A New Flavonol Glycoside Gallate Ester from *Acer okamotoanum* and Its Inhibitory Activity against Human Immunodeficiency Virus-1 (HIV-1) Integrase

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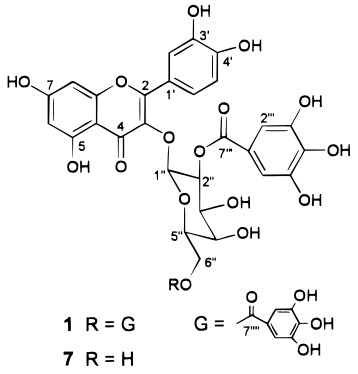
Bioassay-directed chromatographic fractionation of an ethyl acetate extract of the leaves of *Acer okamotoanum* using HIV-1 integrase afforded a new acylated flavonol glycoside, quercetin 3-*O*-(2″,6″-*O*-digalloyl)-*β*-D-galactopyranoside (**1**), together with six known flavonol glycosides and three known phenolic compounds. The structure of the new compound was determined by spectroscopic methods. The most active compounds were quercetin 3-*O*-(2″-galloyl)-α-L-arabinopyranoside (**6**) and **1**, which exhibited IC_{50} values of 18.1 ± 1.3 and 24.2 ± 6.6 μg/mL, respectively, against HIV-1 integrase.

HIV-1 integrase mediates insertion of viral DNA into host cellular DNA that is essential for viral replication and virion production.[1−3] Therefore, the inhibition of this enzyme may be efficacious in anti-AIDS therapy. Despite many efforts to find active compounds from natural products, few HIV-1 integrase inhibitors have been described to date.[4] During a search for biologically active compounds from traditional medicines, a crude extract of the leaves of *Acer okamotoanum* Nakai (Aceraceae) growing on Ullung Island in Korea was found to potently inhibit HIV-1 integrase. Phytochemical studies on this plant have not been carried out previously. By means of bioassay-directed chromatographic fractionation, a new flavonol glycoside gallate ester, quercetin 3-*O*-(2″,6″-*O*-digalloyl)-*β*-D-galactopyranoside (**1**) together with six known flavonol glycosides (**2**−**7**) and three known phenolic compounds **(8**−**10)**, were isolated. Among these compounds, quercetin 3-*O*-(2″-galloyl)-α-L-arabinopyranoside (**6**)[5] and compound **1** showed strong inhibitory activity against HIV-1 integrase (IC_{50} values of 18.1 ± 1.3 and 24.2 ± 6.6 μg/mL, respectively) in our assay system. Herein we report the isolation and characterization of the new flavonol glycoside gallate ester **1** and the anti-HIV-1 integrase activity of the *A. okamotoanum* isolates.

The methanol extract was suspended in water and then consecutively partitioned with dichloromethane, ethyl acetate, and butanol. The ethyl acetate extract showed strong inhibitory activity against HIV-1 integrase (IC_{50} values of 13.6 ± 5.6 μg/mL). The extract was purified by column chromatography on Sephadex LH-20, as well as a combination of chromatography over silica gel, RP-18, and Toyopearl HW-40C to afford the new compound **1**. In addition to **1**, six known flavonol glycosides (**2**−**7**) and three known phenolic compounds (**8**−**10**) were also isolated.

The molecular formula of **1** was established as $C_{35}H_{28}O_{20}$ by ES and HRFAB mass spectrometry. The UV spectrum exhibited absorption maxima at 267 and 355 nm that are characteristic absorption bands of a flavone skeleton.[6] In the IR spectrum, along with the signal for an ester carbonyl (1710 cm^{-1}), signals for a hydroxyl group (3414 cm^{-1}), a conjugated carbonyl (1638 cm^{-1}), and a phenyl group (1618,1498 cm^{-1}) were apparent. The ^1H NMR spectrum suggested that **1** has a quercetin aglycon. Signals at *δ* 6.73 (1H, d, *J* = 8.5 Hz), 7.42 (1H, dd, *J* = 2.2 and 8.5 Hz), and 7.55 (1H, d, *J* = 2.2 Hz) are characteristic for a 3,4-disubstituted B ring of a quercetin unit. Singlets at *δ* 6.92 (2H, s) and 7.18 (2H, s) originated from the galloyl groups. The placement of the sugar residue at C-3 in **1** was suggested from characteristic UV shifts. After addition of NaOAc and $AlCl_3$ + HCl, absorption maxima at 267 and 355 nm shifted to 273, 325, and 415 and to 270, 360, and 405 nm, respectively.[7] Furthermore, the chemical



1 R = G G =

7 R = H

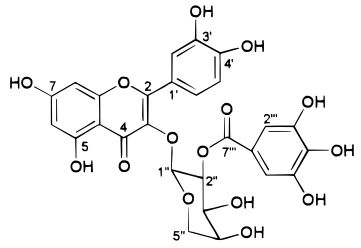


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Table 1. Chemical Shifts and HMBC NMR Correlations of **1** in CD_3OD

position	δ_H	δ_C[a]	HMBC
2		158.1 (s)	
3		134.8 (s)	
4		179.0 (s)	
5		162.7 (s)	
6	6.08 (s, 1H)	100.1 (d)	C-10
7		167.1 (s)	
8	6.22 (s, 1H)	95.1 (d)	
9		158.2 (s)	
10		105.2 (s)	
1′		123.0 (s)	
2′	7.55 (d, 2.2)	117.1 (d)	C-2,C-3′,C-4′,C-6′
3′		145.9 (s)	
4′		149.8 (s)	
5′	6.73 (d, 8.5)	116.3 (d)	C-1′,C-3′,C-4′
6′	7.42 (dd, 2.2, 8.5)	123.2 (d)	C-2, C-2′,C-4′
1″	5.55 (d, 8.0)	101.2 (d)	
2″	5.44 (dd, 8.0, 9.9)	74.4 (d)	C-1″,C-3″,C = O
3″	3.84 (dd, 3.5, 9.9)	73.3 (d)	C- 2″
4″	3.93 (t, 3.4)	70.5 (d)	C-3″,C-5″
5″	3.89 (t, 6.9)	74.6 (d)	C-4″,C-6″
6‴	4.24 (dd, 5.9, 11.2)	63.6 (t)	C-5″,C = O
	4.43 (dd, 7.1, 11.2)		
1‴		121.2 (s)	
1⁗		120.8 (s)	
2‴, 6‴	7.18 (s, 2H)	110.6 (d)	C-3‴,C-4‴,C-5‴
2⁗, 6⁗	6.92 (s, 2H)	110.1 (d)	C-3⁗,C-4⁗,C-5⁗
3‴, 5‴		146.4 (s)	
3⁗, 5⁗		146.3 (s)	
4‴, 4⁗		140.2 (s)	
C = O		168.0 (s)	
C = O		168.2 (s)	

[a] Multiplicities were established using the DEPT pulse sequence.

shifts at C-2 (δ 158.1), C-3 (δ 134.8), and C-4 (δ 179.0) in the ^{13}C NMR spectrum supported the above assignment.[8] The ^1H NMR spectrum of compound **1**, analyzed with the aid of a COSY spectrum, showed characteristic signals assignable to an anomeric proton at δ 5.55 (d, $J = 8.0$ Hz) and methylene protons adjacent to an ester group at δ 4.24 (dd, $J = 5.9, 11.2$ Hz) and 4.43 (dd, $J = 7.1, 11.2$ Hz). Attachment of another galloyl group through an ester linkage at C-2 in galactose was suggested by the downfield shift of H-2″ (δ 5.44) in the ^1H NMR spectrum. Three oxygenated methine protons at δ 3.84 (dd, $J = 3.5, 9.9$ Hz), 3.89 (t, $J = 6.9$ Hz), and 3.93 (t, $J = 3.4$ Hz), together with aromatic protons at δ 6.92 (2H, s) and 7.18 (2H, s) that were assignable to a galloyl group, suggested the presence of a 2,6-*O*-digalloyl galactoside residue in compound **1**. The HMBC NMR spectrum of compound **1** indicated that the carbon signal (δ 168.2, C-7‴) of the galloyl carbonyl units showed $^1H-^{13}C$ long-range correlations with the H-2″ and H-2‴, H-6‴ signals (δ 5.44 and 7.18). The carbon signal (δ 168.0, C-7⁗) of the other galloyl carbonyl was correlated with the proton signals at δ 4.24 (H-6″), 4.43 (H-6″), and 6.92 (H-2⁗ and 6⁗), respectively (Table 1). These data indicated that the galloyl groups were located at C-2″ and C-6″ of galactose. On the basis of the foregoing observations, compound **1** was assigned as quercetin 3-*O*-(2″,6″-digalloyl)-β-D-galactopyranoside. The anti-HIV-1 integrase activities of the *A. okamotoanum* isolates were investigated. As shown in Table 2, compounds **6** and **1** showed strong inhibitory activity against HIV-1 integrase (IC_{50} values of 18.1 ± 1.3 and 24.2 ± 6.6 μg/mL, respectively).

Table 2. HIV-1 Integrase Inhibitory Activities of Compounds **1**−**10**[a]

compd	IC_{50} (μg/mL)	compd	IC_{50} (μg/mL)
1	24.2 ± 6.6	**6**	18.1 ± 1.3
2	64.6 ± 3.9	**7**	27.9 ± 2.4
3	75.2 ± 8.1	**8**	38.5 ± 5.1
4	>100	**9**	28.3 ± 10.2
5	>100	**10**	28.0 ± 2.2

[a] IC_{50} values with standard deviations are from at least three independent experiments.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting apparatus and are uncorrected. Optical rotations were determined on a Autopol III automatic polarimeter (Rudolph Research Co., Flanders, New Jersey). UV spectra were taken with a Shimazu UV 240 UV−vis recording spectrometer. IR spectra were recorded on a Perkin-Elmer 16F-PC FT-IR and a Midac 101025 instrument using potassium bromide pellets. ^1H NMR spectra were recorded on a Gemini Varian-300 (300 MHz) spectrometer, using TMS as internal standard. ^{13}C NMR spectra were recorded on a Gemini Varian-300 (75 MHz) spectrometer. $^1H-^1H$ COSY, HMQC, and HMBC NMR spectra were obtained with the usual pulse sequences, and data processing was performed with the standard Gemini and Bruker software. EIMS were determined on a HP 5890 GC/5988 mass spectometer at 70 eV, electrospray, mass spectra were determined on an Api ES/MS (HP 59987A ES/5989A MS) instrument, and HRFABMS were determined on a JEOL JMS-HX 110/100A (Japan) mass spectometer. Preparative HPLC was performed on a Waters pump (model 510) with UV detector (λ 254 nm, Waters model 486) using a LiChrosorb RP-18 (10 mm i.d. x 25 cm, Merck) column. Cellulose TLC was carried out on precoated cellulose F TLC plates (Merck, art. 5718).

Plant Material. The leaves of *A. okamotoanum* Nakai were collected from Ullung Island, Korea, in July 1995. Voucher specimens (565-12A) have been deposited in the laboratory of Korea Institute of Science & Technology.

Extraction and Isolation. Dried leaves (2.1 kg) were cut into small pieces and percolated three times with MeOH at room temperature to afford 296 g of a dark-green residue on removal of solvent under reduced pressure. The methanol extract was suspended in water and then partitioned in turn with dichloromethane, ethyl acetate, and butanol. The combined EtOAc extract was evaporated under reduced pressure to yield 75 g of a residue. This residue was divided into six fractions by column chromatography on Sephadex LH-20 with a $CHCl_3$−MeOH gradient system. Active fraction 2 was further purified by column chromatography over silica gel with a CH_2Cl_2−EtOAc−MeOH−H_2O gradient system to give nine subfractions. Subfraction 2C was further purified by column chromatography over RP-18 using 50% MeOH as eluent and finally purified by preparative HPLC (LiChrosorb 250-10, RP-18, Merck) eluted with 40% MeOH, followed by increasing percentages of MeOH in H_2O to afford 13 mg of compound **1** as a yellow amorphous powder. Fractions 5, 2C, 2E, 2G, 2H, and 2I were also further purified by column chromatography in a similar manner to yield nine known

compounds. The structures of compounds **2**−**10** were determined by comparison with known compounds.

Quercetin 3-*O*-(2″,6″-digalloyl)-*β*-D-galactopyranoside (1): yellow amorphous powder; mp 222−224 °C dec; $[\alpha]^{21}_D$ −45.9° (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 267 (4.62), 290 (sh), 355 (4.30) nm; (MeOH + $AlCl_3$) λ_{max} 273, 300 (sh), 398 nm; (MeOH + NaOMe) λ_{max} 273, 325, 415 nm; IR (KBr) ν_{max} 3350, 1710, 1638, 1618, 1498, 1448, 1384, 1360, 1304, 1240, 1194 cm^{-1}; ^1H,^{13}C, and HMBC NMR data; see Table 1; ES/MS *m/z* 769 $[M + 1]^+$, HRFABMS $[M + H]^+$ *m/z* 769.1251, calcd for $C_{35}H_{29}O_{20}$ 769.1252. Acid hydrolysis with 10% H_2SO_4 gave quercetin (spectral data compared with the pure compound) and gallic acid. Cellulose TLC of the neutralized hydrolysate in 1-BuOH−benzene−pyridine−H_2O (5:1:3:3, upper layer) gave D-galactose (R_f 0.18).

Quercetin 3-*O*-*β*-D-galactopyranoside (2): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[9]

Quercetin 3-*O*-α-L-rhamnopyranoside (3): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[9,10]

Kaempferol 3-*O*-α-L-rhamnopyranoside (4): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[11]

Kaempferol 3-*O*-α-L-arabinopyranoside (5): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[12]

Quercetin 3-*O*-(2″-galloyl)-α-L-arabinopyranoside (6): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[5]

Quercetin 3-*O*-(2″-galloyl)-*β*-D-galactopyranoside (7): yellow amorphous powder; mp 198−200 °C dec; $[\alpha]^{17}_D$ −109.6° (*c* 1.0, MeOH); ^1H NMR (CD_3OD, 300 MHz) δ 7.64 (1H, d, J = 2.2 Hz, H-2′), 7.44 (1H, dd, J = 2.2, 8.6 Hz, H-6′), 7.16 (2H, s, galloyl H-2, H-6), 6.76 (1H, d, J = 8.6 Hz, H-5′), 6.26 (1H, br s, H-8), 6.11 (1H, br s, H-6), 5.66 (1H, d, J = 8.0 Hz, H-1″), 5.48 (1H, dd, J = 8.2, 9.7 Hz, H-2″), 3.94 (1H, d, J = 3.5 Hz, H-4″), 3.86 (1H, dd, J = 3.5, 9.6 Hz, H-3″), 3.74 (2H, dd, J = 6.5, 11.4 Hz, H-6″), 3.67 (1H, d, J = 6.8 Hz, H-5″); ^{13}C NMR (CD_3OD, 75 MHz) δ 179.0 (s, C-4), 168.3 (s, galloyl C═O), 165.9 (s, C-7), 162.9 (s, C-5), 158.2 (s, C-9), 158.0 (s, C-2), 149.7 (s, C-4′), 149.7 (s, C-4′), 146.3 (s, galloyl C-3, C-5), 145.8 (s, C-3′), 139.9 (s, galloyl C-4), 135.2 (s, C-3), 123.2 (s, C-1′), 123.0 (d, C-6′), 121.6 (s, galloyl C-1), 117.3 (d, C-2′), 116.3 (d, C-5′), 110.8 (d, galloyl C-2, C-6), 105.7 (s, C-10), 101.4 (d, C-1″), 99.9 (d, C-6), 94.9 (d, C-8), 77.4 (d, C-5″), 74.7 (d, C-2″), 73.5 (d, C-3″), 70.7 (d, C-4″), 62.3 (t, C-6″). The UV and MS data of **7** were identical with published values.[13] Acid hydrolysis with 10% H_2SO_4 gave quercetin (spectral data compared with the pure compound) and gallic acid. Cellulose TLC of the neutralized hydrolysate in 1-BuOH−benzene−pyridine−H_2O (5:1:3:3, upper layer) gave D-galactose (R_f 0.18).

Gallic acid methyl ester (8): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[14]

1,2,6-Tri-*O*-galloyl-*β*-D-glucose (9): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[15]

1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucose (10): UV, MS, and ^1H and ^{13}C NMR data were identical with published data.[16]

Bioassays. HIV-1 Integrase. Recombinant human immunodeficiency virus type 1 (HIV-1) integrase was expressed in *Escherichia coli* and purified using a nickel-chelated column in a one-step manner, as described previously.[17] Aliquots of HIV-1 integrase of 0.5 mg/mL as stock solutions were stored at −70 °C until used.

Oligonucleotide Substrates. Two 20-mer oligonucleotides whose sequences resemble the end of U5-LTR were obtained from Korea Biotech., Inc. (Seoul, Korea), namely K16 (U5-LTR, +strand), 5′-TGTGGAAAATCTCTAGCAGT-3′, and K17 (U5-LTR, -strand), 5′-ACTGCTAGA-GATTTTCCACA-3′. The oligonucleotides were purified using 20% polyacrylamide gel before use. To construct the oligonucleotide substrate, oligonucleotide K16 (15 pmol) was labeled at the 5′ end, using [γ-^{32}P]-ATP of 250 μCi (3,000 Ci/mmol; 1 Ci = 37 GBq; Amersham Life Science, Arlington Heights, IL) and T4 polynucleotide kinase (T4 PNK, New England Biolabs, Beverly, MA) of 10 units in 40 μL of reaction buffer (70 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol) for 15 min at 37 °C. The labeling reaction was subjected to 10 mM EDTA and heated to 85 °C for 15 min to inactivate T4 PNK. After the addition of complementary oligonucleotide K17 (30 pmol), the reaction mixture was boiled for 3 min and cooled slowly. Labeled substrate was separated from unincorporated nucleotide by passage through a Biospin 6 instrument (Bio-Rad, Hercules, CA).

HIV-1 Integrase Reaction. A standard reaction assay of endonucleolytic activity was carried out in the presence of potential inhibitor containing 0.1 pmol of duplex oligonucleotide substrate and 15 pmol of HIV-1 integrase in 15 mM Tris−HCl (pH 7.4), 100 mM NaCl, 1 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 2.5 mM CHAPS, 0.1 mM EDTA, 0.1 mM PMSF, 1% glycerol, and 10 mM imidazole in a total volume of 10 μL. Inhibitors or drugs were dissolved in 100% DMSO and added to the reaction mixture, with there being 5% DMSO in the final mixture. Reaction mixtures were incubated at 33 °C for 90 min and stopped by the addition of 4 μL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The reactions were heated to 90 °C for 3 min and electrophoresed on a 20% denaturing polyacrylamide gel. Reaction products were visualized by autoradiography of the wet gel. IC_{50} values were calculated by scanning bands on Kodak-5 film (Image Master VDS, Pharmacia Biotech., Piscataway, NJ).

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