s		2.07 s	
3″-$COCH_3$		2.06 s	2.02 s	
4″-$COCH_3$	2.10 s	1.97 s		1.93 s

δ values in ppm and coupling constants (in parentheses) in Hz.

(sutchuenoside A), which had been isolated from the leaves of *Epimedium sutchuenense* (Berberidaceae), by comparison with the spectral data.[22)]

Crassirhizomoside A (**1**) was obtained as a pale yellow amorphous powder, $[\alpha]_D$ −152°. The high-resolution fast atom bombardment mass (HR-FAB-MS) spectrum revealed the molecular formula of **1** to be $C_{31}H_{34}O_{16}$. The ultraviolet (UV) spectrum exhibited absorption bands at 264, 326 (sh) and 338 nm due to A and B rings of the flavonoid. The bathochromic shift induced by either addition of $AlCl_3$ or NaOAc, suggested the presence of free hydroxyl groups at C-5 and C-4′, respectively.[23)] In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, two broad singlet signals were observed at δ 6.45 and 6.72, assignable to H-6 and H-8 of ring A of the flavonoid unit (Table 1). Two doublets at δ 6.95 and 7.76 (each J=8.5 Hz) indicated the presence of a 4′-substituted phenyl group in ring B. In addition, two anomeric protons at δ 5.53 (d, J=1.9 Hz) and 5.55 (br s), and two methyl protons at δ 0.81 (d, J=6.3 Hz) and 1.25 (d, J=6.0 Hz) in the rhamnose residues were assigned. On acid hydrolysis, **1** gave kaempferol (**5**) and rhamnose, which were identified by comparisons of the ^{1}H- and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra, and the *Rf* values on thin layer chromatography (TLC) with those of authentic samples. By ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H-detected multiple quantum coherence (HMQC) experiments, all the proton and carbon signals due to the two rhamnose residues were well assigned as shown in Tables 1 and 2. The carbon signal assignable to C-2 (δ 159.9) in the aglycone moiety was shifted to a lower-field by about 11.8 ppm, compared with that of kaempferol (**5**, δ 148.1), indicating that one of the two rhamnose residues was located at C-3. The remaining rhamnose residue was deduced to be attached at C-7, on the basis of the chemical shifts of H-6 (δ 6.45) and H-8 (δ 6.72), which were deshielded by 0.27 and 0.33 ppm, respectively, compared with the corresponding signals of kaempferol (δ 6.18 and 6.39, respectively). The connectivites of the sugars were further supported by the presence of correlations between a proton signal at δ 5.53 (H-1″) and a carbon signal at δ 135.3 (C-3), and between a proton signal

Table 2. ^{13}C-NMR Spectral Data of Compounds **1**—**4** in CD_3OD (100 MHz)

	1	**2**	**3**	**4**
2	159.9	159.9	159.8	160.0
3	135.3	135.4	135.6	135.8
4	179.4	179.5	179.6	179.6
5	163.0	163.0	163.0	163.0
6	100.7	100.6	100.7	100.6
7	163.6	163.6	163.6	163.6
8	95.6	95.7	95.6	95.7
9	158.1	158.1	158.1	158.1
10	107.5	107.5	107.5	107.6
1′	122.1	122.2	122.2	122.3
2′	132.0	132.0	132.0	132.0
3′	116.7	116.7	116.8	116.6
4′	162.0	161.9	162.0	161.9
5′	116.7	116.7	116.8	116.6
6′	132.0	132.0	132.0	132.0
1″	99.5	101.9	100.1	102.6
2″	73.0	69.4	70.8	71.7
3″	68.3	72.7	73.0	70.0
4″	74.7	71.8	72.2	74.9
5″	69.6	69.6	70.5	69.6
6″	17.7	17.5	17.7	17.6
1‴	99.9	99.9	99.9	100.6
2‴	71.9	71.7	72.0	71.7
3‴	72.1	72.1	72.1	72.1
4‴	73.6	73.6	73.6	73.6
5‴	71.3	71.3	71.3	71.3
6‴	18.1	18.1	18.1	18.1
2″-$\underline{C}OCH_3$	172.2		171.5	
2″-$CO\underline{C}H_3$	20.8		20.6	
3″-$\underline{C}OCH_3$		172.1	172.3	
3″-$CO\underline{C}H_3$		20.8	20.8	
4″-$\underline{C}OCH_3$	171.8	171.7		172.4
4″-$CO\underline{C}H_3$	20.8	20.7		20.9

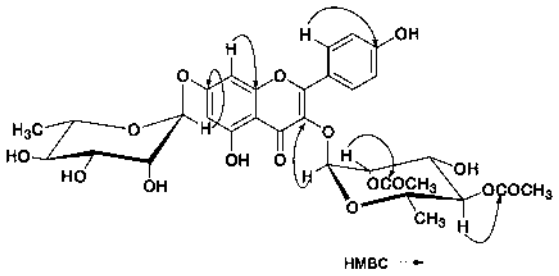

Fig. 1. Long-Range Correlations Observed in the HMBC Spectrum of **1**

at δ 5.55 (H-1‴) and a carbon signal at δ 163.6 (C-7) in the heteronuclear multiple-bond correlation (HMBC) experiment (Fig. 1). The anomeric configurations of the two rhamnose residues in **1** were concluded to both be α- in the preferred conformation of rhamnopyranoside, because of the small J values of their anomeric protons (*anti*-diequatorial). Furthermore, the absolute configuration of the sugar was determined to be L-rhamnose by gas liquid chromatography (GLC) of its pertrimethylsilated L-cysteine methyl ester derivative.[24] The presence of two acetyl groups in **1** were suggested by two singlet signals at δ 2.03 and 2.10 in the ^{1}H-NMR spectrum, as well as four carbon signals at δ 20.8 (quartet), 20.8 (quartet), 172.2 (singlet) and 171.8 (singlet) in the ^{13}C-NMR spectrum. The connectivities of the acetyl groups in **1** were established by the aid of a ^{1}H–^{1}H COSY experiment. Two protons at H-2″ (δ 5.43) and H-4″ (δ 4.75) of the one rhamnose residue were shifted to a lower-field by 1.42 and 1.28 ppm (more than 1 ppm), respectively, compared with the corresponding signals of the unacetylated rhamnose residue in the ^{1}H-NMR spectrum (Table 1). This indicated that two hydroxyl groups at C-2″ and C-4″ of rhamnose, which was linked at C-3 of kaempferol, were acetylated. This was further supported by HMBC correlations observed between a proton signal at δ 5.43 (H-2″) and a carbon signal at δ 172.2 (C-2″-*O*-$\underline{C}OCH_3$), as well as between a proton signal at δ 4.75 (H-4″) and a carbon signal at δ 171.8 (C-4″-*O*-$\underline{C}OCH_3$), respectively (Fig. 1). Consequently, the structure of **1** was determined as kaempferol 3-α-L-(2,4-di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside.

Crassirhizomoside B (**2**) was isolated as a pale yellow amorphous powder, $[\alpha]_D$ −219°, and possessed the same molecular formula as that of **1**, when determined by HR-FAB-MS. The UV absorption bands at 264, 327 (sh) and 342 nm, and bathochromic shifts by addition of $AlCl_3$ and NaOAc, indicated the presence of free hydroxyl groups at C-5 and C-4′ in the flavonoid. Inspection of spectral data of **2** revealed the presence of the same structural moiety as in **1**, including kaempferol as an aglycone, and two rhamnosyl and two acetyl groups. However, the most noticeable change was a higher-field shift of H-2″ (δ 4.34, dd, J=1.7, 3.1 Hz) by 1.19 ppm in **2**, while lower-field shifts of H-1″ (δ 5.61, d, J=1.7 Hz), H-3″ (δ 5.15, dd, J=3.1, 10.0 Hz) and H-4″ (δ 4.99, t, J=10.0 Hz) by 0.8, 1.12 and 0.24 ppm, respectively, compared with the corresponding signals of **1** in the ^{1}H-NMR spectrum. Furthermore, the carbon signals of C-2″ (δ 69.4) and C-4″ (δ 71.8) were shifted to a higher-field by 3.6 and 2.9 ppm, respectively, while those of C-1″ (δ 101.9) and C-3″ (δ 72.7) were shifted to a lower-field by 2.4 and 4.4 ppm, respectively, compared with those of **1** in the ^{13}C-NMR spectrum. These findings indicated that two hydroxyl groups at C-3″ and C-4″ of the rhamnose residue, which was linked at C-3 of the aglycone moiety, were acetylated. This was further confirmed by HMBC correlations between a proton signal at δ 5.15 (H-3″) and a carbon signal at δ 172.1 (C-3″-*O*-$\underline{C}OCH_3$), and between a proton signal at δ 4.99 (H-4″) and a carbon signal at δ 171.7 (C-4″-*O*-$\underline{C}OCH_3$). The stereochemistries of the two rhamnose units were determined on the basis of the coupling constant of the respective anomeric protons (J=*ca.* 1.7 Hz) in the ^{1}H-NMR spectrum and the retention time of the pertrimethylsilated L-cysteine methyl ester derivative in the gas chromatogram. The structure of **2** was established as kaempferol 3-α-L-(3,4-di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside.

Table 3. Inhibition of HIV-1 RT-Associated RDDP, RNase H and DDDP by Kaempferol Glycosides Isolated from *D. crassirhizoma*

Compound	IC_{50} (μM)		
	RDDP	RNase H	DDDP
1	215	>500	25
2	>500	>500	>100
3	240	>500	28
4	405	>500	23
5	110	>500	75
Adriamycin[a]	46		6
Illimaquinone[b]		50	

a) Positive control of HIV-1 RT-associated DNA polymerase activities. *b*) Positive control of HIV-1 RT-associated RNase H activity.

Crassirhizomoside C (**3**) was also obtained as a pale yellow amorphous powder, $[\alpha]_D$ −161°. The molecular formula was determined as $C_{31}H_{34}O_{16}$ by HR-FAB-MS. The UV absorption bands at 264, 325 (sh) and 343 nm, and the bathochromic shifts with $AlCl_3$ and NaOAc, also suggested the presence of kaempferol as an aglycone. The ^{1}H- and ^{13}C-NMR spectra were similar to those of **1** and **2**, except for signals due to the sugar moiety, which was linked at C-3 of kaempferol. The proton signal of H-4″ (δ 3.45, t, J=9.4 Hz) was shifted to a higher-field by 1.30 ppm, while those of H-2″ (δ 5.61, dd, J=1.7, 3.4 Hz), H-3″ (δ 5.08, dd, J=3.4, 9.4 Hz), H-5″ (δ 3.43, m) and H-6″ (δ 0.98, d, J=5.6 Hz) were shifted to a lower-field by 0.18, 1.05, 0.11 and 0.17 ppm, compared with those of **1**; this indicates that two hydroxyl groups at C-2″ and C-3″ of the rhamnose residue were acetylated. This was further supported by HMBC correlations between a proton signal at δ 5.61 (H-2″) and a carbon signal at δ 171.5 (C-2″-*O*-$\underline{C}OCH_3$), and between a proton signal at δ 5.08 (H-3″) and a carbon signal at δ 172.3 (C-3″-*O*-$\underline{C}OCH_3$). Crassirhizomoside C (**3**) was consequently determined as kaempferol 3-α-L-(2,3-di-*O*-acetyl)rhamnopyrano-side-7-α-L-rhamnopyranoside.

Kaempferol glycosides (**1**—**4**) and kaempferol (**5**) were examined for their inhibitory effects on HIV-1 RT-associated DNA polymerase (RDDP, DDDP) and RNase H activities. As shown in Table 3, compounds **1**, **3**, **4** and **5** inhibited RDDP (IC_{50} values, 215, 240, 405 and 110 μM, respectively) and DDDP (IC_{50} values, 25, 28, 23 and 75 μM, respectively) activities of HIV-1 RT, but compound **2** was inactive in both of the assays. Furthermore, compounds **1**—**5** showed no inhibition against RNase H activity at concentrations less than

500 μM. Generally, substances that inhibit *in vitro* HIV RT are likely to fall into one of three categories: i) substances potently blocking both DNA polymerase and RNase H activities, ii) those inhibiting preferably the DNA polymerase activities, and iii) those selectively inhibiting the RNase H activity without any significant affect on the DNA polymerase functions.[25] Compounds **1**, **3**, **4** and **5**, therefore, belong to the second category. The inhibitory potencies of **1**, **3**, **4** and **5** against DDDP [poly(dA)$_n$·oligo (dT)$_{12-18}$-directed DNA synthesis] activity (IC_{50}, 23—75 μM) were stronger than those against RDDP [poly(rA)$_n$·oligo (dT)$_{12-18}$-directed DNA synthesis] activity (IC_{50}, 110—405 μM). Kaempferol (**5**) slightly increased the inhibitory potency against RDDP activity, compared with the kaempferol glycosides (**1**—**4**). The acetyl groups attached at different positions of the rhamnose residue appreciably influenced their inhibitory potencies against DNA polymerase (RDDP, DDDP) activities.

Experimental

Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co.). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu Co.). IR spectra were measured with an FT/IR-230 IR spectrometer (Jasco Co.). ^{1}H- and ^{13}C-NMR spectra were measured with Jeol JNM-LA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; Jeol Co.) spectrophotometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. FAB-MS was measured with a Jeol JMS-AX 300L spectrometer (Jeol Co.) using glycerol as a matrix. Preparative HPLC was carried out on a Gilson HPLC system; pump: model 305 and 306, detector: 119 UV/VIS detector. Column chromatography was carried out on silica-gel (Kieselgel 60, 70—230 mesh, Merck Co.), Sephadex LH-20 (Pharmacia Co.), Amberlite MB-3 (Organo Co.) and ODS (Chromatorex, 100—200 mesh, Fuji Silysia Chemical, Ltd.). TLC was carried out on pre-coated Silica-gel 60 F_{254} plates and RP-18 F_{254S} (0.25 mm, Merck Co.), and spots were detected under a UV light and by spraying with 10% H_2SO_4 followed by heating.

Plant Materials The rhizome of *D. crassirhizoma* was purchased from Zhongshanlu Drug Store of Hohhot, Inner Mongolia of the People's Republic of China. A voucher specimen (TMPW No. 19159) is deposited at the Museum of Materia Medica, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Isolation Procedure The rhizome of *D. crassirhizoma* (5.0 kg) was extracted with MeOH (1500 ml×3) at room temperature for 24 h to give 200 g of an extract. The MeOH extract (190 g) was suspended in H_2O (500 ml) and extracted with hexane (2500 ml×3) to give a hexane-soluble fraction (42 g). The resulting H_2O layer was extracted with $CHCl_3$ (300 ml×3, 62 g), EtOAc (500 ml×3, 10 g) and BuOH (500 ml×3, 26 g), successively. The EtOAc-soluble fraction (10 g) was chromatographed on a column of silica gel (200 g). The column was eluted using a stepwise gradient of $CHCl_3$, MeOH and H_2O to give 7 fractions (fr. A—G; 0.9 g, 1.4 g, 1.8 g, 2.4 g, 1.8 g, 0.9 g and 0.6 g, respectively). Repeated column chromatography of fr. C on silica gel ($CHCl_3$–MeOH, 9 : 1), Sepadex LH-20 ($CHCl_3$–MeOH, 1 : 9) and reversed phase ODS (40% aq. MeOH), was followed by prep. HPLC on TSK-gel ODS-80TM (a linear gradient of CH_3CN, 15%→60%, in 0.2% trichloroacetic acid) afforded **1** (9.3 mg), **2** (7.9 mg) and **3** (4.4 mg). Repeated column chromatography of fr. D on silica gel ($CHCl_3$–MeOH, 9 : 1) and reversed phase ODS (40 % aq. MeOH) furnished **4** (9.6 mg).

Kaempferol 3-α-L-(2,4-Di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside (**1**, Crassirhizomoside A): Pale yellow amorphous powder, $[\alpha]_D$ −152° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3448, 1734, 1654, 1602. UV (MeOH) λ_{max} nm (log ε): 264 (4.3), 326 (sh), 338 (4.1); (MeOH+$AlCl_3$) 274 (4.3), 346 (4.2), 394 (4.1); (MeOH+$AlCl_3$+HCl) 273 (4.3), 338 (4.1), 389 (3.7); (MeOH+NaOAc) 272 (4.3), 345 (sh), 377 (4.2). Negative-ion mode FAB-MS m/z: 661 $[M-H]^-$ (98), 515 $[M-H-C_6H_{10}O_4]^-$ (58), 431 $[M-H-C_{10}H_{14}O_6]^-$ (67), 285 $[M-H-C_{16}H_{24}O_{10}]^-$ (100). HR negative-ion mode FAB-MS m/z: 661.1811 ($[M-H]^-$, Calcd for $C_{31}H_{33}O_{16}$: 661.1768). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

Kaempferol 3-α-L-(3,4-Di-*O*-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside (**2**, Crassirhizomoside B): Pale yellow amorphous powder, $[\alpha]_D$ −219° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3448, 1734, 1656, 1604. UV (MeOH) λ_{max} nm (log ε): 264 (4.4), 327 (sh), 342 (4.2); (MeOH+$AlCl_3$) 273 (4.4), 348 (4.2), 395 (4.2); (MeOH+$AlCl_3$+HCl) 274 (4.4), 340 (4.2), 390 (4.1); (MeOH+NaOAc) 272 (4.3), 350 (sh), 377 (4.3). Negative-ion mode FAB-MS m/z: 661 $[M-H]^-$ (51), 515 $[M-H-C_6H_{10}O_4]^-$ (66), 431 $[M-H-C_{10}H_{14}O_6]^-$ (33), 285 $[M-H-C_{16}H_{24}O_{10}]^-$ (100). HR negative-ion mode FAB-MS m/z: 661.1810 ($[M-H]^-$, Calcd for $C_{31}H_{33}O_{16}$: 661.1768). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

Kaempferol 3-α-l-(2,3-Di-O-acetyl)rhamnopyranoside-7-α-l-rhamnopyranoside (**3**, Crassirhizomoside C): Pale yellow amorphous powder, $[\alpha]_D$ −161° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3422, 1718, 1654, 1602. UV (MeOH) λ_{max} nm (log ε): 264 (4.3), 325 (sh), 343 (4.1); (MeOH+$AlCl_3$) 274 (4.3), 348 (4.2), 395 (4.1); (MeOH+$AlCl_3$+HCl) 274 (4.3), 342 (4.1), 390 (4.0); (MeOH+NaOAc) 273 (4.2), 350 (sh), 378 (4.2). Negative-ion mode FAB-MS m/z: 661 $[M-H]^-$ (10), 515 $[M-H-C_6H_{10}O_4]^-$ (12), 431 $[M-H-C_{10}H_{14}O_6]^-$ (11), 285 $[M-H-C_{16}H_{24}O_{10}]^-$ (39). HR negative-ion mode FAB-MS m/z: 661.1811 ($[M-H]^-$, Calcd for $C_{31}H_{33}O_{16}$: 661.1768). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

Kaempferol 3-α-L-(4-*O*-Acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside (**4**)[22]: Pale yellow amorphous powder, $[\alpha]_D$ −170° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3460, 1654, 1608. UV (MeOH) λ_{max} nm (log ε): 264 (4.3), 326 (sh), 343 (4.2); (MeOH+$AlCl_3$) 273 (4.4), 348 (4.2), 395 (4.2); (MeOH+$AlCl_3$+HCl) 274 (4.3), 340 (4.2), 392 (4.0); (MeOH+NaOAc) 273 (4.3), 346 (sh), 378 (4.2). Negative-ion mode FAB-MS m/z: 619 $[M-H]^-$ (53), 473 $[M-H-C_6H_{10}O_4]^-$ (48), 431 $[M-H-C_8H_{12}O_5]^-$ (52), 285 $[M-H-C_{14}H_{22}O_9]^-$ (100). HR negative-ion mode FAB-MS m/z: 619.1694 ($[M-H]^-$, Calcd for $C_{29}H_{31}O_{15}$: 619.1662). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

Acid Hydrolysis of 1 Compound **1** (5 mg) was refluxed in 1 N-HCl (5 ml) at 80 °C for 3 h, neutralized with 1 N-NaOH. The reaction mixture was extracted with $CHCl_3$ (10 ml×3) and evaporated. The residue was chromatographed on silica gel eluting with hexane : acetone (3 : 2) to give kaempferol (**5**, 2 mg).

Kaempferol (**5**): Pale yellow amorphous powder. ^{1}H-NMR ($CDCl_3$) δ: 6.18 (d, J=2.2 Hz, H-6), 6.39 (d, J=2.2 Hz, H-8), 6.90 (d, J=8.9 Hz, H-3′, 5′), 8.08 (d, J=8.9 Hz, H-2′, 6′). ^{13}C-NMR ($CDCl_3$) δ: 148.1 (C-2), 137.1 (C-3), 177.4 (C-4), 162.5 (C-5), 99.3 (C-6), 165.7 (C-7), 94.5 (C-8), 158.3 (C-9), 104.5 (C-10), 123.8 (C-1′), 130.7 (C-2′, 6′), 116.3 (C-3′, 5′), 160.6 (C-4′).

Determination of Sugar Each sample of **1**, **2** or **3** (2 mg) was refluxed with 4 N HCl–dioxane (1 : 1, 2 ml) for 2 h. The mixture was extracted with $CHCl_3$ (5 ml×3). The resulting water layer was desalted with Amberlite MB-3 and dried to give a residue. The residue was dissolved in pyridine (1 ml), to which a solution of L-cysteine methyl ester hydrochloride (0.1 M) in pyridine (2 ml) was added. The mixture was kept at 60 °C for 1.5 h. After the solvent was evaporated under reduced pressure, the residue was trimethylsilylated with hexamethyldisilazane–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 GLC (column, DB-1, J & W Scientific, 0.25 mm×30 m; column temperature, 50→300 °C, 10 °C/min then 300 °C, 5 min; carrier gas, He). The sugar derivative thus obtained showed a retention time of 21.2 min, identical to that of authentic L-rhamnose.

The water layers were also checked by silica gel TLC (EtOAc–MeOH–H_2O–AcOH, 65 : 20 : 15 : 15). The spots on the TLC plate were visualized by an anisaldehyde-H_2SO_4 reagent.

RNase H Activity Assay For the assay of HIV-1-RT-associated RNase H,[4] HIV-1 RT was adjusted to 3.3 U/μl with a solution of 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$ and 2.5 mM dithiothreitol (DTT). A mixture (20 μl) containing 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 2.5 mM DTT, 7.2 nM of [^{3}H]poly(rA)·poly(dT) (370 kBq/ml), and a test compound in dimethyl sulfoxide (DMSO; final concentration of 5%) was preincubated at 37 °C for 5 min. The reaction mixture was allowed to keep at 37 °C for 2 h. A blank reaction was carried out under the same conditions without adding the enzyme, and a control reaction was done in the absence of the test compound. The reaction was terminated by the addition of 20 μl of 0.02 M ethylenediamine tetraacetate (EDTA). The mixture was applied onto a Whatman DE81 paper disc, which was washed batchwise with 3 ml of 5% Na_2HPO_4, distilled water three times, ethanol once and ether once. The paper disc was then dried and immersed in 3 ml of scintillation fluid. The RNase H activity was measured by the degradation of ^{3}H-labeled RNA in a hybrid in the presence of the tested compound. The percentage of the inhibition was calculated as follows:

$$\text{Inhibition (\%)} = [1-(\text{dpm}_{\text{blank}}-\text{dpm}_{\text{comp.}})/(\text{dpm}_{\text{blank}}-\text{dpm}_{\text{contl.}})]\times 100$$

Illimaquinone was used as a positive control which inhibited the RNase H

activity with an IC_{50} of 50 μM under the above conditions.

DNA–RNA Hybrid Preparation A mixture of 0.57 nM poly(dT), 0.32 nM poly(rA) and 5 pM [^{3}H]poly(rA) in 50 mM Tris–HCl (pH 8.0) was heated up to 90 °C for 5 min, allowed to cool gradually to 37 °C in 30 min, kept at room temperature for 30 min and finally stored at −20 °C.[26)]

RDDP Activity Assay For the assay of RDDP activity,[27,28)] HIV-1 RT was adjusted to 0.01 U/μl 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 μl) containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.25 μg/ml (*ca.* 16 nM) poly(rA)·oligo$(dT)_{12-18}$ as a templateprimer, 250 nM dTTP, 100 nM [methyl-^{3}H]dTTP (18.5 MBq/ml), 0.01 U/μl of RT, and 1.0 μl 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 μl of 0.02 M EDTA. The resulting mixture was applied onto a Whatman DE81 paper disc and washed in a similar manner described above. The paper disc was then dried and immersed in 3 ml of scintillation fluid. The amount of a 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:

$$\text{Inhibition (\%)} = [1 - (\text{dpm}_{\text{comp.}}/\text{dpm}_{\text{contl.}})] \times 100$$

Adriamycin was used as a positive control, which inhibited the RDDP activity with an IC_{50} of 46 μM under the above conditions.

DDDP Activity Assay For the assay of DDDP activity, HIV-1 RT was adjusted to 0.1 U/μl 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 μl) containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.25 μg/ml (*ca.* 16 nM) poly(dA)·oligo$(dT)_{12-18}$ as a templateprimer, 250 nM dTTP, 200 nM [methyl-^{3}H]dTTP (18.5 MBq/ml), 0.1 U/μl of RT, and 1.0 μl of the 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 μl of 0.02 M EDTA. The resulting mixture was applied onto a Whatman DE81 paper disc and washed in a similar manner as described above. The radioactivity of a polymer fraction on the paper disc was determined in a scintillation counter. The calculation of the inhibitory potency for the test compound was carried out as follows:

$$\text{inhibition (\%)} = [1 - (\text{dpm}_{\text{comp.}}/\text{dpm}_{\text{contl.}})] \times 100$$

Adriamycin was used as a positive control, which inhibited DDDP activity with an IC_{50} of 6 μM under the above conditions.

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