HETEROCYCLES, Vol. 71, No. 5, 2007, pp. 1193 - 1201. © The Japan Institute of Heterocyclic Chemistry
Received, 14th February, 2007, Accepted, 29th March, 2007, Published online, 29th March, 2007. COM-07-11028

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),[1-7] 42 saponins such as theasaponins A_1–A_5, B_1, C_1, E_1–E_{13}, F_1–F_3, H_1, G_1, and G_2, assamsaponins A–D, F, and I, camelliasaponins B_1 and C_1, floratheasaponins A—C, and foliatheasaponins I—V were isolated from the seeds, flowers, and leaves of Japanese tea plant. Among them, theasaponins A_2, E_1, E_2, and E_5, and assamsaponins A, C, and D, were found to show protective effects on ethanol-induced gastric lesions in rats[2,3] and floratheasaponins A—C were found to show anti-hyperlipidemic activity.[6] Furthermore, foliatheasaponins II and III were found to inhibit release of β-hexosaminidase, as a marker of antigen-induced degranulation, in RBL-2H3 cells.[7] 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,[2] was purified by HPLC to give **1** (0.013%), **2** (0.041%), **3** (0.022%), and **4** (0.41%).

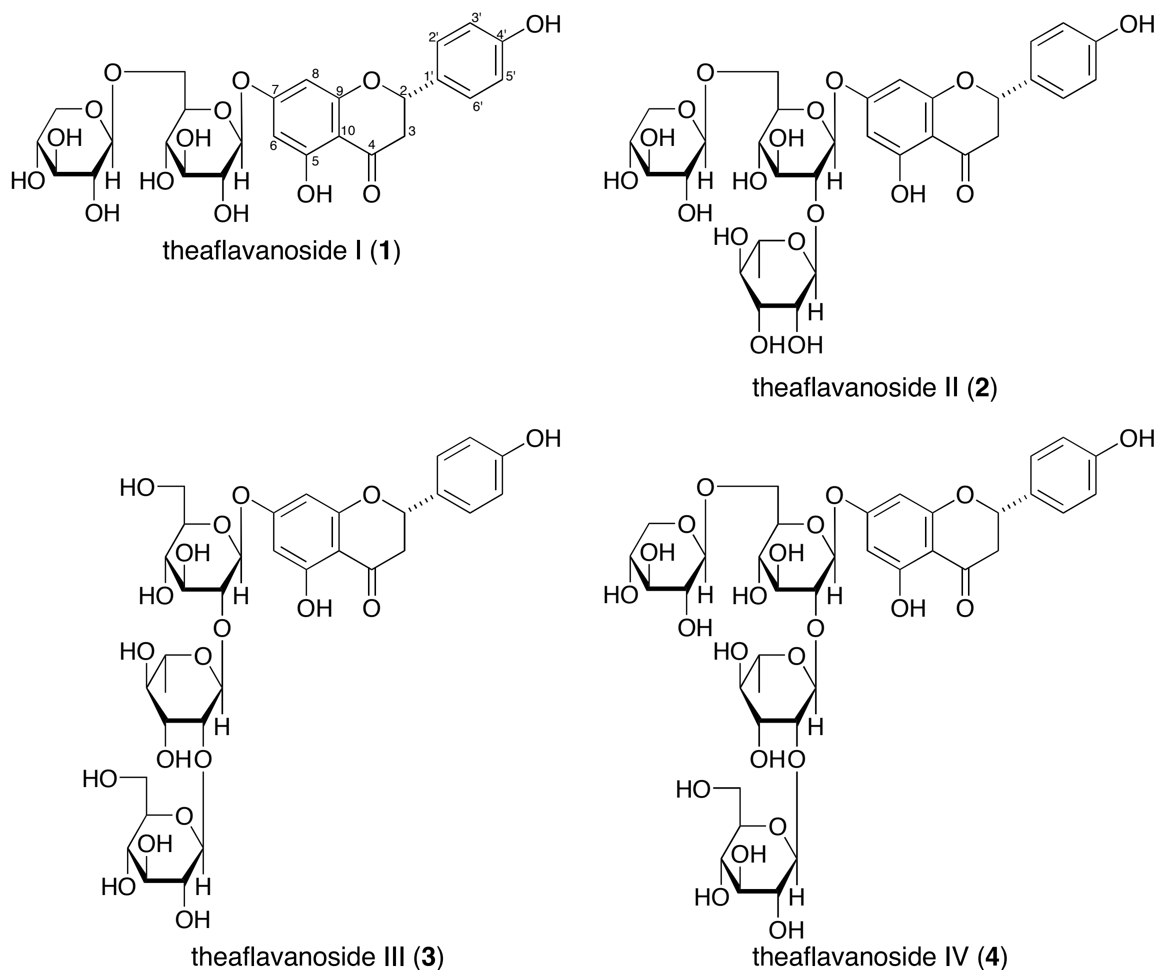

Chart 1

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° 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 ε 4.30) and 329 (3.60) nm, suggestive of the flavanone structure.[8,9] 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.[10–12] The ^{1}H- (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[13] showed signals assignable to a dihydropyrone moiety in flavanone structure by a characteristic ABX type coupling pattern {δ [2.75 (1H, dd, J = 2.8, 17.1 Hz), 3.35 (1H, m), 3-H_2], 5.51 (1H, dd, J = 2.8, 12.2 Hz, 2-H)}, two singlet aromatic protons [δ 6.15, 6.17 (1H each, both br s, 6, 8-H)], *ortho*-coupled A_2B_2 type aromatic protons [δ 6.81, 7.35 (2H each, both d, J = 8.6 Hz, 3',5' and 2',6'-H)], and a chelated hydroxyl proton [δ 12.05 (1H, br s, 5-OH)] together with a xylopyranosyl and a glucopyranosyl parts [δ 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}C-NMR Data for Theaflavanosides I (**1**), II (**2**), III (**3**), and IV (**4**) (125 MHz, 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: β-D-glucopyranosyl; Rha: α-L-rhamnopyranosyl; Xyl: β-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 (δ_C 164.9); Xyl-1-H and Glc-6-C (δ_C 68.0). The circular dichroic (CD) spectrum of **1** showed negative Cotton effect [($\Delta\varepsilon$ = −3.53) at 278 nm in MeOH], which indicated the absolute configuration of the 2-position to be *S*.[8,9,14] On the basis of the above-mentioned evidence, the structure of theaflavanoside I was determined to be (2*S*)-naringenin 7-*O*-β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**1**).

Theaflavanoside II (**2**) was obtained as a pale yellow powder with negative optical rotation ($[\alpha]_D^{27}$ −88.4° in MeOH). The IR spectrum of **2** showed absorption bands at 1638, 1454, 1375, and 1170 cm^{-1} ascribable to chelated carbonyl function and aromatic ring, and broad bands at 3453 and 1074 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 $[M+Na]^+$ and *m/z* 711 $[M-H]^-$, and high-resolution positive-ion FAB-MS analysis revealed the molecular formula of **2** to be $C_{32}H_{40}O_{18}$. Furthermore, in the negative-ion FAB-MS of **2** showed the fragment ion peaks at *m/z* 579 $[M-C_5H_9O_4]^-$ and *m/z* 565 $[M-C_6H_{11}O_4]^-$, 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.[10–12] The ^{1}H- (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra[13] of **2** showed signals assignable to a naringenin part {δ [2.74 (1H, dd, J = 2.7, 16.6 Hz), 3.34 (1H, m), 3-H_2], 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 [δ 1.15 (3H, d, J = 6.1 Hz, Rha-6-H_3), 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}H- and ^{13}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}C-NMR data for **2** with those for **1** revealed a glycosylation shift around the 2-position of the glucopyranosyl moiety [**2**: δ_C 97.0 (Glc-1), 76.3 (Glc-2), 76.8 (Glc-3); **1**: δ_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 (δ_C 164.6); Rha-1-H and Glc-2-C (δ_C 76.3); and Xyl-1-H and Glc-6-C (δ_C 67.8)]. The CD spectrum of **2** showed negative Cotton effect [($\Delta\varepsilon$ = −3.91) at 284 nm in MeOH], which indicated the absolute configuration of the 2-position to be *S*.[8,9,14] Consequently, the structure of theaflavanoside II was elucidated to be (2*S*)-naringenin 7-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-xylopyranosyl(1→6)]-β-D-glucopyranoside (**2**).

Theaflavanosides III (**3**) with negative optical rotation ($[\alpha]_D^{27}$ −40.4° in MeOH) was also isolated as a pale yellow powder. The molecular formula $C_{33}H_{42}O_{19}$ of **3** was also determined from the positive- and negative-ion FAB-MS (*m/z* 765 $[M+Na]^+$, *m/z* 741 $[M-H]^-$) and by high-resolution positive-ion MS measurement. Furthermore, the fragment ion peaks at *m/z* 579 $[M-C_6H_{11}O_5]^-$ and *m/z* 433 $[M-C_{12}H_{21}O_9]^-$, 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.[10–12] The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra[13] of **3** indicated the presence of the following functions: a naringenin part {δ [2.73 (1H, dd, *J* = 2.0, 16.2 Hz), 3.35 (1H, m), 3-H_2], 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 [δ 1.20 (3H, d, *J* = 5.5 Hz, Rha-6-H_3), 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 (δ_C 164.6); Rha-1-H and *inner*-Glc-2-C (δ_C 75.9); *terminal*-Glc-1-H and Rha-2-C (δ_C 81.7)]. The 2*S* orientation in **3** was elucidated by the CD spectrum [($\Delta\varepsilon$ = −3.84) at 280 nm in MeOH],[8,9,14] and thus the structure of theaflavanoside III was determined to be (2*S*)-naringenin 7-*O*-β-D-glucopyranosyl(1→2)-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside (**3**).

Theaflavanoside IV (**4**) was obtained as a pale yellow powder with a negative optical rotation ($[\alpha]_D^{27}$ −97.6° in MeOH). In the positive- and negative-ion FAB-MS of **4**, quasimolecular ion peaks were observed at *m/z* 897 $[M+Na]^+$, and 873 $[M-H]^-$, and high-resolution FAB-MS analysis revealed the molecular formula of **4** to be $C_{38}H_{50}O_{23}$. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR (Table 1) spectra[13] of **4** were found to be similar to those of **3**, expect for the additional signals due to a xylopyranosyl part: a naringenin part: {δ [2.72 (1H, dd, *J* = 2.8, 16.5 Hz), 3.40 (1H, m), 3-H_2], 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 [δ 1.18 (3H, d, *J* = 6.0 Hz, Rha-6-H_3), 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 (δ_C 164.4); Rha-1-H and *inner*-Glc-2-C (δ_C 75.9); Xyl-1-H and *inner*-Glc-6-C (δ_C 67.7); *terminal*-Glc-1-H

and Rha-2-C (δ_C 81.7)]. Finally, the absolute configuration of the 2-position in **4** was clarified on the basis of the CD spectrum [($\Delta\varepsilon$ = −3.97) at 279 nm in MeOH].[8,9,14] Consequently, the structure of theaflavanoside IV was determined to be (2*S*)-naringenin 7-*O*-*β*-D-glucopyranosyl(1→2)-*α*-L-rhamnopyranosyl(1→2)-[*β*-D-xylopyranosyl(1→6)]-*β*-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*,[15] *Bupleurum scorzonerifolium*,[16,17] *Curcuma zedoaria*,[18-20] *Angelica furcijuga*,[21,22] *Betula platyphylla* var. *japonica*,[23] *Pisum sativum*,[24] *Salacia reticulata*,[25] *Tilia argentea*,[26] *Anastatica hierochuntica*,[9] *Panax notoginseng*,[27] *Cyperus longus*,[28] and *Erycibe expansa*.[29] Since the aglycon flavanone, naringenin, was also showed hepatoprotective effect,[9] 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.[9,30,31] 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 μM	3 μM	10 μM	30 μM	100 μM
theaflavanoside I (**1**)	0.0 ± 1.0	-2.3 ± 2.1	0.7 ± 1.3	0.1 ± 0.5	-3.8 ± 1.7
theaflavanoside II (**2**)	0.0 ± 2.0	-1.8 ± 1.8	-0.2 ± 1.4	0.7 ± 0.8	3.6 ± 1.5
theaflavanoside III (**3**)	0.0 ± 2.9	1.1 ± 1.0	0.4 ± 0.6	5.0 ± 1.6	13.8 ± 0.8**
theaflavanoside IV (**4**)	0.0 ± 1.9	-0.7 ± 1.2	-0.2 ± 0.5	1.6 ± 1.4	3.1 ± 0.6
naringenin	0.0 ± 0.9	2.9 ± 0.4	9.3 ± 2.1	43.1 ± 0.4**	98.2 ± 5.3**
silybin[a]	0.0±0.3	4.8±1.1	7.7±0.7	45.2±8.8**	77.0±5.5**

Each value represents the mean±S.E.M. (*N*=4).
Significantly different from the control, **p*<0.05, ***p*<0.01.
[a]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; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV-VIS detectors; and HPLC column, YMC-Pack ODS-A (250 × 4.6 mm i.d.) and (250 × 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_{254} (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm) (reversed-phase) and detection was achieved by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4, 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.[2] 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° (*c* 0.60, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{26}H_{30}O_{14}Na$ $[M+Na]^+$: 589.1533. Found: 589.1538. CD [MeOH, nm ($\Delta\varepsilon$)]: 229 (–3.56), 278 (–3.53), 332 (–3.32). UV [MeOH nm, (log ε)]: 226 (4.50), 283 (4.30), 329 (3.60). IR (KBr): 3453, 1638, 1456, 1375, 1169, 1046 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: [2.75 (1H, dd, J = 2.8, 17.1 Hz), 3.35 (1H, m), 3-H_2], 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). ^{13}C-NMR (DMSO-d_6, 125 MHz) δc: given in Table 1. Positive-ion FAB-MS: *m/z* 589 $[M+Na]^+$. Negative-ion FAB-MS: *m/z* 565 $[M–H]^-$.

Theaflavanoside II (**2**): a pale yellow powder, $[\alpha]_D^{27}$ –88.4° (*c* 1.70, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{32}H_{40}O_{18}Na$ $[M+Na]^+$: 735.2112. Found: 735.2119. CD [MeOH, nm ($\Delta\varepsilon$)]: 232 (–3.72), 284 (–3.91), 331 (–3.50). UV [MeOH nm, (log ε)]: 226 (4.48), 283 (4.28), 330 (3.57). IR (KBr): 3453, 1638, 1454, 1375, 1170, 1074 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 1.15 (3H, d, J = 6.1 Hz, Rha-6-H_3), [2.74 (1H, dd, J = 2.7, 16.6 Hz), 3.34 (1H, m), 3-H_2], 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). ^{13}C-NMR (DMSO-d_6, 125 MHz) δc: given in Table 1. Positive-ion FAB-MS: *m/z* 735 $[M+Na]^+$. Negative-ion FAB-MS: *m/z* 711 $[M–H]^-$, 579 $[M–C_5H_9O_4]^-$, 565 $[M–C_6H_{11}O_4]^-$.

Theaflavanoside III (**3**): a pale yellow powder, $[\alpha]_D^{27}$ –40.4° (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{33}H_{42}O_{19}Na$ $[M+Na]^+$: 765.2218. Found: 765.2213. CD [MeOH, nm ($\Delta\varepsilon$)]: 223 (–3.99), 280 (–3.84), 331 (–3.53). UV [MeOH nm, (log ε)]: 226 (4.48), 283 (4.29), 330 (3.58). IR (KBr): 3453, 1647, 1458, 1375, 1175, 1074 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 1.20 (3H, d, J = 5.5 Hz, Rha-6-H_3), [2.73 (1H, dd, J = 2.0, 16.2 Hz), 3.35 (1H, m), 3-H_2], 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}C-NMR (DMSO-d_6, 125 MHz) δc: given in Table 1. Positive-ion FAB-MS: m/z 765 [M+Na]$^+$. Negative-ion FAB-MS: m/z 741 [M–H]$^-$, 579 [M–$C_6H_{11}O_5$]$^-$, 433 [M–$C_{12}H_{21}O_9$]$^-$.

Theaflavanoside IV (**4**): a pale yellow powder, $[\alpha]_D^{27}$ –97.6° (c 1.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{38}H_{50}O_{23}$Na [M+Na]$^+$: 897.2641 Found: 897.2634. CD [MeOH, nm ($\Delta\varepsilon$)]: 232 (–3.89), 279 (–3.97), 332 (–3.74). UV [MeOH nm, (log ε)]: 226 (4.57), 283 (4.38), 330 (3.70). IR (KBr): 3569, 1638, 1458, 1375, 1175, 1075 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 1.18 (3H, d, J = 6.0 Hz, Rha-6-H_3), [2.72 (1H, dd, J = 2.8, 16.5 Hz), 3.40 (1H, m), 3-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}C-NMR (DMSO-d_6, 125 MHz) δc: given in Table 1. Positive-ion FAB-MS: m/z 897 [M+Na]$^+$. Negative-ion FAB-MS: m/z 873 [M–H]$^-$, 741 [M–$C_5H_9O_4$]$^-$, 711 [M–$C_6H_{11}O_5$]$^-$.

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. × 250 mm; detection, UV (254 nm); mobile phase, MeOH–H_2O (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_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 NH_2-60-5, 4.6 mm i.d. × 250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, MeCN–H_2O (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_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.[32] The cell suspension at 4×10^4 cells in 100 μL William's E medium containing fetal calf serum (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate, and precultured for 4 h at 37°C under a 5% CO_2 atmosphere. The fresh medium (100 μL) 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 μL of the fresh medium, and 10 μL of MTT (5 mg/mL in phosphate buffered saline) solution was

added to the medium. After 4 h culture, the medium was removed, 100 μL 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.

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

Statistics

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

ACKNOWLEDGMENTS

This research was supported by the 21st COE program, Academic Frontier Project, and Grand-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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