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

Byung-Sun MIN, Miyuki TOMIYAMA, Chao-Mei MA, Norio NAKAMURA, and Masao HATTORI*

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan.

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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)rhamnopyranoside-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 herpanin,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 (IC50) of 25 μg/ml, while it more potently inhibited RT activity (IC50, 4.0 μg/ml). The rhizome of D. crassirhizoma is a well-known Chinese herbal medicine used as a tea niace.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 activity19 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 acetylramnosides (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 Dryopteris 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

* To whom correspondence should be addressed. e-mail: saibo421@ms.toyama-mpu.ac.jp © 2001 Pharmaceutical Society of Japan
Crassirhizomoside A (1) was obtained as a pale yellow amorphous powder, \([\alpha]_D^{20} -152^\circ\). 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 AlCl3 or NaOAc, suggested the presence of free hydroxyl groups at C-5 and C-4', respectively. In the proton nuclear magnetic resonance (1H-NMR) spectrum, two broad singlet signals were observed at \(\delta\) 6.45 and 6.72, assignable to H-6 and H-8. In addition, two doublets at \(\delta\) 6.95 and 7.76 (each \(J = 8.5\)) indicated the presence of a 4'-substituted phenyl group in ring B. In detail, two anomic protons at \(\delta 5.53 (d, J = 1.9\) Hz) and 5.55 (br s), and two methyl protons at \(\delta 0.81 (d, J = 6.3\) 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 comparison of the 1H- and carbon-13 nuclear magnetic resonance (1H-NMR) spectra, and the \(R_f\) values on thin layer chromatography (TLC) with those of authentic samples. By 1H-1H correlation spectroscopy (COSY) and 1H-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 (\(\delta 159.9\)) in the aglycone moiety was shifted to a lower-field by about 11.8 ppm, compared with that of kaempferol (5, \(\delta 148.1\)), 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 (\(\delta 6.45\)) and H-8 (\(\delta 6.72\)), which were deshielded by 0.27 and 0.33 ppm, respectively, compared with the corresponding signals of kaempferol (\(\delta 6.18\) and 6.39, respectively). The connectivities of the sugars were further supported by the presence of correlations between a proton signal at \(\delta 5.53 (H-1')\) and a carbon signal at \(\delta 135.3 (C-3)\), and between a proton signal...
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.25) The presence of two acetyl groups in 1 were suggested by two singlet signals at δ 2.03 and 2.10 in the 1H-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 13C-NMR spectrum. The connectivities of the acetyl groups in 1 were established by the aid of a 1H–1H 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 unacylated rhamnose residue in the 1H-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-ÇOCH3), as well as between a proton signal at δ 4.75 (H-4") and a carbon signal at δ 171.8 (C-4"-O-ÇOCH3), respectively (Fig. 1). Consequently, the structure of 1 was determined as kaempferol 3-α-L-(2,3-di-O-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside.

Crassirhizmoside B (2) was isolated as a pale yellow amorphous powder, [α]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 with AlCl₃ and NaOAc, also suggested the presence of free hydroxy 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 1H-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 13C-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-ÇOCH3), and between a proton signal at δ 4.99 (H-4") and a carbon signal at δ 171.7 (C-4"-O-ÇOCH3). 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 1H-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.

Crassirhizmoside C (3) was also obtained as a pale yellow amorphous powder, [α]D −161°. The molecular formula was determined as C₂₁H₂₄O₁₀ by HR-FAB-MS. The UV absorption bands at 264, 325 (sh) and 343 nm, and the bathochromic shifts with AlCl₃ and NaOAc, also suggested the presence of kaempferol as an aglycone. The 1H- and 13C-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-ÇOCH3), and between a proton signal at δ 5.08 (H-3") and a carbon signal at δ 172.3 (C-3"-O-ÇOCH3). Crassirhizmoside C (3) was consequently determined as kaempferol 3-α-L-(2,3-di-O-acetyl)rhamnopyranoside-7-α-L-rhamnopyranoside.

Kaempferol glycosides (1—4) and kaempferol (5) were examined in 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 (IC₅₀ values, 215, 240, 405 and 110 μM, respectively) and DDDP (IC₅₀ 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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>RDDP</th>
<th>RNase H</th>
<th>DDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>215</td>
<td>&gt;500</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>240</td>
<td>&gt;500</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>405</td>
<td>&gt;500</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>110</td>
<td>&gt;500</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Adriamycin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illimaquinone&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Positive control of HIV-1 RT-associated DNA polymerase activities.  
<sup>b</sup> Positive control of HIV-1 RT-associated RNase H activity.
ILIMAQUINONE was used as a positive control which inhibited the RNAase H activity was measured by the degradation of 3H-labeled RNA in a hybrid in the presence of the test compound. The percentage of the inhibition was calculated as follows:

\[
\text{Inhibition} = \frac{1 - (dpm_{\text{blank}} - dpm_{\text{core}})}{dpm_{\text{blank}} - dpm_{\text{core}}} \times 100
\]
activity with an IC<sub>50</sub> of 50 μM under the above conditions.

**DNA–RNA Hybrid Preparation** A mixture of 0.57 nmol poly(dT), 0.32 nmol poly(rA) and 5 pmol [3H]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.20

**RDDP Activity Assay** For the assay of RDDP activity, HIV-1 RT was adjusted to 0.01 U/μl with a solution of 0.2 mM 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<sub>2</sub>, 5 mM DTT, 1.25 μg/ml (ca. 16 nm) poly(rA)-oligo(dT)<sub>12–18</sub> as a template primer, 250 nM dTTP, 100 nM [methyl-3H]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 mM 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 imersed in 3 ml of scintillation fluid. The amount of a polymer fraction, including 3H-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:

\[
\text{Inhibition} = \left(1 - \frac{\text{dpm}_{\text{comp.}}}{\text{dpm}_{\text{cont.}}} \right) \times 100
\]

Adriamycin was used as a positive control, which inhibited the RDDP activity with an IC<sub>50</sub> 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 mM 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<sub>2</sub>, 5 mM DTT, 1.25 μg/ml (ca. 16 nm) poly(dA)-oligo(dT)<sub>12–18</sub> as a template primer, 250 nM dTTP, 200 nM [methyl-3H]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 mM 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 3H-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:

\[
\text{Inhibition} = \left(1 - \frac{\text{dpm}_{\text{comp.}}}{\text{dpm}_{\text{cont.}}} \right) \times 100
\]

Adriamycin was used as a positive control, which inhibited DDDP activity with an IC<sub>50</sub> of 6 μM under the above conditions.

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**References**