Bioactive Saponins and Glycosides. XXVIII.[1] New Triterpene Saponins, Foliatheasaponins I, II, III, IV, and V, from Tencha (the Leaves of *Camellia sinensis*)

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New triterpene saponins, foliatheasaponins I—V, were isolated from the methanolic extract of Tencha [the leaves of *Camellia sinensis* (L.) O. KUNTZE (Theaceae)]. The chemical structures of these new saponins were elucidated on the basis of chemical and physicochemical evidence. Among the new saponins, foliatheasaponins II and III, were found to inhibit release of β-hexosaminidase, as a marker of antigen-induced degranulation, in RBL-2H3 cells.

Key words *Camellia sinensis*; Tencha; foliatheasaponin; degranulation inhibitor; triterpene saponin; tea plant

Green tea is the most popular drink in Japan and there are several varieties such as Sencha (煎茶 in Japanese), Gyokuro (玉露), and Tencha (碾茶), *etc*, by the difference of foliar cultivation and preparation. Among them, Tencha is made from young leaves of *Camellia sinensis* (L.) O. KUNTZE (Theaceae) and prepared by the following procedure; 1) cultivation under shaded condition for about 3 weeks to avoid direct rays of the sun; 2) plucked of the leaves then processed steaming and drying. Previously, we have reported the isolation and structure elucidation of theasaponins A_1—A_5, C_1, E_1—E_{13}, F_1—F_3, G_1, G_2, and H_1 together with assamsaponins A—D, F, and I, camelliasaponins B_1 and C_1, and floratheasaponin A from the seeds of *C. sinensis*.[2—6] Among the saponin constituents, theasaponins A_2, E_1, E_2, and E_5 and assamsaponins A, C, and D,[7,8] were found to show gastroprotective activity induced by ethanol in rats.[4,5] Furthermore, floratheasaponins A—C with anti-hyperlipidemic activity were isolated from the flowers of *C. sinensis*.[9] However, the isolation studies on saponin constituents from the leaves of *C. sinensis* were reported unsatisfactorily.[10—15] As a continuing study on the saponin constituents from *C. sinensis*, we have isolated new acylated triterpene oligoglycosides named foliatheasaponins I (**1**), II (**2**), III (**3**), IV (**4**), and V (**5**) from the methanolic extract of Tencha together with two saponins, theasaponin B_1[10,16] (**6**) and floratheasaponin A[9] (**7**), seven flavonoids, kaempferol 3-*O*-β-D-galactopyranoside[9,17] (**8**), isovitexin[18] (**9**), vitexin[19] (**10**), vicetin-2[19,20] (**11**), isoschaftoside[20] (**12**), kaempferol 3-*O*-β-D-galactopyransyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside[9,21] (**13**), and kaempferol 3-*O*-β-D-galactopyransyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside[9,22] (**14**), five catechins, (−)-epicatechin[9,23,24] (**15**), (−)-epicatechin 3-*O*-gallate[9,23,24] (**16**), (−)-epigallocatechin[23,24] (**17**), (+)-gallocatechin[25] (**18**), and (−)-epigallocatechin 3-*O*-gallate[9,23,24] (**19**), and benzyl β-D-glucopyranoside[26] (**20**), benzylalcohol β-D-xylopyranosyl(1→6)-β-D-glucopyranoside[27] (**21**), icariside[28] (**22**), phenetylalcohol β-D-xylopyranosyl(1→6)-β-D-glucopyranoside[27] (**23**), methylgallate,[29] caffeine,[24] and L-theanine.[24] In addition, **2** and **3** were found to inhibit release of β-hexosaminidase, as a marker of antigen-induced degranuration, in RBL-2H3 cells. This paper deals with the isolation of chemical constituents and structure elucidation of five new saponins (**1**—**5**) from Tencha as well as the inhibitory effects of the isolated saponins on the release of β-hexosaminidase in RBL-2H3 cells.

Extraction and Isolation Tencha (cultivated in Kyoto prefecture, Japan) was extracted with methanol to give a methanolic extract (34.5%). The methanolic extract was partitioned into an EtOAc and water mixture to give an EtOAc-soluble fraction (21.3%) and an aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give *n*-BuOH- and H_2O-soluble fractions (7.6 and 5.6%, respectively). The EtOAc-soluble fraction was subjected to normal- and reversed-phase column chromatographies, and finally HPLC to give **16** (0.37%), **17** (1.12%), **19** (1.39%), methylgallate (0.020%), and caffeine (0.074%). From the *n*-BuOH-soluble fraction, **1** (0.0019%), **2** (0.0067%), **3** (0.0015%), **4** (0.0036%), **5** (0.0018%), **6** (0.0029%), **7** (0.0017%), **8** (0.0062%), **9** (0.0097%), **10** (0.0052%), **11** (0.0031%), **12** (0.053%), **13** (0.089%), **14** (0.048%), **15** (0.0025%), **17** (0.82%), **18** (0.0051%), **19** (0.21%), **20** (0.014%), **21** (0.045%), caffeine (1.04%), and L-theanine (1.10%) were isolated by normal- and reversed-phase column chromatographies, and finally HPLC. Through the similar procedure, the H_2O-soluble fraction was purified by normal- and reversed-phase column chromatographies, and finally HPLC to give **20** (0.0009%), **21** (0.0018%), **22** (0.0009%), and **23** (0.0018%).

Structures of Foliatheasaponins I (1), II (2), III (3), IV (4), and V (5) Foliatheasaponin I (**1**) was also obtained as colorless fine crystals from $CHCl_3$–MeOH with mp 214.0—215.0 °C with negative optical rotation ($[\alpha]_D^{27}$ −18.3° in MeOH). The IR spectrum of **1** showed absorption bands at 3475, 1719, 1655, and 1078 cm^{-1} ascribable to hydroxyl, carbonyl, α,β-unsaturated ester, and ether functions. The molecular formula, $C_{61}H_{94}O_{27}$, of **1** was determined from the positive- and negative-ion FAB-MS [*m/z* 1281 $(M+Na)^+$ and *m/z* 1257 $(M-H)^-$] and by high-resolution positive-ion FAB-MS. Treatment of **1** with 10% aqueous potassium hydroxide (KOH)–50% aqueous 1,4-dioxane (1 : 1, v/v) liberated de-

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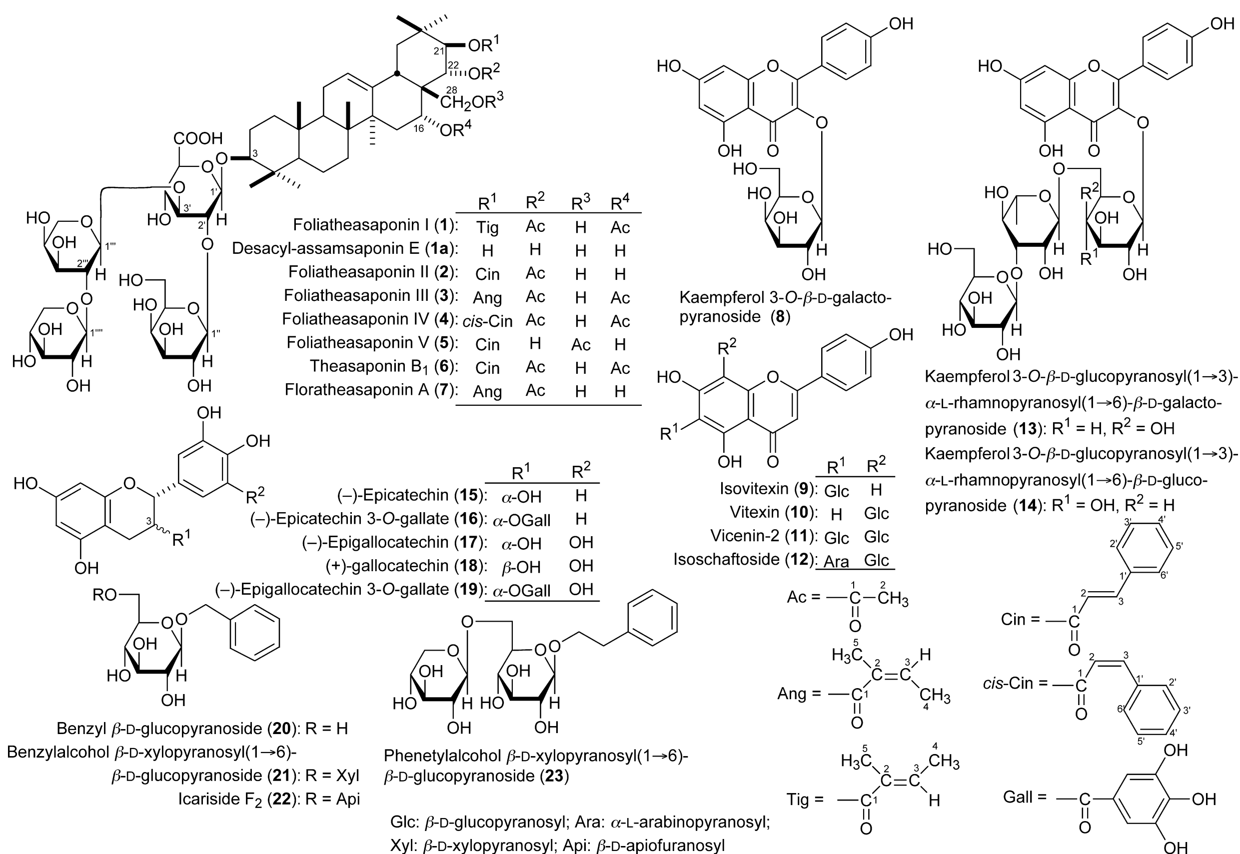

Chart 1

sacyl-assamsaponin E[7] (**1a**) and two organic acids, acetic acid and tiglic acid, which were identified by HPLC analysis of their *p*-nitrobenzyl derivatives.[2,4—9] The ^{1}H- (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[30] indicated the presence of the following functions: a theasapogenol B part {seven methyls [δ 0.78, 0.80, 1.07, 1.13, 1.28, 1.29, 1.50 (3H each, all s, 26, 25, 29, 24, 30, 23, 27-H_3)], a methylene and four methines bearing an oxygen function [δ 3.24 (1H, dd, J=4.1, 11.7 Hz, 3-H), 3.47, 3.60 (1H each, both d, J=10.3 Hz, 28-H_2), 5.61 (1H, br s, 16-H), 5.85 (1H, d, J=10.3 Hz, 21-H), 6.14 (1H, d, J=10.3 Hz, 22-H)], and an olefin [δ 5.41 (1H, br s, 12-H)]}, a tetrasaccharide moiety [δ 4.92 (1H, d, J=7.2 Hz, 1′-H), 5.03 (1H, d, J=7.6 Hz, 1⁗-H), 5.75 (1H, d, J=7.2 Hz, 1″-H), 5.75 (1H, d, J=6.5 Hz, 1‴-H)], and two acetyl and a tigloyl moieties [δ 1.64 (3H, d, J=7.2 Hz, Tig-4-H_3), 1.92 (3H, d, J=1.0 Hz, Tig-5-H_3), 2.01, 2.51 (3H each, both s, Ac), 7.06 (1H, dq-like, Tig-3-H)]. In the heteronuclear multiple bond connectivity (HMBC) experiment of **1**, long-range correlations were observed between the 16-proton and acetyl carbonyl carbon (δ_C 169.8), between the 21-proton and the tigloyl carbonyl carbon (δ_C 168.0), and between the 22-proton and the acetyl carbonyl carbon (δ_C 170.5). Consequently, the structure of foliatheasaponin I was determined to be 16,22-di-*O*-acetyl-21-*O*-tigloyltheasapogenol B 3-*O*-β-D-galactopyranosyl(1→2)[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (**1**).

Foliatheasaponin II (**2**) was obtained as colorless fine crystals with negative optical rotations ($[\alpha]_D^{27}$ −6.3°, MeOH). The molecular formula of **2** was determined to be $C_{63}H_{92}O_{26}$ by positive- and negative-ion FAB-MS [m/z 1287 $(M+Na)^+$ and m/z 1263 $(M-H)^-$] and by high-resolution positive-ion FAB-MS. Alkaline hydrolysis of **2** liberated **1a**[7] and two organic acids, acetic acid and *trans*-cinnamic acid,[24] which were identified by HPLC analysis of their *p*-nitrobenzyl derivatives.[2,4—9] The proton and carbon signals in the ^{1}H- (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[30] of **2** were similar to those of theasaponin B_1 (**6**), except for the signals due to the 16-position. The positions of two acyl groups in **2** were clarified by the HMBC experiments, in which long-range correlations were observed between the 21-proton and the *trans*-cinnamoyl carbonyl carbon (δ_C 167.0) and 22-proton and the acetyl carbonyl carbon (δ_C 171.1). On the basis of these evidence, the structure of foliatheasaponin II was elucidated to be 22-*O*-acetyl-21-*O*-angeloyltheasapogenol B 3-*O*-β-D-galactopyranosyl(1→2)[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (**2**).

Foliatheasaponins III (**3**) and IV (**4**) were obtained as colorless fine crystals both with negative optical rotations (**3**: $[\alpha]_D^{27}$ −10.0°; **4**: $[\alpha]_D^{27}$ −9.6°, MeOH). The molecular formula of **3** and **4** were determined to be $C_{61}H_{94}O_{27}$ and $C_{65}H_{94}O_{27}$, respectively, by high-resolution positive-ion FAB-MS. Alkaline hydrolysis of **3** and **4** liberated **1a**[7] and organic acids, acetic acid (from **3** and **4**), angelic acid (from **3**), and *cis*-cinnamic acid[31] (from **4**), which were identified by HPLC analysis of their *p*-nitrobenzyl derivatives.[2,4—9] The proton and carbon signals in the ^{1}H- (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[30] of **3** resembled those of **1**, except for the signals due to the angeloyl group: [δ 1.98 (3H, s, Ang-5-H_3), 2.06 (3H, d, J=7.3 Hz, Ang-4-H_3), 5.99 (1H, dq-

Table 1. ^{13}C-NMR (150 MHz) Data of Foliatheasaponins I (**1**), II (**2**), III (**3**), IV (**4**), and V (**5**) and Theasaponin B_1 (**6**)

C-	1	2	3	4	5	6
1	39.8	38.8	38.7	38.6	38.8	38.8
2	26.5	26.5	26.5	26.5	26.5	26.5
3	89.6	89.6	89.5	89.5	89.6	89.6
4	39.6	39.6	39.5	39.5	39.6	39.6
5	55.7	55.7	55.6	55.6	55.7	55.8
6	18.3	18.4	18.3	18.2	18.4	18.3
7	33.1	33.1	33.0	33.0	33.1	33.1
8	40.0	40.1	40.0	39.9	40.0	40.1
9	46.9	46.8	46.8	46.8	46.9	46.9
10	36.8	36.7	36.7	36.7	36.8	36.8
11	23.8	23.8	23.7	23.7	23.9	23.8
12	125.2	123.9	125.1	125.1	123.8	125.2
13	141.0	143.0	140.9	140.8	142.7	141.0
14	41.2	41.7	41.1	41.1	41.8	41.2
15	31.1	34.6	31.0	30.9	34.7	31.1
16	71.4	68.0	71.4	71.3	67.7	71.4
17	46.9	48.0	46.9	46.9	47.1	47.3
18	39.6	40.0	39.6	39.5	40.6	39.6
19	47.3	47.2	47.2	47.2	47.3	47.0
20	36.1	36.4	35.9	35.9	36.3	36.1
21	78.8	79.7	78.3	79.1	81.9	79.1
22	73.4	74.3	73.3	73.3	71.0	73.3
23	28.0	28.0	27.9	27.9	28.0	28.0
24	16.7	16.7	16.7	16.7	16.8	16.8
25	15.6	15.6	15.6	15.6	15.6	15.6
26	16.8	16.8	16.7	16.7	17.0	16.8
27	26.9	27.4	26.9	26.9	27.4	27.0
28	63.8	63.7	63.7	63.7	66.4	63.8
29	29.4	29.5	29.3	29.4	29.8	29.4
30	19.5	20.1	19.7	19.4	20.1	19.5
16-*O*-Ac-1	169.8		169.9	169.8		169.8
2	22.0		22.0	22.0		22.0
21-*O*-acyl-1	168.0	167.0	167.8	166.3	167.5	167.1
2	129.1	119.5	128.4	120.0	120.1	118.9
3	137.4	144.7	138.1	143.9	144.1	145.3
4	14.2		16.0			
5	12.3		20.9			
1′		135.3		135.8	135.4	135.2
2′,6′		129.2		130.6	129.2	129.3
3′,5′		128.5		128.5	128.4	128.6
4′		130.7		129.5	130.6	130.6
22-*O*-Ac-1	170.5	171.1	170.4	170.5		170.6
2	20.8	20.9	20.9	20.8		20.8
28-*O*-Ac-1					170.7	
2					20.7	
GlcA-1′	105.6	105.6	105.7	105.7	105.6	105.6
2′	79.0	79.0	79.0	79.0	79.0	79.1
3′	84.1	83.9	84.0	84.0	84.0	84.1
4′	71.0	71.0	71.0	71.0	71.0	71.0
5′	77.3	77.3	77.4	77.0	77.3	77.3
6′	172.0	172.1	172.0	172.1	172.1	172.0
Gal-1″	103.5	103.5	103.5	103.5	103.5	103.5
2″	73.8	73.7	73.3	73.7	73.8	73.8
3″	75.2	75.1	75.1	75.1	75.1	75.2
4″	70.1	70.0	70.1	70.1	70.1	70.1
5″	76.4	76.4	76.4	76.4	76.4	76.4
6″	62.0	61.9	61.9	61.9	61.9	62.0
Ara-1‴	101.7	101.7	101.7	101.7	101.7	101.8
2‴	82.0	82.0	82.1	82.0	82.0	82.0
3‴	73.4	73.3	73.8	73.4	73.3	73.4
4‴	68.3	68.3	68.3	68.3	68.3	68.3
5‴	66.0	66.0	66.0	65.9	65.9	66.0
Xyl-1⁗	106.9	106.8	106.9	106.8	106.9	106.8
2⁗	75.7	75.7	75.7	75.7	75.8	75.7
3⁗	78.2	78.2	78.2	78.4	78.2	78.2
4⁗	70.8	70.7	70.7	70.7	70.7	70.8
5⁗	67.4	67.4	67.5	67.4	67.5	67.5

Measured in pyridine-d_5.

like, Ang-3-H)]. The position of the angeloyl group in **3** was clarified by the HMBC experiments, which showed a long-range correlation between the 21-proton [δ 5.87 (1H, d, J=10.4 Hz)] and the angeloyl carbonyl carbon (δ_C 167.8). Consequently, the structure of foliatheasaponin III was determined to be 16,22-di-*O*-acetyl-21-*O*-angeloyltheasapogenol B 3-*O*-β-D-galactopyranosyl(1→2)[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (**3**). On the other hand, proton and carbon signals in the ^{1}H-(pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[30] of **4** were superimposable on those of **6**, except for the signals due to the *cis*-cinnamoyl group: [δ 6.16, 7.00 (1H each, both d, J=12.7 Hz, Cin-2-H and Cin-3-H), 7.29 (1H, br t, J=*ca.* 8 Hz, Cin-4′-H), 7.33 (2H, dd, J=7.9, 8.2 Hz, Cin-3′,5′-H), 7.86 (2H, dd, J=1.0, 8.2 Hz, Cin-2′,6′-H)]. The position of the *cis*-cinnamoyl group in **4** was clarified by the HMBC experiments, which showed a long-range correlation between the 21-proton [δ 5.81 (1H, d, J=10.3 Hz)] and the *cis*-cinnamoyl carbonyl carbon (δ_C 166.3). Thus the structure of foliatheasaponin IV was determined to be 16,22-di-*O*-acetyl-21-*O*-*cis*-cinnamoyltheasapogenol B 3-*O*-β-D-galactopyranosyl(1→2)[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (**4**).

Foliatheasaponin V (**5**) was isolated as colorless fine crystals of mp 234.0—235.5 °C (from $CHCl_3$–MeOH) with positive optical rotation ($[\alpha]_D^{27}$ +8.4° in MeOH). The IR spectrum of **5** showed absorption bands due to hydroxyl, carbonyl, α,β-unsaturated ester, and ether functions at 3475, 1731, 1656, and 1078 cm^{-1}. In the positive- and negative-ion FAB-MS, quasimolecular ion peaks were observed at m/z 1287 $(M+Na)^+$ and m/z 1263 $(M-H)^-$, respectively, and high-resolution positive-ion FAB-MS revealed the molecular formula of **5** to be $C_{63}H_{92}O_{26}$. Alkaline hydrolysis of **5** with 10% aqueous KOH–50% aqueous 1,4-dioxane (1 : 1, v/v) provided **1a**[7] and two organic acids, acetic acid and *trans*-cinnamic acid,[24] which were identified by HPLC analysis of their *p*-nitrobenzyl derivatives.[2,4—9] The ^{1}H- (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[30] of **5** showed signals assignable to seven methyls [δ 0.81, 0.98, 1.11, 1.16, 1.28, 1.35, 1.84 (3H each, all s, 25, 26, 24, 29, 23, 30, 27-H_3)], a methylene and four methines bearing an oxygen function [δ 3.28 (1H, dd, J=4.5, 11.7 Hz, 3-H), 4.28 (2H, br s, 28-H_2), 4.58 (1H, d, J=9.6 Hz, 22-H), 4.78 (1H, br s, 16-H), 6.57 (1H, d, J=9.6 Hz, 21-H)], an olefin [δ 5.45 (1H, br s, 12-H)], and four glycopyranosyl moieties {a β-D-glucuronopyranosyl [δ 4.95 (1H, d, J=7.9 Hz, 1′-H)], a β-D-xylopyranosyl [δ 5.02 (1H, d, J=7.9 Hz, 1⁗-H)], a β-D-galactopyranosyl [δ 5.77 (1H, d, J=7.6 Hz, 1″-H)], an α-L-arabinopyranosyl [δ 5.78 (1H, d, J=6.2 Hz, 1‴-H)]} together with an acetyl and a *trans*-cinnamoyl moieties [δ 1.99 (3H, s, Ac), 6.81, 7.94 (1H each, both d, J=16.1 Hz, Cin-2-H and Cin-3-H), 7.33 (3H, br s, Cin-2′,6′-H and Cin-4′-H), 7.53 (2H, br s, Cin-3′,5′-H)]. The position of the acyl groups in **5** was clarified on the basis of the HMBC experiment. Thus, long-range correlations were observed between the 21-proton and the cinnamoyl carbonyl carbon (δ_C 167.5) and between the 28-protons and the acetyl carbonyl carbon (δ_C 170.7). Very recently,[33] isotheasaponin B_1 was isolated from the leaves of *C. sinensis* and the structure, which was the same as that of foliatheasaponin V, was deduced by spectral analysis. On the basis of this evidence, the structure of isotheasaponin B_1 (fo-

liatheasaponin V) was confirmed to be 28-*O*-acetyl-21-*O*-*trans*-cinnamoyltheasapogenol B 3-*O*-β-D-galactopyranosyl-(1→2)[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (**5**).

Inhibitory Effects of Saponin Constituents on the Release of β-Hexosaminidase in RBL-2H3 Cells Histamine, which is released from mast cells on stimulation by an antigen or a degranulation inducer, is usually determined as a degranulation marker *in vitro* experiments on immediate allergic reactions. β-Hexosaminidase is also stored in the secretory granules of mast cells and is released concomitantly with histamine when mast cells are immunologically activated.[34,35] Therefore it is generally accepted that β-hexosaminidase is a degranulation marker of mast cells. As a part of our characterization studies of bioactive components from natural medicines, we previously reported several inhibitors of the release of β-hexosaminidase such as diarylheptanoids,[36—38] sesquiterpenes,[39—41] diterpenes,[42] flavonoids,[43] anthraquinones,[44] stilbenes,[45] phenanthrenes,[45] phenylpropanoids,[46] and alkaloids.[47,48] As a continuing study of the antiallergic constituents from the medicinal foods, effects of foliatheasaponins on the release of β-hexosaminidase in RBL-2H3 cells were examined. Among the new saponins, foliatheasaponins II [**2**, inhibition: 46.3±3.5% at 3 μM and 55.7±2.3% at 6 μM] and III [**3**, 22.8±1.8% at 6 μM and 47.0±4.3% at 10 μM], inhibited the release of β-hexosaminidase and those activities were stronger than those of antiallergic compounds, tranilast[38] (inhibition: 22.4±2.5% at 100 μM, IC_{50}=282 μM) and ketotifen fumarate[38] (inhibition: 27.6±2.2% at 100 μM, IC_{50}=158 μM).

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (*l*=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JNM-ECA600 (600 MHz) spectrometer; ^{13}C-NMR spectra, JNM-ECA600 (150 MHz) spectrometer with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV–VIS detectors.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reverse-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh) and Diaion HP-20 (Nippon Rensui); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reverse phase); reverse-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Plant Material Tencha (the leaves of *C. sinensis*) was cultivated in Kyoto prefecture, Japan and purchased by Maiko no Cha (Maiko Tea Company), Kyoto, Japan. A voucher of this plant material is on file in our laboratory.

Extraction and Isolation Tencha (2.9 kg) was extracted three times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a methanolic extract (1000 g, 34.5%), and an aliquot (500 g) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (308 g, 21.3%) and an aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (110 g, 7.6%) and an H_2O-soluble fraction (81 g, 5.6%).

The EtOAc-soluble fraction (100 g) was subjected to normal-phase silica gel column chromatography [2.5 kg, $CHCl_3$→$CHCl_3$–MeOH (5 : 1→1 : 1→1 : 5, v/v)→MeOH] to give seven fractions [Fr. 1 (3.4 g), Fr. 2 (0.4 g), Fr. 3 (29.9 g), Fr. 4 (3.5 g), Fr. 5 (42.1 g), Fr. 6 (14.6 g), and Fr. 7 (4.6 g)]. Fraction 4 (3.5 g) was subjected to reversed-phase silica gel column chromatography [100 g, H_2O→MeOH–H_2O (30 : 70→50 : 50→70 : 30, v/v)→MeOH] to furnish methylgallate (89 mg, 0.020%) and caffeine (336 mg, 0.074%). Fraction 5 (14.0 g) was subjected to reversed-phase silica gel column chromatography [300 g, MeOH–H_2O (30 : 70→50 : 50→70 : 30, v/v)→MeOH] to afford eight fractions [Fr. 5-1=(−)-epigallocatechin (**17**, 588 mg, 0.39%), Fr. 5-2 (9.70 g), Fr. 5-3 (2.00 g), Fr. 5-4=(−)-epicatechin 3-*O*-gallate (**16**, 568 mg, 0.37%), Fr. 5-5 (300 mg), Fr. 5-6 (90 mg), Fr. 5-7 (632 mg), and Fr. 5-8 (453 mg)]. Fraction 5-2 (1.00 g) was purified by HPLC [YMC-pack ODS-A, 250×20 mm i.d., MeOH–1% aqueous AcOH (30 : 70, v/v)] to give **17** (114 mg, 0.73%) and (−)-epigallocatechin 3-*O*-gallate (**19**, 208 mg, 1.30%). Fraction 7 (14.0 g) was subjected to reversed-phase silica gel column chromatography [330 g, MeOH–H_2O (30 : 70→50 : 50→80 : 20, v/v)→MeOH] to give seven fractions [Fr. 7-1 (433 mg), Fr. 7-2=**19** (398 mg, 0.087%), Fr. 7-3 (419 mg), Fr. 7-4 (170 mg), Fr. 7-5 (142 mg), Fr. 7-6 (1282 mg), and Fr. 7-7 (309 mg)].

The *n*-BuOH-soluble fraction (100 g) was subjected to normal-phase silica gel column chromatography [2.5 kg, $CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer→6 : 4 : 1, v/v/v)→MeOH] to give ten fractions [Fr. 1 (0.1 g), Fr. 2=caffeine (10.1 g, 0.79%), Fr. 3 (4.1 g), Fr. 4 (1.2 g), Fr. 5 (11.1 g), Fr. 6 (2.2 g), Fr. 7 (9.9 g), Fr. 8 (19.7 g), Fr. 9 (30.5 g), and Fr. 10 (12.4 g)]. Fraction 3 (3.5 g) was subjected to reversed-phase silica gel column chromatography [100 g, H_2O→MeOH–H_2O (30 : 70→70 : 30, v/v)→MeOH] to furnish caffeine (2.7 g, 0.25%). Fraction 5 (10.0 g) was subjected to reversed-phase silica gel column chromatography [100 g, H_2O→MeOH–H_2O (30 : 70→70 : 30→90 : 10, v/v)→MeOH] to afford ten fractions [Fr. 5-1 (510 mg), Fr. 5-2 (528 mg), Fr. 5-3 (160 mg), Fr. 5-4 (534 mg), Fr. 5-5 (369 mg), Fr. 5-6 (315 mg), Fr. 5-7 (764 mg), Fr. 5-8 (1779 mg), Fr. 5-9 (2483 mg), and Fr. 5-10 (2068 mg)]. Fraction 5-2 (528 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to give benzyl β-D-glucopyranoside (**20**, 159 mg, 0.014%). Fraction 7 (9.0 g) was subjected to reversed-phase silica gel column chromatography [330 g, MeOH–H_2O (10 : 90→30 : 70→50 : 50→70 : 30→80 : 20→90 : 10, v/v)→MeOH→$CHCl_3$] to afford ten fractions [Fr. 7-1 (980 mg), Fr. 7-2 (938 mg), Fr. 7-3 (493 mg), Fr. 7-4 (756 mg), Fr. 7-5 (141 mg), Fr. 7-6 (91 mg), Fr. 7-7 (127 mg), Fr. 7-8 (493 mg), Fr. 7-9 (901 mg), and Fr. 7-10 (3588 mg)]. Fraction 7-2 (938 mg) was purified by HPLC [MeOH–1% aqueous AcOH (20 : 80, v/v)] to give benzyl β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**21**, 52 mg, 0.0045%) and **15** (30 mg, 0.0025%). Fraction 7-4 (756 mg) was further separated by HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to give kaempferol 3-*O*-β-D-glucopyranoside (**8**, 73 mg, 0.0062%), isovitexin (**9**, 113 mg, 0.0097%), and vitexin (**10**, 61 mg, 0.0052%). Fraction 8 (15.0 g) was subjected to reversed-phase silica gel column chromatography [450 g, MeOH–H_2O (10 : 90→30 : 70→50 : 50→70 : 30→90 : 10, v/v)→MeOH→$CHCl_3$] to afford twelve fractions [Fr. 8-1 (300 mg), Fr. 8-2 (314 mg), Fr. 8-3 (501 mg), Fr. 8-4=**17** (5344 mg, 0.53%), Fr. 8-5 (863 mg), Fr. 8-6 (835 mg), Fr. 8-7 (1.20 g), Fr. 8-8 (1.60 g), Fr. 8-9 (1.12 g), Fr. 8-10 (2.24 g), Fr. 8-11 (1.20 g), and Fr. 8-12 (170 mg)]. Fraction 8-3 (501 mg) was separated by HPLC [MeOH–1% aqueous AcOH (10 : 90, v/v)] to give (+)-gallocatechin (**18**, 51 mg, 0.0051%). Fraction 8-5 (60 mg) was purified by HPLC [MeOH–1% aqueous AcOH (20 : 80, v/v)] to furnish **17** (71 mg, 0.010%) and **19** (121 mg, 0.017%). Fraction 8-6 (250 mg) was separated by HPLC [MeOH–1% aqueous AcOH (30 : 70, v/v)] to give isoschaftoside (**12**, 61 mg, 0.021%). Fraction 8-8 (500 mg) was purified by HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to give kaempferol 3-*O*-β-D-galactopyransyl(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**13**, 186 mg, 0.060%) and kaempferol 3-*O*-β-D-galactopyransyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**14**, 61 mg, 0.020%). Faction 8-9 (500 mg) was purified by HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to furnish **14** (43 mg, 0.0095%). Fraction 8-10 (2.00 g) was purified by HPLC [MeOH–1% aqueous AcOH (70 : 30, v/v)] to furnish five fractions [Fr. 8-10-1 (120 mg), Fr. 8-10-2 (230 mg), Fr. 8-10-3 (196 mg), Fr. 8-10-4 (88 mg), and Fr. 8-10-5 (260 mg)]. Fraction 8-10-1 (120 mg) was further purified by HPLC [CH_3CN–1% aqueous AcOH (40 : 60, v/v)] to furnish foliatheasaponin I (**1**, 20 mg, 0.0019%) and floratheasaponin A (**7**, 17 mg, 0.0017%). Fraction 8-10-2 (189 mg) further purified by HPLC [CH_3CN–1% aqueous AcOH (40 : 60, v/v)] to give foliatheasaponin III (**3**, 16 mg, 0.0015%). Fraction 8-10-3 (196 mg) was further purified by HPLC [CH_3CN–1% aqueous AcOH (50 : 50, v/v)] to furnish foliatheasaponins II (**2**, 68 mg, 0.0067%) and V (**5**, 18 mg, 0.0018%). Fraction 8-10-4 (88 mg) was further purified by HPLC [CH_3CN–1% aqueous AcOH (40 : 60, v/v)] to furnish foliatheasaponin IV (**4**, 37 mg, 0.0036%). Fraction 8-10-5 (260 mg) was further purified by HPLC [CH_3CN–1% aqueous AcOH (45 : 55, v/v)] to furnish theasaponin B_1 (**6**, 29 mg, 0.0029%). Fraction 9 (25.0 g) was subjected to reversed-phase silica gel column

chromatography [750 g, MeOH–H_2O (10 : 90→20 : 80→30 : 70→40 : 60→50 : 50→70 : 30, v/v)→MeOH→$CHCl_3$] to give ten fractions [Fr. 9-1=L-theanine (10.60 g, 1.10%), Fr. 9-2=**17** (2.90 g, 0.28%), Fr. 9-3=**19** (2.00 g, 0.19%), Fr. 9-4 (0.68 g), Fr. 9-5 (1.69 g), Fr. 9-6 (2.77 g), Fr. 9-7 (1.99 g), Fr. 9-8 (0.17 g), Fr. 9-9 (0.29 g), and Fr. 9-10 (0.63 g)]. Fraction 9-4 (683 mg) was separated by HPLC [MeOH–1% aqueous AcOH (30 : 70, v/v)] to give vicenin-2 (**11**, 32 mg, 0.0031%). Fraction 9-5 (1.00 g) was purified by HPLC [MeOH–1% aqueous AcOH (35 : 65, v/v)] to furnish **12** (200 mg, 0.032%). Fraction 9-7 (500 mg) was separated by HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to give **13** (77 mg, 0.029%) and **14** (49 mg, 0.018%).

The H_2O-soluble fraction (81.2 g) was subjected to Diaion HP-20 column chromatography (3.0 kg, H_2O→MeOH→acetone) to give H_2O-, MeOH-, and acetone-eluted fractions (75.4, 5.9, and 0.2 g, respectively). The MeOH-eluted fraction (5.9 g) was subjected to normal-phase silica gel column chromatography [180 g, $CHCl_3$→$CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer→6 : 4 : 1, v/v/v)→MeOH] to give six fractions [Fr. 1 (43 mg), Fr. 2 (676 mg), Fr. 3 (506 mg), Fr. 4 (2147 mg), Fr. 5 (1305 mg), and Fr. 6 (949 mg)]. Fraction 3 (506 mg) was separated by HPLC [MeOH–1% aqueous AcOH (40 : 60, v/v)] to give 2 fractions [Fr. 3-1 (120 mg) and Fr. 3-2=phenetylalcohol β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**23**, 25 mg, 0.0018%)]. Fraction 3-1 (120 mg) was separated by HPLC [MeOH–1% aqueous AcOH (20 : 80, v/v)] to give benzyl β-D-glucopyranoside (**20**, 13 mg, 0.0009%), benzylalcohol β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**21**, 34 mg, 0.0024%), and icariside F_2 (**22**, 13 mg, 0.0009%).

The known compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, MS, and ^1H- and ^{13}C-NMR) with reported values[9,13,17—23,25—29] of commercial samples.[24]

Foliatheasaponin I (**1**): Colorless fine crystals (from $CHCl_3$–MeOH), mp 214.0—215.0 °C, $[\alpha]_D^{27}$ −18.3° (c=0.97, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{61}H_{94}O_{27}Na$ $(M+Na)^+$: 1281.5880. Found: 1281.5885. IR (KBr): 3475, 1719, 1655, 1078 cm^{-1}. ^1H-NMR (600 MHz, pyridine-d_5) δ: 0.78, 0.80, 1.07, 1.13, 1.28, 1.29, 1.50 (3H each, all s, 26, 25, 29, 24, 30, 23, 27-H_3), 1.64 (3H, d, J=7.2 Hz, 21-O-Tig-4-H_3), 1.92 (3H, d, J=1.0 Hz, 21-O-Tig-5-H_3), 2.01, 2.51 (3H each, both s, 22, 16-O-Ac), 3.01 (1H, dd, J=3.8, 14.1 Hz, 18-H), 3.24 (1H, dd, J=4.1, 11.7 Hz, 3-H), 3.47, 3.60 (1H each, both d, J=10.3 Hz, 28-H_2), 4.92 (1H, d, J=7.2 Hz, 1′-H), 5.03 (1H, d, J=7.6 Hz, 1′′′′-H), 5.41 (1H, br s, 12-H), 5.61 (1H, br s, 16-H), 5.75 (1H, d, J=7.2 Hz, 1″-H), 5.75 (1H, d, J=6.5 Hz, 1‴-H), 5.85 (1H, d, J=10.3 Hz, 21-H), 6.14 (1H, d, J=10.3 Hz, 22-H), 7.06 (1H, dq-like, 21-O-Tig-3-H). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 1281 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1257 $(M-H)^-$, 1125 $(M-C_5H_9O_4)^-$, 1095 $(M-C_6H_{11}O_5)^-$, 993 $(M-C_{10}H_{17}O_8)^-$, 831 $(M-C_{16}H_{27}O_{13})^-$.

Foliatheasaponin II (**2**): Colorless fine crystals (from $CHCl_3$–MeOH), mp 235.5—236.5 °C, $[\alpha]_D^{27}$ −6.3° (c=0.24, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{63}H_{92}O_{26}Na$ $(M+Na)^+$: 1287.5775. Found: 1287.5768. UV [MeOH, nm (log ε)]: 277 (4.18). IR (KBr): 3475, 1719, 1655, 1078 cm^{-1}. ^1H-NMR (600 MHz, pyridine-d_5) δ: 0.81, 0.85, 1.14, 1.14, 1.30, 1.38, 1.85 (3H each, all s, 25, 26, 29, 24, 23, 30, 27-H_3), 1.91 (3H, s, 22-O-Ac), 3.10 (1H, m, 18-H), 3.28 (1H, dd, J=4.6, 11.5 Hz, 3-H), 3.40, 3.64 (1H each, both d, J=10.6 Hz, 28-H_2), 4.46 (1H, br s, 16-H), 4.95 (1H, d, J=7.5 Hz, 1′-H), 5.02 (1H, d, J=7.5 Hz, 1′′′′-H), 5.40 (1H, br s, 12-H), 5.77 (1H, d, J=7.8 Hz, 1″-H), 5.78 (1H, d, J=7.0 Hz, 1‴-H), 6.33 (1H, d, J=10.1 Hz, 22-H), 6.68 (1H, d, J=10.1 Hz, 21-H), 6.88, 8.05 (1H each, both d, J=16.1 Hz, Cin-2-H and Cin-3-H), 7.33 (3H, br s, Cin-2′,6′-H and Cin-4′-H), 7.58 (2H, br s, Cin-3′,5′-H). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 1287 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1263 $(M-H)^-$.

Foliatheasaponin III (**3**): Colorless fine crystals (from $CHCl_3$–MeOH), mp 220.4—220.8 °C, $[\alpha]_D^{27}$ −10.0° (c=2.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{61}H_{94}O_{27}Na$ $(M+Na)^+$: 1281.5880. Found: 1281.5886. IR (KBr): 3453, 1731, 1647, 1080 cm^{-1}. ^1H-NMR (600 MHz, pyridine-d_5) δ: 0.75, 0.77, 1.07, 1.12, 1.26, 1.28, 1.49 (3H each, all s, 26, 25, 29, 24, 30, 23, 27-H_3), 1.98 (3H, s, 21-O-Ang-5-H_3), 2.04, 2.51 (3H each, both s, 22, 16-O-Ac), 2.06 (3H, d, J=7.3 Hz, 21-O-Ang-4-H_3), 3.00 (1H, dd-like, 18-H), 3.20 (1H, dd-like, 3-H), 3.46, 3.59 (1H each, both d, J=10.4 Hz, 28-H_2), 4.91 (1H, d, J=7.4 Hz, 1′-H), 5.02 (1H, d, J=7.4 Hz, 1′′′′-H), 5.39 (1H, br s, 12-H), 5.61 (1H, br s, 16-H), 5.76 (1H, d, J=8.0 Hz, 1″-H), 5.77 (1H, d, J=6.1 Hz, 1‴-H), 5.87 (1H, d, J=10.4 Hz, 21-H), 5.99 (1H, dq-like, 21-O-Ang-3-H), 6.13 (1H, d, J=10.4 Hz, 22-H). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 1281 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1257 $(M-H)^-$, 1125 $(M-C_5H_9O_4)^-$, 963 $(M-C_{11}H_{19}O_9)^-$.

Foliatheasaponin IV (**4**): Colorless fine crystals (from $CHCl_3$–MeOH), mp 236.5—237.0 °C, $[\alpha]_D^{27}$ −9.6° (c=1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{65}H_{94}O_{27}Na$ $(M+Na)^+$: 1329.5880. Found: 1329.5886. UV [MeOH, nm (log ε)]: 274 (4.09). IR (KBr): 3475, 1719, 1655, 1078 cm^{-1}. ^1H-NMR (600 MHz, pyridine-d_5) δ: 0.72, 0.75, 1.07, 1.11, 1.19, 1.27, 1.47 (3H each, all s, 26, 25, 29, 24, 30, 23, 27-H_3), 2.03, 2.43 (3H each, both s, 22, 16-O-Ac), 2.97 (1H, dd, J=3.8, 14.1 Hz, 18-H), 3.19 (1H, dd, J=4.1, 11.7 Hz, 3-H), 3.45, 3.58 (1H each, both d, J=11.6 Hz, 28-H_2), 4.91 (1H, d, J=7.6 Hz, 1′-H), 5.02 (1H, d, J=7.6 Hz, 1′′′′-H), 5.37 (1H, br s, 12-H), 5.60 (1H, br s, 16-H), 5.76 (1H, d, J=7.6 Hz, 1″-H), 5.79 (1H, d, J=6.9 Hz, 1‴-H), 5.81 (1H, d, J=10.3 Hz, 21-H), 6.15 (1H, d, J=10.3 Hz, 22-H), 6.16, 7.00 (1H each, both d, J=12.7 Hz, Cin-2-H and Cin-3-H), 7.29 (1H, br t, J=*ca.* 8 Hz, Cin-4′-H), 7.33 (2H, dd, J=7.9, 8.2 Hz, Cin-3′,5′-H), 7.86 (2H, dd, J=1.0, 8.2 Hz, Cin-2′,6′-H). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 1329 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1305 $(M-H)^-$, 1173 $(M-C_5H_9O_4)^-$, 1143 $(M-C_6H_{11}O_5)^-$, 1041 $(M-C_{10}H_{17}O_8)^-$, 879 $(M-C_{16}H_{27}O_{13})^-$.

Foliatheasaponin V (**5**): Colorless fine crystals (from $CHCl_3$–MeOH), mp 234.0—235.5 °C, $[\alpha]_D^{27}$ +8.4° (c=0.68, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{63}H_{92}O_{26}Na$ $(M+Na)^+$: 1287.5775. Found: 1287.5768. UV [MeOH, nm (log ε)]: 277 (4.36). IR (KBr): 3475, 1731, 1656, 1078 cm^{-1}. ^1H-NMR (600 MHz, pyridine-d_5) δ: 0.81, 0.98, 1.11, 1.16, 1.28, 1.35, 1.84 (3H each, all s, 25, 26, 24, 29, 23, 30, 27-H_3), 1.99 (3H, s, 28-O-Ac), 2.87 (1H, dd, J=4.5, 13.7 Hz, 18-H), 3.28 (1H, dd, J=4.5, 11.7 Hz, 3-H), 4.28 (2H, br s, 28-H_2), 4.58 (1H, d, J=9.6 Hz, 22-H), 4.78 (1H, br s, 16-H), 4.95 (1H, d, J=7.9 Hz, 1′-H), 5.02 (1H, d, J=7.9 Hz, 1′′′′-H), 5.45 (1H, br s, 12-H), 5.77 (1H, d, J=7.6 Hz, 1″-H), 5.78 (1H, d, J=6.2 Hz, 1‴-H), 6.57 (1H, d, J=9.6 Hz, 21-H), 6.81, 7.94 (1H each, both d, J=16.1 Hz, Cin-2-H and Cin-3-H), 7.33 (3H, br s, Cin-2′,6′-H and Cin-4′-H), 7.53 (2H, br s, Cin-3′,5′-H). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 1287 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1263 $(M-H)^-$.

Alkaline Hydrolysis of Foliatheasaponins I—V (1—5) A solution of foliatheasaponins I—V (**1**—**5**) (4.7, 15.5, 14.1, 10.6, and 8.2 mg, respectively) in 50% aqueous 1,4-dioxane (1.0 ml) was treated with 10% aqueous KOH (1.0 ml) and the whole was stirred at 37 °C for 1 h. Removal of the solvent under reduced pressure gave a reaction mixture. A part of the reaction mixture was dissolved in $(CH_2)_2Cl_2$ (2.0 ml) and the solution was treated with p-nitrobenzyl-N-N'-diisopyopylisourea (10 mg), then the whole was stirred at 80 °C for 1 h. The reaction solution was subjected to HPLC analysis [column: YMC-Pack ODS-A, 250×4.6 mm i.d.; mobile phase: CH_3CN–H_2O (50 : 50, v/v); detection: UV (254 nm); flow rate: 1.0 ml/min] to identify the p-nitrobenzyl esters of acetic acid (**a**, t_R 9.6 min) from **1**—**5**, tiglic acid (**b**, t_R 27.5 min) from **1**, angelic acid (**c**, t_R 30.6 min) from **3**, *trans*-cinnamic acid (**d**, t_R 37.8 min) from **5**, and *cis*-cinnamic acid[31] (**e**, t_R 43.9 min) from **4**. The rest of the reaction mixture was neutralized with Dowex HCR W2 (H^+ form) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a product, which was subjected to ordinary-phase silica gel column chromatography [500 mg, $CHCl_3$–MeOH–H_2O (6 : 4 : 1, v/v/v)] to give desacyl-assamsaponin E[7] (**1a**, 3.2 mg from **1**, 10.5 mg from **2**, 12.8 mg from **3**, 7.5 mg from **4**, and 6.1 mg from **5**).

Bioassay. Inhibitory Effect on the Release of β-Hexosaminidase in RBL-2H3 Cells The inhibitory effects of the test samples on the release of β-hexosaminidase from RBL-2H3 cells [Cell No. JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] were evaluated by a method reported previously.[36—48] Briefly, RBL-2H3 cells were dispensed into 24-well plates at a concentration of 2×10^5 cells/well using Eagle's minimum essential medium (MEM, Sigma) containing fetal calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.45 μg/ml of anti-DNP IgE, and these were incubated overnight at 37 °C in 5% CO_2 for sensitization of the cells. Then the cells were washed twice with 500 μl of Siraganian buffer [119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), and 40 mM NaOH, pH 7.2], and incubated in 160 μl of Siraganian buffer [5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albmin (BSA) were added] for an additional 10 min at 37 °C. Aliquots (20 μl) of test sample solution were added to each well and incubated for 10 min, followed by the addition of 20 μl of antigen (DNP-BSA, final concentration 10 μg/ml) at 37 °C for 10 min to stimulate the cells to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μl) was transferred into a 96-well microplate and incubated with 50 μl of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured using a

microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration 0.1%).

The percent inhibition of the release of β-hexosaminidase by the test material was calculated using the following equation, and IC_{50} values were determined graphically:

$$\text{inhibition (\%)} = \left(1 - \frac{T - B - N}{C - N}\right) \times 100$$

Control (C): DNP-BSA (+), test sample (−); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (−), test sample (+); Normal (N): DNP-BSA (−), test sample (−).

Under these conditions, it was calculated that 40—60% of β-hexosaminidase was released from the cells in the control groups by determination of the total β-hexosaminidase activity after sonication of the cell suspension.

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