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**NEW FLAVANONE OLIGOGLYCOSIDES, THEAFLAVANOSIDES I, II, III, AND IV, WITH HEPATOPROTECTIVE ACTIVITY FROM THE SEEDS OF TEA PLANT (*CAMELLIA SINENSIS*)**

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**Abstract** — Four new flavanone oligoglycosides, theaflavanosides I, II, III, and IV, were isolated from the seeds of *Camellia sinensis*. The structures of theaflavanosides were elucidated on the basis of chemical and physicochemical evidence. Among them, theaflavanoside III was found to show hepatoprotective effect on D-galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes.

In previous our studies on the bioactive constituents from tea plant, *Camellia sinensis* (L.) O. KUNTZE (Theaceae),<sup>1-7</sup> 42 saponins such as theasaponins A<sub>1</sub>–A<sub>5</sub>, B<sub>1</sub>, C<sub>1</sub>, E<sub>1</sub>–E<sub>13</sub>, F<sub>1</sub>–F<sub>3</sub>, H<sub>1</sub>, G<sub>1</sub>, and G<sub>2</sub>, assamsaponins A–D, F, and I, camelliasaponins B<sub>1</sub> and C<sub>1</sub>, floratheasaponins A–C, and foliatheasaponins I–V were isolated from the seeds, flowers, and leaves of Japanese tea plant. Among them, theasaponins A<sub>2</sub>, E<sub>1</sub>, E<sub>2</sub>, and E<sub>5</sub>, and assamsaponins A, C, and D, were found to show protective effects on ethanol-induced gastric lesions in rats<sup>2,3</sup> and floratheasaponins A–C were found to show anti-hyperlipidemic activity.<sup>6</sup> Furthermore, foliatheasaponins II and III were found to inhibit release of  $\beta$ -hexosaminidase, as a marker of antigen-induced degranulation, in RBL-2H3 cells.<sup>7</sup> As a continuing study on the seeds of *C. sinensis*, we have isolated four new flavanone oligoglycosides named theaflavanosides I (**1**), II (**2**), III (**3**), and IV (**4**). This paper deals with the structure elucidation of theaflavanosides (**1**–**4**) and the hepatoprotective effects of theaflavanosides on D-galactosamine (D-GalN)-induced cytotoxicity in primary cultured mouse hepatocytes.

The methanolic extract from tea seeds (cultivated in Shizuoka prefecture, Japan), which was described previously,<sup>2</sup> was purified by HPLC to give **1** (0.013%), **2** (0.041%), **3** (0.022%), and **4** (0.41%).

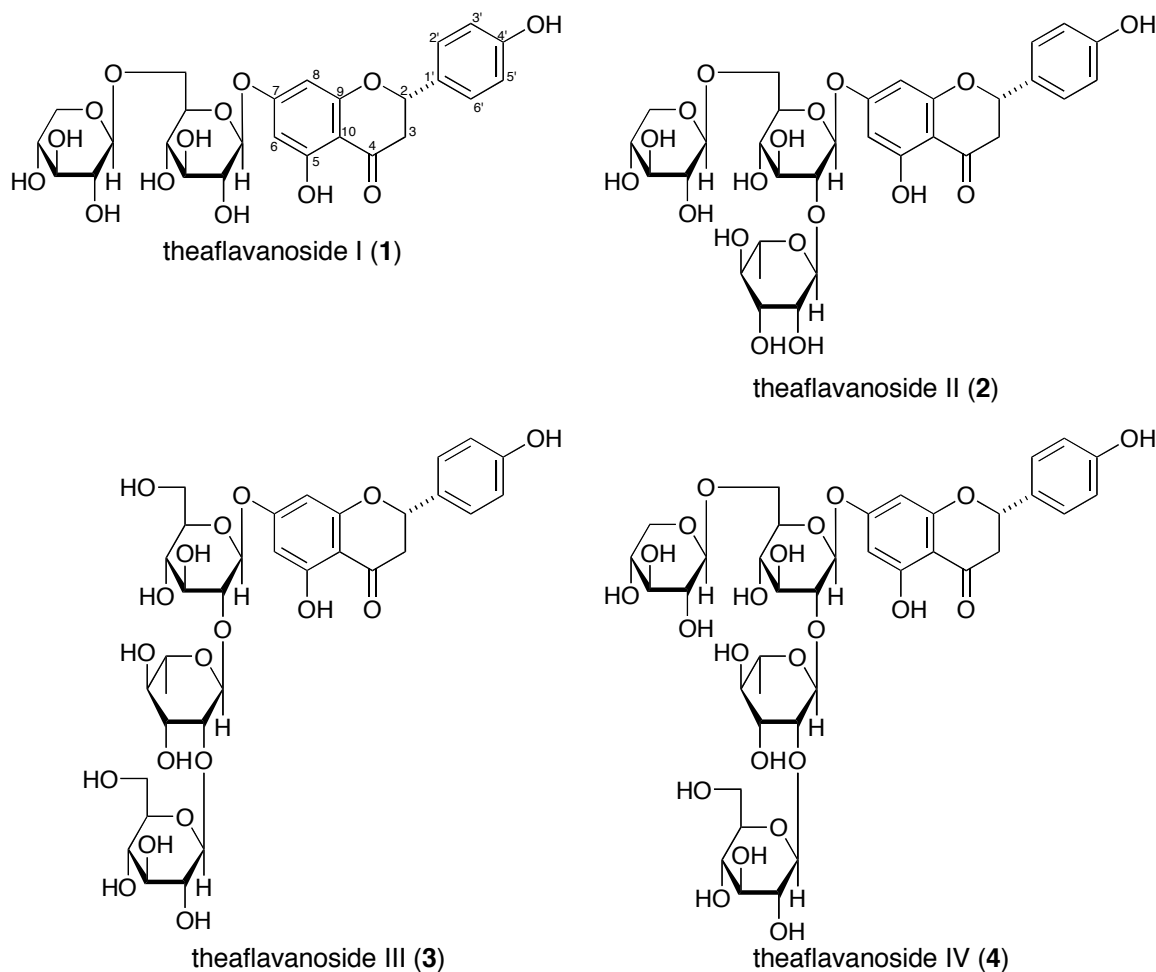


Chart 1

### Structures of Theaflavanosides I (1), II (2), III (3), and IV (4)

Theaflavanoside I (1) was isolated as a pale powder with negative optical rotation ( $[\alpha]_D^{27} -34.5^\circ$  in MeOH). The positive-ion fast atom bombardment (FAB)-MS of 1 showed a quasimolecular ion peak at  $m/z$  589  $[M+Na]^+$ , while a quasimolecular ion peak was observed at  $m/z$  565  $[M-H]^-$  in the negative-ion FAB-MS. The molecular formula  $C_{26}H_{30}O_{14}$  of 1 was determined by high-resolution MS measurement. The IR spectrum of 1 showed absorption bands at 3453, 1638, 1456, 1375, 1169, and 1046  $cm^{-1}$  ascribable to hydroxyl, chelated carbonyl, aromatic ring, and ether functions. In the UV spectrum of 1, absorption maxima were observed at 283 ( $\log \epsilon$  4.30) and 329 (3.60) nm, suggestive of the flavanone structure.<sup>8,9</sup> Acid hydrolysis of 1 with 1.0 M hydrochloric acid (HCl) liberated naringenin as an aglycon together with D-xylose and D-glucose, which were identified by HPLC analysis using an optical rotation detector.<sup>10–12</sup> The  $^1H$ - (DMSO- $d_6$ ) and  $^{13}C$ -NMR (Table 1) spectra of 1, which were assigned by various NMR experiments,<sup>13</sup> showed signals assignable to a dihydropyrone moiety in flavanone structure by a characteristic ABX type coupling pattern  $\{\delta [2.75$  (1H, dd,  $J = 2.8, 17.1$  Hz), 3.35 (1H, m), 3-H<sub>2</sub>], 5.51 (1H, dd,  $J = 2.8, 12.2$  Hz, 2-H) $\}$ , two singlet aromatic protons [ $\delta$  6.15, 6.17 (1H each, both br s, 6, 8-H)], *ortho*-coupled  $A_2B_2$  type aromatic protons [ $\delta$  6.81, 7.35 (2H each, both d,  $J = 8.6$  Hz, 3',5' and 2',6'-H)], and a chelated hydroxyl proton [ $\delta$  12.05 (1H, br s, 5-OH)] together with a xylopyranosyl and a glucopyranosyl parts [ $\delta$  4.15 (1H, d,  $J = 7.7$  Hz, Xyl-1-H), 5.00 (1H, d,  $J = 7.7$  Hz, Glc-1-H)]. The

**Table 1.**  $^{13}\text{C}$ -NMR Data for Theaflavanosides I (1), II (2), III (3), and IV (4) (125 MHz,  $\text{DMSO-}d_6$ )

	1	2	3	4		1	2	3	4
2	78.5	78.4	78.5	78.4	Rha-1	100.3	100.4	100.4	
3	41.8	41.8	41.9	41.8	2	70.2	81.7	81.7	
4	197.1	197.1	197.2	197.2	3	70.3	69.2	69.2	
5	162.9	162.9	162.8	162.9	4	71.7	70.7	70.7	
6	96.3	96.1	96.1	96.1	5	68.2	67.7	67.7	
7	164.9	164.6	164.6	164.4	6	17.9	17.8	17.8	
8	95.3	94.9	94.9	94.9	Xyl-1	103.8	103.8		103.7
9	162.6	162.6	162.7	162.7	2	73.2	73.2		73.2
10	103.2	103.2	103.2	103.2	3	76.4	76.3		76.6
1'	128.5	128.5	128.5	128.5	4	69.4	69.4		69.4
2',6'	128.4	128.4	128.4	128.4	5	65.5	65.5		65.5
3',5'	115.1	115.1	115.1	115.1	terminal-Glc-1			104.4	104.4
4'	157.8	157.7	157.8	157.7	2			73.9	73.9
inner-Glc-1	99.2	97.0	97.0	96.7	3			76.1	76.3
2	72.8	76.3	75.9	75.9	4			69.5	69.6
3	76.0	76.8	76.7	76.4	5			76.9	76.4
4	69.1	69.2	69.5	69.6	6			60.7	60.8
5	75.3	75.1	76.4	75.1					
6	68.0	67.8	60.3	67.7					

Glc:  $\beta$ -D-glucopyranosyl; Rha:  $\alpha$ -L-rhamnopyranosyl; Xyl:  $\beta$ -D-xylopyranosyl

position of the oligosaccharide moiety in **1** was clarified on the basis of the HMBC experiment. Thus, long-range correlations were observed between the following proton and carbon pairs: Glc-1-H and 7-C ( $\delta_{\text{C}}$  164.9); Xyl-1-H and Glc-6-C ( $\delta_{\text{C}}$  68.0). The circular dichroic (CD) spectrum of **1** showed negative Cotton effect [ $\Delta\epsilon = -3.53$ ] at 278 nm in MeOH], which indicated the absolute configuration of the 2-position to be *S*.<sup>8,9,14</sup> On the basis of the above-mentioned evidence, the structure of theaflavanoside I was determined to be (2*S*)-naringenin 7-*O*- $\beta$ -D-xylopyranosyl(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside (**1**).

Theaflavanoside II (**2**) was obtained as a pale yellow powder with negative optical rotation ( $[\alpha]_{\text{D}}^{27} -88.4^\circ$  in MeOH). The IR spectrum of **2** showed absorption bands at 1638, 1454, 1375, and 1170  $\text{cm}^{-1}$  ascribable to chelated carbonyl function and aromatic ring, and broad bands at 3453 and 1074  $\text{cm}^{-1}$  suggestive of an oligoglycoside structure. In the positive- and negative-ion FAB-MS of **2**, quasimolecular ion peaks were observed at  $m/z$  735  $[\text{M}+\text{Na}]^+$  and  $m/z$  711  $[\text{M}-\text{H}]^-$ , and high-resolution positive-ion FAB-MS analysis revealed the molecular formula of **2** to be  $\text{C}_{32}\text{H}_{40}\text{O}_{18}$ . Furthermore, in the negative-ion FAB-MS of **2** showed the fragment ion peaks at  $m/z$  579  $[\text{M}-\text{C}_5\text{H}_9\text{O}_4]^-$  and  $m/z$  565  $[\text{M}-\text{C}_6\text{H}_{11}\text{O}_4]^-$ , which were formed by the cleavages at the terminal pentose and hexose units, respectively. Acid hydrolysis of **2** with 1.0 M HCl liberated naringenin together with L-rhamnose, D-xylose, and D-glucose, which were identified by HPLC analysis using an optical rotation detector.<sup>10-12</sup> The  $^1\text{H}$ - ( $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$ -NMR (Table 1) spectra<sup>13</sup> of **2** showed signals assignable to a naringenin part  $\{\delta$  [2.74 (1H, dd,  $J = 2.7$ , 16.6 Hz), 3.34 (1H, m), 3-H<sub>2</sub>], 5.53 (1H, dd,  $J = 2.7$ , 12.0 Hz, 2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.80, 7.35 (2H each, both d,  $J = 8.6$  Hz, 3',5' and 2',6'-H), 12.03 (1H, br s, 5-OH)] together with three glycopyranosyl moieties [ $\delta$  1.15 (3H, d,  $J = 6.1$  Hz, Rha-6-H<sub>3</sub>), 4.13 (1H, d,  $J = 7.3$  Hz, Xyl-1-H), 5.09 (1H, br s, Rha-1-H), 5.22 (1H, d,  $J = 7.0$  Hz, Glc-1-H)]. The proton and carbon signals in the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR (Table 1) spectra of **2** were found to be similar to those of **1**, expect for the additional signals

due to a rhamnopyranosyl part. Comparison of the  $^{13}\text{C}$ -NMR data for **2** with those for **1** revealed a glycosylation shift around the 2-position of the glucopyranosyl moiety [**2**:  $\delta_{\text{C}}$  97.0 (Glc-1), 76.3 (Glc-2), 76.8 (Glc-3); **1**:  $\delta_{\text{C}}$  99.2 (Glc-1), 72.8 (Glc-2), 76.0 (Glc-3)]. In the HMBC experiment of **2**, long-range correlations were observed between the following protons and carbons [Glc-1-H and 7-C ( $\delta_{\text{C}}$  164.6); Rha-1-H and Glc-2-C ( $\delta_{\text{C}}$  76.3); and Xyl-1-H and Glc-6-C ( $\delta_{\text{C}}$  67.8)]. The CD spectrum of **2** showed negative Cotton effect [ $\Delta\epsilon = -3.91$ ] at 284 nm in MeOH, which indicated the absolute configuration of the 2-position to be *S*.<sup>8,9,14</sup> Consequently, the structure of theaflavoside II was elucidated to be (2*S*)-naringenin 7-*O*- $\alpha$ -L-rhamnopyranosyl(1 $\rightarrow$ 2)-[ $\beta$ -D-xylopyranosyl(1 $\rightarrow$ 6)]- $\beta$ -D-glucopyranoside (**2**).

Theaflavosides III (**3**) with negative optical rotation ( $[\alpha]_{\text{D}}^{27} -40.4^\circ$  in MeOH) was also isolated as a pale yellow powder. The molecular formula  $\text{C}_{33}\text{H}_{42}\text{O}_{19}$  of **3** was also determined from the positive- and negative-ion FAB-MS ( $m/z$  765 [ $\text{M}+\text{Na}$ ] $^+$ ,  $m/z$  741 [ $\text{M}-\text{H}$ ] $^-$ ) and by high-resolution positive-ion MS measurement. Furthermore, the fragment ion peaks at  $m/z$  579 [ $\text{M}-\text{C}_6\text{H}_{11}\text{O}_5$ ] $^-$  and  $m/z$  433 [ $\text{M}-\text{C}_{12}\text{H}_{21}\text{O}_9$ ] $^-$ , which indicated the loss of mono-hexose and di-hexose units, were observed in the negative-ion FAB-MS. Acid hydrolysis of **3** with 1.0 M HCl liberated L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector.<sup>10-12</sup> The  $^1\text{H}$ -NMR ( $\text{DMSO}-d_6$ ) and  $^{13}\text{C}$ -NMR (Table 1) spectra<sup>13</sup> of **3** indicated the presence of the following functions: a naringenin part { $\delta$  [2.73 (1H, dd,  $J = 2.0, 16.2$  Hz), 3.35 (1H, m), 3-H<sub>2</sub>], 5.52 (1H, br d,  $J = ca. 14$  Hz, 2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.81, 7.35 (2H each, both d,  $J = 8.6$  Hz, 3',5' and 2',6'-H), 12.05 (1H, br s, 5-OH)} together with two glucopyranosyl and a rhamnopyranosyl moieties [ $\delta$  1.20 (3H, d,  $J = 5.5$  Hz, Rha-6-H<sub>3</sub>), 4.32 (1H, d,  $J = 7.7$  Hz, *terminal*-Glc-1-H), 5.13 (1H, br s, Rha-1-H), 5.24 (1H, d,  $J = 7.1$  Hz, *inner*-Glc-1-H)]. The structure of the oligoglycosyl moiety and the position of oligosugar linkage to the aglycone were characterized by HMBC experiments. Thus, long-range correlations were observed between the following proton and carbon pairs [*inner*-Glc-1-H and 7-C ( $\delta_{\text{C}}$  164.6); Rha-1-H and *inner*-Glc-2-C ( $\delta_{\text{C}}$  75.9); *terminal*-Glc-1-H and Rha-2-C ( $\delta_{\text{C}}$  81.7)]. The 2*S* orientation in **3** was elucidated by the CD spectrum [ $\Delta\epsilon = -3.84$ ] at 280 nm in MeOH,<sup>8,9,14</sup> and thus the structure of theaflavoside III was determined to be (2*S*)-naringenin 7-*O*- $\beta$ -D-glucopyranosyl(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside (**3**).

Theaflavoside IV (**4**) was obtained as a pale yellow powder with a negative optical rotation ( $[\alpha]_{\text{D}}^{27} -97.6^\circ$  in MeOH). In the positive- and negative-ion FAB-MS of **4**, quasimolecular ion peaks were observed at  $m/z$  897 [ $\text{M}+\text{Na}$ ] $^+$ , and 873 [ $\text{M}-\text{H}$ ] $^-$ , and high-resolution FAB-MS analysis revealed the molecular formula of **4** to be  $\text{C}_{38}\text{H}_{50}\text{O}_{23}$ . The proton and carbon signals in the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR (Table 1) spectra<sup>13</sup> of **4** were found to be similar to those of **3**, except for the additional signals due to a xylopyranosyl part: a naringenin part: { $\delta$  [2.72 (1H, dd,  $J = 2.8, 16.5$  Hz), 3.40 (1H, m), 3-H<sub>2</sub>], 5.51 (1H, dd,  $J = 2.8, 12.8$  Hz, 2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.81, 7.35 (2H each, both d,  $J = 8.5$  Hz, 3',5' and 2',6'-H), 12.07 (1H, br s, 5-OH)} together with tetraglycosyl moieties [ $\delta$  1.18 (3H, d,  $J = 6.0$  Hz, Rha-6-H<sub>3</sub>), 4.13 (1H, d,  $J = 7.2$  Hz, Xyl-1-H), 4.32 (1H, d,  $J = 7.6$  Hz, *terminal*-Glc-1-H), 5.13 (1H, br s, Rha-1-H), 5.23 (1H, d,  $J = 7.4$  Hz, *inner*-Glc-1-H)]. In the HMBC experiment of **4**, long-range correlations were observed between the following proton and carbon pairs [*inner*-Glc-1-H and 7-C ( $\delta_{\text{C}}$  164.4); Rha-1-H and *inner*-Glc-2-C ( $\delta_{\text{C}}$  75.9); Xyl-1-H and *inner*-Glc-6-C ( $\delta_{\text{C}}$  67.7); *terminal*-Glc-1-H

and Rha-2-C ( $\delta_C$  81.7)]. Finally, the absolute configuration of the 2-position in **4** was clarified on the basis of the CD spectrum [ $\Delta\epsilon = -3.97$ ] at 279 nm in MeOH].<sup>8,9,14</sup> Consequently, the structure of theaflavanoside IV was determined to be (2*S*)-naringenin 7-*O*- $\beta$ -D-glucopyranosyl(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl(1 $\rightarrow$ 2)-[ $\beta$ -D-xylopyranosyl(1 $\rightarrow$ 6)]- $\beta$ -D-glucopyranoside (**4**).

### Protective Effects of Theaflavanosides I–IV (1–4) on D-GalN-induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

Previously, we have reported the isolation and structure elucidation of several constituents with hepatoprotective effects from *Hovenia dulcis*,<sup>15</sup> *Bupleurum scorzonerifolium*,<sup>16,17</sup> *Curcuma zedoaria*,<sup>18–20</sup> *Angelica furcijuga*,<sup>21,22</sup> *Betula platyphylla* var. *japonica*,<sup>23</sup> *Pisum sativum*,<sup>24</sup> *Salacia reticulata*,<sup>25</sup> *Tilia argentea*,<sup>26</sup> *Anastatica hierochuntica*,<sup>9</sup> *Panax notoginseng*,<sup>27</sup> *Cyperus longus*,<sup>28</sup> and *Erycibe expansa*.<sup>29</sup> Since the aglycon flavanone, naringenin, was also showed hepatoprotective effect,<sup>9</sup> the inhibitory effects of naringenin glycosides, theaflavanosides I–IV (**1–4**), on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes were examined. As shown in Table 2, theaflavanoside III (**3**) was found to show inhibitory activity. However, the hepatoprotective activity of **3** was weaker than that of aglycon part, naringenin, and commercial silybin, which is well known to show potent hepatoprotective activity.<sup>9,30,31</sup> This evidence suggested that the glycosyl moieties in flavanones reduced the hepatoprotective effect in this model.

**Table 2.** Inhibitory Effects of Theaflavanosides I–IV (**1–4**) on D-GalN-induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

	Inhibition (%)				
	0 $\mu$ M	3 $\mu$ M	10 $\mu$ M	30 $\mu$ M	100 $\mu$ M
theaflavanoside I ( <b>1</b> )	0.0 $\pm$ 1.0	-2.3 $\pm$ 2.1	0.7 $\pm$ 1.3	0.1 $\pm$ 0.5	-3.8 $\pm$ 1.7
theaflavanoside II ( <b>2</b> )	0.0 $\pm$ 2.0	-1.8 $\pm$ 1.8	-0.2 $\pm$ 1.4	0.7 $\pm$ 0.8	3.6 $\pm$ 1.5
theaflavanoside III ( <b>3</b> )	0.0 $\pm$ 2.9	1.1 $\pm$ 1.0	0.4 $\pm$ 0.6	5.0 $\pm$ 1.6	13.8 $\pm$ 0.8**
theaflavanoside IV ( <b>4</b> )	0.0 $\pm$ 1.9	-0.7 $\pm$ 1.2	-0.2 $\pm$ 0.5	1.6 $\pm$ 1.4	3.1 $\pm$ 0.6
naringenin	0.0 $\pm$ 0.9	2.9 $\pm$ 0.4	9.3 $\pm$ 2.1	43.1 $\pm$ 0.4**	98.2 $\pm$ 5.3**
silybin <sup>a</sup>	0.0 $\pm$ 0.3	4.8 $\pm$ 1.1	7.7 $\pm$ 0.7	45.2 $\pm$ 8.8**	77.0 $\pm$ 5.5**

Each value represents the mean $\pm$ S.E.M. ( $N=4$ ).

Significantly different from the control, \* $p < 0.05$ , \*\* $p < 0.01$ .

<sup>a</sup>Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

## EXPERIMENTAL

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ( $l = 5$  cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600; IR spectra, Shimadzu FTIR-8100 spectrophotometer; FAB-MS and high-resolution FAB-MS, JEOL JMS-SX 102A mass spectrometer; <sup>1</sup>H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; <sup>13</sup>C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index and SPD-10Avp UV-VIS detectors; and HPLC column, YMC-Pack ODS-A (250  $\times$  4.6 mm i.d.) and (250  $\times$  20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: normal-phase column chromatography; silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh), reversed-phase column chromatography; Diaion HP-20 (Mitsubishi Chemical): TLC, pre-coated TLC plates with silica gel 60F<sub>254</sub> (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F<sub>254S</sub> (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with silica gel RP-18 WF<sub>254S</sub> (Merck, 0.25 mm) (reversed-phase) and detection was achieved by spraying with 1% Ce(SO<sub>4</sub>)<sub>2</sub>-10% aqueous H<sub>2</sub>SO<sub>4</sub>, followed by heating.

#### Isolation of Theaflavanosides I (1), II (2), III (3), and IV (4)

Fraction 1 (3.50 g) was obtained from the methanol-eluted fraction (16.0 g, 6.34% from the seeds) of the seeds of *C. sinensis* (1.0 kg, cultivated in Shizuoka prefecture, Japan) as reported previously.<sup>2</sup> Fraction 1 (3.50 g) was purified by HPLC [MeCN–1% aqueous AcOH (20 : 80, v/v)] to give six fractions {Fr. 1-1 (1256 mg), Fr. 1-2 (43 mg), Fr. 1-3 (33 mg), Fr. 1-4 [= theaflavanoside IV (4, 1043 mg, 0.41%)], Fr. 1-5 [= theaflavanoside II (2, 104 mg, 0.041%)], and Fr. 1-6 (235 mg)}. Fraction 1-6 (235 mg) was further purified by HPLC [MeCN–MeOH–1% aqueous AcOH (16 : 8 : 76, v/v/v)] to give theaflavanosides I (1, 32 mg, 0.013%) and III (3, 56 mg, 0.022%).

Theaflavanoside I (1): a pale yellow powder,  $[\alpha]_D^{27} -34.5^\circ$  (*c* 0.60, MeOH). High-resolution positive-ion FAB-MS: Calcd for C<sub>26</sub>H<sub>30</sub>O<sub>14</sub>Na [M+Na]<sup>+</sup>: 589.1533. Found: 589.1538. CD [MeOH, nm ( $\Delta\epsilon$ ): 229 (–3.56), 278 (–3.53), 332 (–3.32). UV [MeOH nm, (log  $\epsilon$ ): 226 (4.50), 283 (4.30), 329 (3.60). IR (KBr): 3453, 1638, 1456, 1375, 1169, 1046 cm<sup>–1</sup>. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$ : [2.75 (1H, dd, *J* = 2.8, 17.1 Hz), 3.35 (1H, m), 3-H<sub>2</sub>], 4.15 (1H, d, *J* = 7.7 Hz, Xyl-1-H), 5.00 (1H, d, *J* = 7.7 Hz, Glc-1-H), 5.51 (1H, dd, *J* = 2.8, 12.2 Hz, 2-H), 6.15, 6.17 (1H each, both br s, 6, 8-H), 6.81, 7.35 (2H each, both d, *J* = 8.6 Hz, 3',5' and 2',6'-H), 12.05 (1H, br s, 5-OH). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz)  $\delta$ c: given in Table 1. Positive-ion FAB-MS: *m/z* 589 [M+Na]<sup>+</sup>. Negative-ion FAB-MS: *m/z* 565 [M–H]<sup>–</sup>.

Theaflavanoside II (2): a pale yellow powder,  $[\alpha]_D^{27} -88.4^\circ$  (*c* 1.70, MeOH). High-resolution positive-ion FAB-MS: Calcd for C<sub>32</sub>H<sub>40</sub>O<sub>18</sub>Na [M+Na]<sup>+</sup>: 735.2112. Found: 735.2119. CD [MeOH, nm ( $\Delta\epsilon$ ): 232 (–3.72), 284 (–3.91), 331 (–3.50). UV [MeOH nm, (log  $\epsilon$ ): 226 (4.48), 283 (4.28), 330 (3.57). IR (KBr): 3453, 1638, 1454, 1375, 1170, 1074 cm<sup>–1</sup>. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$ : 1.15 (3H, d, *J* = 6.1 Hz, Rha-6-H<sub>3</sub>), [2.74 (1H, dd, *J* = 2.7, 16.6 Hz), 3.34 (1H, m), 3-H<sub>2</sub>], 4.13 (1H, d, *J* = 7.3 Hz, Xyl-1-H), 5.09 (1H, br s, Rha-1-H), 5.22 (1H, d, *J* = 7.0 Hz, Glc-1-H), 5.53 (1H, dd, *J* = 2.7, 12.0 Hz, 2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.80, 7.35 (2H each, both d, *J* = 8.6 Hz, 3',5' and 2',6'-H), 12.03 (1H, br s, 5-OH). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz)  $\delta$ c: given in Table 1. Positive-ion FAB-MS: *m/z* 735 [M+Na]<sup>+</sup>. Negative-ion FAB-MS: *m/z* 711 [M–H]<sup>–</sup>, 579 [M–C<sub>5</sub>H<sub>9</sub>O<sub>4</sub>]<sup>–</sup>, 565 [M–C<sub>6</sub>H<sub>11</sub>O<sub>4</sub>]<sup>–</sup>.

Theaflavanoside III (3): a pale yellow powder,  $[\alpha]_D^{27} -40.4^\circ$  (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for C<sub>33</sub>H<sub>42</sub>O<sub>19</sub>Na [M+Na]<sup>+</sup>: 765.2218. Found: 765.2213. CD [MeOH, nm ( $\Delta\epsilon$ ): 223 (–3.99), 280 (–3.84), 331 (–3.53). UV [MeOH nm, (log  $\epsilon$ ): 226 (4.48), 283 (4.29), 330 (3.58). IR (KBr): 3453, 1647, 1458, 1375, 1175, 1074 cm<sup>–1</sup>. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$ : 1.20 (3H, d, *J* = 5.5 Hz, Rha-6-H<sub>3</sub>), [2.73 (1H, dd, *J* = 2.0, 16.2 Hz), 3.35 (1H, m), 3-H<sub>2</sub>], 4.32 (1H, d, *J* = 7.7 Hz, terminal-Glc-1-H), 5.13 (1H, br s, Rha-1-H), 5.24 (1H, d, *J* = 7.1 Hz, inner-Glc-1-H), 5.52 (1H, br d, *J* = ca. 14 Hz,

2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.81, 7.35 (2H each, both d,  $J = 8.6$  Hz, 3',5' and 2',6'-H), 12.05 (1H, br s, 5-OH).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz)  $\delta$ : given in Table 1. Positive-ion FAB-MS:  $m/z$  765  $[\text{M}+\text{Na}]^+$ . Negative-ion FAB-MS:  $m/z$  741  $[\text{M}-\text{H}]^-$ , 579  $[\text{M}-\text{C}_6\text{H}_{11}\text{O}_5]^-$ , 433  $[\text{M}-\text{C}_{12}\text{H}_{21}\text{O}_9]^-$ . Theaflavanoside IV (**4**): a pale yellow powder,  $[\alpha]_{\text{D}}^{27} -97.6^\circ$  ( $c$  1.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for  $\text{C}_{38}\text{H}_{50}\text{O}_{23}\text{Na}$   $[\text{M}+\text{Na}]^+$ : 897.2641 Found: 897.2634. CD [MeOH, nm ( $\Delta\epsilon$ ): 232 (-3.89), 279 (-3.97), 332 (-3.74). UV [MeOH nm, ( $\log \epsilon$ ): 226 (4.57), 283 (4.38), 330 (3.70). IR (KBr): 3569, 1638, 1458, 1375, 1175, 1075  $\text{cm}^{-1}$ .  $^1\text{H-NMR}$  (DMSO- $d_6$ , 500 MHz)  $\delta$ : 1.18 (3H, d,  $J = 6.0$  Hz, Rha-6- $\text{H}_3$ ), [2.72 (1H, dd,  $J = 2.8, 16.5$  Hz), 3.40 (1H, m), 3- $\text{H}_2$ ], 4.13 (1H, d,  $J = 7.2$  Hz, Xyl-1-H), 4.32 (1H, d,  $J = 7.6$  Hz, terminal-Glc-1-H), 5.13 (1H, br s, Rha-1-H), 5.23 (1H, d,  $J = 7.4$  Hz, inner-Glc-1-H), 5.51 (1H, dd,  $J = 2.8, 12.8$  Hz, 2-H), 6.10, 6.12 (1H each, both br s, 6, 8-H), 6.81, 7.35 (2H each, both d,  $J = 8.5$  Hz, 3',5' and 2',6'-H), 12.07 (1H, br s, 5-OH).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz)  $\delta$ : given in Table 1. Positive-ion FAB-MS:  $m/z$  897  $[\text{M}+\text{Na}]^+$ . Negative-ion FAB-MS:  $m/z$  873  $[\text{M}-\text{H}]^-$ , 741  $[\text{M}-\text{C}_5\text{H}_9\text{O}_4]^-$ , 711  $[\text{M}-\text{C}_6\text{H}_{11}\text{O}_5]^-$ .

#### Acid Hydrolysis of **1**–**4**

A solution of **1**–**4** (3 mg each) in 1 M HCl (1.0 mL) was heated under reflux for 3 h. After cooling, the reaction mixture was extracted with EtOAc. The EtOAc extract was subjected to HPLC analysis under the following conditions, respectively: HPLC column, YMC-Pack ODS-A, 4.6 mm i.d.  $\times$  250 mm; detection, UV (254 nm); mobile phase, MeOH– $\text{H}_2\text{O}$  (60:40, v/v); flow rate 0.8 mL/min. Identification of naringenin from **1**–**4** present in the EtOAc extract was carried out by comparison of its retention time with that of authentic sample ( $t_{\text{R}}$ : 11.2 min), respectively. On the other hand, the aqueous layer was subjected to HPLC analysis under the following conditions, respectively: HPLC column, Kaseisorb LC  $\text{NH}_2$ -60-5, 4.6 mm i.d.  $\times$  250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, MeCN– $\text{H}_2\text{O}$  (85:15, v/v); flow rate 0.8 mL/min. Identification of L-rhamnose (i) from **2**–**4**, D-xylose (ii) from **1**, **2**, and **4**, and D-glucose (iii) from **1**–**4** present in the aqueous layer was carried out by comparison of its retention time and optical rotation with those of authentic samples,  $t_{\text{R}}$ : (i) 7.8 min (L-rhamnose, negative optical rotation), (ii) 9.5 min (D-xylose, positive optical rotation), and (iii) 13.9 min (D-glucose, positive optical rotation), respectively.

#### Protective Effect on Cytotoxicity Induced by D-GalN in Primary Cultured Mouse Hepatocytes

The hepatoprotective effects of the constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes. Hepatocytes were isolated from male ddY mice (30–35 g) by collagenase perfusion method.<sup>32</sup> The cell suspension at  $4 \times 10^4$  cells in 100  $\mu\text{L}$  William's E medium containing fetal calf serum (10%), penicillin G (100 units/mL), and streptomycin (100  $\mu\text{g}/\text{mL}$ ) was inoculated in a 96-well microplate, and precultured for 4 h at  $37^\circ\text{C}$  under a 5%  $\text{CO}_2$  atmosphere. The fresh medium (100  $\mu\text{L}$ ) containing D-GalN (2 mM) and a test sample were added and the hepatocytes were cultured for 44 h. The medium was exchanged with 100  $\mu\text{L}$  of the fresh medium, and 10  $\mu\text{L}$  of MTT (5 mg/mL in phosphate buffered saline) solution was

added to the medium. After 4 h culture, the medium was removed, 100  $\mu\text{L}$  of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was obtained by following formula.

$$\text{Inhibition (\%)} = [(O.D.(\text{sample}) - O.D.(\text{control})) / (O.D.(\text{normal}) - O.D.(\text{control}))] \times 100$$

### Statistics

Values are expressed as means  $\pm$  S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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