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Anti-allergic activity of stilbenes from Korean rhubarb (*Rheum undulatum* L.): structure requirements for inhibition of antigen-induced degranulation and their effects on the release of TNF-α and IL-4 in RBL-2H3 cells

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Abstract—Stilbenes isolated from the rhizomes of *Rheum undulatum* (Korean rhubarb) and the related compounds were investigated on their anti-allergic activities. The results revealed that 3,5,4′-trimethylpiceatannol exhibited the most potent inhibition against β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with IC_{50} of 2.1 μM, followed by trimethylresveratrol (IC_{50} = 5.1 μM). Structural requirements of stilbenes for the activity are as follows: (1) The oxygen functions (–OCH_3, –OH), especially methoxyl groups, are essential and their positions on aromatic rings are important for the activity; (2) the α–β double bond increased the activity; (3) the glycoside moiety dramatically decreased the activity; and (4) the substitution group at the 3′-position in trimethylresveratrol (3,5,4′-trimethoxystilbene) was preferably OH > H > OCH_3 for the activity. Several active stilbenes (piceatannol, 3,5,4′-trimethylpiceatannol, resveratrol, trimethylresveratrol) also inhibited ionomycin-induced β-hexosaminidase release, suggesting that inhibition of Ca^{2+} influx or degranulation mechanisms after Ca^{2+} influx is important for their activities. Piceatannol, 3,5,4′-trimethylpiceatannol, resveratrol, and trimethylresveratrol also significantly inhibited antigen-induced release of TNF-α and IL-4 in RBL-2H3 cells.

1. Introduction

Korean rhubarb, *Rheum undulatum* L., is a plant belonging to the Polygonaceae family and its rhizomes have been used as a remedy for blood stagnation syndrome as well as a purgative agent in Japanese, Korean and Chinese traditional medicines. This rhubarb has been considered to have a less purgative effect but to be more effective on blood stagnation syndrome than other rhubarbs,[1] and contains stilbenes (e.g., rhapontiticin, piceatannol 3′-*O*-β-D-glucopyranose, rhapontigenin, piceatannnol, etc.) as the principal constituents.[2,3] Stilbenes have been reported to show nitric oxide production inhibitory, anti-bacterial, anti-fungal, anti-oxidant, anti-inflammatory, anti-cancer, and anti-malarial activities.[2–8]

An allergic reaction known as a hypersensitivity type I occurs when a person's immune system mounts a defense against normally harmless substances. Substances that cause allergic reactions are called allergens, such as dust mites, pollen, cosmetics, food, and mold spores. Histamine, which is released from mast cells and basophils stimulated by an allergen, is usually determined as a degranulation marker in immediate allergic reactions in in vitro experiments. When granules in mast cells or basophils degranulate, an enzyme β-hexosaminidase is also released along with histamine. Thus, this enzyme activity is used as a marker of mast cell degranulation.[9] We previously reported anti-oxidant activity of stilbenes isolated from *R. undulatum* and their inhibitory effects on nitric oxide production in lipopolysaccharide (LPS)-activated macrophages.[2,3] In addition, effects of principal stilbene constituents on antigen-induced histamine release from rat peritoneal mast cells and on 48-h homologous passive cutaneous anaphylaxis in rats (type I allergic model) and on sheep blood cell-induced delayed-type hypersensitivity in mice (type IV

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allergic model) were also reported.[10] However, structure–activity relationships of stilbenes for anti-allergic activity have not been studied sufficiently.

In the present study, the methanolic extract also inhibited antigen-induced degranulation in rat basophilic leukemia (RBL-2H3) cells (IC_{50} = 42 μg/mL). We therefore examined effects of 9 stilbenes isolated from the rhizomes of *R. undulatum* and their related compounds on the antigen-induced degranulation in RBL-2H3 cells. Furthermore, we investigated the effects of the active compounds **7**, **7b**, **8**, and **8b** on ionomycin-induced degranulation, and on antigen-induced TNF-α and IL-4 release that participate in the late phase of type I allergic reactions.[11]

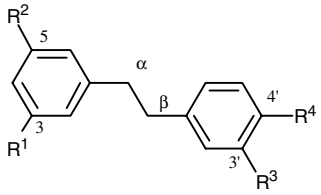
2. Results and discussion

2.1. Structural requirements of stilbenes on the antigen-induced release of β-hexosaminidase from RBL-2H3 cells

To clarify the structure–activity relationships of stilbenes for the anti-allergic activity, we investigated the inhibitory effects of 15 stilbenes and 4 dihydrostilbenes on the release of β-hexosaminidase of RBL-2H3 cells. As shown in Table 1, 3,5,4′-trimethylpiceatannol (**7b**, IC_{50} = 2.1 μM) exhibited the most potent activity, followed by trimethylresveratrol (**8b**, 5.1 μM), rhapontigenin (**5**, 11 μM), isorhapontigenin (**6**, 12 μM), tetramethylpiceatannol (**7d**, 13 μM), 3,3′,4′-trimethylpiceatannol (**7c**, 16 μM), resveratrol (**8**, 17 μM), desoxyrhapontigenin (**9**, 18 μM), and piceatannol (**7**, 24 μM) and their activities were stronger than those of two anti-allergic compounds, tranilast (0.28 mM) and ketotifen fumarate (0.22 mM).[12] *trans*-Stilbene (**10**, >100 μM), which lacks oxygen functions on the ring, was inactive. Thus, the effects of substitutes on aromatic rings of stilbenes indicated that stilbenes substituted with methoxyl groups at the 3-, 5-, and 4′-positions exhibited higher activity than those with hydroxyl groups. Moreover, the activity was dramatically decreased when there is no substitute on aromatic rings. Substitution of glycoside moiety on the ring dramatically decreased the activity, which could be observed from the inhibitory activities of rhaponticin (**1**, >100 μM), isorhapontin (**3**, >100 μM), and piceatannol 3′-*O*-β-D-glucopyranoside (**4**, >100 μM) compared with those of rhapontigenin (**5**, 11 μM), isorhapontigenin (**6**, 12 μM), and piceatannol (**7**, 24 μM), respectively. Rhaponticin methylether (**1b**, >100 μM) and rhaponticin 2″-*O*-gallate (**2**, >100 μM), which substituted for a glycoside moiety at the 3-position, also showed weak activities.

Effects of substituted positions on aromatic rings can be observed from the inhibitory activity of compounds **7b** and **7c**. These two compounds contain three methoxyl groups and one hydroxyl group in different positions. However, they both showed quite a difference in IC_{50}

Table 1. Inhibitory effects of stilbenes (**1**–**10**, **1b**, **7b**–**7d**, **8b**) and dihydrostilbenes (**1a**, **4a**, **7a**, **8a**) on antigen-induced release of β-hexosaminidase from RBL-2H3 cells



Compound	α–β	R^1	R^2	R^3	R^4	IC_{50} (μM)	Enzyme inhibition (%)[a]
Rhaponticin (**1**)	C=C	*O*-Glc	OH	OH	OCH_3	>100 (9.4)	—
1a	C–C	*O*-Glc	OH	OH	OCH_3	>100 (6.0)	—
1b	C=C	*O*-Glc(CH_3)$_4$	OCH_3	OCH_3	OCH_3	>100 (45.6)	—
Rhaponticin 2″-*O*-gallate (**2**)	C=C	*O*-Glc(2-Gallate)	OH	OH	OCH_3	>100 (10.3)	—
Isorhapontin (**3**)	C=C	*O*-Glc	OH	OCH_3	OH	>100 (23.5)	—
Piceatannol 3′-*O*-Glc (**4**)	C=C	OH	OH	*O*-Glc	OH	>100 (−7.1)	—
4a	C–C	OH	OH	*O*-Glc	OH	>100 (10.7)	—
Rhapontigenin (**5**)	C=C	OH	OH	OH	OCH_3	**11**	11.5
Isorhapontigenin (**6**)	C=C	OH	OH	OCH_3	OH	**12**	33.9
Piceatannol (**7**)	C=C	OH	OH	OH	OH	**24**	7.2
7a	C–C	OH	OH	OH	OH	81	—
7b	C=C	OCH_3	OCH_3	OH	OCH_3	**2.1**	−0.9
7c	C=C	OCH_3	OH	OCH_3	OCH_3	**16**	9.8
7d	C=C	OCH_3	OCH_3	OCH_3	OCH_3	**13**	2.9
Resveratrol (**8**)	C=C	OH	OH	H	OH	**17**	7.7
8a	C–C	OH	OH	H	OH	>100 (15.9)	—
8b	C=C	OCH_3	OCH_3	H	OCH_3	**5.1**	4.0
Desoxyrhapontigenin (**9**)	C=C	OH	OH	H	OCH_3	**18**	33.1
trans-Stilbene (**10**)	C=C	H	H	H	H	>100 (−13.4)	—

Glc; β-D-glucopyranosyl. Values in parentheses represent the inhibition (%) at 100 μM.

[a] Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

values. Compound **7b** (2.1 μM), with methoxyl groups at the 3-, 5-, and 4′-positions, exhibited activity ca. eight times higher than that of compound **7c** (16 μM) with 3, 3′, and 4′-methoxyl groups. These results suggested that the positions of the methoxyl groups of stilbenes were important for their anti-allergic activity. In addition, three compounds **7b**, **7d**, and **8b**, which have three methoxyl groups at the 3-, 5-, and 4′-positions but have a different group substituted at the 3′-position (OH, OCH_3, and H, respectively), also showed different IC_{50} values. Compound **7b** (2.1 μM) exhibited activity ca. two times higher than that of compound **8b** (5.1 μM) and ca. six times higher than that of compound **7d** (13 μM). Therefore, it is indicated that the 3′-hydroxyl substitute enhanced the activity of trimethylresveratrol, but the 3′-methoxyl substitute reduced the activity.

Inamori et al. reported that the inhibitory effect of piceatannol (**7**) was stronger than that of the α,β-dihydroderivative (**7a**) on antigen-induced histamine release in rat peritoneal mast cells.[13] In agreement with the previous report, our results showed that the activity of **7** (24 μM) was higher than that of **7a** (81 μM). This effect was also observed from the activity of resveratrol (**8**, 17 μM) compared with that of dihydroresveratrol (**8a**, >100 μM). Thus, the compounds containing the α–β double bond exhibited higher activity than those of non-containing ones.

The effects of test compounds on β-hexosaminidase were also examined to clarify whether their effects were due to the inhibition of enzyme activity or of degranulation. As a result, the active compounds showed weak or less inhibition against the enzyme activity of β-hexosaminidase.

The inhibitory effects of hydroxystilbenes on the release of β-hexosaminidase were previously reported by Cheong et al.[14] They concluded that (1) the anti-allergic effect decreased in proportion to the number of methoxyl groups, and hydroxyl substituents on the benzene rings might be important for the anti-allergic activity; and (2) glycoside substitution of stilbenes decreased the activity. The results of the present study supported the previous reports and indicated some additional or revised structural requirements of stilbenes for the activity: (1) The oxygen functions (–OCH_3, –OH) are essential and their positions on aromatic rings are important for the activity, especially that of methoxyl groups, which were different from the previous report;[14] (2) the glycoside moiety dramatically decreased the activity; (3) the α–β double bond increased the activity; and (4) the substitution group at the 3′-position in trimethylresveratrol was preferably OH > H > OCH_3 for the activity.

Our previous study on the effects of stilbenes against nitric oxide production in LPS-activated macrophages indicated similar structural requirements to those of the anti-allergic activity, except that the α–β double bond of stilbenes did not affect nitric oxide production,[3] whereas the α–β double bond is essential for the anti-allergic activity.

In the present study, the most active stilbene **7b** (2.1 μM) is discovered for the first time on anti-allergic activity against the antigen-induced degranulation, and several active stilbenes (**7**, **8**, and **8b**) as well as **7b** were also investigated on ionomycin-induced release of β-hexasaminidase, and on antigen-induced release of TNF-α and IL-4 in RBL-2H3 cells.

2.2. Inhibitory effects of stilbenes on ionomycin-induced release of β-hexosaminidase in RBL-2H3 cells

As shown in Table 2, compounds **7**, **7b**, **8**, and **8b** inhibited the release of β-hexosaminidase by ionomycin, and their IC_{50} values (IC_{50} = 31, 2.9, 23, and 8.5 μM, respectively) were similar to those by antigen (24, 2.1, 17, and 5.1 μM, respectively) in RBL-2H3 cells.

In RBL-2H3 cells, tyrosin kinase Syk recruited by aggregated FcεRI phosphorylates phospholipase Cγ, which leads to the generation of inositol 1,4,5-triphosphate (IP_3). IP_3 causes the release of Ca^{2+} from intracellular Ca^{2+} stores and activates Ca^{2+} influx via Ca^{2+} release-activated Ca^{2+} (CRAC) channels to replenish the depleted Ca^{2+} stores. The Ca^{2+} influx is essential for the degranulation and cytokine production in RBL-2H3 cells.[15] Ionomycin has been widely used for inducing an increase in intracellular Ca^{2+} concentrations as a Ca^{2+} ionophore. Recently, Nakata et al. reported that ionomycin can activate Ca^{2+} influx via CRAC channels by depleting Ca^{2+} stores rather than acting as an ionophore in RBL-2H3 cells, namely, ionomycin may cause Ca^{2+} influx in a similar way, as does antigen.[16]

In the present study, compounds **7**, **7b**, **8**, and **8b** inhibited the degranulation of RBL-2H3 cells by both the antigen and ionomycin at similar IC_{50} values. These results suggested that the active compounds might inhibit Ca^{2+} influx via CRAC channels or mechanisms after Ca^{2+} influx. Piceatannol (**7**) was reported to be a selective inhibitor of Syk in RBL-2H3 cells, and the inhibition of Syk has been considered as an inhibitory

Table 2. Inhibitory effects of stilbenes (**7**, **7b**, **8**, **8b**) on ionomycin-induced release of β-hexosaminidase from RBL-2H3 cells

Compounds	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Piceatannol (**7**)	0.0 ± 1.0	—	3.4 ± 2.2	10.4 ± 1.7**	46.1 ± 2.6**	94.6 ± 0.5**	31
7b	0.0 ± 2.6	25.0 ± 2.4**	55.6 ± 2.6**	79.7 ± 1.2**	95.7 ± 0.5**	100.2 ± 0.5**	2.9
Resveratrol (**8**)	0.0 ± 1.5	—	2.0 ± 0.6	19.8 ± 2.9**	57.8 ± 2.7**	92.8 ± 0.7**	23
8b	0.0 ± 1.1	−1.9 ± 1.7	13.2 ± 1.9**	55.1 ± 3.1**	89.8 ± 0.6**	100.1 ± 1.5**	8.5

Each value represents the mean ± SEM (N = 4). Significantly different from the control, $^{**}p < 0.01$.

mechanism of degranulation by **7**.[17] However, our results suggested that the inhibition of Syk was partly involved in the inhibitory activity of **7**, but the inhibition of Ca^{2+} influx or degranulation mechanisms after Ca^{2+} influx was important.

2.3. Inhibitory effects of stilbenes on antigen-induced release of TNF-α and IL-4 in RBL-2H3 cells

Inhibitory effects of several stilbenes (**7**, **7b**, **8**, and **8b**) with the degranulation inhibitory activity on the release of TNF-α and IL-4 in RBL-2H3 cells were also investigated. The results showed that 3,5,4′-trimethylpiceatannol (**7b**) again exhibited the potent activity against TNF-α and IL-4 release with IC_{50} of 10 and 39 μM, respectively, whereas resveratrol (**8**) exhibited the most potent activity against IL-4 with IC_{50} of 32 μM (Table 3). Trimethylresveratrol (**8b**), which is active against the release of β-hexosaminidase (IC_{50} = 5.1 μM), exhibited only moderate and mild activities against the release of TNF-α and IL-4 (64 μM and >100 μM for TNF-α and IL-4, respectively). This might revealed that the mechanism of action of compound **8b** on the degranulation is somewhat different from that on the release of TNF-α and IL-4. Piceatannol (**7**) exhibited moderate activity against both TNF-α and IL-4, with IC_{50} values of 30 and 72 μM, respectively.

3. Experimental

3.1. Isolation of stilbenes from the rhizomes of *Rheum undulatum* and synthesis of related stilbenes

Stilbene constituents (**1**–**9**) from the rhizomes of *R. undulatum* and related compounds (**1a**, **1b**, **2a**, **7a**–**7d**, **8a**, **8b**, **10**) were prepared with some chemical modifications, as described previously.[2,3]

3.2. Bioassay methods

3.2.1. Reagents. Minimum Essential Medium Eagle (MEM) and anti-DNP IgE (Monoclonal Anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; the ELISA kit for determination of TNF-α (TNF-α, rat, code 3012) was from Biosource International Co., Ltd, ELISA kit for determination of IL-4 (IL-4, rat, code 2737) was from Amersham Pharmacia Biotech Co., Ltd; dinitrophenylated bovine albumin (DNP-BSA) was prepared as described previously.[18] Other chemicals were from Wako; 24-well and 96-well microplates were from Sumitomo Bakelite Co., Ltd.

3.2.2. Inhibitory effects on the antigen-induced release of β-hexosiminidase in RBL-2H3 cells. Inhibitory effects on the release of β-hexosiminidase in RBL-2H3 cells [Cell No JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] were evaluated by the following method;[12] RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using MEM containing 10% FCS, penicillin (100 units/mL), streptomycin (100 μg/mL), and anti-DNP IgE (0.45 μg/mL), then incubated overnight at 37 °C in 5% CO_2 for sensitization of the cells. 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] supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (incubation buffer) and then incubated in 160 μL of the incubation buffer for an additional 10 min at 37 °C. After that, 20 μL of test sample solution was added to each well and incubated for 10 min, followed by an addition of 20 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) at 37 °C for 10 min to stimulate the cells to degranulate. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μL) was transferred into 96-well plate 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 with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to the incubation buffer (final DMSO concentration was 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

Table 3. Inhibitory effects of stilbenes (**7**, **7b**, **8**, **8b**) on the release of TNF-α and IL-4 from RBL-2H3 cells

Compounds	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
	Inhibition (%) on TNF-α					
Piceatannol (**7**)	0.0 ± 7.3	—	28.8 ± 3.5*	40.7 ± 1.8**	83.1 ± 1.1**	30
7b	0.0 ± 2.6	28.1 ± 2.4**	52.4 ± 4.1**	64.2 ± 2.7**	94.6 ± 3.0**	10
Resveratrol (**8**)	0.0 ± 9.3	—	12.6 ± 4.8	23.8 ± 2.9	64.8 ± 5.0**	74
8b	0.0 ± 4.3	—	5.9 ± 3.6	32.5 ± 4.9**	60.1 ± 3.9**	64
	Inhibition (%) on IL-4					
Piceatannol (**7**)	0.0 ± 1.6	—	−13.5 ± 2.4	8.3 ± 1.2	76.5 ± 1.1**	72
7b	0.0 ± 2.5	—	−13.9 ± 2.0	31.9 ± 0.5**	99.8 ± 0.1**	39
Resveratrol (**8**)	0.0 ± 2.0	—	19.1 ± 1.4**	38.7 ± 1.5**	90.5 ± 1.3**	32
8b	0.0 ± 2.5	—	—	−12.8 ± 0.3	44.2 ± 1.0**	>100

Each value represents the mean ± SEM (N = 4). Significantly different from the control, $^{*}p < 0.05$, $^{**}p < 0.01$.

$$\text{Inhibition } (\%) = [1 - (T - B - N)/(C - N)] \times 100$$

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 40–70% 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.

3.2.3. Inhibitory effects on the ionomycin-induced release of β-hexosiminidase in RBL-2H3 cells. The inhibitory effects on the ionomycin-induced release of β-hexsosaminidase in RBL-2H3 cells were evaluated similarly to that of antigen-induced degranulation. However, in the case of ionomycin, anti-DNP IgE and DNP-BSA were not added to the wells. Briefly, the cells were incubated in 160 μL of the incubation buffer for 10 min at 37 °C. After that, 20 μL of test sample solution was added to each well and incubated for 10 min, followed by an addition of 20 μL of ionomycin (final concentration was 1 μM) at 37 °C for 10 min to stimulate the cells to degranulate. The reaction was stopped by cooling in an ice bath, and β-hexsosaminidase activity in the medium was determined as described above. Under these conditions, it was calculated that 50–70% of β-hexosaminidase was released from the cells.

3.2.4. β-Hexosiminidase inhibitory activity. In order to clarify that the anti-allergic effects of samples were due to the inhibition on hexosaminidase release, but not the false positive by the inhibition of β-hexosaminidase activity, the following assay was carried out.

The cell suspension (5×10^7 cells) in 6 mL of PBS was sonicated. The solution was then centrifuged, and the supernatant was diluted with the incubation buffer and adjusted to equal the enzyme activity of the degranulation tested above. The enzyme solution (45 μL) and test sample solution (5 μL) were transferred into a 96-well microplate and incubated with 50 μL of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μL of the stop solution. The absorbance was measured using a microplate reader at 405 nm.

3.2.5. Inhibitory effect on antigen-induced TNF-α and IL-4 release from RBL-2H3 cells. Inhibitory effects on the release of TNF-α and IL-4 from RBL-2H3 cells were evaluated by the following method.[12] RBL-2H3 cells (2×10^5 cells/well) were sensitized with anti-DNP IgE as described above. The cells were washed twice with 500 μL of MEM containing 10% FCS, penicillin (100 units/mL), and streptomycin (100 μg/mL), and exchanged with 320 μL of fresh medium. Then, 40 μL of test sample solution and 40 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) were added to each well and incubated at 37 °C for 4 h. The supernatant (50 μL) was transferred into 96-well ELISA plates and then TNF-α and IL-4 concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO concentration was 0.1%).

The inhibition of production of TNF-α and IL-4 from cells was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } (\%) = [1 - (T - N)/(C - N)] \times 100$$

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

3.3. Statistics

Values are expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis.

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