Constituents in Watercress: Inhibitors of Histamine Release from RBL-2H3 Cells Induced by Antigen Stimulation

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Histamine release inhibitors in watercress (*Nasturtium officinale*) were isolated using a monitoring system with antigen-stimulated RBL-2H3 cells. Of the 15 compounds isolated, flavonols and megastigmanes significantly inhibited histamine release. Two flavonols, 3-*O*-sophorosides of rhamnetin and rhamnazin, were new compounds. To investigate the inhibitory mechanism, the effects of rhamnetin, rhamnetin 3-*O*-sophoroside and an isolated megastigmane glucoside on the increase in the intracellular free calcium concentration were examined at a concentration providing 60% inhibition of histamine release. The results suggest that these compounds did not affect the calcium influx at that concentration. The structure–activity relationships of the megastigmanes on histamine release were also investigated.

Key words *Nasturtium officinale*; histamine release inhibitor; flavonol; megastigmane; RBL-2H3 cell; watercress

In recent years it has been recognized that foods have a tertiary function,¹⁾ which contributes to disease prevention by modulating the human defense system, apart from their nutritional and organoleptic ones. Immune response is one of the most important defense systems and allergenic symptoms occur when the defense system is disordered by a specific allergen. Much information has been accumulated on specific allergens in foods; however, little data has been provided regarding the anti-allergic activity of foods except for teas,²⁾ citrus fruits³⁾ and perilla.⁴⁾ Therefore, we decided to focus our attention on foods possessing anti-allergic activities.⁵⁾

The release of histamine from basophils or mast cells induced by antigen stimulation is an essential step in the immediate type allergic reaction. Rat basophilic leukemia 2H3 (RBL-2H3) cells have receptors for IgE and can be stimulated to release histamine by aggregation of the receptors with the antigen. Thus, we have considered histamine release from RBL-2H3 cells induced by an antigen as an *in vitro* model for *in vivo* allergic reactions and have utilized it to search for anti-allergic constituents in foods and food stuffs. In a screening study,^{5c)} we found that the 70% methanol extract of vegetable watercress (*Nasturtium officinale*) had an inhibitory activity on the histamine release from the cells. Watercress is a Cruciferae plant and its volatile flavor components⁶⁾ and surface wax components⁷⁾ were investigated by several researchers, while the more polar constituents were not studied. In an earlier study,^{5c)} we partitioned the watercress extract between aqueous methanol and hexane to remove the volatile and wax components and found that the aqueous methanol layer still showed strong inhibitory effects. Therefore, we focused our work on the isolation of the histamine release inhibitors from watercress.

RESULTS AND DISCUSSION

The methanolic extract from watercress was applied onto a Diaion HP-20 column after removal of the hexane soluble portion. The methanol eluate of the column was further

chromatographed to give 15 compounds (**1**–**15**). Of the total compounds, three compounds were known megastigmanes and were identified as (9*R*)-4-oxo- β -ionol β -D-glucopyranoside (**1**),⁸⁾ (6*R*,9*R*)-3-oxo- α -ionol β -D-glucopyranoside (**2**)^{8b,9)} and (6*S*,9*R*)-6-hydroxy-3-oxo- α -ionol 9-*O*- β -D-glucopyranoside (**3**),¹⁰⁾ by comparison with their spectral data in the literature. Compounds **4**, **5**, **7** and **8** were identified as phenethylamine, L-phenylalanine, 1,2,3,4-tetrahydro- β -carboline-1-carboxylic acid¹¹⁾ and 1-methyl-1,2,3,4-tetrahydro- β -carboline-1-carboxylic acid, respectively, by comparison with their spectral data with authentic samples or those already reported. Another 8 compounds were characterized as flavonols by spectroscopy. Of them, quercetin 3-*O*-sophoroside 7-*O*- β -glucopyranoside (**6**),¹²⁾ rhamnazin 3-*O*- β -glucopyranoside (**11**),¹³⁾ rhamnetin 3-*O*- β -glucopyranoside (**12**),¹⁴⁾ kaempferol 3-*O*- β -glucopyranoside (**13**), isorhamnetin 3-*O*- β -glucopyranoside (**14**) and rhamnazin (**15**) were known compounds, and they were identified by comparison of their spectral data with those of authentic samples and their related compounds or those already reported.

Compound **9** showed a quasi-molecular ion peak at m/z 641 ($[M+H]^+$) in FAB-MS and electrospray ionization (ESI)-MS and at m/z 642 ($[M+2H]^+$) in ESI-MS. The molecular formula was determined to be C₂₈H₃₂O₁₇ based on the high-resolution (HR) FAB-MS data of $[M+H]^+$. Sugar analysis using the pyridylation reaction¹⁵⁾ showed the presence of glucose. The ¹H-NMR and ¹³C-NMR spectra showed signals due to two glucopyranoses and a 5,7,3',4'-substituted trihydroxy-monomethoxy-flavonol. In order to confirm the substituted position in the flavonol moiety and the connecting positions of the sugars and the aglycone were confirmed using the heteronuclear multiple bond connectivity (HMBC) technique. In the HMBC spectrum, correlations were observed from the methoxyl proton signal (δ 3.88) and a pair of meta-coupled proton signals (δ 6.32 and 6.57) to the carbon signal (δ 167.3) at the 7-position, from the anomeric proton signal (δ 5.38) of the inner glucose (glucose A) to the carbon signal (δ 135.4) at the 3-position, from

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Chart 1

the anomeric proton signal (δ 4.76) of the outer glucose (glucose B) to the carbon signal (δ 83.1) at the 2-position in glucose A, and from the proton signal (δ 3.78) at the 2-position in glucose A to the carbon signal (δ 105.1) at the 1-position in glucose B. Consequently, the structure of **9** was determined to be rhamnetin 3-*O*-sophoroside (3',4',5-trihydroxy-7-methoxyflavonol 3-*O*-(2-*O*- β -glucopyranosyl- β -glucopyranoside)).

Compound **10** showed a quasi-molecular ion peak at m/z 655 ($[M+H]^+$) in FAB-MS and ESI-MS and at m/z 656 ($[M+2H]^+$) in ESI-MS. The molecular formula was determined to be C₂₉H₃₄O₁₇ based on the HR-FAB-MS data of $[M+H]^+$. Sugar analysis using the pyridylation reaction showed the presence of glucose. The ¹H-NMR and ¹³C-NMR spectra were almost completely identical to those of **9** except for the signals due to one additional methoxyl group. These data suggested that **10** was a 5,7,3',4'-substituted dihydroxydimethoxy-flavonol glucosylglucoside. In the HMBC spectrum, the methoxyl proton signal (δ 3.89) and a pair of meta-coupling proton signals (δ 6.34 and 6.63) correlated to the carbon signal (δ 167.3) at the 7-position, while the other methoxyl proton signal (δ 3.96) and proton signals (δ 7.83 and 6.93) at the 2'- and 5'-positions correlated to the carbon signal (δ 148.6) at the 3'-position. These data suggested that the aglycone of **10** was rhamnazin (4',5-dihydroxy-3',7-dimethoxyflavonol). The connecting positions of the building blocks (rhamnazin and two glucoses) were also determined by HMBC correlation similar to those described in the case of **9**. On the basis of these data, the structure of **10** was confirmed as rhamnazin 3-*O*-sophoroside (4',5-dihydroxy-3',7-dimethoxyflavonol 3-*O*-(2-*O*- β -glucopyranosyl- β -glucopyra-

Table 1. Inhibitory Effects of Flavonols on Histamine Release from RBL-2H3 Cells

Trivial name	IC ₅₀ (μ M)
Rhamnazin (15)	40
Rhamnazin 3-glucoside (11)	70
Rhamnazin 3-sophoroside (10)	0% inh. at 160 μ M
Rhamnetin (17)	60
Rhamnetin 3-glucoside (12)	80
Rhamnetin 3-sophoroside (9)	95
Isoquercitrin (quercetin-3-glucoside, 16)	195
Quercetin 3-sophoroside 7-glucoside (6)	170
Kaempferol 3-glucoside (13)	155
Isorhamnetin 3-glucoside (14)	125

noside)).

The major constituents from watercress were the 3',4',5,7-substituted or 4',5,7-substituted flavonol 3-*O*- β -glucopyranoside, and **9** and **10** were newly identified as natural products. Also, all other identified compounds were for the first time isolated from watercress.

All of the isolated compounds (**1**–**15**) were subjected to *in vitro* assay of histamine release from RBL-2H3 cells induced by antigen-stimulation. Isoquercitrin (**16**), which was detected by HPLC and ¹H-NMR in a fraction (data not shown), and rhamnetin (**17**) were also subjected to the assay. The IC₅₀ values of the megastigmanes (**1**–**3**) were 180, 115, and 115 μ M, respectively. The IC₅₀ values of the flavonols (**6**, **9**–**17**) are summarized in Table 1. The other isolated compounds did not show any effects on histamine release at 1 mM (**4**, **5**) or 300 μ M (**7**, **8**).

Chart 2

Many researchers have reported the flavonol inhibitory effect on histamine release from basophilic and mast cells stimulated by various agents.^{3a,16} However, little data has been provided regarding the effect of the flavonol glycosides. Middleton *et al.* reported that rutin (quercetin 3-rhamnosylglucoside) lacked inhibitory activity on human basophil histamine release stimulated by various agents.^{3a,16a-c} Yamasaki *et al.*^{16f} and Sankawa *et al.*^{16h} reported no or weak inhibitory activity of kaempferol 3-glucoside (**13**) and 3-rhamnosylglucoside on histamine release from rat peritoneal mast cells stimulated by compound 48/80 and concanavalin A. In our current study, we investigated the inhibitory effects of 10 flavonols including 8 glucosides. Among them, three 7-methoxy flavonol glucosides (**9**, **11** and **12**) showed relatively strong activity, though the 7-methoxy flavonol aglycones (**15** and **17**) showed the strongest activity and the former showed the same IC_{50} value as ketotifen fumarate, an anti-allergic drug. Furthermore, kaempferol 3-glucoside (**13**), which is the same as Yamasaki *et al.* and Sankawa *et al.* tested, showed moderate activity. Yamasaki *et al.* and Sankawa *et al.* used non-immunologic stimuli for the histamine release and the mechanism of the release is thought to be somewhat different from that induced by an antigen.^{16c} This might be the reason for the difference between their results and ours. In our study, most of the isolated flavonol glucosides showed inhibitory activity (IC_{50} values $<200 \mu M$) on the histamine release stimulated by an immunological system, suggesting their possible use for the prevention of allergen-IgE-induced allergic reactions.

The isolated megastigmane glucosides (**1**–**3**) possess both an enone function in the cyclohexane ring and a carbon–carbon double bond in the side chain. It is of interest to clarify whether these functions are essentially required for the inhibition of histamine release. Therefore, we investigated the inhibitory effects of various naturally occurring megastigmane glucosides (**18**–**25**), of which the structures are shown in Chart 2. In contrast to the relatively strong inhibitory activity of **1**–**3**, all of the other glucosides, which do not have both functions had no effect on histamine release at the concentration of $300 \mu M$. Namely, the megastigmane glucosides with a completely reduced skeleton such as **18**, **19** and **21** did not inhibit histamine release and even the compounds possessing

either of these function (**23**, **24**) did not show any effects. These data strongly suggest that both the enone function and the double bond in the side chain are essential to the inhibitory effect of the megastigmanes.

Yoshikawa *et al.* isolated **3** from *Corchorus olitorius* along with several megastigmane glucosides.¹⁷ They examined their inhibitory activities on histamine release from rat peritoneal exudate cells induced by the antigen–antibody reaction. Among them, **3** showed the most potent activity and the compound without the enone function showed very weak activity. These results also support our hypothesis.

A number of biochemical events following the antigen stimulation precede histamine release from RBL-2H3 cells.¹⁸ Among the events, a rise in the intracellular free calcium concentration by calcium influx to cells is believed to be an essential step in the cascade leading to the histamine release. Therefore, it is of interest to observe the effect of the histamine release inhibitor on calcium influx from a mechanistic point of view.

We preincubated Fura-2-AM loaded RBL-2H3 cells with **3**, **9**, or **17** at the concentration which showed 60% inhibition of histamine release, and then observed the calcium influx induced by the antigen. The intracellular calcium ion concentration was monitored as the ratio of the emission fluorescence (495 nm) excited at the two wavelengths of 335 and 362 nm. The addition of ionomycin, a calcium specific ionophore, increases the intracellular calcium concentration relative to the external level. As shown in Fig. 1, the flavonols (**9** and **17**) hardly affected any increase in the intracellular calcium concentration. This fact suggests that their inhibitory activity against histamine release was not caused by the inhibition of calcium influx and that these compounds affected the downstream portion of the calcium influx stage in the cascade. As described above, many authors reported that flavonols inhibited histamine release, however, no one showed that they did not affect the calcium influx.

In the case of the megastigmane glucoside (**3**), the increment of the ratio of fluorescence to the first plateau after antigen stimulation was reduced by 60% in comparison with the control and superficially this suggested that **3** inhibited calcium influx. However, the ratio at the second plateau after the addition of ionomycin was also reduced by 60% in compari-

Fig. 1. Calcym Influx in RBL-2H3 Cells Induced by Antigen (DNP-BSA) Stimulation in the Presence of **9** (100 μ M, Top), **17** (80 μ M, Middle), and **3** (150 μ M, Bottom)

son with the control (Fig. 1). These results indicated that **3** did not affect calcium influx but only affected the ratio of fluorescence. Consequently, it was deduced that the inhibition of megastigmane on histamine release was due to an effect on the downstream portion of the calcium influx.

Kanda *et al.* reported that apple condensed tannin, namely polymeric polyphenol mixtures, inhibited histamine release from RBL-2H3 cells and they ascribed the effect to their inhibitory activity on calcium influx.^{5d)} On the other hand Matsuo *et al.* reported that a small polyphenol, epigallocatechin gallate, did not inhibit calcium influx of the cells, while it inhibited the histamine release at the same concentration.¹⁹⁾ In the present study, we also showed inhibition of histamine release by small polyphenols, namely **9** and **17**, did not account for the inhibition of calcium influx. It is of interest that the inhibition of histamine release from RBL-2H3 cells by these small polyphenols was not due to interference with the calcium influx through the cell membrane.

All the isolated compounds except for **15** showed activity

weaker than that of ketotifen fumarate. This result is not particularly surprising in view of the fact that watercress is a vegetable and is not a medicinal plant. Considering the yield, it is deduced that the methanol fraction of the HP-20 column contains more than 5% flavonols. Therefore, most of the activity found in the original fraction seemed to be explained by flavonols, though their activities are not excessively strong.

Recently, our group found carrot juice orally administrated to mice to reduce the specific IgE production in mice. Both carrot and watercress are sources of vegetable juice.^{5e)} Therefore, there is a possibility that the ingestion of vegetable juice multi-functionally modulates the allergenic symptom. Despite the high potential intake in humans, the vegetable components possessing physiological activity have not been well studied except for vitamins and minerals. Therefore, further studies should be done to clarify the latent function of vegetables and their components.

MATERIALS AND METHODS

General Melting points were determined on a Yanagimoto micro hot-stage melting point apparatus and are uncorrected. UV and circular dichroism (CD) spectra were taken on a Hitachi U-2000 A spectrophotometer and a Jasco J-720 spectropolarimeter, respectively. FAB-MS were measured on a JEOL JMS-SX102 A spectrometer. ESI-MS were recorded on an HP 5989B MS engine equipped with an HP 59987A API electrospray interface and an HP 1090 HPLC. ¹H- and ¹³C-NMR spectra were determined on a JEOL A-600 or JEOL A-500 spectrometer with tetramethylsilane as the internal standard. Preparative HPLC was performed on a Tosoh CCPS 8020 system (Tosoh CCPS $\times$ 2, Tosoh UV-8020, Shimadzu CTO-6A, Shimadzu C-R4A), a Shimadzu LC-10AT system (LC-10AT $\times$ 2, SPD-10AV, CTO-10A, C-R7A) or a Jasco 880 LC system (Jasco 880-PU, 870-UV, Tosoh CO-8000, Shimadzu C-R4A) at 40 $^{\circ}$ C. Fluorospectrophotometry was performed on a Hitachi F2500.

Plant Material The aerial part of fresh watercress (*Nasturtium officinale*) for vegetable juice was kindly supplied by the Kagome Co., Ltd.

Assay on Histamine Release The methods of *in vitro* assay on histamine are basically the same as those reported in previous papers.^{5c,d)} A HEPES buffer containing 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1.0 mM CaCl₂, 5.5 mM glucose, 0.1% bovine serum albumin (BSA) and 5 mM HEPES was used in the assay. Test solutions of water insoluble samples were prepared using a water soluble polyvinylpyrrolidone (PVP) of molecular weight 40000 as a co-solubilizing reagent. A water insoluble sample was dissolved in an appropriate organic solvent such as chloroform or acetone with 5 times the weight of PVP. The solvent was evaporated and completely removed *in vacuo*. The HEPES buffer was added to the residue and sonicated to give a clear sample solution. Water soluble samples were directly dissolved in the HEPES buffer.

Cells (3×10^5 cells/well) were precultured at 37 $^{\circ}$ C for 24 h in a 24-well flat-bottomed microtiter plate in 0.5 ml α -minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) per well of the medium. The supernatants were discarded and the cells were incubated at 37 $^{\circ}$ C for 1.5 h with

0.5 ml α -MEM containing 2% FBS and anti-trinitrophenyl (anti-TNP) IgE (1000-fold diluted ascites). The cells were washed three times with the HEPES buffer to eliminate free IgE. Then, the test solution (0.4 ml) and dinitrophenyl (DNP)-BSA (0.1 mg) dissolved in the HEPES buffer (0.1 ml) were added to the cells. After 35 min incubation at 37 °C, the plate was put on ice, and the cold HEPES buffer (0.6 ml) was added to each well to stop the reaction. The supernatant was taken out from each well and 20 μ l of perchloric acid was added. After centrifugation at 12000 rpm for 30 min, each supernatant was passed through a 0.45- μ m filter and collected. The amount of released histamine was analyzed by HPLC according to the post column conversion method using *o*-phthalaldehyde. The total histamine content in intact cells was regarded as the sum of the released and remaining histamine. The amount of remaining histamine was analyzed as follows. The residual cells were dispersed with saline (0.25 ml) containing 2% EDTA-Na and 0.25% trypsin and incubated at 37 °C for 20 min. After addition of 0.75 ml of the HEPES buffer to the well, the solution was taken out from the well and 20 μ l of perchloric acid was added. After centrifuging and filtration in a similar manner described above, the amount of histamine was analyzed by the post column HPLC. About 30% of total cell histamine was released by the antigen stimulation.

Ketotifen fumarate, which was used as a positive control, was purchased from Fujisawa Pharmaceutical Co. (Osaka). The IC₅₀ value of ketotifen fumarate was 40 μ M.

Isolation and Identification of Substances The aerial part of fresh watercress (1.2 kg) was extracted 3 times with MeOH and the crude extract was partitioned by two extractions with hexane to afford the hexane and MeOH layers. After evaporation of the solvents, the hexane extract (0.24 g) and MeOH extract (8.5 g) were obtained. The MeOH extract was applied to a Diaion HP-20 column (54×305 mm) and successively eluted with water and MeOH to afford the water and MeOH eluates. After evaporation of the solvents, the water fraction (7.4 g) and MeOH fraction (1.1 g) were obtained. The inhibitory effect of the hexane extract, the water fraction and the MeOH fraction on histamine release were 0%, 56%, and 74% at the concentration of 0.5 mg/ml, respectively. Since the methanol fraction showed appreciable inhibitory effect, this fraction was chromatographed on a Sephadex LH-20 column (54×620 mm) using MeOH as the eluant to give fractions 1 (0.39 g), 2 (0.19 g), 3 (0.20 g), 4 (0.19 g) and 5 (0.05 g). These fractions were further chromatographed by repeated preparative HPLC using an Ultrasphere ODS column (10×250 mm) or a Capcell pack C18 SG120 column (20×250 mm) with an adequate mixture of MeOH and 0.1% trifluoroacetic (TFA) (or 0.1% AcOH) as the mobile phase. Finally, the fractions 1, 2, 3, 4 and 5 gave **1** (3.0 mg), **2** (2.4 mg), **3** (2.9 mg), **4** (6.9 mg) and **5** (10.4 mg); **6** (2.3 mg), **7** (2.0 mg) and **8** (2.8 mg); **9** (19.3 mg) and **10** (9.3 mg); **9** (1.7 mg), **11** (7.7 mg) and **12** (2.7 mg); and **13** (0.2 mg), **14** (0.8 mg) and **15** (1.1 mg), respectively. Compound **16** was detected in a fraction (1.4 mg) separated from the fraction 5.

Authentic samples of kaempferol 3-*O*- β -D-glucopyranoside (**13**), isorhamnetin 3-*O*- β -D-glucopyranoside (**14**), quercetin 3-*O*- β -D-glucopyranoside (isoquercitrin, **16**) and rhamnetin (**17**) were purchased from Funakoshi Co. (Tokyo).

Authentic samples of β -phenethylamine (**4**), L-phenylalanine (**5**) and glucose were purchased from Wako Pure Chem. Ind. Ltd. (Osaka). Authentic (1-methyl-1,2,3,4-tetrahydro- β -carbonyl)carboxylic acid (**8**) was kindly supplied by Showa Denko. Authentic rhamnazin (**15**) was obtained by acid hydrolysis of **10**.

Though some compounds are known, their spectroscopical data obtained by modern technique are shown below to provide references to later researchers.

(9*R*)-4-Oxo- β -ionol β -D-Glucopyranoside (**1**): Colorless powder. liquid chromatography (LC)-ESI-MS (positive, 40% MeOH, flow injection analysis (FIA)) *m/z*: 393 [M+Na]⁺, 371 [M+H]⁺. ¹H-NMR (600 MHz, MeOH-*d*₄) δ : 1.86 (2H, t, *J*=7.2 Hz, H-2ab), 2.49 (2H, t, *J*=7.2 Hz, H-3ab), 6.31 (1H, d, *J*=16.2 Hz, H-7), 5.77 (1H, dd, *J*=6.0, 16.2 Hz, H-8), 4.50 (1H, t, *J*=6.0 Hz, H-9), 1.36 (3H, d, *J*=6.0 Hz, Me-10), 1.17 and 1.18 (each 3H, s, Me-11 and Me-12), 1.78 (3H, m, Me-13), 4.40 (1H, d, *J*=7.8 Hz, Glc-1), 3.20 (1H, t, *J*=7.8 Hz, Glc-2), 3.35 (1H, dd, *J*=7.8, 9.0 Hz, Glc-3), 3.31 (1H, t, *J*=9.0 Hz, Glc-4), 3.28 (1H, m, Glc-5), 3.67 (1H, dd, *J*=5.4, 12.0 Hz, Glc-6a), 3.82 (1H, dd, *J*=2.4, 12.0 Hz, Glc-6b). ¹³C-NMR (150 MHz, MeOH-*d*₄) δ : 36.7 (C-1), 38.3 (C-2), 35.2 (C-3), 201.9 (C-4), 163.8 (C-5), 130.8 (C-6), 127.6 (C-7), 140.4 (C-8), 77.3 (C-9), 21.0 (C-10), 27.7 (C-11 and C-12), 13.7 (C-13), 102.8 (Glc-1), 75.4 (Glc-2), 78.2 (Glc-3), 71.6 (Glc-4), 78.0 (Glc-5), 62.7 (Glc-6).

(6*R*, 9*R*)-3-Oxo- α -ionol β -D-Glucopyranoside (**2**): Colorless powder. LC-ESI-MS (positive, 40% MeOH, FIA) *m/z*: 393 [M+Na]⁺, 371 [M+H]⁺. CD (MeOH, nm, rel.int.): 243 (+55.2), 322 (−2.0). ¹H-NMR (600 MHz, MeOH-*d*₄) δ : 2.04, 2.43 (each 1H, d, *J*=16.5 Hz, H-2a and H-2b), 5.85 (1H, br s, H-4), 2.67 (1H, d, *J*=9.5 Hz, H-6), 5.64 (1H, ddd, *J*=1.0, 9.5, 16.5 Hz, H-7), 5.78 (1H, dd, *J*=6.5, 16.5 Hz, H-8), 4.40 (1H, br t, *J*=6.5 Hz, H-9), 1.29 (3H, d, *J*=6.5 Hz, Me-10), 1.00 and 1.03 (each 3H, s, Me-11 and Me-12), 1.93 (3H, d, *J*=1.0 Hz, Me-13), 4.35 (1H, d, *J*=8.0 Hz, Glc-1), 3.18 (1H, dd, *J*=8.0, 9.0 Hz, Glc-2), 3.34 (1H, t, *J*=9.0 Hz, Glc-3), 3.31 (1H, t, *J*=9.0 Hz, Glc-4), 3.22 (1H, m, Glc-5), 3.66 (1H, dd, *J*=5.0, 12.0 Hz, Glc-6a), 3.82 (1H, dd, *J*=2.0, 12.0 Hz, Glc-6b). ¹³C-NMR (150 MHz, MeOH-*d*₄) δ : 37.2 (C-1), 48.4 (C-2), 202.0 (C-3), 126.2 (C-4), 165.9 (C-5), 56.8 (C-6), 128.9 (C-7), 138.3 (C-8), 77.0 (C-9), 21.1 (C-10), 27.6, 28.1 (C-11 and C-12), 23.8 (C-13), 102.5 (Glc-1), 75.3 (Glc-2), 78.2 (Glc-3), 71.6 (Glc-4), 78.0 (Glc-5), 62.8 (Glc-6).

(6*S*,9*R*)-6-Hydroxy-3-oxo- α -ionol 9-*O*- β -D-Glucopyranoside (**3**): Colorless powder. LC-ESI-MS (positive, 40% MeOH, FIA) *m/z*: 409 [M+Na]⁺, 387 [M+H]⁺. CD (MeOH, nm, rel.int.): 240 (+53.0), 320 (−1.9). ¹H-NMR (600 MHz, MeOH-*d*₄) δ : 2.15, 2.52 (each 1H, d, *J*=17.5 Hz, H-2a and H-2b), 5.87 (1H, br s, H-4), 5.86 (2H, m, H-7 and H-8), 4.42 (1H, br t, *J*=5.8 Hz, H-9), 1.29 (3H, d, *J*=5.8 Hz, Me-10), 1.03 and 1.04 (each 3H, s, Me-11 and Me-12), 1.92 (3H, d, *J*=1.0 Hz, Me-13), 4.34 (1H, d, *J*=7.5 Hz, Glc-1), 3.17 (1H, dd, *J*=7.5, 8.8 Hz, Glc-2), 3.35 (1H, t, *J*=8.8 Hz, Glc-3), 3.25 (1H, t, *J*=8.8 Hz, Glc-4), 3.22 (1H, m, Glc-5), 3.63 (1H, dd, *J*=5.5, 11.5 Hz, Glc-6a), 3.85 (1H, dd, *J*=1.5, 11.5 Hz, Glc-6b). ¹³C-NMR (150 MHz, MeOH-*d*₄) δ : 42.5 (C-1), 50.8 (C-2), 201.3 (C-3), 127.2 (C-4), 166.3 (C-5), 80.2 (C-6), 131.6 (C-7), 135.3 (C-8), 77.3 (C-9), 21.2 (C-10), 24.7, 23.5 (C-11 and C-12), 19.6 (C-13), 102.8 (Glc-1), 75.3

(Glc-2), 78.2 (Glc-3), 71.5 (Glc-4), 78.1 (Glc-5), 62.9 (Glc-6).

Quercetin 3-*O*-sophoroside 7-*O*- β -Glucopyranoside (6): Pale yellowish oil. LC-ESI-MS (positive, 0.1% TFA in 40% MeOH, FIA) m/z : 789 $[M+H]^+$. 1H -NMR (600 MHz, MeOH- d_4) δ : 6.49, 6.76 (each 1H, d, $J=2.4$ Hz, H-6 and H-8), 7.70 (1H, d, $J=2.4$ Hz, H-2'), 6.89 (1H, d, $J=8.4$ Hz, H-5'), 7.57 (1H, dd, $J=2.4, 8.4$ Hz, H-6'), 5.40 (1H, d, $J=7.8$ Hz, Glc A-1), 3.77 (1H, m, Glc A-2), 3.59 (1H, m, Glc A-3), 3.39 (1H, m, Glc A-4), 3.20 (1H, m, Glc A-5), 3.52 (1H, dd, $J=5.4, 12.0$ Hz, Glc A-6a), 3.68 (1H, dd, $J=3.6, 12.0$ Hz, Glc A-6b), 4.76 (1H, d, $J=7.2$ Hz, Glc B-1), 3.39 (1H, m, Glc B-2), 3.40 (1H, m, Glc B-3), 3.40 (1H, m, Glc B-4), 3.32 (1H, m, Glc B-5), 3.72 (1H, m, Glc B-6a), 3.80 (1H, m, Glc B-6b), 5.07 (1H, d, $J=7.8$ Hz, Glc C-1), 3.49 (1H, m, Glc C-2), 3.51 (1H, m, Glc C-3), 3.40 (1H, m, Glc C-4), 3.50 (1H, m, Glc C-5), 3.70 (1H, m, Glc C-6a), 3.92 (1H, dd, $J=3.0, 12.6$ Hz, Glc C-6b). ^{13}C -NMR (150 MHz, MeOH- d_4) δ : 159.4 (C-2), 135.4 (C-3), 179.9 (C-4), 107.6 (C-4a), 162.8 (C-5), 100.8 (C-6), 164.7 (C-7), 95.8 (C-8), 158.1 (C-8a), 122.9 (C-1'), 117.8 (C-2'), 146.0 (C-3'), 150.0 (C-4'), 116.2 (C-5'), 123.2 (C-6'), 101.1 (Glc A-1), 83.1 (Glc A-2), 77.9 (Glc A-3), 71.1 (Glc A-4), 78.4 (Glc A-5), 62.4 (Glc A-6), 105.1 (Glc B-1), 75.6 (Glc B-2), 77.9 (Glc B-3), 71.3 (Glc B-4), 78.1 (Glc B-5), 62.5 (Glc B-6) 101.7 (Glc C-1), 74.7 (Glc C-2), 78.3 (Glc C-3), 71.1 (Glc C-4), 77.9 (Glc C-5), 62.5 (Glc C-6).

1,2,3,4-Tetrahydro- β -carboline-1-carboxylic Acid (7): Colorless amorphous. LC-ESI-MS (positive, 20% MeOH, FIA) m/z : 217 $[M+H]^+$, 144. 1H -NMR (600 MHz, D_2O): 4.41 and 4.45 (each 1H, d, $J=16.8$ Hz, H-1ab), 4.01 (1H, dd, $J=5.4, 10.8$ Hz, H-3), 3.35 (1H, dd, $J=5.4, 15.9$ Hz, H-4a), 2.99 (1H, dd, $J=10.8, 15.9$ Hz, H-4b), 7.55 (1H, d, $J=7.8$ Hz, H-5), 7.10 (1H, t, $J=7.8$ Hz, H-6), 7.19 (1H, t, $J=7.8$ Hz, H-7), 7.41 (1H, d, $J=7.8$ Hz, C-8).

1-Methyl-1,2,3,4-tetrahydro- β -carboline-1-carboxylic Acid (8): Colorless amorphous. LC-ESI-MS (positive, 20% MeOH, FIA) m/z : 231 $[M+H]^+$, 158. 1H -NMR (600 MHz, MeOH- d_4): 1.75 (3H, d, $J=6.6$ Hz, Me), 4.71 (1H, br q, $J=6.6$ Hz, H-1), 4.53 (1H, brs, NH), 3.96 (1H, dd, $J=5.4, 12.6$ Hz, H-3), 3.44 (1H, ddd, $J=1.8, 5.4, 16.2$ Hz, H-4a), 3.03 (1H, ddd, $J=2.4, 12.6, 16.2$ Hz, H-4b), 7.48 (1H, d, $J=7.8$ Hz, H-5), 7.05 (1H, t, $J=7.8$ Hz, H-6), 7.14 (1H, t, $J=7.8$ Hz, H-7), 7.34 (1H, d, $J=7.8$ Hz, C-8). ^{13}C -NMR (125 MHz, MeOH- d_4): 18.7 (Me), 50.7 (C-1), 60.2 (C-3), 23.8 (C-4), 108.4 (C-4a), 128.1 (C-4b), 118.9 (C-5), 120.1 (C-6), 122.7 (C-7), 112.1 (C-8), 138.4 (C-8a), 173.7 (COOH).

Rhamnetin 3-*O*-Sophoroside (9): Pale yellowish oil. LC-ESI-MS (positive, MeOH-0.1% HCOOH 20 : 80, FIA) m/z : 641 $[M+H]^+$, 642 $[M+2H]^+$. FAB-MS (positive, gly + *m*-nitrobenzylalcohol (*m*-NBA)) m/z : 641 $[M+H]^+$. HR-FAB-MS m/z : 641.1719 (Calcd for $C_{28}H_{33}O_{17}$: 641.1718). UV λ_{max}^{MeOH} nm: 255, 298, 355. 1H -NMR (600 MHz, MeOH- d_4) δ : 6.32, 6.57 (each 1H, d, $J=1.8$ Hz, H-6 and H-8), 7.70 (1H, d, $J=2.1$ Hz, H-2'), 6.89 (1H, d, $J=8.4$ Hz, H-5'), 7.55 (1H, dd, $J=2.1, 8.4$ Hz, H-6'), 3.88 (3H, s, OMe), 5.38 (1H, d, $J=7.3$ Hz, Glc A-1), 3.78 (1H, dd, $J=7.3, 8.9$ Hz, Glc A-2), 3.60 (1H, t, $J=8.9$ Hz, Glc A-3), 3.40 (1H, m, Glc A-4), 3.20 (1H, m, Glc A-5), 3.53 (1H, dd, $J=5.4, 12.0$ Hz, Glc A-6a), 3.69 (1H, dd, $J=2.2, 12.0$ Hz, Glc A-6b), 4.76 (1H, d, $J=7.3$ Hz,

Glc B-1), 3.38 (1H, m, Glc B-2), 3.41 (1H, m, Glc B-3), 3.40 (1H, m, Glc B-4), 3.32 (1H, m, Glc B-5), 3.71 (1H, dd, $J=4.9, 11.0$ Hz, Glc B-6a), 3.80 (1H, dd, $J=2.4, 11.0$ Hz, Glc B-6b). ^{13}C -NMR (150 MHz, MeOH- d_4) δ : 159.3 (C-2), 135.4 (C-3), 179.9 (C-4), 106.7 (C-4a), 162.9 (C-5), 99.1 (C-6), 167.3 (C-7), 93.2 (C-8), 158.4 (C-8a), 123.1 (C-1'), 117.9 (C-2'), 146.1 (C-3'), 150.0 (C-4'), 116.2 (C-5'), 123.2 (C-6'), 56.5 (OMe), 101.2 (Glc A-1), 83.1 (Glc A-2), 78.0 (Glc A-3), 71.1 (Glc A-4), 78.3 (Glc A-5), 62.5 (Glc A-6), 105.1 (Glc B-1), 75.7 (Glc B-2), 78.0 (Glc B-3), 71.2 (Glc B-4), 78.1 (Glc B-5), 62.4 (Glc B-6).

Rhamnazin 3-*O*-Sophoroside (10): Pale yellowish oil. LC-ESI-MS (positive, MeOH-0.1% HCOOH 20 : 80, FIA) m/z : 655 $[M+H]^+$, 656 $[M+2H]^+$. FAB-MS (positive, gly + *m*-NBA) m/z : 655 $[M+H]^+$. HR-FAB-MS m/z 655.1917 (Calcd for $C_{29}H_{35}O_{17}$: 655.1874). UV λ_{max}^{MeOH} nm: 254, 351. 1H -NMR (600 MHz, MeOH- d_4) δ : 6.34, 6.63 (each 1H, d, $J=2.2$ Hz, H-6 and H-8), 7.83 (1H, d, $J=2.1$ Hz, H-2'), 6.93 (1H, d, $J=8.3$ Hz, H-5'), 7.67 (1H, dd, $J=2.1, 8.3$ Hz, H-6'), 3.89, 3.96 (each 3H, s, 7-OMe and 3'-OMe), 5.60 (1H, d, $J=7.2$ Hz, Glc A-1), 3.76 (1H, dd, $J=7.2, 8.9$ Hz, Glc A-2), 3.66 (1H, t, $J=8.9$ Hz, Glc A-3), 3.35 (1H, m, Glc A-4), 3.24 (1H, m, Glc A-5), 3.51 (1H, dd, $J=5.5, 12.0$ Hz, Glc A-6a), 3.71 (1H, dd, $J=2.2, 12.0$ Hz, Glc A-6b), 4.78 (1H, d, $J=7.2$ Hz, Glc B-1), 3.33 (1H, m, Glc B-2), 3.38 (1H, m, Glc B-3), 3.34 (1H, m, Glc B-4), 3.26 (1H, m, Glc B-5), 3.63 (1H, dd, $J=5.9, 12.5$ Hz, Glc B-6a), 3.74 (1H, dd, $J=2.2, 12.5$ Hz, Glc B-6b). ^{13}C -NMR (150 MHz, MeOH- d_4) δ : 159.2 (C-2), 135.1 (C-3), 179.7 (C-4), 106.8 (C-4a), 162.9 (C-5), 99.1 (C-6), 167.5 (C-7), 93.2 (C-8), 158.4 (C-8a), 123.1 (C-1'), 114.3 (C-2'), 148.6 (C-3'), 151.0 (C-4'), 116.2 (C-5'), 124.4 (C-6'), 56.5 (C-7-OMe), 57.0 (C-3'-OMe), 100.9 (Glc A-1), 82.4 (Glc A-2), 77.9 (Glc A-3), 71.3 (Glc A-4), 78.4 (Glc A-5), 62.4 (Glc A-6), 104.4 (Glc B-1), 75.5 (Glc B-2), 77.9 (Glc B-3), 71.3 (Glc B-4), 78.1 (Glc B-5), 62.5 (Glc B-6).

Rhamnazin 3-*O*- β -Glucopyranoside (11): Pale yellowish needles (from ethanol). mp 245–246 °C. LC-ESI-MS (positive, MeOH-0.1% HCOOH 20 : 80, FIA) m/z : 493 $[M+H]^+$, 515 $[M+Na]^+$. 1H -NMR (600 MHz, MeOH- d_4) δ : 6.32, 6.59 (each 1H, d, $J=2.2$ Hz, H-6 and H-8), 7.93 (1H, d, $J=1.8$ Hz, H-2'), 6.90 (1H, d, $J=8.4$ Hz, H-5'), 7.62 (1H, dd, $J=1.8, 8.4$ Hz, H-6'), 3.88, 3.95 (each 3H, s, 7-OMe and 3'-OMe), 5.42 (1H, d, $J=7.3$ Hz, Glc-1), 3.47 (1H, dd, $J=7.3, 9.2$ Hz, Glc-2), 3.45 (1H, t, $J=9.2$ Hz, Glc-3), 3.31 (1H, dd, $J=8.8, 9.2$ Hz, Glc-4), 3.25 (1H, m, Glc-5), 3.56 (1H, dd, $J=5.5, 11.9$ Hz, Glc-6a), 3.73 (1H, dd, $J=2.2, 11.9$ Hz, Glc-6b). ^{13}C -NMR (150 MHz, MeOH- d_4) δ : 159.0 (C-2), 135.6 (C-3), 179.5 (C-4), 106.7 (C-4a), 162.8 (C-5), 99.1 (C-6), 167.4 (C-7), 93.1 (C-8), 158.4 (C-8a), 123.0 (C-1'), 114.5 (C-2'), 151.0 (C-3'), 148.4 (C-4'), 116.0 (C-5'), 124.0 (C-6'), 56.5 (7-OMe), 56.8 (3'-OMe), 103.6 (Glc-1), 76.0 (Glc-2), 78.0 (Glc-3), 71.5 (Glc-4), 78.6 (Glc-5), 62.6 (Glc-6).

Rhamnetin 3-*O*- β -Glucopyranoside (12): Pale yellowish oil. LC-ESI-MS (positive, 50% MeOH, FIA) m/z : 501 $[M+Na]^+$. 1H -NMR (600 MHz, MeOH- d_4) δ : 6.35, 6.60 (each 1H, d, $J=1.8$ Hz, H-6 and H-8), 7.73 (1H, d, $J=2.1$ Hz, H-2'), 6.88 (1H, d, $J=8.4$ Hz, H-5'), 7.61 (1H, dd, $J=2.1, 8.4$ Hz, H-6'), 3.88 (3H, s, 7-OMe), 5.28 (1H, d, $J=6.6$ Hz, Glc-1), 3.48 (1H, dd, $J=7.7, 9.1$ Hz, Glc-2), 3.42 (1H, t, $J=9.1$ Hz, Glc-3), 3.35 (1H, m, Glc-4), 3.22 (1H, m, Glc-5), 3.57 (1H, dd, $J=2.4, 12.0$ Hz, Glc-6a), 3.73 (1H, dd, $J=5.3,$

12.0 Hz, Glc-6b).

Kaempferol 3-*O*- β -Glucopyranoside (13): Pale yellowish oil. LC-ESI-MS (positive, MeOH–0.1% HCOOH 20:80, FIA) *m/z*: 449 [M+H]⁺, 471 [M+Na]⁺. ¹H-NMR (600 MHz, MeOH-*d*₄) δ : 6.21, 6.41 (each 1H, d, *J*=2.8 Hz, H-6 and H-8), 6.89 (2H, d, *J*=8.4 Hz, H-3' and H-5'), 8.07 (2H, d, *J*=8.4 Hz, H-2' and H-6'), 5.21 (1H, d, *J*=7.0 Hz, Glc-1), 3.44 (1H, dd, *J*=7.0, 8.9 Hz, Glc-2), 3.41 (1H, t, *J*=8.9 Hz, Glc-3), 3.33 (1H, m, Glc-4), 3.20 (1H, m, Glc-5), 3.57 (1H, dd, *J*=5.7, 12.2 Hz, Glc-6a), 3.68 (1H, dd, *J*=2.4, 12.2 Hz, Glc-6b).

Isorhamnetin 3-*O*- β -Glucopyranoside (14): Pale yellowish oil. LC-ESI-MS (positive, MeOH–0.1% HCOOH 20:80, FIA) *m/z*: 479 [M+H]⁺. ¹H-NMR (600 MHz, MeOH-*d*₄) δ : 6.21, 6.41 (each 1H, d, *J*=2.0 Hz, H-6 and H-8), 7.95 (1H, d, *J*=2.4 Hz, H-2'), 6.92 (1H, d, *J*=8.4 Hz, H-5'), 7.60 (1H, dd, *J*=2.4, 8.4 Hz, H-6'), 3.95 (3H, s, OMe), 5.40 (1H, d, *J*=8.0 Hz, Glc-1), 3.47 (1H, dd, *J*=7.0, 8.0 Hz, Glc-2), 3.44 (1H, t, *J*=7.0 Hz, Glc-3), 3.52 (1H, m, Glc-4), 3.24 (1H, m, Glc-5), 3.56 (1H, dd, *J*=5.6, 12.0 Hz, Glc-6a), 3.73 (1H, dd, *J*=2.2, 12.0 Hz, Glc-6b).

Sugar Analysis Using Pyridylamination Reaction The analysis was performed according to the method described by Hase *et al.*¹⁵⁾ with some modifications. Purification of the pyridylaminated sugars was carried out by the method described by Kondo.²⁰⁾ The HPLC conditions to analyze the pyridylaminated sugars were as follows. Column: Asahipack NH₂P-50 (4.6×250 mm); mobile phase: CH₃CN–50 mM Tris–HCl buffer (pH 8.5) containing 25 mM boric acid (10/2); temperature: 45 °C; flow rate: 1.0 ml/min; detection: fluorescence (Ex. 320 nm. Em, 400 nm).

Megastigmanes Alangionoside A (18),²¹⁾ E (22),²²⁾ and L (24)²³⁾ were isolated from *Alangium premnifolium*. Compound 19 was isolated from *A. platanifolium* var. *platanifolium*.²⁴⁾ Ampelosiosionoside (20) was isolated from *Myrsine seguini* and identified by comparing its spectroscopic data with those in the literature.²⁵⁾ Dendranthemoside A (21) was isolated from *Dendranthema shiwogiku*.²⁶⁾ Blumenol C glucoside (23)²⁷⁾ and grasshopper ketone glucoside (25) were isolated from *Glochidion zeylanicum*. The details of the structural identification of 23 and 25 will be reported elsewhere.

Monitoring Calcium Influx with a Fluorospectrophotometer Compounds 3 and 9 were selected as representatives of megastigmanes and flavonol glucosides in watercress, respectively, considering their isolated amounts and inhibitory activities. Compound 17, the aglycone of 9, was also chosen to investigate the effect of a non-glycosidic flavonol. Calcium influx was monitored by the method described by T. Kanda *et al.*^{5d)} with some modifications. RBL-2H3 cells were suspended in a PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1.0 mM CaCl₂, 5.5 mM glucose, 0.1% BSA and 10 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES) at pH 7.4) containing 6 μ M Fura-2-AM and anti-TNP IgE for 15 min at 37 °C. The cells were washed three times with the buffer, resuspended in fresh buffer in a quartz cuvette and continuously stirred at 37 °C, with a magnetic stirrer. A test sample was added to the cuvette with a microsyringe. After incubating for 5 min at 37 °C, the cells were challenged with DNP-BSA. The intracellular free calcium ion concentration was monitored as the ratio of fluorescence excited at two

wavelengths, 335 and 362 nm. The emission wavelength was 495 nm. Ionomycin calcium (0.5 μ M) was added to the cuvette 210 s after the antigen challenge.

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