

# Isolation and Characterization of Dammarane-Type Saponins from *Gynostemma pentaphyllum* and Their Inhibitory Effects on IL-6-Induced STAT3 Activation

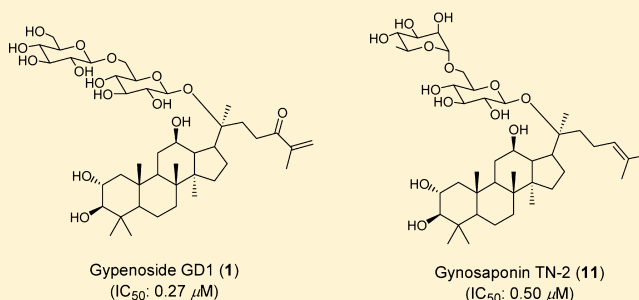
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## Supporting Information

**ABSTRACT:** Five new dammarane-type saponins, gypenosides GD1–GD5 (1–5), along with six known saponins (6–11), were isolated from the aerial parts of *Gynostemma pentaphyllum* using various chromatographic methods. Their structures were elucidated by a combination of spectroscopic and spectrometric data, including 1D and 2D NMR and HRESIMS. All isolates were tested for their inhibitory effects on IL-6-induced STAT3 promoter activity in Hep3B cells. Compounds 1, 9, and 11 displayed potent inhibitory effects, with IC<sub>50</sub> values ranging from 0.27 to 0.59  $\mu$ M.



*Gynostemma pentaphyllum* (Thunb.) Makino, belonging to the family Cucurbitaceae, is a perennial creeping plant mainly distributed in Korea, mainland China, and Japan. It has been widely used as a vegetable, a dietary supplement, and an herbal tea.<sup>1</sup> Previous phytochemical studies on *G. pentaphyllum* indicated the presence of dammarane saponins and flavonoid glycosides, of which some have various bioactivities such as antilipidemic, anti-inflammatory, hepatoprotective, and cardiovascular effects.<sup>2–5</sup> Up to the present, approximately 170 dammarane-type glycosides have been isolated from *G. pentaphyllum*. In contrast, about 30 ginsenosides have been reported from *Panax ginseng* C.A. Mey. (Araliaceae).<sup>1</sup> Moreover, the saponins from *G. pentaphyllum*, known as gypenosides, can be more easily isolated from the *n*-BuOH-soluble fraction of the plant of origin than the ginsenosides.<sup>6</sup> Due to this reason, *G. pentaphyllum* has continuously attracted the interest of numerous research groups.

Interleukin-6 (IL-6), a multifunctional cytokine that preferentially activates signal transducer and activator of transcription 3 (STAT3), is well recognized for its role in initiating and amplifying inflammatory processes. Previous investigations have reported that an increased level of IL-6 is implicated closely in the pathology of immune and inflammatory disorders such as rheumatoid arthritis, osteoporosis, cancer, cachexia, hypercalcemia, and multiple myeloma.<sup>7–13</sup> Therefore, inhibition of IL-6-induced STAT3 has been suggested to be a potential approach for the treatment of human immune and inflammatory diseases. In a preliminary bioassay, it was found that an *n*-BuOH-soluble fraction of *G. pentaphyllum* possessed an inhibitory effect on IL-6-induced

STAT3 promoter activity in Hep3B cells (IC<sub>50</sub> value: 7.2  $\mu$ g/mL). The bioassay-guided investigation of this fraction afforded five new dammarane-type saponins, gypenosides GD1–GD5 (1–5), along with six known saponins (6–11). Herein are described the structural elucidation of compounds 1–5, along with the evaluation of the inhibitory effects on IL-6-induced STAT3 activation of compounds 1–11.

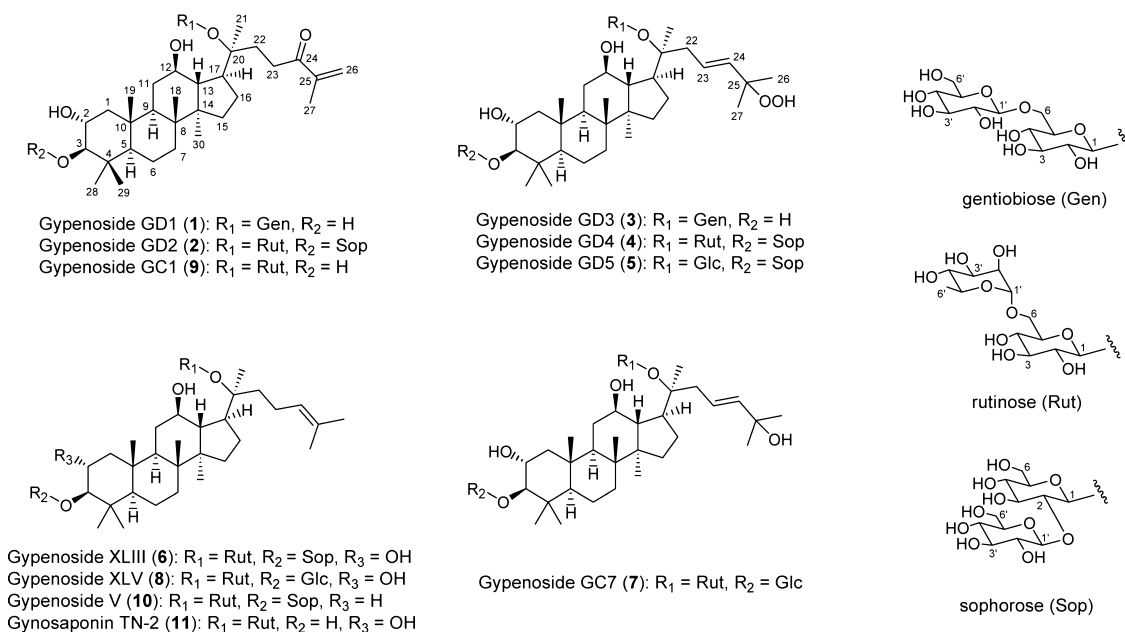
## RESULTS AND DISCUSSION

Compound 1 was isolated as an amorphous powder. The molecular formula, C<sub>42</sub>H<sub>70</sub>O<sub>15</sub>, was deduced from its HRESIMS data (*m/z* 837.4601 [M + Na]<sup>+</sup>, calcd for C<sub>42</sub>H<sub>70</sub>O<sub>15</sub>Na, 837.4612) and <sup>13</sup>C NMR data (Table 1). The IR spectrum showed the presence of hydroxy (3387 cm<sup>-1</sup>) and  $\alpha,\beta$ -unsaturated carbonyl (1675 cm<sup>-1</sup>) groups. The <sup>1</sup>H NMR spectrum (Table 1) exhibited signals for seven methyl groups at  $\delta_H$  1.81, 1.53, 1.24, 1.07, 0.95, 0.93, and 0.91, two germinal coupled protons at  $\delta_H$  6.36 (1H, s) and 5.73 (1H, d, *J* = 1.0 Hz), three oxygenated methines at  $\delta_H$  4.19 (1H, m), 4.09 (1H, m), and 3.35 (1H, d, *J* = 9.5 Hz), and two anomeric protons at  $\delta_H$  5.10 (1H, d, *J* = 7.5 Hz) and 5.02 (1H, d, *J* = 8.0 Hz). The <sup>13</sup>C NMR data including DEPT and HSQC spectra of 1 showed 42 carbon resonances comprising seven quaternary carbons, 17 methines, 11 methylenes, and seven methyls, of which 30 were assigned to the aglycone 2 $\alpha,3\beta,12\beta,20$ (S)-tetrahydroxydammar-25-en-24-one<sup>14</sup> and 12 to the sugar moieties. Acid hydrolysis of 1 gave a D-glucose, which was

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Chart 1



clarified by GC analysis. The chemical shifts and the values of coupling constants of the sugar moieties in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data suggested the presence of two  $\beta$ -glucopyranosyl signals, which were confirmed by the analysis of their HMBC and COSY spectra (Figure 1). Its  $\beta$ -glycosidic linkages were evident from the  $J$  values in the  $^1\text{H}$  NMR spectrum ( $J_{1'2'} = 7.5$  Hz and  $J_{1''2''} = 8.0$  Hz), allowing the sugar part to be determined as a gentiobiose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside) residue. The location of the gentiobiose moiety was assigned at C-20 by the observed HMBC correlations between H-1' ( $\delta_{\text{H}}$  5.10) and C-20 ( $\delta_{\text{C}}$  83.3) and between H-1'' ( $\delta_{\text{H}}$  5.02) and C-6' ( $\delta_{\text{C}}$  70.4). The configuration of 1 was determined from a NOESY experiment and from the  $J$  values, which showed NOE cross-peaks (H-3 $\alpha$ /Me-28, H-3 $\alpha$ /H-5 $\alpha$ , H-2 $\beta$ /Me-29, Me-29/Me-19, Me-19/Me-18, H-12 $\alpha$ /H-30, H-30/H-17 $\alpha$ , H-17 $\alpha$ /Me-21; Figure 2) and a large coupling constant between H-2 and H-3 ( $J = 9.5$  Hz). These data permitted the H-2, H-3, H-12, and C-20 to be established as  $\beta$ ,  $\alpha$ ,  $\alpha$ , and S, respectively.<sup>16,17</sup> Thus, compound 1 was determined as 2 $\alpha$ ,3 $\beta$ ,12 $\beta$ ,20(S)-tetrahydroxydammar-25-en-24-one-20-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside and given the trivial name gypenoside GD1.

Compound 2 was isolated as an amorphous powder. The molecular formula of  $\text{C}_{54}\text{H}_{90}\text{O}_{24}$  was assigned from the HRESIMS data ( $m/z$  1145.5713 [ $\text{M} + \text{Na}$ ] $^+$ , calcd for  $\text{C}_{54}\text{H}_{90}\text{O}_{24}\text{Na}$ , 1145.5720). Its IR spectrum was similar to that of 1, suggesting that compound 2 also possesses hydroxy (3385  $\text{cm}^{-1}$ ) and  $\alpha,\beta$ -unsaturated carbonyl (1676  $\text{cm}^{-1}$ ) functionalities. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data were also closely comparable to those of 1 for the aglycone moiety; however, a difference was observed in that the C-3 signal was strongly deshielded to  $\delta_{\text{C}}$  95.6 (2) (vs  $\delta_{\text{C}}$  83.8 for compound 1) (Table 1). This difference was attributed to the attachment of the sugar moiety at C-3, suggesting that the aglycone part of 2 was identical to that of 1.<sup>14,16</sup> Moreover, acid hydrolysis of 2 gave L-rhamnose and D-glucose in a ratio of 1:3 by GC-MS analysis, and the composition of the sugar moieties was identified as one rutinose ( $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside) unit and one sophorose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-

glucopyranoside) unit by detailed analysis of the HMBC and COSY spectra. The locations of these disaccharides were determined as at the C-20 and C-3 positions by the observed HMBC correlations between H-1' ( $\delta_{\text{H}}$  5.10) and C-20 ( $\delta_{\text{C}}$  83.1) and between H-1'' ( $\delta_{\text{H}}$  4.92) and C-3 ( $\delta_{\text{C}}$  95.6), respectively. The configuration of 2 was elucidated as H-2 $\beta$ , H-3 $\alpha$ , H-12 $\alpha$ , and 20(S) in the same manner as for 1.<sup>4,5</sup> Accordingly, compound 2 (gypenoside GD2) was characterized as 2 $\alpha$ ,3 $\beta$ ,12 $\beta$ ,20(S)-tetrahydroxydammar-25-en-24-one-3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranosyl-20-O- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside.

Compound 3 was isolated as an amorphous powder. Its molecular formula of  $\text{C}_{42}\text{H}_{72}\text{O}_{16}$  was determined from the HRESIMS data ( $m/z$  855.4703 [ $\text{M} + \text{Na}$ ] $^+$ , calcd for  $\text{C}_{42}\text{H}_{72}\text{O}_{16}\text{Na}$ , 855.4718). Acid hydrolysis of 3 yielded D-glucose by GC-MS analysis, and the  $^1\text{H}$  and  $^{13}\text{C}$  NMR, HMBC, and COSY spectra displayed signals assignable to a gentiobiose moiety. The  $^{13}\text{C}$  NMR spectrum exhibited 42 resonances, of which 12 signals were attributed to the gentiobiose unit and 30 signals to the aglycone. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data were similar to those of 1, except for signals due to the side chain from C-22 to C-27 (Table 1). Compound 3 exhibited signals for two olefinic methine groups ( $\delta_{\text{H}}$  6.14 and 6.12/ $\delta_{\text{C}}$  138.1 and 126.7) and a hydroperoxy group ( $\delta_{\text{C}}$  81.4) instead of an  $\alpha,\beta$ -unsaturated carbonyl ( $\delta_{\text{C}}$  202.7) and a 1,1-disubstituted olefinic group ( $\delta_{\text{H}}$  6.36, and 5.73/ $\delta_{\text{C}}$  144.4, and 125.7) as found in compound 1. Furthermore, additional supporting data for the side chain included the COSY cross-peaks observed from H-22 through H-24 [H-22 ( $\delta_{\text{H}}$  3.09, 2.88), H-23 ( $\delta_{\text{H}}$  6.14), and H-24 ( $\delta_{\text{H}}$  6.12)] and the HMBC correlations observed from H-24 ( $\delta_{\text{H}}$  6.12) and H-26, 27 ( $\delta_{\text{H}}$  1.59) to C-25 ( $\delta_{\text{C}}$  81.4) (Figure 1). The results obtained suggested that the aglycone of 3 is identical to those of gypenoside GC4.<sup>14,16</sup> The location of disaccharide was determined at C-20 by the observed HMBC correlation between H-1' ( $\delta_{\text{H}}$  5.16) and C-20 ( $\delta_{\text{C}}$  83.3). The configuration of 3 was determined as H-2 $\beta$ , H-3 $\alpha$ , H-12 $\alpha$ , and 20(S) by the NOESY correlations (H-3 $\alpha$ /Me-28, H-3 $\alpha$ /H-5 $\alpha$ , H-2 $\beta$ /Me-29, Me-29/Me-19, Me-19/Me-18, H-12 $\alpha$ /H-30, H-30/H-17 $\alpha$ , H-17 $\alpha$ /Me-21; Figure 2) and the coupling constant

Table 1. <sup>1</sup>H and <sup>13</sup>C NMR Data for Compounds 1–5<sup>a</sup>

|                | no.  | 1 <sup>b</sup>                 |                     | 2 <sup>c</sup>                 |                | 3 <sup>b</sup>                                   |                | 4 <sup>b</sup>                                   |                | 5 <sup>c</sup>                                   |                |
|----------------|------|--------------------------------|---------------------|--------------------------------|----------------|--|----------------|--|----------------|--|----------------|
|                |      | δ <sub>H</sub>                 | δ <sub>C</sub>      | δ <sub>H</sub>                 | δ <sub>C</sub> | δ <sub>H</sub>                                   | δ <sub>C</sub> | δ <sub>H</sub>                                   | δ <sub>C</sub> | δ <sub>H</sub>                                   | δ <sub>C</sub> |
| aglycone       | 1    | 1.21<br>2.38 dd (3.5,<br>12.5) | 48.6 t <sup>d</sup> | 1.05<br>2.38 dd (4.2,<br>13.3) | 48.0 t         | 1.26, 2.41                                       | 48.4 t         | 1.02, 2.33                                       | 48.0 t         | 1.08<br>2.39 dd (4.2,<br>13.3)                   | 48.0 t         |
|                | 2    | 4.09                           | 68.8 d              | 4.03                           | 66.7 d         | 4.11   | 68.8 d         | 4.02   | 66.9 d         | 4.04   | 66.8 d         |
|                | 3    | 3.35 d (9.5)                   | 83.8 d              | 3.18 d (9.1)                   | 95.6 d         | 3.38 d (9.3)                                     | 83.8 d         | 3.15 d (9.5)                                     | 95.9 d         | 3.20 d (9.1)                                     | 95.7 d         |
|                | 4    |                                | 40.0 s              |                                | 41.0 s         |  | 40.1 s         |  | 40.2 s         |  | 40.2 s         |
|                | 5    | 0.95                           | 56.6 d              | 0.77 d (12.6)                  | 56.2 d         | 0.97   | 56.5 d         | 0.77 d (13.5)                                    | 56.4 d         | 0.80 br d<br>(11.9)                              | 56.3 d         |
|                | 6    | 1.45–1.60                      | 18.9 t              | 1.42–1.62                      | 18.7 t         | 1.49, 1.56                                       | 18.9 t         | 1.49–1.65  | 18.9 t         | 1.41, 1.50                                       | 18.6 t         |
|                | 7    | 1.21, 1.50                     | 35.2 t              | 1.18, 1.45                     | 35.0 t         | 1.22, 1.51                                       | 35.1 t         | 1.18, 1.42                                       | 35.2 t         | 1.21, 1.46                                       | 35.1 t         |
|                | 8    |                                | 40.2 s              |                                | 40.0 s         |  | 40.2 s         |  | 41.2 s         |  | 41.1 s         |
|                | 9    | 1.55                           | 50.5 d              | 1.47                           | 50.2 d         | 1.57   | 50.3 d         | 1.46   | 50.3 d         | 1.49   | 50.2 d         |
|                | 10   |                                | 38.6 s              |                                | 37.8 s         |  | 38.6 s         |  | 38.0 s         |  | 38.0 s         |
|                | 11   | 1.58, 2.11                     | 31.1 t              | 1.55, 2.10                     | 31.0 t         | 1.61, 2.17                                       | 31.0 t         | 1.53, 2.09                                       | 31.0 t         | 1.54, 2.12                                       | 31.2 t         |
|                | 12   | 4.19                           | 70.7 t              | 4.23                           | 70.0 t         | 4.05   | 70.1 t         | 3.96   | 70.7 t         | 3.99   | 70.5 t         |
|                | 13   | 2.02                           | 49.6 d              | 2.01                           | 49.6 d         | 2.05   | 49.7 d         | 1.99   | 49.7 d         | 1.98   | 49.8 d         |
|                | 14   |                                | 51.5 s              |                                | 51.4 s         |  | 51.5 s         |  | 51.7 s         |  | 51.6 s         |
|                | 15   | 0.95, 1.54                     | 30.7 t              | 0.98, 1.54                     | 30.6 t         | 0.97, 1.61                                       | 30.5 t         | 0.94, 1.60                                       | 30.6 t         | 0.98, 1.55                                       | 30.7 t         |
|                | 16   | 1.33, 1.80                     | 26.7 t              | 1.32, 1.84                     | 26.6 t         | 1.47, 1.78                                       | 26.3 t         | 1.42, 1.76                                       | 26.4 t         | 1.45, 1.76                                       | 26.5 t         |
|                | 17   | 2.56 br q (9.5)                | 52.2 d              | 2.61 q (9.8)                   | 51.9 d         | 2.44   | 51.9 d         | 2.39   | 52.2 d         | 2.43   | 52.2 d         |
|                | 18   | 0.93 s                         | 16.2 q              | 0.91 s                         | 16.0 q         | 1.05 s   | 16.2 q         | 1.00 s   | 16.2 q         | 1.00 s   | 16.0 q         |
|                | 19   | 0.95 s                         | 17.7 q              | 0.88 s                         | 17.4 q         | 1.00 s   | 17.6 q         | 0.88 s   | 17.6 q         | 0.91 s   | 17.6 q         |
|                | 20   |                                | 83.3 s              |                                | 83.1 s         |  | 83.3 s         |  | 83.4 s         |  | 83.3 s         |
|                | 21   | 1.53 s                         | 21.8 q              | 1.52 s                         | 21.5 q         | 1.57 s   | 23.3 q         | 1.56 s   | 23.2 q         | 1.62 s   | 23.3 q         |
|                | 22   | 2.08, 2.74                     | 29.9 t              | 2.04, 2.74                     | 29.8 t         | 2.88 dd (7.5,<br>14.5)<br>3.09 dd (5.0,<br>14.5) | 39.9 t         | 2.78 dd (8.5,<br>14.5)<br>3.02 dd (5.5,<br>14.5) | 40.2 t         | 2.78 dd (6.3,<br>14.0)<br>3.07 dd (8.4,<br>14.0) | 40.2 t         |
|                | 23   | 3.16, 3.45                     | 32.8 t              | 3.10, 3.45                     | 32.7 t         | 6.14   | 126.7 d        | 6.13   | 126.6 d        | 6.20   | 126.7 d        |
|                | 24   |                                | 202.7 s             |                                | 202.5 s        | 6.12 d (16.0)                                    | 138.1 d        | 6.11 d (17.0)                                    | 137.9 d        | 6.09 d (15.4)                                    | 138.2 d        |
|                | 25   |                                | 144.4 s             |                                | 144.4 s        |  | 81.4 s         |  | 81.5 s         |  | 81.5 s         |
|                | 26   | 5.73 d (1.0)<br>6.36 s         | 125.7 t             | 5.76 s<br>6.33 s               | 125.3 t        | 1.59 s   | 25.1 q         | 1.59 s   | 25.3 q         | 1.61 s   | 25.3 q         |
|                | 27   | 1.81 s                         | 17.9 q              | 1.86 s                         | 17.8 q         | 1.59 s   | 25.5 q         | 1.59 s   | 25.7 q         | 1.61 s   | 25.6 q         |
|                | 28   | 1.24 s                         | 29.3 q              | 1.29 s                         | 28.3 q         | 1.25 s   | 29.2 q         | 1.28 s   | 28.5 q         | 1.30 s   | 28.4 q         |
|                | 29   | 1.07 s                         | 17.6 q              | 1.19 s                         | 17.6 q         | 1.08 s   | 17.5 q         | 1.16 s   | 17.8 q         | 1.21 s   | 17.8 q         |
|                | 30   | 0.91 s                         | 17.4 q              | 0.96 s                         | 17.4 q         | 0.85 s   | 17.2 q         | 0.81 s   | 17.3 q         | 0.88 s   | 17.2 q         |
| 20-O-<br>inner | 1'   | 5.10 d (7.5)                   | 98.1 d              | 5.10 d (7.7)                   | 98.0 d         | 5.16 d (7.5)                                     | 98.2 d         | 5.12 d (7.5)                                     | 98.6 d         | 5.22 d (7.7)                                     | 98.4 d         |
|                | 2'   | 3.88                           | 74.9 d              | 3.99                           | 74.9 d         | 3.92   | 75.1 d         | 3.95   | 75.6 d         | 4.01   | 75.4 d         |
|                | 3'   | 4.14                           | 79.6 d              | 4.22                           | 79.7 d         | 4.18   | 78.9 d         | 4.17   | 79.3 d         | 4.25   | 79.1 d         |
|                | 4'   | 4.07                           | 71.4 d              | 4.10                           | 71.8 d         | 4.10   | 71.5 d         | 4.03   | 71.5 d         | 4.13   | 71.4 d         |
|                | 5'   | 4.02                           | 76.8 d              | 4.17                           | 76.9 d         | 4.10   | 77.1 d         | 4.13   | 76.9 d         | 4.29   | 78.5 d         |
|                | 6'   | 4.25<br>4.77 dd (2.0,<br>11.0) | 70.4 t              | 4.07<br>4.68 d (9.8)           | 68.0 t         | 4.34<br>4.72 dd (1.0,<br>12.0)                   | 70.4 t         | 3.98, 4.58                                       | 66.9 t         | 4.28, 4.48                                       | 63.1 t         |
| 20-O-term      | 1''  | 5.02 d (8.0)                   | 105.7 d             | 5.52 s                         | 102.3 d        | 5.10 d (8.0)                                     | 105.1 d        | 5.39 s   | 101.8 d        |  |                |
|                | 2''  | 4.03                           | 75.3 d              | 4.58                           | 72.4 d         | 4.05   | 75.3 d         | 4.54   | 72.4 d         |  |                |
|                | 3''  | 3.92                           | 78.6 d              | 4.52                           | 72.8 d         | 3.97   | 78.4 d         | 4.48   | 73.2 d         |  |                |
|                | 4''  | 4.19                           | 71.8 d              | 4.30                           | 74.1 d         | 4.20   | 71.8 d         | 4.17   | 74.6 d         |  |                |
|                | 5''  | 4.21                           | 78.5 d              | 4.34                           | 69.8 d         | 4.22   | 78.3 d         | 4.33   | 69.6 d         |  |                |
|                | 6''  | 4.36<br>4.52 dd (2.5,<br>11.0) | 62.9 t              | 1.66 d (5.6)                   | 18.7 q         | 4.33<br>4.52 dd (2.5,<br>11.0)                   | 62.8 t         | 1.64 d (6.5)                                     | 18.9 t         |  |                |
| 3-O-inner      | 1''' |                                |                     | 4.92 d (7.0)                   | 104.6 d        |  |                | 4.88 d (7.0)                                     | 104.8 d        | 4.92 d (7.0)                                     | 104.7 d        |
|                | 2''' |                                |                     | 4.32                           | 82.3 d         |  |                | 4.26   | 82.6 d         | 4.31   | 82.4 d         |
|                | 3''' |                                |                     | 4.20                           | 78.6 d         |  |                | 4.16   | 78.8 d         | 4.20   | 78.7 d         |
|                | 4''' |                                |                     | 4.12                           | 71.3 d         |  |                | 4.08   | 71.5 d         | 4.15   | 71.7 d         |
|                | 5''' |                                |                     | 3.97                           | 78.4 d         |  |                | 3.93   | 78.5 d         | 4.02   | 78.5 d         |
|                | 6''' |                                |                     | 4.42                           | 62.9 t         |  |                | 4.42, 4.48                                       | 63.2 t         | 4.42   | 63.1 t         |

Table 1. continued

|          | no.              | 1 <sup>b</sup>      |                     | 2 <sup>c</sup>      |                     | 3 <sup>b</sup>      |                     | 4 <sup>b</sup>      |                     | 5 <sup>c</sup>      |                     |
|----------|------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|          |                  | $\delta_{\text{H}}$ | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ | $\delta_{\text{C}}$ |
| 3-O-term | 1 <sup>'''</sup> |                     |                     | 4.48 dd (4.2, 10.2) |                     |                     |                     |                     |                     | 4.47 dd (4.2, 10.2) |                     |
|          | 2 <sup>'''</sup> |                     |                     | 5.53 d (7.7)        | 105.7 d             |                     |                     | 5.47 d (7.5)        | 105.9 d             | 5.53 d (7.7)        | 105.8 d             |
|          | 3 <sup>'''</sup> |                     |                     | 4.18                | 76.8 d              |                     |                     | 4.14                | 76.8 d              | 4.16                | 76.9 d              |
|          | 4 <sup>'''</sup> |                     |                     | 4.30                | 78.2 d              |                     |                     | 4.25                | 78.4 d              | 4.32                | 78.3 d              |
|          | 5 <sup>'''</sup> |                     |                     | 4.31                | 71.9 d              |                     |                     | 4.25                | 72.2 d              | 4.31                | 72.0 d              |
|          | 6 <sup>'''</sup> |                     |                     | 4.05                | 78.5 d              |                     |                     | 4.00                | 78.6 d              | 4.05                | 78.6 d              |
|          |                  |                     |                     | 4.29, 4.61          | 62.4 t              |                     |                     | 4.26, 4.54          | 62.6 t              | 4.25, 4.54          | 62.5 t              |

<sup>a</sup>TMS was used as an internal standard; chemical shifts ( $\delta$ ) are expressed in ppm;  $J$  values are given in parentheses. <sup>b</sup>Data were measured at 500 and 125 MHz. <sup>c</sup>Data were measured at 700 and 175 MHz. <sup>d</sup>Carbon multiplicity.

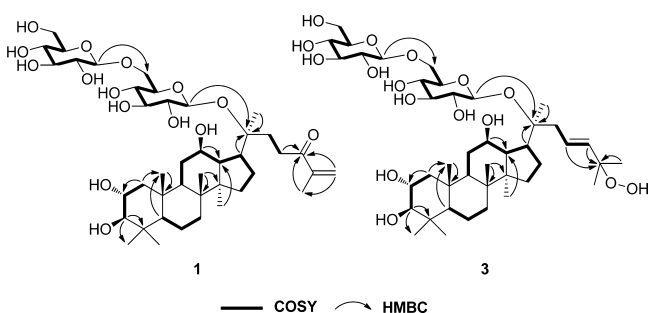


Figure 1. COSY (bold) and key HMBC (arrow) correlations of compounds 1 and 3.

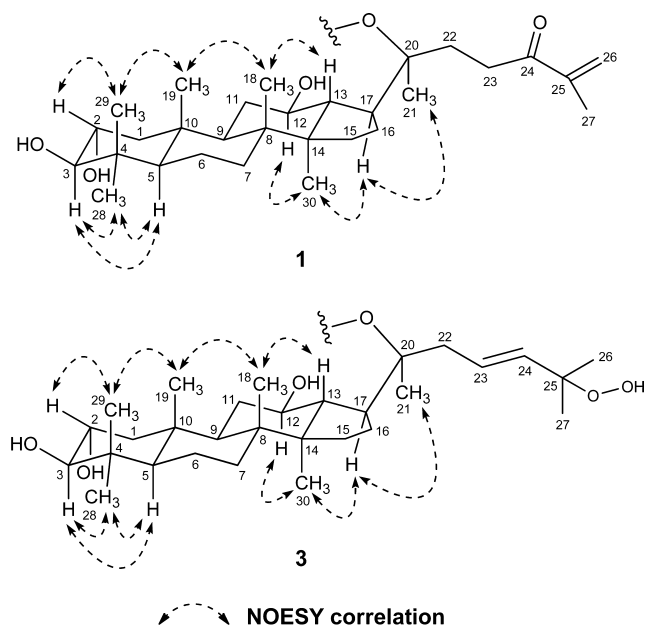


Figure 2. Key NOESY correlations of compounds 1 and 3.

with a  $J_{2,3}$  value of 9.3 Hz. On the basis of the above spectroscopic data, compound 3 (gypenoside GD3) was elucidated as  $2\alpha,3\beta,12\beta,20(S)$ -tetrahydroxy-25-hydroperoxydammar-23-ene-20- $O$ - $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside.

Compound 4 was obtained as an amorphous powder. Its molecular formula of  $\text{C}_{54}\text{H}_{92}\text{O}_{25}$  was determined by HRESIMS ( $m/z$  1163.5792 [ $\text{M} + \text{Na}$ ]<sup>+</sup>, calcd for  $\text{C}_{54}\text{H}_{92}\text{O}_{25}\text{Na}$ , 1163.5825). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data resembled those of 3 for the aglycone moiety; however, the signal of C-3 was shifted downfield to  $\delta_{\text{C}}$  95.9 (vs  $\delta_{\text{C}}$  83.8 for compound 3)

(Table 1). These results indicated that the aglycone of 4 is identical to that of 3, with the differences being due to the sugar moieties at C-3 and C-20. Acid hydrolysis of 4 yielded L-rhamnose and D-glucose in the ratio of 1:3 by GC-MS analysis, and the identification of the two disaccharide units present was confirmed as a rutinose and a sophorose by the analysis of the 1D and 2D NMR data. HMBC correlations from one anomeric proton at  $\delta_{\text{H}}$  5.12 to  $\delta_{\text{C}}$  83.4 and from the other at  $\delta_{\text{H}}$  4.88 to  $\delta_{\text{C}}$  95.9 were observed, indicating that sugar moieties could be located as 20- $O$ -rutinose and 3- $O$ -sophorose substituents, respectively. The configurations of 4 were also elucidated as H-2 $\beta$ , H-3 $\alpha$ , H-12 $\alpha$ , and 20( $S$ ) by a combination of a NOESY experiment and coupling constant ( $J_{2,3} = 9.5$  Hz) information. Thus, compound 4 (gypenoside GD4) was characterized as  $2\alpha,3\beta,12\beta,20(S)$ -tetrahydroxy-25-hydroperoxydammar-23-ene-3- $O$ - $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranosyl-20- $O$ - $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside.

Compound 5 was purified as an amorphous powder. Its molecular formula of  $\text{C}_{48}\text{H}_{82}\text{O}_{21}$  was deduced from the HRESIMS data ( $m/z$  1017.5230 [ $\text{M} + \text{Na}$ ]<sup>+</sup>, calcd for  $\text{C}_{48}\text{H}_{82}\text{O}_{21}\text{Na}$ , 1017.5246). Acid hydrolysis of 5 yielded only D-glucose from the GC-MS analysis conducted. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of 5 and 4 indicated these two compounds to have an identical aglycone moiety and to differ only in the signals due to the sugar moieties (Table 1). These results, including the HMBC and COSY data, indicated that the rutinose unit of 4 was replaced by a glucose moiety in 5. The location of the each sugar moiety was determined from the HMBC data ( $\delta_{\text{H}}$  5.22/ $\delta_{\text{C}}$  83.3 and  $\delta_{\text{H}}$  4.92/ $\delta_{\text{C}}$  95.7), indicating that the two sugar moieties (a glucose and a gentiobiose) could be located at C-20 and C-3, respectively. From the above spectroscopic data, compound 5 (gypenoside GD5) was elucidated as  $2\alpha,3\beta,12\beta,20(S)$ -tetrahydroxy-25-hydroperoxydammar-23-ene-3- $O$ - $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranosyl-20- $O$ - $\beta$ -D-glucopyranoside.

The six known dammarane-type triterpenoids were identified by comparison of their reported spectroscopic data as gypenoside XLIII (6),<sup>17</sup> gypenoside GC7 (7),<sup>14</sup> gypenoside XLV (8),<sup>17</sup> gypenoside GC1 (9),<sup>14</sup> gypenoside V (10),<sup>18</sup> and gynosaponin TN-2 (11).<sup>19</sup>

All isolates were evaluated for their inhibitory effect on IL-6-induced STAT3 activation in Hep3B cells (Table 2). Hep3B cells stably expressing pSTAT3-luciferase were stimulated with IL-6 (10 ng/mL) for 12 h in the presence or absence of the test compounds, and pSTAT3-inducible luciferase activity was then measured. Of these, compounds 1, 9, and 11 showed potent inhibitory effects, with  $\text{IC}_{50}$  values of 0.27, 0.59, and 0.50  $\mu\text{M}$ , respectively, as compared with positive controls, statin<sup>20–22</sup>



**Table 2. Inhibitory Effects of Compounds 1–11 on IL-6-Induced STAT3 Activation<sup>a</sup>**

| compound | IC <sub>50</sub> (μM) <sup>b</sup> | compound | IC <sub>50</sub> (μM) |
|----------|------------------------------------|----------|-----------------------|
| 1        | 0.27                               | 7        | 17.1                  |
| 2        | 19.2                               | 8        | 17.6                  |
| 3        | >30                                | 9        | 0.59                  |
| 4        | >30                                | 10       | 4.0                   |
| 5        | >30                                | 11       | 0.50                  |
| 6        | >30                                |          |                       |

<sup>a</sup>Statin (IC<sub>50</sub> value of 0.51 μM) and genistein (IC<sub>50</sub> value of 15.1 μM) were used as the positive controls. <sup>b</sup>Results are expressed as the mean IC<sub>50</sub> values in μM from triplicate experiments.

(IC<sub>50</sub> value of 0.51 μM) and genistein (IC<sub>50</sub> value of 15.1 μM). It was also found that no cytotoxic effect was observed at their effective concentration used experimentally, indicating that this inhibitory activity was not due to cellular cytotoxicity (data not shown). Considering these results with respect to preliminary structural requirements for activity, three partial structures responsible for the bioactivity could be suggested as follows: a sugar moiety at C-3, a side chain from C-22 to C-27, and a hydroxy group at C-2. Compounds 1, 9, and 11 without a sugar moiety at C-3 exhibited potent bioactivity (<0.6 μM), whereas compounds 2 and 8 showed moderate activity (2: 19.2 μM, 8: 17.6 μM). Compounds 3, 4, and 5, possessing a hydroperoxy group, showed weak inhibitory effects (>30 μM). Compound 10, lacking a hydroxy group at C-2, displayed higher activity, with an IC<sub>50</sub> value of 4.1 μM, than compounds 6 and 7 (>30 and 17.2 μM).

In conclusion, five new and six known dammarane-type saponins (1–11) were isolated from the *n*-BuOH-soluble fraction of *G. pentaphyllum*. It was demonstrated that gypenosides GD1 (1) and GC1 (9) and gynosaponin TN-2 (11) exhibited potent inhibitory effects on IL-6-induced STAT3 promoter activity for the first time. On the basis of these findings, compounds 1, 9, and 11 may be regarded as promising IL-6/STAT3 inhibitors and worthy of further study.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were measured on a JASCO DIP-1000 polarimeter. IR spectra were obtained on a JASCO FT/IR-4100. NMR spectra were recorded on Bruker DRX 500 and Avance III 700 MHz NMR spectrometers. NMR spectra were recorded in pyridine-*d*<sub>5</sub>. HRESIMS were measured on a Bruker maXis 4G spectrometer. Semipreparative HPLC was performed using a Waters HPLC system equipped with two Waters 515 pumps and a 2996 photodiode array detector using a YMC J'sphere ODS-H80 column (4 μm, 150 × 20 mm, i.d., flow rate 6 mL/min). GC-MS was performed using an Agilent 6890/5973i gas chromatograph. MPLC was performed on a Biotage Isolera Prime chromatography system. Open column chromatography was performed using silica gel (70–230 mesh, Merck), Lichroprep RP-18 (40–63 μm, Merck), Sephadex LH-20 (25–100 μm, Pharmacia), and MCI gel (75–150 μm, Mitsubishi). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F<sub>254</sub> (0.25 mm, Merck) plates, and spots were detected by a 10% vanillin-H<sub>2</sub>SO<sub>4</sub> in water spray reagent.

**Plant Material.** The aerial parts of *G. pentaphyllum* were collected at Geochang, Gyeongsangnam-do, Korea, in October 2011. One of the authors (B.Y.H) performed the botanical identification, and a voucher specimen (CBNU-2011-GP) has been deposited at the Herbarium of the College of Pharmacy, Chungbuk National University, Korea.

**Extraction and Isolation.** The air-dried and powdered aerial parts of *G. pentaphyllum* (6 kg) were extracted with MeOH (24 L × 3) at room temperature, and then the solution was evaporated in vacuo. The

residue was suspended in distilled water and partitioned with *n*-hexane, EtOAc, and *n*-BuOH, respectively. The *n*-BuOH-soluble extract (34.8 g) was chromatographed on a Diaion HP-20 column eluted with a step gradient of H<sub>2</sub>O–MeOH (20:80 to 0:100) to give five fractions (GPB1–GPB5). Fraction GPB4 was chromatographed further on a silica gel column eluted with a gradient of CH<sub>2</sub>Cl<sub>2</sub>–MeOH–H<sub>2</sub>O (8:2:1 to 3:3:1, lower layer) to give seven subfractions (GPB41–GPB47). GPB41 was chromatographed on an RP-18 MPLC column (MeCN–H<sub>2</sub>O, 45:55) to afford compound 11 (130 mg). GPB43 was purified by semipreparative HPLC (MeCN–H<sub>2</sub>O, 35:65 to 50:50) to yield compound 6 (11 mg). GPB44 was further purified using an MCI gel column (acetone–H<sub>2</sub>O, 50:50 to 90:10) followed by semipreparative HPLC (MeCN–H<sub>2</sub>O, 25:75 to 45:55) to give compounds 1 (5 mg), 3 (6 mg), and 9 (14 mg). GPB45 was purified by semipreparative HPLC (MeCN–H<sub>2</sub>O, 35:65 to 45:55) to yield compound 7 (8 mg). GPB47 was chromatographed on an MCI gel column (acetone–H<sub>2</sub>O, 50:50 to 90:10) to give five subfractions (GPB47A–GPB47E). GPB47B was purified using a Sephadex LH-20 column (MeOH) followed by semipreparative HPLC (MeCN–H<sub>2</sub>O, 20:80 to 40:60) to afford compounds 2 (4 mg) and 5 (5 mg). GPB47C was further purified by semipreparative HPLC (MeCN–H<sub>2</sub>O, 20:80 to 45:55) to afford compounds 4 (14 mg), 8 (20 mg), and 10 (18 mg).

**Gypenoside GD1 (1):** white-yellow, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> +10.1 (c 0.1, MeOH); IR (KBr)  $\nu_{\max}$  3387, 2936, 1675, 1516, 1386, 1038 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 500 and 125 MHz), see Table 1; HRESIMS *m/z* 837.4601 [M + Na]<sup>+</sup> (calcd for C<sub>42</sub>H<sub>70</sub>O<sub>15</sub>Na, 837.4612).

**Gypenoside GD2 (2):** white-yellow, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> +6.5 (c 0.1, MeOH); IR (KBr)  $\nu_{\max}$  3385, 2937, 1676, 1516, 1386, 1038 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 500 and 125 MHz), see Table 1; HRESIMS *m/z* 1145.5713 [M + Na]<sup>+</sup> (calcd for C<sub>54</sub>H<sub>90</sub>O<sub>24</sub>Na, 1145.5720).

**Gypenoside GD3 (3):** white, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> +12.3 (c 0.1, MeOH); IR (KBr)  $\nu_{\max}$  3345, 2929, 1657, 1375, 1038 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 500 and 125 MHz), see Table 1; HRESIMS *m/z* 855.4703 [M + Na]<sup>+</sup> (calcd for C<sub>42</sub>H<sub>72</sub>O<sub>16</sub>Na, 855.4718).

**Gypenoside GD4 (4):** white, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> +7.4 (c 0.1, MeOH); IR (KBr)  $\nu_{\max}$  3350, 2928, 1654, 1370, 1038 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 500 and 125 MHz), see Table 1; HRESIMS *m/z* 1163.5792 [M + Na]<sup>+</sup> (calcd for C<sub>54</sub>H<sub>92</sub>O<sub>25</sub>Na, 1163.5825).

**Gypenoside GD5 (5):** white, amorphous powder; [ $\alpha$ ]<sub>D</sub><sup>25</sup> +9.6 (c 0.1, MeOH); IR (KBr)  $\nu_{\max}$  3338, 2929, 1655, 1370, 1039 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 700 and 175 MHz), see Table 1; HRESIMS *m/z* 1017.5230 [M + Na]<sup>+</sup> (calcd for C<sub>48</sub>H<sub>82</sub>O<sub>21</sub>Na, 1017.5246).

**Acid Hydrolysis of Compounds 1–5 and Determination of Sugar Components.** Compounds 1–5 (each 2.0 mg) were dissolved in 1.0 N HCl (1 mL) and heated at 80 °C for 3 h in a water bath. The solvent was removed under a vacuum, and each mixture was then extracted with CHCl<sub>3</sub> (2 mL, 3 times). The residue (sugar portion) was dissolved in dry pyridine (0.1 mL), followed by addition of L-cysteine methyl ester hydrochloride in dry pyridine (0.06 M, 0.1 mL). After heating the mixture at 60 °C for 2 h, NaBH<sub>4</sub> (2.0 mg) was added and the mixture was stirred for 1 h at room temperature. Trimethylsilylimidazole solution (0.1 mL) was added, and the reaction mixture was then heated at 60 °C for 2 h. The dried product was partitioned with *n*-hexane and water, and the *n*-hexane layer was then analyzed by GC-MS: column DB-5MS (0.25 mm × 30 m), column temperature 60 to 280 °C (10 °C/min), injector temperature 260 °C, detector temperature 280 °C, carrier gas He (2 mL/min). Under these conditions, the standard sugars gave peaks at *t*<sub>R</sub> (min) 16.94 for L-rhamnose and 17.77 for D-glucose, respectively.

**IL-6-Induced pSTAT3 Luciferase Assay.** The stable cell line expressing pSTAT3-luciferase was established as described previously.<sup>23</sup> Hep3B cells expressing pSTAT3-luciferase were seeded into 96-well culture plates, 2 × 10<sup>4</sup> cells/well, and cultured for 24 h. After this, the culture medium was replaced with serum-free medium, and the cells were then treated with IL-6 (10 ng/mL) with the absence or presence of samples for 12 h. The luciferase activity was then

measured according to the manufacturer's protocol (Promega Corp., Madison, WI, USA). The purity of the isolated compounds used for biological evaluation was more than 96%, as determined by HPLC and NMR spectroscopy.

**Cell Viability Assay.** Hep3B cells were seeded and cultured for 24 h in 96-well plates, and then the culture medium was replaced with serum-free medium. The cells were treated with samples at the indicated concentrations for 48 h, and the cell viability (MTT assay) was measured according to MTT assay manufacturer's protocol (Sigma Chemical Co., St. Louis, MO, USA).

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

1D NMR, 2D NMR, and HRESIMS spectra of compounds 1–5 are available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on April 20, 2015, with errors in the Table of Contents graphic, the Abstract graphic, and Chart 1. The corrected version was reposted on May 12, 2015.