

## Three New Stereoisomers of Condensed Tannins from the Roots of *Rosa multiflora*

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**Three new stereoisomers of condensed tannins (1–3), and four known phenolic compounds (4–7) were isolated from the 80% acetone extract of the roots of *Rosa multiflora* Thunberg. The structures of these compounds were elucidated using 1D/2D NMR, high resolution (HR)-MS, and circular dichroism (CD) spectra. In order to evaluate their anti-oxidative and anti-inflammatory activities, their 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and inhibitory activity on nitric oxide (NO) production were determined.**

**Key words** *Rosa multiflora*; condensed tannin; proguibourtinidin; profisetinidin; phlobatannin

*Rosa multiflora* Thunberg (RM), also known as the Multiflora Rose, the Baby Rose, or the Rambler Rose, is native to China, Japan, and Korea. RM was first introduced in the West in 1866 as a rootstock for ornamental roses and has been sometimes been viewed as a weed because of its tenacious nature and prolific growth characteristics.<sup>1)</sup> However, all parts of RM, that is, hips, leaves, stems, flowers, and roots, have been used as folk medicines in Korea. In particular, the roots of RM, the plant material utilized in this study, have traditionally been used to treat hemorrhage and rheumatism.<sup>2)</sup> Recently, some lignans and triterpenes were isolated from these roots.<sup>3–6)</sup> In the present study, we describe the isolation and elucidation of the three new structural isomers of condensed tannins that are related to proanthocyanidins (proguibourtinidin, profisetinidin and phlobatannin) and possess unique absolute configurations. In addition, their anti-oxidative and anti-inflammatory activities are evaluated.

### Results and Discussion

The 80% acetone extract of the roots of RM was subjected to repeated liquid column chromatography using Sephadex LH-20, MCI-gel CHP20P and reversed phase medium pressure liquid chromatography (MPLC) to afford seven compounds including 3 new condensed tannins (Fig. 1). The known compounds (4–7) were identified as gallic acid, cat chin, gallo catechin and procyanidin B-3, respectively by comparing their spectroscopic (MS and NMR) data with literature values.<sup>7,8)</sup>

Compound **1** was obtained as an amorphous red-brown powder. High resolution (HR)-negative FAB-MS ( $m/z$  545.1453 [ $M-H$ ]<sup>-</sup>, Calcd for  $C_{30}H_{25}O_{10}$ , 545.1448) indicated its molecular formula was  $C_{30}H_{26}O_{10}$ . The FT-IR spectrum of **1** exhibited vibration bands corresponding to free hydroxyl ( $3350\text{ cm}^{-1}$ ), aliphatic ( $2920\text{ cm}^{-1}$ ) and aromatic ( $1475\text{ cm}^{-1}$ ) components. During thin layer chromatography (TLC), **1** was detected using the UV lamp at 254 nm, as a dark green spot by spraying with  $FeCl_3$  solution, or as an orange spot by spraying with anisaldehyde- $H_2SO_4$  solution and heating.  $^1H$ -NMR showed the typical proton signals of flavan-3-ol, aromatic signals at 6.0–7.0 ppm, and aliphatic signals at 2.4–4.6 ppm.  $^1H$ - and  $^{13}C$ -NMR spectra contained signals indicating a rotational conformer.<sup>9)</sup> All the above data suggested that compound **1** was a proanthocyanidin (Table 1).

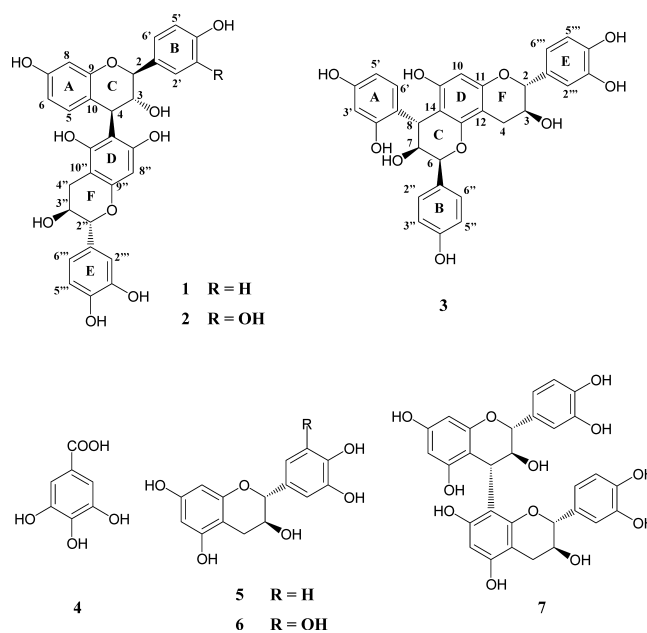


Fig. 1. Structures of Compounds 1–7

Careful analysis of the  $^1H$ -NMR spectrum of compound **1** showed an aromatic ABX-spin system [ $\delta_H$  6.48 (1H, d,  $J=9.0$  Hz, H-5), 6.17 (1H, dd,  $J=2.4, 9.0$  Hz, H-6) and 6.07 (1H, d,  $J=2.4$  Hz, H-8)] and an aromatic  $A_2B_2$ -spin system [ $\delta_H$  7.17 (2H, d,  $J=8.4$  Hz, H-2', H-6') and 6.72 (2H, d,  $J=8.4$  Hz, H-3', H-5')] (Table 1). The aliphatic group was assigned to three correlated methines [ $\delta_H$  4.34 (1H, d,  $J=9.3$  Hz, H-4)  $\leftrightarrow$  4.48 (1H, t,  $J=9.3$  Hz, H-3)  $\leftrightarrow$  4.44 (1H, d,  $J=9.3$  Hz, H-2)] by correlation spectroscopy (COSY), and their carbon signals were assigned to  $\delta_C$  83.1 (C-2), 68.2 (C-3) and 40.7 (C-4) by heteronuclear single quantum coherence (HSQC) (Fig. 2, Table 1). These findings suggested a guibourtinidol moiety.

Another aromatic ABX-spin system [ $\delta_H$  6.75 (1H, d,  $J=1.8$  Hz, H-2''), 6.67 (1H, d,  $J=8.4$  Hz, H-5'') and 6.63 (1H, dd,  $J=1.8, 8.4$  Hz, H-6'')] and an additional aromatic singlet signal [ $\delta_H$  5.90 (1H, s, H-6'' or 8'')] were observed in the  $^1H$ -NMR spectrum of **1** (Table 1). Another aliphatic chain was assigned to a methylene [ $\delta_H$  2.80 (1H, dd,  $J=5.4, 15.9$  Hz, H-4<sub>eq</sub>'') and 2.41 (1H, dd,  $J=8.4, 15.9$  Hz, H-4<sub>ax</sub>'')] and two

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methines [ $\delta_{\text{H}}$  4.51 (1H, d,  $J=7.8$  Hz, H-2'') and 3.77 (1H, ddd,  $J=5.4, 7.8, 8.4$  Hz, H-3'')] by COSY, and their carbon signals were assigned as  $\delta_{\text{C}}$  81.6 (C-2''), 67.1 (C-3'') and 28.9 (C-4'') by HSQC (Fig. 2). These findings suggested the presence of a catechin moiety.

The proton signal,  $\delta_{\text{H}}$  4.34 of H-4 on the guibourtinidol moiety (upper unit), showed correlations with some carbon signals on the catechin moiety (lower unit), that is,  $\delta_{\text{C}}$  154.5, 154.1 and 106.7 by heteronuclear multiple bond coherence (HMBC). Of these carbon signals,  $\delta_{\text{C}}$  106.7 represents a connecting point of the lower unit and may be due to C-6'' or C-8'' (D-ring). Based on the HMBC, the correlations between additional prominent aromatic singlet signal at  $\delta_{\text{H}}$  5.90,  $\delta_{\text{H}}$  4.51 (H-2'' as confirmed by COSY) and  $\delta_{\text{C}}$  155.6 (oxygen

bearing aromatic carbon) indicate that the signals at  $\delta_{\text{C}}$  106.7,  $\delta_{\text{C}}$  155.6 and  $\delta_{\text{H}}$  5.90 should be assigned as C-6'', C-9'' and H-8'', respectively, and also prove the existence of a 4 $\rightarrow$ 6'' interflavanoid linkage (Fig. 2).

Thus, the putative structure of **1** was similar to that of guibourtinidol-(4 $\alpha$  $\rightarrow$ 6)-catechin, which has already been reported.<sup>9)</sup> It showed negative Cotton effect at 200–220 nm in the circular dichroism (CD) spectrum defined an  $\alpha$  orientation at C-4, that is, the 4*S* configuration.<sup>9)</sup> However, **1** showed a high-amplitude positive Cotton effect at 215 nm in the CD spectrum defined as a  $\beta$  orientation at C-4, that is, the 4*R* configuration.<sup>9)</sup> In addition, the absolute configurations of 2*S*,3*R* were determined based on the large coupling constant of the C-ring. Thus, the above results indicated that compound **1** was *ent*-guibourtinidol-(4 $\beta$  $\rightarrow$ 6)-catechin.

Compound **2** was obtained as an amorphous red-brown powder. Aspects of its TLC, FT-IR, and NMR spectra were similar to those of compound **1**. HR-negative FAB-MS ( $m/z$  561.1401 [ $\text{M}-\text{H}$ ]<sup>-</sup>, Calcd for  $\text{C}_{30}\text{H}_{25}\text{O}_{11}$ , 561.1397) revealed a molecular formula of  $\text{C}_{30}\text{H}_{26}\text{O}_{11}$ , which indicated the presence of one more oxygen than in compound **1**. The <sup>1</sup>H-NMR spectrum of **2** showed three sets of ABX-typed aromatic rings, indicating that the proanthocyanidin contained fiseitinidol and catechin moieties compared with **1** (Fig. 2, Table 1). Furthermore, with the exception of an ABX-typed aromatic ring instead of an A<sub>2</sub>B<sub>2</sub>-spin system, the 1D/2D NMR spectrum of **2** was almost identical to that of **1**.

Based on the above, the structure of **2** appeared to be

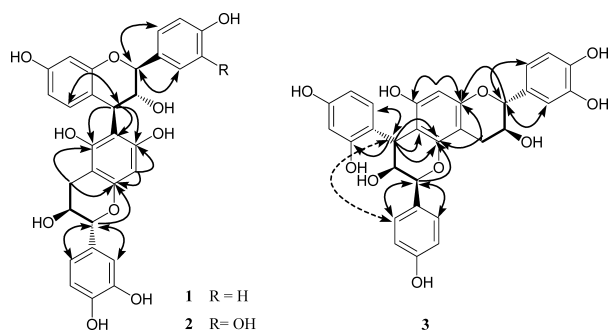


Fig. 2. The Main Correlations of COSY (H—H), HMBC (H $\rightarrow$ C) and NOESY (H $\rightarrow$ H) Correlations for Compounds **1**, **2** and **3**

Table 1. <sup>1</sup>H- and <sup>13</sup>C-NMR Data of **1** and **2** (in DMSO-*d*<sub>6</sub>+D<sub>2</sub>O)

| Positions     | <b>1</b>   |                                   | <b>2</b>   |                                   |             |
|---------------|--|-----------------------------------|--|-----------------------------------|-------------|
|               | $\delta_{\text{H}}$ (mult, $J$ , Hz) <sup>a)</sup> | $\delta_{\text{C}}$ <sup>b)</sup> | $\delta_{\text{H}}$ (mult, $J$ , Hz) <sup>a)</sup> | $\delta_{\text{C}}$ <sup>b)</sup> |             |
| A and C Rings | 2  | 4.44 (d, 9.3)                     | 83.1   | 4.36 (d, 9.6)                     | 83.4        |
|               | 3  | 4.48 (t, 9.3)                     | 68.2   | 4.46 (t, 9.6)                     | 68.2        |
|               | 4 <sub>eq</sub>                                    | 4.34 (d, 9.3)                     | 40.7   | 4.32 (d, 9.6)                     | 40.7        |
|               | 5  | 6.48 (d, 9.0)                     | 128.9  | 6.48 (d, 8.4)                     | 128.9       |
|               | 6  | 6.17 (dd, 2.4, 9.0)               | 108.5  | 6.17 (dd, 2.4, 8.4)               | 108.5       |
|               | 7  |                                   | 155.4  |                                   | 155.3       |
|               | 8  | 6.07 (d, 2.4)                     | 102.3  | 6.07 (d, 2.4)                     | 102.3       |
|               | 9  |                                   | 155.8  |                                   | 155.9       |
|               | 10   |                                   | 118.5  |                                   | 118.5       |
|               | B ring   | 1'                                |  | 130.7                             |             |
| 2'            |  | 7.17 (d, 8.4)                     | 129.6  | 6.79 (d, 1.8)                     | 115.6       |
| 3'            |  | 6.72 (d, 8.4)                     | 115.0  |                                   | 145.0–145.2 |
| 4'            |  |                                   | 157.1  |                                   |             |
| 5'            |  | 6.72 (d, 8.4)                     | 115.0  | 6.68 (d, 7.8)                     | 115.4       |
| 6'            |  | 7.17 (d, 8.4)                     | 129.6  | 6.64 (m)                          | 120.0       |
| D and F rings | 2''  | 4.51 (d, 7.8)                     | 81.6   | 4.51 (d, 7.8)                     | 81.6        |
|               | 3''  | 3.77 (ddd, 5.4, 7.8, 8.4)         | 67.1   | 3.78 (m)                          | 67.2        |
|               | 4 <sub>ax</sub> ''                                 | 2.41 (dd, 8.4, 15.9)              | 28.9   | 2.42 (dd, 8.4, 15.9)              | 28.9        |
|               | 4 <sub>eq</sub> ''                                 | 2.80 (dd, 5.4, 15.9)              |  | 2.81 (dd, 5.1, 15.9)              |             |
|               | 5''  |                                   | 154.5  |                                   | 155.0       |
|               | 6''  |                                   | 106.7  |                                   | 106.8       |
|               | 7''  |                                   | 154.1  |                                   | 154.2       |
|               | 8''  | 5.90 (s)                          | 96.1   | 5.91 (s)                          | 96.2        |
|               | 9''  |                                   | 155.6  |                                   | 155.4       |
|               | 10''   |                                   | 99.8   |                                   | 99.0        |
| E ring        | 1'''   |                                   | 130.9  |                                   | 131.6       |
|               | 2'''   | 6.75 (d, 1.8)                     | 114.9  | 6.76 (d, 1.8)                     | 115.0       |
|               | 3'''   |                                   | 144.6  |                                   | 145.0–145.2 |
|               | 4'''   |                                   | 145.0  |                                   |             |
|               | 5'''   | 6.67 (d, 8.4)                     | 115.3  | 6.68 (d, 8.4)                     | 115.2       |
|               | 6'''   | 6.63 (dd, 1.8, 8.4)               | 118.8  | 6.64 (br d, 8.4)                  | 118.8       |

a) Recorded at 600 MHz. b) Recorded at 150 MHz.

closely related to that of fisetinidol-(4 $\alpha$ →6)-catechin, which has already been reported.<sup>10</sup> It showed negative Cotton effect at 200–220 nm in the CD spectrum defined as an  $\alpha$  orientation at C-4, that is, the 4*S* configuration. However, the CD spectrum of compound **2** showed a strong positive Cotton effect at 216 nm, which defined a 4 $\beta$  orientation.<sup>9</sup> The large coupling constant of the C-ring and the CD spectrum indicated an absolute stereochemistry of 2*S*,3*R*,4*R*. Hence, the structure of compound **2** was determined as *ent*-fisetinidol-(4 $\beta$ →6)-catechin.

Compound **3** was obtained as an off-white powder and its TLC, FT-IR, and NMR characteristics were similar to those of compound **1**. HR-negative FAB-MS ( $m/z$  545.1450 [M-H]<sup>-</sup>, Calcd for C<sub>30</sub>H<sub>25</sub>O<sub>10</sub>, 545.1448) indicated a molecular formula of C<sub>30</sub>H<sub>26</sub>O<sub>10</sub>, which was identical to that of compound **1**.

<sup>1</sup>H-NMR spectrum of **3** provided an aromatic ABX-spin system [ $\delta_{\text{H}}$  6.30 (1H, d,  $J=8.4$  Hz, H-6'), 6.13 (1H, br d,  $J=8.4$  Hz, H-5') and 6.31 (1H, br s, H-3')] in the A-ring, an aromatic A<sub>2</sub>B<sub>2</sub>-spin system [ $\delta_{\text{H}}$  7.00 (2H, d,  $J=8.4$  Hz, H-2'', 6''), 6.50 (2H, d,  $J=8.4$  Hz, H-3'', 5'')] in the B-ring and three methines [ $\delta_{\text{H}}$  5.24 (1H, br s, H-6), 4.41 (1H, d,  $J=2.7$  Hz, H-8) and 4.38 (1H, br d,  $J=2.7$  Hz, H-7)] in the C-ring. HMBC showed correlation peaks between the A, B and C-rings (Fig. 2, Table 2). The small coupling constants ( $J_{6,7}<1.0$ ,  $J_{7,8}=2.7$  Hz) of these three methines indicated a *cis*-*trans* configuration of the heterocyclic C-ring, and these findings suggested an epiguibourtinidol moiety.<sup>11</sup> In addition, <sup>1</sup>H-NMR spectrum of **3** also showed the other ABX-type aromatic system [ $\delta_{\text{H}}$  6.68 (1H, d,  $J=8.4$  Hz, H-5'''), 6.60 (1H, br s, H-2''') and 6.33 (1H, br d,  $J=8.4$  Hz, H-6''')] in the E-ring, an additional aromatic proton in the D-ring ( $\delta_{\text{H}}$  5.90, 1H, s), and an aliphatic group (a methylene and two methines) [ $\delta_{\text{H}}$  4.40 (1H, d,  $J=7.8$  Hz, H-2), 3.70 (1H, m, H-3), 2.65 (1H, m, H-4<sub>eq</sub>) and 2.41 (1H, dd,  $J=7.2, 15.6$  Hz, H-4<sub>ax</sub>)] in the F-ring. HMBC provided the nature of the connection between the D, E and F-rings (Fig. 2, Table 2). The large coupling constants between H-2 and 3 ( $J_{2,3}=7.8$  Hz) indicated a *trans* configuration, and these findings suggested the presence of a catechin moiety.

In particular, the signals indicating a rotational conformer of **3** were not observed on NMR spectrum even though its configuration was similar to that of compound **1**, and this finding suggested that compound **3** was a phlobatannin related to **1**.<sup>12</sup> Interestingly, the carbon signal (C-8) corresponding with C-4, which was the linking point in the upper unit of compound **1**, was observed up-field ( $\delta_{\text{C}}$  31.2) of that observed in compound **1** ( $\delta_{\text{C}}$  40.7) in the <sup>13</sup>C-NMR spectrum of **3** (Table 2). Moreover, HMBC revealed characteristic cross-peaks between the additional aromatic proton in the D-ring at  $\delta_{\text{H}}$  5.90,  $\delta_{\text{H}}$  4.40 (H-2 which was confirmed by COSY) and  $\delta_{\text{C}}$  155.2 (the oxygen-bearing carbon signal), as well as between one of the methine protons of the C-ring ( $\delta_{\text{H}}$  5.24, H-6), the methylene protons of the F-ring ( $\delta_{\text{H}}$  2.41, 2.65, H-4) and  $\delta_{\text{C}}$  153.9 (another oxygen bearing carbon signal). Thus, these correlations proved the existence of tetrahydropyrano[2,3-*f*]chromene and the signals at  $\delta_{\text{C}}$  155.2,  $\delta_{\text{C}}$  153.9 and  $\delta_{\text{H}}$  5.90 were assigned as C-11, C-13 and H-10, respectively (Fig. 2).

In addition, the negative Cotton effect at 210 nm in its CD spectrum indicated an 8 $\alpha$  linkage and a *cis*-*trans* configured

Table 2. <sup>1</sup>H- and <sup>13</sup>C-NMR Data of **3** (in DMSO-*d*<sub>6</sub>+D<sub>2</sub>O)

| Positions    | <b>3</b>   |                                   |       |
|--------------|--|-----------------------------------|-------|
|              | $\delta_{\text{H}}$ (mult, $J$ , Hz) <sup>a)</sup> | $\delta_{\text{C}}$ <sup>b)</sup> |       |
| C, D, F-Ring | 2  | 4.40 (d, 7.8)                     | 81.0  |
|              | 3  | 3.70 (m)                          | 66.7  |
|              | 4 <sub>ax</sub>                                    | 2.41 (dd, 7.2, 15.6)              | 28.2  |
|              | 4 <sub>eq</sub>                                    | 2.65 (m)                          |       |
|              | 6  | 5.24 (br s)                       | 79.9  |
|              | 7  | 4.38 (br d, 2.7)                  | 70.4  |
|              | 8  | 4.41 (d, 2.7)                     | 31.2  |
|              | 9  |                                   | 155.4 |
|              | 10   | 5.90 (s)                          | 97.2  |
|              | 11   |                                   | 155.2 |
|              | 12   |                                   | 99.8  |
|              | 13   |                                   | 153.9 |
|              | 14   |                                   | 104.6 |
|              | A-ring   | 1'                                |       |
| 2'           |  |                                   | 155.6 |
| 3'           |  | 6.31 (br s)                       | 102.7 |
| 4'           |  |                                   | 154.0 |
| 5'           |  | 6.13 (br d, 8.4)                  | 108.2 |
| 6'           |  | 6.30 (d, 8.4)                     | 129.0 |
| B-ring       | 1''  |                                   | 130.3 |
|              | 2''  | 7.00 (d, 8.4)                     | 126.5 |
|              | 3''  | 6.50 (d, 8.4)                     | 115.6 |
|              | 4''  |                                   | 156.7 |
|              | 5''  | 6.50 (d, 8.4)                     | 115.6 |
|              | 6''  | 7.00 (d, 8.4)                     | 126.5 |
| E-ring       | 1'''   |                                   | 130.8 |
|              | 2'''   | 6.60 (br s)                       | 114.5 |
|              | 3'''   |                                   | 144.8 |
|              | 4'''   |                                   | 144.8 |
|              | 5'''   | 6.68 (d, 8.4)                     | 115.6 |
|              | 6'''   | 6.33 (br d, 8.4)                  | 117.7 |

a) Recorded at 600 MHz. b) Recorded at 150 MHz.

Table 3. DPPH Radical Scavenging Activities and NO Production Inhibition by Compounds **1**–**7**

| Compounds | DPPH radical scavenging activity ( $\mu\text{M}$ ) | Inhibitory activity on NO production ( $\mu\text{M}$ ) |
|-----------|--|--|
| <b>1</b>  | 25.76±0.39 <sup>d</sup>                            | 26.67±6.92 <sup>b</sup>                                |
| <b>2</b>  | 13.45±0.17 <sup>a</sup>                            | 23.43±2.57 <sup>b</sup>                                |
| <b>3</b>  | 18.39±0.65 <sup>c</sup>                            | 25.82±5.95 <sup>b</sup>                                |
| <b>4</b>  | 16.07±0.32 <sup>b</sup>                            | 23.36±1.20 <sup>b</sup>                                |
| <b>5</b>  | 33.31±0.56 <sup>e</sup>                            | 33.72±2.08 <sup>b</sup>                                |
| <b>6</b>  | 16.12±0.51 <sup>b</sup>                            | 26.50±5.39 <sup>b</sup>                                |
| <b>7</b>  | 13.76±0.06 <sup>a</sup>                            | 23.39±0.74 <sup>b</sup>                                |
| Vit. C    | 13.30±0.31 <sup>a</sup>                            | —  |
| L-NMMA    | —  | 7.83±1.07 <sup>a</sup>                                 |

Values represent means±S.D. of three determinations. Values bearing different superscripts in same columns are significantly different ( $p<0.05$ ).

heterocyclic C-ring, which indicated an absolute stereochemistry of 6*S*,7*S*,8*R*.<sup>12</sup> Furthermore, an association between H-2'', 6'' in the B-ring and H-8 in the C-ring by nuclear Overhauser effect spectroscopy (NOESY) indicated that the C-ring had a 'sofa conformation' and supported the above absolute stereochemistry.<sup>13</sup> Accordingly, compound **3** was identified as (6*S*,7*S*,8*R*)-2-(3,4-dihydroxyphenyl)-6-(4-hydroxyphenyl)-8-(2,4-dihydroxyphenyl)-2,3-*trans*-6,7-*cis*-7,8-*trans*-3,4,7,8-tetrahydro-2*H*,6*H*-pyrano[2,3-*f*]chromene-3,7,9-triol. The phlobatannins composed of guibourtinidol and catechin moieties have already been reported as 8,9-



*trans*-9,10-*cis*-tetrahydropyrano[2,3-*h*]chromene, which had 10 $\alpha$  (negative Cotton effect at 210 nm in its CD) or 10 $\beta$  (positive Cotton effect at 210 nm in its CD) linkage. However, the structural and regional isomer, **3**, is reported here for the first time.<sup>14)</sup>

The naturally occurring phlobatannins are known to be made from 5-dehydroxy-type condensed tannins such as proguibourtinidin and profisetinidin *via* quinone-methide intermediate. Based on the biomimetic synthesis under mild basic conditions, recyclization involving 5''-OH (in the D-ring) and C-2 (in the C-ring) requires rotation of the C(3)–C(4) bond, which will invariably convert the 3,4-*trans* to the 3,4-*cis* configuration.<sup>12)</sup> Thus, **3** might have been a metabolic product of proguibourtinidin (guibourtinidol-catechin) such as **1** on the root of RM in our present study. Furthermore, this result indicated the existence of an enzyme that could recognize unique 5-dehydroxy-type condensed tannins and produce distinct phlobatannins on the root of RM.

These new stereoisomers of the condensed tannins (**1–3**), along with the four known phenolic compounds isolated from the root of RM, were evaluated for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and inhibitory activity on nitric oxide (NO) production in order to determine their anti-oxidative and anti-inflammatory activity. Among the 5-dehydroxy-condensed tannins, **2** (IC<sub>50</sub> = 13.45 ± 0.17  $\mu$ M) was superior to **1** (IC<sub>50</sub> = 25.76 ± 0.39  $\mu$ M) significantly ( $p < 0.05$ ) because of the 3,4-dihydroxybenzene B-ring, which plays a very important role in radical scavenging activity.<sup>15–16)</sup> Due to the additional separate A-ring that possessed a hydroxyl group, **3** (IC<sub>50</sub> = 18.39 ± 0.65  $\mu$ M), which was a metabolic product of **1**, showed more potent free radical scavenging activity than **1** significantly ( $p < 0.05$ ). Among known condensed tannins, the dimer of flavan-3-ol, **7** (IC<sub>50</sub> = 13.76 ± 0.06  $\mu$ M) was superior to the monomers (**5**, **6**), and **6** (IC<sub>50</sub> = 16.12 ± 0.51  $\mu$ M) exhibited more potent free radical scavenging activity than **5** (IC<sub>50</sub> = 33.31 ± 0.56  $\mu$ M) significantly ( $p < 0.05$ ) (Table 3). These findings indicated that the free radical scavenging activities of condensed tannins were dependent on the number of phenolic hydroxyl groups. In addition, all compounds that did not exhibit toxicity at 100  $\mu$ g/ml or below on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown) moderately inhibited NO production. Free radicals such as peroxynitrite (ONOO<sup>-</sup>) produced from NO are known to play a key role in enhancing inflammation through the activation of nuclear factor (NF)-kappa B and activator protein 1 (AP)-1 transcription factors, development of cyclooxygenase-2 (COX-2), and expression of inducible nitric oxide synthase (iNOS) in various inflammatory diseases.<sup>17)</sup> Thus, these results suggest that the condensed tannins isolated from the root of RM might be developed as anti-oxidative and anti-inflammatory agents.

## Experimental

**General Experimental Methods** Sephadex LH-20 (10–25  $\mu$ m, GE Healthcare Bio-Science AB, Uppsala, Sweden) and MCI-gel CHP 20P (75–150  $\mu$ m, Mitsubishi Chemical, Tokyo, Japan) and were used for column chromatography. Reversed-phase MPLC using Water 650E HPLC system equipped with a Gilson 110UV/VIS detector was carried out on ODS columns (Ultra Pack ODS-SM-50B, 300 × 26 mm i.d., 50  $\mu$ m, Yamazen, Osaka, Japan) with the MeOH/H<sub>2</sub>O solvent system. TLC was carried out on pre-coated silica gel 60 F<sub>254</sub> plate (Merck, Darmstadt, Germany) and spots were detected under UV radiation (254 nm) and by spraying with FeCl<sub>3</sub> solution and 10% H<sub>2</sub>SO<sub>4</sub> or anisaldehyde-H<sub>2</sub>SO<sub>4</sub> solution followed by heating.

NMR spectra were recorded on a Varian VNS (<sup>1</sup>H-NMR, 600 MHz; <sup>13</sup>C-NMR, 150 MHz) instrument. FT-IR spectra were obtained using Magna 750. HR-FAB-MS was measured with JMS-AX505WA. Optical rotations and circular dichroism spectra were measured on Autopol III polarimeter and J-715 spectropolarimeter, respectively.

**Plant Material** The roots of RM, which had already been cut, were purchased on Gyeongdong Market (Seoul, Korea) in March 2008 and the identity of this plant material was confirmed by TLC by comparison with another root of RM (Yesan, Korea), certified by Prof. Lee (Pharmacognosy Lab, College of Pharmacy, Chung-Ang University). The voucher specimen (RM2008) was deposited at the herbarium of the College of Pharmacy, Chung-Ang University.

**Extraction and Isolation** Two kilograms of the roots of RM were extracted several times with 80% acetone at room temperature. After removing the acetone under vacuum, the residual aqueous solution was filtered. The filtrate was then concentrated (160 g), applied to Sephadex LH-20 (2 kg, 10 × 80 cm) and eluted with water containing increasing proportions of MeOH to afford 3 sub-fractions. Sixteen grams of fraction 2 was subjected to MCI-gel CHP20P (600 g, 5 × 60 cm) with a gradient elution system of water–methanol to yield **4** (42 mg), **5** (2500 mg) and three additional sub-fractions. Repeated column chromatography of these sub-fractions using reversed-phase MPLC (300 × 26 mm i.d., 50  $\mu$ m, 0–30% MeOH in H<sub>2</sub>O) yielded **1** (237 mg), **2** (143 mg), **3** (141 mg), **6** (20 mg) and **7** (150 mg), respectively.

*ent*-Guibourtinidol-(4 $\beta$ →6)-catechin (**1**): Amorphous red-brown powder.  $[\alpha]_D^{25} + 100^\circ$  ( $c = 0.0014$ , MeOH). IR (KBr) cm<sup>-1</sup>: 3350, 1600, 1475. HR-FAB-MS  $m/z$ : 545.1453 [M–H]<sup>-</sup> (Calcd for C<sub>30</sub>H<sub>25</sub>O<sub>10</sub>, 545.1448). c.d.  $[\theta]_{286}^{25} 3.15$ ,  $[\theta]_{283}^{25} 0$ ,  $[\theta]_{274}^{25} -6.32$ ,  $[\theta]_{251}^{25} 0$ ,  $[\theta]_{240}^{25} 6.69$ ,  $[\theta]_{215}^{25} 62.07$ ,  $[\theta]_{200}^{25} 0$ . <sup>1</sup>H- and <sup>13</sup>C-NMR data: see Table 1.

*ent*-Fisetinidol-(4 $\beta$ →6)-catechin (**2**): Amorphous red-brown powder.  $[\alpha]_D^{25} + 130^\circ$  ( $c = 0.0015$ , MeOH). IR (KBr) cm<sup>-1</sup>: 3350, 1600, 1475. HR-FAB-MS  $m/z$ : 561.1401 [M–H]<sup>-</sup> (Calcd for C<sub>30</sub>H<sub>25</sub>O<sub>11</sub>, 561.1397). c.d.  $[\theta]_{286}^{25} 4.59$ ,  $[\theta]_{282}^{25} 0$ ,  $[\theta]_{273}^{25} -7.16$ ,  $[\theta]_{247}^{25} 0$ ,  $[\theta]_{240}^{25} 6.78$ ,  $[\theta]_{216}^{25} 53.27$ ,  $[\theta]_{210}^{25} 0$ . <sup>1</sup>H- and <sup>13</sup>C-NMR data: see Table 1.

(6*S*,7*S*,8*R*)-2-(3,4-Dihydroxyphenyl)-6-(4-hydroxyphenyl)-8-(2,4-dihydroxyphenyl)-2,3-*trans*-6,7-*cis*-7,8-*trans*-3,4,9,10-tetrahydro-2*H*,6*H*-pyrano[2,3-*f*]chromene-3,7,9-triol (**3**): Amorphous off-white powder.  $[\alpha]_D^{25} - 100^\circ$  ( $c = 0.0014$ , MeOH). IR (KBr) cm<sup>-1</sup>: 3350, 1600, 1475. HR-FAB-MS  $m/z$ : 545.1450 [M–H]<sup>-</sup> (Calcd for C<sub>30</sub>H<sub>25</sub>O<sub>10</sub>, 545.1448). c.d.  $[\theta]_{278}^{25} 5.14$ ,  $[\theta]_{250}^{25} 0$ ,  $[\theta]_{236}^{25} -20.50$ ,  $[\theta]_{210}^{25} -11.91$ ,  $[\theta]_{203}^{25} 0$ . <sup>1</sup>H- and <sup>13</sup>C-NMR data: see Table 2.

**Measurement of DPPH Radical Scavenging Activity** DPPH (Sigma, St. Louis, MO, U.S.A.) radical scavenging assays were performed in order to evaluate the anti-oxidative activity of each component isolated from the roots of RM. Briefly, 20  $\mu$ l of each sample in absolute EtOH was added to 180  $\mu$ l of a DPPH solution (0.1 mM, in absolute EtOH). After mixing gently for 30 min, optical densities were measured at 540 nm using a microplate reader (TECAN, Salzburg, Austria). L-Ascorbic acid was used as a positive control.

**Cell Culture** Mouse macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank. These cells were grown at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>) in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum, 100 IU/ml penicillin G and 100 mg/ml streptomycin (Gibco BRL, Grand Island, NY, U.S.A.).

**Measurement of Cell Viability** Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, which is based on the reduction of MTT to formazan by mitochondrial dehydrogenase. Briefly, the cells (3 × 10<sup>4</sup>/200  $\mu$ l medium) were treated with each sample and incubated for 24 h at 37 °C. After MTT reagent (0.5 mg/ml) (Sigma, St. Louis, MO, U.S.A.) was added to medium and incubated for an additional 4 h, the medium was then removed and the MTT-formazan was dissolved in 200  $\mu$ l dimethylsulfoxide (DMSO). The extent of the reduction of MTT to formazan was quantified by measuring the absorbance at 540 nm using a microplate reader.

**Measurement of Inhibitory Effect on NO Production** NO liberated from RAW 264.7 cells was measured by Griess assay. Briefly, RAW 264.7 cells (3 × 10<sup>4</sup>/200  $\mu$ l medium) stimulated by 1  $\mu$ g/ml lipopolysaccharide (LPS) were treated with each sample and incubated for 24 h at 37 °C. The medium was then mixed with an equal volume of Griess reagent (Sigma, St. Louis, MO, U.S.A.). The amount of NO was quantified by measuring the absorbance at 540 nm using a microplate reader and was determined from a sodium nitrite standard curve.

**Statistical Analysis** All data are expressed means ± S.D. Analysis was

performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (S–N–K) test. Results were considered to be significantly different when *p* values were less than 0.05. Values bearing different superscripts indicate significant differences.

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