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Antiallergic Principles from *Alpinia galanga*: Structural Requirements of Phenylpropanoids for Inhibition of Degranulation and Release of TNF-α and IL-4 in RBL-2H3 Cells

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Abstract—The 80% aqueous acetone extract of the rhizomes of *Alpinia galanga* was found to inhibit release of β-hexosaminidase, as a marker of antigen-IgE-mediated degranulation in RBL-2H3 cells. Nine known phenylpropanoids and *p*-hydroxybenzaldehyde were isolated from the extract. Among them, 1′*S*-1′-acetoxychavicol acetate and 1′*S*-1′-acetoxyeugenol acetate exhibited potent inhibitory activity with IC_{50} values of 15 and 19 μM. From the effects of various related compounds, both the 1′- and 4-acetoxyl groups of 1′*S*-1′-acetoxychavicol acetate and 1′*S*-1′-acetoxyeugenol acetate were essential for their strong activity, and the 2′–3′ double bond enhanced the activity. In addition, 1′*S*-1′-acetoxychavicol acetate and 1′*S*-1′-acetoxyeugenol acetate inhibited ear passive cutaneous anaphylaxis reactions in mice and the antigen-IgE-mediated TNF-α and IL-4 production, both of which participate in the late phase of type I allergic reactions, in RBL-2H3 cells.

Most known medicines for the treatment of type I allergy strongly antagonize chemical mediators (e.g., histamine, serotonin, and leukotrienes) which are released from basophils and mast cells in type I allergy, but they exhibit only weak inhibition for the degranulation of basophils and mast cells. Therefore, strong degranulation inhibitors have been expected as new lead compounds for antiallergic medicines. In the course of our studies on bioactive constituents from natural resources, we reported various antiallergic constituents such as isocoumarins, benzylidenephtalide, diterpenes, ionone glucosides, triterpenes, and triterpenoid saponins from *Hydrangea macrophylla* var. *thunbergii*, *Sagittaria trifolia*, *Hovenia dulcis*, *Corchorus olitorius*, *Benincasa hispida*, and *Phaseolus vulgaris* by determination of histamine release induced by an antigen or degranulation inducers from rat peritoneal mast cells or by examination of passive cutaneous anaphylaxis (PCA) in rats.[1–6]

Recently, β-hexosaminidase activity released in the medium has been used as a marker of mast cell degranulation, since the enzyme is also stored in secretory granules of mast cells, and is released concomitantly with histamine when mast cells are immunologically activated.[7,8] Using the inhibitory activity, we previously reported the isolation and structural elucidation of many constituents from natural medicines, such as the bark of *Myrica rubra*, the fruit of *Alpinia oxyphylla*, and the rhizomes of *Hedychium coronarium*, and so on.[9–13] In our continuing studies on antiallergic constituents from natural resources, the aqueous acetone extract of the rhizomes of *Alpinia galanga* Swartz (Zingiberaceae) was found to show inhibitory activity (IC_{50} = 19 μg/mL) stronger than those of synthetic antiallergic compounds, tranilast[14] [161 μg/mL (=492 μM)] and ketotifen fumalate[15] (91 μg/mL (=216 μM)].

A. galanga is widely cultivated in India, China and southeast Asian countries, such as Thailand, Indonesia, and Philippines. The rhizomes of this plant are extensively used as spice or ginger substitutes for flavoring foods, and also in traditional medicine for several purposes, such as stomachic in China, or for carminative, antiflatulent, antifungal, and anti-itching in Thailand. In chemical and pharmacoloical studies of *A. galanga*, the pungent principal compound, 1′*S*-1′-acetoxychavicol acetate (**1**), was reported to possess antitumor,

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anti-inflammatory, pungency, antifungal, gastroprotective, and xanthine oxidase inhibitory activities.[16–21] However, antiallergic constituents of this plant have not been reported.

In the present study, we examined the effects of constituents from *A. galanga* and related compounds on the release of β-hexosaminidase in rat basophilic leukemia (RBL-2H3) cells, and the structural requirements of active compounds. In addition, the principal active constituents on ear PCA reaction in mice and on antigen-induced production of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4), which participate in the late phase of type I allergic reaction,[22,23] from RBL-2H3 cells are described.

Results and Discussion

Isolation of active constituents from the rhizomes of *A. galanga*

The dried rhizomes of *A. galanga* (2.1 kg) were extracted with 80% aqueous acetone three times under room temperature. The aqueous acetone extract (6.6% from this natural medicine) was subjected to ordinary-phase silica-gel (SiO_2) [*n*-hexane–ethyl acetate (EtOAc) (10:1→5:1)→EtOAc] and reversed-phase silica-gel (ODS) column chromatographies [methanol (MeOH)–H_2O] and finally HPLC [YMC-Pack ODS-5-A, 250×20 mm i.d., MeOH–H_2O or acetonitrile (CH_3CN)–H_2O] to give 1′*S*-1′-acetoxychavicol acetate (**1**,[21] 1.10%), 1′*S*-1′-acetoxyeugenol acetate (**2**,[21] 0.0129%), 1′*S*-1′-hydroxychavicol acetate (**4**,[24] 0.0479%), methyleugenol (**10**, 0.0006%), chavicol β-D-glucopyranoside (**13**,[25] 0.023%), *trans*-*p*-hydroxycinnamaldehyde (**14**,[26] 0.0275%), *trans*-*p*-hydroxycinnamyl acetate (**15**,[27] 0.0211%), *trans*-*p*-coumaryl alcohol (**16**,[28] 0.0519%), *trans*-*p*-coumaryl diacetate (**17**,[21] 0.0030%), and *p*-hydroxybenzaldehyde (0.0047%). Known compounds were identified by comparison of their physical data with those of commercial samples (**10** and *p*-hydroxybenzaldehyde) or with reported values.[21,24–28]

Related compounds

Demethyleugenol (**11**) was isolated from the leaves of *Piper betle*.[29] Chavicol (**12**) was obtained by hydrolysis of chavicol β-D-glucopyranoside (**13**) and acetylation of **12** afforded chavicol acetate (**5**).[29] Compound **7** was obtained by the Grignard reaction of vinylmagnesium bromide with benzaldehyde.[29] Compounds **6** and **8** were obtained by acetylation of **9** and **7**, respectively.[29] Dihydro-(1′*S*)-1′-acetoxychavicol acetate (**3**) was obtained by hydrogenation of **1**.[29] Eugenol (**9**), *trans*-cinnamic acid (**18**), *trans*-*o*-coumaric acid (**19**), *trans*-*p*-coumaric acid (**21**), and caffeic acid (**22**) were purchased from Nacalai Tesque. 3,4-Dimethoxycinnamic acid (**24**) and 3,5-dimethoxy-4-hydroxycinnamic acid (**25**) were from Sigma. *trans*-*m*-Coumaric acid (**20**) was from Tokyo Kasei Kogyo. Methyleugenol (**10**), *p*-methoxycinnamic acid (**23**), and *p*-hydroxybenzaldehyde were from Wako Pure Chemical.

Effects of phenylpropanoids from *A. galanga* and related compounds on β-hexosaminidase in RBL-2H3 cells

Among the phenylpropanoids from *A. galanga*, 1′*S*-1′-acetoxychavicol acetate (**1**) and 1′*S*-1′-acetoxyeugenol acetate (**2**) showed inhibitory activity with IC_{50} of 15 and 19 μM without affecting the enzyme activity (inhibition: 2.1 and −3.0% at 100 μM), and their inhibitory activities were stronger than those of tranilast and ketotifen fumarate. Furthermore, *trans*-*p*-hydroxycinnamaldehyde (**14**) and *trans*-*p*-coumaryl diacetate (**17**) weakly inhibited the degranulation by ca. 20% at 100 μM (Table 1).

To clarify the structure–activity relations of the active phenylpropanoids **1** and **2** for the activity, we examined the inhibitory effects of natural and synthetic phenylpropanoids (**3**–**13**, **15**, **16**, **18**–**25**) on the release of β-hexosaminidase in RBL-2H3 cells. 1′*S*-1′-Hydroxychavicol acetate (**4**) lacking the 1′-acetyl group and chavicol acetate (**5**) and **6** lacking the 1′-acetoxyl group did not show any effects. In addition, compound **8** lacking the 4-acetoxyl group and **7** lacking both the 1′-acetyl and 4-acetoxyl groups did not inhibit the granulation. On the other hand, the dihydro-(1′*S*)-1′-acetoxychavicol acetate (**3**) showed less activity than **1**. Coumaric acid-type phenylpropanoids (**14**–**25**) also did not inhibit the granulation, except for **14** and **17**. These results suggested that both of the 1′- and 4-acetoxyl groups in **1** and **2** were essential for the activity and the 2′-3′ double bond enhanced the activity. Recently, Kim et al. reported that eugenol (**9**) inhibited histamine release induced by compound 48/80 or antigen in rat peritoneal mast cells.[30] However, under our conditions, **9** in addition to other related compounds, except for **11**, did not inhibit the release of β-hexosaminidase at 100 μM.

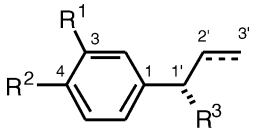
Effects of 1′*S*-1′-acetoxychavicol acetate (1) and 1′*S*-1′-acetoxyeugenol acetate (2) on ear PCA reaction in mice

Next, to clarify the efficacy of 1′*S*-1′-acetoxychavicol acetate (**1**) and 1′*S*-1′-acetoxyeugenol acetate (**2**) in vivo, effects of **1** and **2** on PCA reaction using both ears of mice, which is an experimental model of type I allergy, were examined. As shown in Table 2, compounds **1** and **2** dose dependently inhibited the leakage of dye 30 min after challenge at doses of 6.25–50 mg/kg, and their potencies were stronger than that of a reference compound, tranilast. These results suggest that **1** and **2** are effective for the immediate phase reactions in type I allergy.

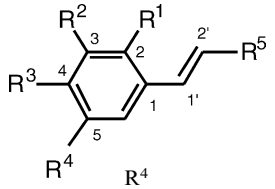
Effects of 1′*S*-1′-acetoxychavicol acetate (1) and 1′*S*-1′-acetoxyeugenol acetate (2) on antigen-induced TNF-α and IL-4 production in RBL-2H3 cells

Recently, the biphasic allergic reaction mediated by antigen-IgE antibody has been reported. After challenge with an antigen, sensitized animals and atopic individuals exhibit immediate responses, such as the appearance of wheals and flares on the skin and bronchoconstriction of the airways, and late phase responses such as edema and erythema usually persist

Table 1. Effects of phenylpropanoids in the rhizomes of *A. galanga* and related compounds on the release of β-hexosaminidase from RBL-2H3 cells



	2′-3′	R^1	R^2	R^3	IC_{50} (μM)
1′*S*-1′-Acetoxychavicol acetate (**1**)	C=C	H	OAc	OAc	15
1′*S*-1′-Acetoxyeugenol acetate (**2**)	C=C	OCH_3	OAc	OAc	19
Dihydro-(1′*S*)-1′-acetoxychavicol acetate (**3**)	C–C	H	OAc	OAc	60
1′*S*-1′-Hydroxychavicol acetate (**4**)	C=C	H	OAc	OH	— (4%)[a]
Chavicol acetate (**5**)	C=C	H	OAc	H	— (11%)
6	C=C	OCH_3	OAc	H	— (−9%)
7	C=C	H	H	OH[b]	— (−3%)
8	C=C	H	H	OAc[c]	— (−9%)
Eugenol (**9**)	C=C	OCH_3	OH	H	— (4%)
Methyleugenol (**10**)	C=C	OCH_3	OCH_3	H	— (1%)
Demethyleugenol (**11**)	C=C	OH	OH	H	>100 (17%**)
Chavicol (**12**)	C=C	H	OH	H	— (3%)
Chavicol β-D-glucopyranoside (**13**)	C=C	H	*O*-Glc	H	— (0%)



	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)
trans-p-Hydroxycinnamaldehyde (**14**)	H	H	OH	H	CHO	>100 (22%**)[a]
trans-p-Hydroxycinnamyl acetate (**15**)	H	H	OH	H	CH_2OAc	— (6%)
trans-p-Coumaryl alcohol (**16**)	H	H	OH	H	CH_2OH	— (2%)
trans-p-Coumaryl diacetate (**17**)	H	H	OAc	H	CH_2OAc	>100 (21%**)
trans-Cinnamic acid (**18**)	H	H	H	H	COOH	— (7%)
trans-o-Coumaric acid (**19**)	OH	H	H	H	COOH	— (11%)
trans-m-Coumaric acid (**20**)	H	OH	H	H	COOH	— (−1%)
trans-p-Coumaric acid (**21**)	H	H	OH	H	COOH	— (6%)
Caffeic acid (**22**)	H	OH	OH	H	COOH	— (5%)
p-Methoxycinnamic acid (**23**)	H	H	OCH_3	H	COOH	— (11%)
3,4-Dimethoxycinnamic acid (**24**)	H	OCH_3	OCH_3	H	COOH	— (−9%)
3,5-Dimethoxy-4-hydroxycinnamic acid (**25**)	H	OCH_3	OH	OCH_3	COOH	— (10%)

[a]Values in parentheses represent the inhibition (%) at 100 μM ($n=4$). Significantly different from the control, $^{**}p<0.01$.
[b]The mixture of 1′*S*- and 1′*R*-hydroxyl groups.
[c]The mixture of 1′*S*- and 1′*R*-acetoxyl groups.

Table 2. Inhibitory effects of 1′*S*-1′-acetoxychavicol acetate (**1**), and 1′*S*-1′-acetoxyeugenol acetate (**2**), and tranilast on ear PCA reaction in mice

	Dose (mg/kg, po)	*n*	Leakage of dye (% of control)
Control (PBS)	—	10	32.8±8.2
Control (anti DNP-IgE)	—	13	100.0±3.8
1′*S*-1′-Acetoxychavicol acetate (**1**)	6.25	10	72.4±8.5
	12.5	9	65.5±4.8*
	25	10	49.5±5.9**
	50	10	44.3±4.9**
1′*S*-1′-Acetoxyeugenol acetate (**2**)	6.25	10	65.2±11.7*
	12.5	9	58.4±9.8**
	25	9	54.7±6.3**
	50	9	37.8±5.8**
Tranilast	100	10	66.0±9.7*
	200	9	49.8±5.8**
	400	8	42.7±7.0**

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

Table 3. Inhibitory effects of 1′*S*-1′-acetoxychavicol acetate (**1**) and 1′*S*-1′-acetoxyeugenol acetate (**2**) on the release of TNF-α and IL-4 in RBL-2H3 cells

	Inhibition (%)					
Concn (μM):	0	3	10	30	100	IC_{50}
TNF-α release						
1′*S*-1′-Acetoxychavicol acetate (**1**)	0.0±2.6	−5.4±1.3	32.4±5.5**	66.9±3.8**	87.2±0.8**	17
1′*S*-1′-Acetoxyeugenol acetate (**2**)	0.0±8.8	—	30.9±8.2	71.9±8.6**	78.7±20.0**	15
Luteolin	0.0±3.1	25.0±3.0**	89.0±1.3**	101.3±1.2**	—	5.8
IL-4 release						
1′*S*-1′-Acetoxychavicol acetate (**1**)	0.0±9.3	39.4±2.8**	45.9±3.3**	98.1±1.3**	99.9±0.7**	12
1′*S*-1′-Acetoxyeugenol acetate (**2**)	0.0±15.4	—	43.2±6.2**	99.1±1.9**	100.0±0.2**	12
Luteolin	0.0±2.1	41.6±1.3**	89.4±1.0**	99.2±0.2**	—	3.7

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

over a 6–24 h period at the site of challenge in the skin and airways.[22,23] The immediate responses are mainly due to small molecule chemical mediators (e.g., histamine, serotonin) from mast cells. Mast cells also produce cytokines including TNF-α, IL-4, and IL-5, and these cytokines play an important role in the late phase reactions.[22,23] From natural resources, several flavones (e.g., luteolin, apigenin, diosmetin, quercetin) were reported to inhibit release of TNF-α and IL-4.[13,22] However, there have been no studies reported about the inhibitory effects of phenylpropanoids on the release of TNF-α and IL-4 from mast cells. In the present study, the effects of 1′*S*-1′-acetoxychavicol acetate (**1**) and 1′*S*-1′-acetoxyeugenol acetate (**2**), which exhibited strong inhibitory effects against the release of β-hexosaminidase, on release of TNF-α and IL-4 from RBL-2H3 cells 4 h after challenge were examined. As shown in Table 3, **1** and **2** inhibited releases of TNF-α and IL-4 with IC_{50} values of 12–17 μM. These findings suggest that these active phenylpropanoids (**1**, **2**) are also effective against the late phase reactions.

In conclusion, the 80% aqueous acetone extract of the rhizomes of *A. galanga* was found to inhibit release of β-hexosaminidase, as a marker of antigen-IgE-mediated degranulation in RBL-2H3 cells. Nine known phenylpropanoids and *p*-hydroxybenzaldehyde were isolated from the extract. Among them, 1′*S*-1′-acetoxychavicol acetate (**1**) and 1′*S*-1′-acetoxyeugenol acetate (**2**) were found to inhibit degranulation of RBL-2H3 cells induced by antigen. With regard to the structural requirements for the activity, both the 1′- and 4-acetoxyl groups of **1** and **2** were essential for their strong activity, and the 2′–3′ double bond enhanced the activity. Both compounds inhibited PCA reaction in mice and release of TNF-α and IL-4 from RBL-2H3 4 h after the challenge. These findings suggest that **1** and **2** are a novel class of inhibitor against type I allergy.

Bioassay Methods

Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells

Inhibitory effects of test samples on the release of β-hexosaminidase from RBL-2H3 cells [Cell No. JCRB0023, obatined from Health Science Research Resources Bank (Osaka, Japan)] were evaluated by the method reported previously.[9–13] Briefly, RBL-2H3 cells dispensed into 24-well plates at a concentration of 2×10^5 cells/well using Eagle's Minimum Essential Medium (MEM) containing 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 μg/mL), and anti-DNP IgE (0.45 μg/mL), and incubated overnight at 37 °C in 5% CO_2 for sensitization of the cells. Then, 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), 40 mM NaOH, pH 7.2] and incubated in 160 μL of Siraganian buffer [supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA)] for an additional 10 min at 37 °C. Then, aliquots of 20 μL of the test sample solution were added to each well and incubated for 10 min, followed by 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 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 with 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 was 0.1%). The inhibition% of the release of β-hexosaminidase by the test samples 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 (−).

The cell suspension (5×10^7 cells) in 6 mL of PBS was sonicated. For β-hexosaminidase inhibitory activity, the solution was then centrifuged and the supernatant was

diluted with Siraganian buffer and adjusted to equal the enzyme activity of the degranulation test described 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.

Inhibitory effect on antigen-induced TNF-α and IL-4 production in RBL-2H3 cells

Inhibitory effects of test samples on TNF-α and IL-4 production from RBL-2H3 cells were evaluated by the method reported previously.[13] 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 the 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 a 96-well ELISA plate and TNF-α and IL-4 concentrations were determined using commercial kits (TNF-α, rat, ELISA system, code 2734; IL-4, rat, ELISA system, code 2737, Amersham Pharmacia Biotech Co., Ltd.). The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO concentration was 0.1%). To estimate the production of TNF-α or IL-4 from cells, the same procedure was followed (Normal), but without addition of antigen. Thus, the inhibition % of the production of TNF-α or IL-4 by the test sample was calculated using the following equation, and IC_{50} values were determined graphically:

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

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

Ear passive cutaneous anaphylaxis (PCA) reaction in mice

The ear PCA reaction was performed according to the method reported by Inagaki et al.[31] with slight modification. Briefly, 10 μL of anti-DNP IgE diluted in PBS (20 μg/mL), or PBS alone (normal group) was injected intradermally into both ears of male ddY mice (4 weeks old). Forty-seven hours later, test compounds suspended in 5% acasia solution was administered orally. After 1 h, 0.25 mL of PBS which contain 0.5% Evans blue and 0.25 mg of DNP-BSA was injected into the vein. Thirty min later, mice were killed by cervical dislocation and the aures externa was removed and incubated with 1 M KOH solution overnight at 37 °C to dissolve them. The solution was then mixed with 4.5 mL of a mixture of acetone–0.2 M H_3PO_4 (15:3). After centrifugation at 4000 rpm for 10 min, absorbance was measured at 620 nm using a spectrophotometer (Beckmann DU 530). The results were expressed as % of control. Tranilast was used as a reference compound.

Statistics

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

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