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Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the 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 *Curcuma zedoaria* cultivated in Thailand (Thai zedoary) was found to inhibit release of β-hexosaminidase, as a marker of antigen-IgE-mediated degranulation, in RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice. From the active fraction, four curcuminoids (curcumin, dihydrocurcumin, tetrahydrodemethoxycurcumin, and tetrahydrobisdemethoxycurcumin) were isolated together with two bisabolane-type sesquiterpenes, and the effects of four curcuminoids from Thai zedoary and several related compounds on the degranulation were examined. Among them, curcumin showed the highest activity against β-hexosaminidase release with IC_{50} of 5.3 μM, followed by bisdemethoxycurcumin (IC_{50} = 11 μM). With regard to the structural requirements of curcuminoids for the activity, the conjugated olefins at the 1–7 positions and the 4′- or 4″-hydroxyl groups of curcuminoids were suggested to be essential for the strong activity, whereas the 3′- or 3″-methoxyl group only enhanced the activity. Furthermore, effects of curcumin and bisdemethoxycurcumin on calcium ionophores (A23187 and ionomycin)-induced degranulation and antigen-induced release of TNF-α and IL-4 were examined.

1. Introduction

Type I allergy is induced by certain types of antigens such as foods, dust mites, medicines, cosmetics, mold spores, and pollen. This class of antigens induces the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophils. Recently, the biphasic reactions, the early phase and the late phase reactions, have been reported in type I allergy. The early phase reaction in type I allergy occurs within minutes and then the mediators such as histamine and serotonin are released from the cell. These mediators induce vasodilation, mucous secretion, and bronchoconstriction. β-Hexosaminidase is also stored in the secretory granules of mast cells and basophils, and released along with histamine when mast cells and basophils are activated. Therefore, this enzyme activity is used as a marker of mast cell or basophil degranulation,[1] and this assay has been used for evaluation of the anti-allergic compounds as alternative for passive cutaneous anaphylaxis (PCA) reactions in laboratory animals.[2] The late phase reaction occurs within 4–6 h after the early phase reaction in type I allergy. The mediators such as cytokines (TNF-α, IL-4, etc.) from the cells are involved in the late phase. These mediators increase endothelial cell adhesion and recruitment of inflammatory cells to the affected site.[3]

The rhizomes of *Curcuma zedoaria* ROSCOE (so called 'zedoary') have been used as anti-flatulence and anti-inflammatory in Thai traditional medicine. Moreover, the rhizomes of this plant also have been used as fragrance and spice in Asian countries. Regarding the pharmacological studies of this plant, anti-inflammatory effects of the extract and sesquiterpenes were reported.[4] In our previous studies, we reported the hepatoprotective, vasodilative, and nitric oxide production inhibitory activities of various sesquiterpenes and curcuminoids isolated from the rhizomes of *C. zedoaria* cultivated in China.[5]

As a part of our studies on bioactive constituents from natural medicines, we previously reported various

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inhibitors against degranulation induced by antigen in RBL-2H3 cells.[6] In the present study, the 80% aqueous acetone extract of the dried rhizomes of *C. zedoaria* cultivated in Thailand (Thai zedoary)[7] was found to inhibit release of β-hexosaminidase, as a marker of antigen-IgE-mediated degranulation, in RBL-2H3 cells and ear PCA reaction in mice. In the present study, four curcuminoids and two bisabolane-type sesquiterpenes were isolated from the active fraction, and we examined the effects of curcuminoids and their derivatives on the antigen-induced degranulations in RBL-2H3 cells to discuss structural requirements of curcuminoids for the activity. In addition, we describe effects of two active curcuminoids [curcumin (**1**) and bisdemethoxycurcumin (**6**)] on calcium ionophores-induced degranulation and on antigen-induced release of TNF-α and IL-4 in RBL-2H3 cells.

2. Results and discussion

2.1. Isolation of active constituents from Thai zedoary

The dried rhizomes of *C. zedoaria* (1.45 kg) were extracted with 80% aqueous acetone three times under room temperature. The 80% aqueous acetone extract (12.7% from this natural medicine) significantly inhibited ear PCA reaction in mice (Table 1). The extract also inhibited the release of β-hexosaminidase in RBL-2H3 cells (IC_{50} = 48 μg/mL, Table 2) and this activity was stronger than those of anti-allergic compounds tranilast [282 μM (=93 μg/mL)] and ketotifen fumarate [158 μM (=67 μg/mL)] (Table 2). Therefore, the inhibition against the release of β-hexosaminidase was used for the following bioassay-guided separation of active compounds.

The extract was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble fraction (9.8%) and water-soluble fraction (2.9%). The EtOAc-soluble fraction, which showed potent activity (IC_{50} = 45 μg/mL), was subjected to normal-phase silica gel (SiO_2) [*n*-hexane–AcOEt (10:1 → 5:1 → 2:1 → 1:1) → AcOEt → MeOH] and reversed-phase silica gel (ODS) column chromatographies (MeOH–H_2O) and finally HPLC (YMC-Pack ODS-A, 250 × 20 mm i.d., CH_3CN–H_2O or MeOH–H_2O) to give four curcuminoids [curcumin (**1**,[8] 1.40% from the dried rhizomes), dihydrocurcumin (**2**,[8] 0.20%), tetrahydrodemethoxycurcumin (**3**,[9] 0.043%), tetrahydrobisdemethoxycurcumin (**4**,[9] 0.055%)], and two bisabolene-type sesquiterpenes [4,5-dihydrobisabola-2,10-diene (**5**,[10] 0.021%) and bisacurone[10] (0.046%)].

Related curcuminoids, bisdemethoxycurcumin (**6**) isolated from Chinese zedoary[5] and tetrahydrocurcumin (**7**),[11] hexahydrocurcumin (**8**),[12] monomethylcurcumin (**9**),[13] and dimethylcurcumin (**10**)[13] derived from **1** in the usual way were examined to clarify the structure–activity relationships for curcuminoids (Fig. 1).

2.2. Inhibitory effects of compounds 1–5 isolated from Thai zedoary and related curcuminoids (6–10) on the release of β-hexosaminidase from RBL-2H3 cells

As shown in Table 3, curcumin (**1**), bisdemethoxycurcumin (**6**), and monomethylcurcumin (**9**) exhibited potent inhibitory activities with IC_{50} of 5.3, 11, and 15 μM,

Table 1. Inhibitory effects of the 80% aq acetone extract from Thai zedoary and its fractions on ear PCA reactions in mice

	Dose (mg/kg, p.o.)	*n*	Leakage of dye (O.D. at 620 nm)	Inhibition (%)
Control (PBS)	—	7	0.039 ± 0.003**	—
Control (anti-DNP-IgE)	—	12	0.398 ± 0.055	—
80% Aq acetone ext.	100	7	0.299 ± 0.033	27.6
	200	7	0.285 ± 0.032	31.5
	400	7	0.276 ± 0.034*	34.0
EtOAc-soluble fraction	100	5	0.304 ± 0.066	26.2
	200	8	0.226 ± 0.021**	47.9
H_2O-soluble fraction	400	6	0.365 ± 0.053	9.2
Control (PBS)	—	5	0.050 ± 0.009**	—
Control (anti-DNP-IgE)	—	8	0.310 ± 0.054	—
Tranilast	100	7	0.177 ± 0.019*	51.2
	200	7	0.126 ± 0.017**	70.8

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

Table 2. Inhibitory effects of the 80% aq acetone extract from Thai zedoary and its fractions on antigen-induced release of β-hexosaminidase in RBL-2H3 cells

	Inhibition (%)				IC_{50} (μg/mL)	Enzyme inhibition at 100 μg/mL
Concn:	0 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL		
80% Aq acetone extract	0.0 ± 4.8	5.1 ± 3.3	26.8 ± 2.8**	87.9 ± 0.8**	48	18%
EtOAc-soluble fraction	0.0 ± 0.5	4.3 ± 2.0	35.4 ± 3.2**	97.6 ± 1.9**	35	27%
H_2O-soluble fraction	0.0 ± 3.1	7.8 ± 2.9	0.0 ± 5.2	4.8 ± 3.1	—	—

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

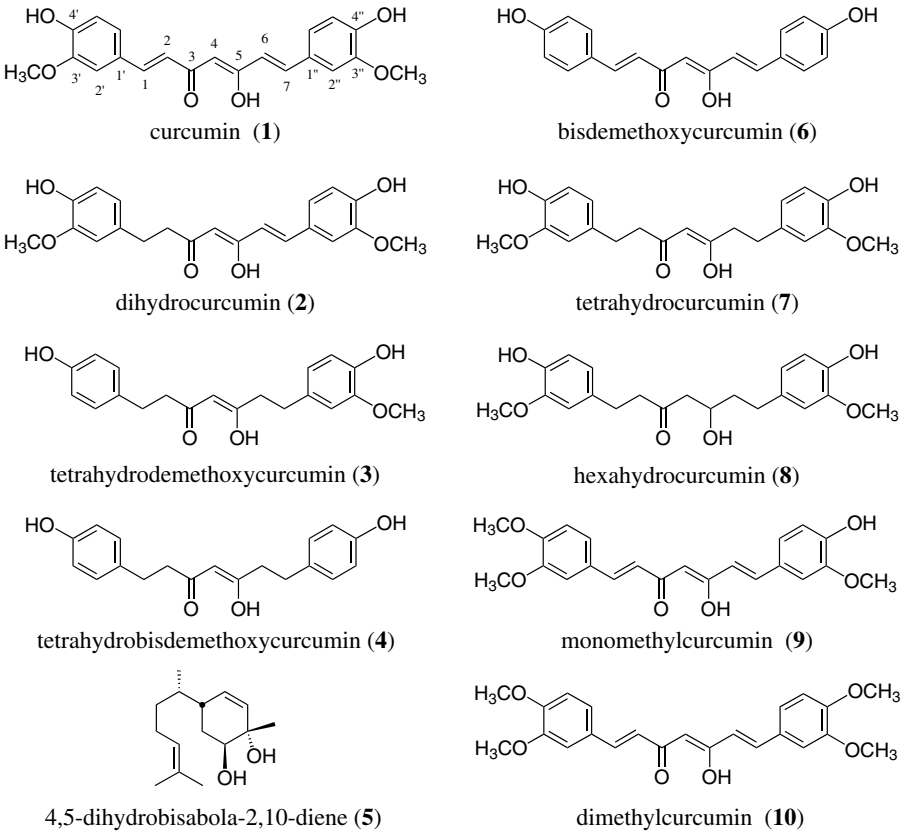

Figure 1. Chemical structures of compounds **1**–**5** isolated from the rhizomes of *C. zedoaria* and related compounds **6**–**10**.

Table 3. Inhibitory effects of compounds **1**–**5** isolated from Thai zedoary and related compounds **6**–**10** on antigen-induced release of β-hexosaminidase in RBL-2H3 cells

Compounds	Inhibition (%)					IC_{50} (μM)	Enzyme inhibition at 100 μM
Concn:	0 μM	3 μM	10 μM	30 μM	100 μM		
1	0.0 ± 0.5	28.5 ± 5.9**	80.4 ± 4.7**	100.1 ± 1.1**	102.3 ± 0.8**	5.3	27%
2	0.0 ± 8.6	—	9.1 ± 1.1	39.0 ± 6.6**	81.5 ± 6.0**	39	16%
3	0.0 ± 1.8	—	13.5 ± 4.7	27.1 ± 2.6*	78.0 ± 9.7**	62	2%
4	0.0 ± 0.5	—	9.1 ± 4.5	22.2 ± 5.4*	41.6 ± 7.1**	>100	—
5	0.0 ± 5.6	—	19.6 ± 3.4**	25.1 ± 3.2**	22.4 ± 3.9**	>100	—
6	0.0 ± 7.5	7.3 ± 7.2	41.0 ± 9.3*	91.2 ± 4.6**	92.5 ± 0.8**	11	16%
7	0.0 ± 2.3	—	3.0 ± 3.0	21.0 ± 6.6**	92.7 ± 3.4**	58	2%
8	0.0 ± 2.6	—	7.8 ± 2.6	11.4 ± 6.1	30.5 ± 3.3**	>100	—
9	0.0 ± 4.1	10.6 ± 1.6	47.4 ± 4.4**	67.5 ± 3.6**	88.6 ± 4.9**	15	30%
10	0.0 ± 3.1	—	3.1 ± 2.5	1.0 ± 3.4	8.5 ± 0.6	>100	—
	0 μM	30 μM	10 μM	300 μM	1000 μM		
Tranilast	0.0 ± 1.7	8.2 ± 1.8	22.4 ± 2.5*	56.9 ± 3.4**	75.0 ± 0.6**	282	—
Ketotifen fumarate	0.0 ± 1.8	7.4 ± 1.5	27.6 ± 2.2*	80.7 ± 1.8**	100.7 ± 1.1**	158	—

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

respectively. Whereas, compounds **2**–**5**, **7**, **8**, and **10** showed moderate or weak activity. Effects of the active compounds **1**–**3**, **6**, **7**, and **9** were also examined on the enzyme activity of β-hexosaminidase. As a result, these active compounds showed weak or less inhibition against β-hexosaminidase at 100 μM. These results indicated that these compounds inhibited the antigen-induced degranulation but not enzyme activity of β-hexosaminidase.

Yano and co-workers reported that the extract of the rhizomes of *Curcuma longa* and several curcuminoids isolated from the active fraction inhibited compound 48/80, concanavalin A, and/or calcium ionophore A23187-induced histamine release in rat peritoneal mast cells.[14] However, structure–activity relationships of curcuminoids for anti-allergic activity have not been sufficiently studied.

With regard to the structure–activity relationships of curcuminoids on anti-allergic activity, when the conjugated olefins at the 1–7 positions and the 3′ and 3″-methoxyl groups decreased in number, the activities were decreased. This indicated the importance of the conjugated olefins and methoxyl groups of curcuminoids. However, the conjugated olefins seemed to affect the activity more than the methoxyl groups, which can be observed from the IC_{50} of compound **1**, which was only two times higher than that of compound **6** (lacks of two methoxyl groups) [**1** (IC_{50} = 5.3 μM) > **6** (11 μM)], but eight times higher than that of compound **2** (lacks of one olefinic group at the 1–2 positions), and 11 times higher than that of compound **7** (lack of two olefinic groups at the 1–2 and 6–7 positions) [**1** (5.3 μM) > **2** (39 μM) > **7** (58 μM) > **8** (100 μM)]. Yano and co-workers reported that **6** showed stronger activity than **1** against compound 48/80-induced histamine release in rat peritoneal mast cells.[14] However, in the present study, the activity of **1** was stronger than that of **6** against antigen-IgE-mediated degranulation in RBL-2H3 cells. The methylation of the 4′-hydroxyl group of **1** reduced the activity ca. three times less than that of **1**. Moreover, the methylation of both the 4′- and 4″-hydroxyl groups of **1** dramatically decreased the activity [**1** (5.3 μM) > **9** (15 μM) > **10** (>100 μM)]. These results indicated that at least one *p*-hydroxyl group of aromatic rings of **1** was essential to exhibit the activity.

The structure–activity relationships of curcuminoids on anti-allergic activity can be concluded that the conjugated olefins at the 1–7 positions and the 4′- and 4″-hydroxyl groups of curcuminoids are essential for the activity, whereas 3′- and 3″-methoxyl groups only enhanced the activity.

2.3. Inhibitory effects of curcumin (1) and bisdemethoxycurcumin (6) on the release of β-hexosaminidase induced by calcium ionophores

Calcium ionophores are compounds that enhance Ca^{2+} influx into the cell by increasing membrane permeability of Ca^{2+}. The increase in intracellular Ca^{2+} induces the movement of granules to plasma membrane followed by the degranulation of mast cells or basophils and activates the formation of inflammatory mediators such as prostaglandins and leukotrienes.[3] Previously, curcumin (**1**) was reported to inhibit concanavalin A, compound 48/80, and A23187-induced histamine release in rat peritoneal mast cells and concluded that curcumin (**1**) potently suppressed the histamine release probably through the inhibition of the degranulation process following a rise in intracellular Ca^{2+} levels.[14]

In the present study, effects of curcumin (**1**) and bisdemethoxycurcumin (**6**) on A23187 and ionomycin-induced degranulations of RBL-2H3 cells were examined. In agreement with the previous report,[14] compounds **1** and **6** inhibited A23187 and ionomycin-induced degranulation of RBL-2H3 cells, and their IC_{50} values (7.2 and 13 μM for A23187, 6.0 and 15 μM for ionomycin) were similar to those by antigen (5.3 and 11 μM) (Table 4).

Recently, ionomycin was reported to cause the activation of calcium release-activated calcium channels (CRAC) by depleting intracellular calcium stores rather than acting as an ionophore and may cause calcium influx in a similar way, as does antigen.[15] In the present study, compounds **1** and **6** inhibited the degranulation of RBL-2H3 cells by both the antigen and ionomycin at similar IC_{50} values. These results suggested that both the compounds might inhibit Ca^{2+} influx via CRAC channels or mechanisms after Ca^{2+} influx.

2.4. Inhibitory effects of curcumin (1) and bisdemethoxycurcumin (6) on antigen-induced TNF-α and IL-4 production in RBL-2H3 cells

Curcumin (**1**) was previously reported to inhibit the production of TNF-α and IL-1 in a human macrophage cell line by LPS and inhibit IL-12 production in mouse spleen macrophages induced by LPS,[16] but the effects of **1** on the antigen-induced production of cytokines in basophils was not investigated so far. To investigate whether curcumin (**1**) and bisdemethoxycurcumin (**6**) can inhibit the late phase reaction or not, inhibitory effects of **1** and **6** on the release of TNF-α and IL-4 were examined. As a result, **1** inhibited the release of TNF-α and IL-4 with IC_{50} of 20 and 18 μM, respectively. Whereas those of **6** were 38 and 34 μM (Table 5). IC_{50} values of both compounds **1** and **6** for release of TNF-α and IL-4 were ca. four times lower than those for release of β-hexosaminidase. These findings suggested that **1** and **6** might exhibit weaker effects on the late phase reactions than the early phase reactions.

Table 4. Inhibitory effects of curcumin (**1**) and bisdemethoxycurcumin (**6**) on the release of β-hexosaminidase induced by calcium ionophores, A23187 and ionomycin

	Inhibition (%)					IC_{50}
Concn:	0 μM	3 μM	10 μM	20 μM	30 μM	(μM)
A23187 (1 μM)						
Curcumin (**1**)	0.0 ± 2.4	12.6 ± 0.4**	58.1 ± 6.7	98.1 ± 0.9**	110.0 ± 1.6**	7.2
6	0.0 ± 2.3	—	33.2 ± 4.1**	77.1 ± 0.8**	95.4 ± 0.1**	13
Ionomycin (1 μM)						
Curcumin (**1**)	0.0 ± 2.3	16.8 ± 2.8**	79.5 ± 1.6**	100.9 ± 0.5**	101.0 ± 0.3**	6.0
6	0.0 ± 2.3	—	24.7 ± 2.0**	72.3 ± 1.3**	90.5 ± 0.4**	15

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

Table 5. Inhibitory effects of curcumin (**1**) and bisdemethoxycurcumin (**6**) on antigen-induced TNF-α and IL-4 production from RBL-2H3

	Inhibition (%)						IC_{50}
Concn:	0 μM	3 μM	10 μM	20 μM	30 μM	100 μM	(μM)
TNF-α Release							
Curcumin (**1**)	0.0 ± 4.4	—	12.7 ± 3.8	37.8 ± 2.6**	98.8 ± 1.1**	110.0 ± 1.6**	20
6	0.0 ± 8.8	—	7.0 ± 7.0	5.2 ± 4.6	47.0 ± 5.3**	110.6 ± 1.4**	38
Luteolin	0.0 ± 3.1	25.0 ± 3.0**	89.0 ± 1.3**	—	101.3 ± 1.2**	—	5.8[6c]
IL-4 Release							
Curcumin (**1**)	0.0 ± 1.1	—	5.0 ± 2.1	56.0 ± 1.9**	97.6 ± 1.5**	—	18
6	0.0 ± 1.4	—	3.7 ± 1.7	14.7 ± 0.8**	55.6 ± 1.6**	101.8 ± 0.4**	34
Luteolin	0.0 ± 2.1	41.6 ± 1.3**	89.4 ± 1.0**	—	99.2 ± 0.2**	—	3.7[6c]

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

In conclusion, the 80% aqueous acetone extract of Thai zedoary was found to inhibit antigen-IgE-mediated degranulation in RBL-2H3 cells and ear PCA reaction in mice. By bioassay-guided separation, three curcuminoids (**1**–**3**) were isolated as active compounds. Regarding to the structural requirements of curcuminoids for antigen-induced degranulation, the conjugated olefins at the 1–7 positions and the 4′- or 4″-hydroxyl groups of curcuminoids were suggested to be essential for the strong activity, whereas the 3′- or 3″-methoxyl group only enhanced the activity. In addition, curcumin (**1**) and bisdemethoxycurcumin (**6**) were suggested to inhibit Ca^{2+} influx via CRAC channels or the mechanisms after Ca^{2+} influx. Furthermore, curcumin (**1**) inhibited antigen-induced release of TNF-α and IL-4, both of which participate in the late phase of type I allergic reaction, in RBL-2H3. These results can support that the rhizomes of *C. zedoaria* have been used as anti-inflammatory agents in Thai traditional medicine, and the detail mechanisms of action of curcuminoids including experimental animal models need to be studied further.

3. Experimental

3.1. Instruments

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-LA500 (500 MHz) spectrometer and JEOL EX-270 (270 MHz); ^{13}C NMR spectra, JNM-LA500 (125 MHz) and JEOL EX-270 (68 MHz) spectrometer with tetramethylsilane as an internal standard.

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

3.2. Plant material

The rhizomes of *C. zedoaria* were purchased in Thailand in July 2000, and were identified by Dr. Yutana Pongpiriyadacha (Faculty of Nakhon si thammarat, Rajamangala Institute of Technology, Thailand) and one of the authors (Dr. Supinya Tewtrakul). A voucher specimen (No. T-11) of this herbal medicine is on file in our laboratory.

3.3. Extraction and isolation

The dried rhizomes of *C. zedoaria* (1.45 kg) were extracted with 80% aqueous acetone three times under room temperature to give the 80% aqueous acetone extract (184.4 g, 12.7% from this natural medicine). The aqueous acetone extract (129.9 g) was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble fraction (100.5 g, 9.8%) and water-soluble fraction (29.4 g, 2.9%). The EtOAc-soluble fraction (76 g) was subjected to ordinary-phase silica gel [2.3 kg, *n*-hexane–AcOEt (10:1 → 5:1 → 2:1 → 1:1) → AcOEt → MeOH] to give 13 fractions [Fr. 1 (0.066 g), Fr. 2 (6.419 g), Fr. 3 (9.149 g), Fr. 4 (0.746 g), Fr. 5 (3.904 g), Fr. 6 (0.956 g), Fr. 7 (0.892 g), Fr. 8 (2.093 g), Fr. 9 (3.411 g), Fr. 10 (10.099 g), Fr. 11 (19.188 g), Fr. 12 (12.483 g), and Fr. 13 (6.594 g)]. Fr. 10 (10.099 g) was separated by reversed-phase silica gel (ODS) column chromatography [300 g, MeOH–H_2O (50:50 → 60:40 → 85:15) → MeOH] to furnish seven fractions [Fr.10–1 (0.429 g), Fr. 10–2 (4.555 g), Fr. 10–3 (0.414 g), Fr. 10–4 (2.345 g), Fr. 10–5 (1.501 g), Fr. 10–6 (0.425 g), and Fr. 10–7 (0.430 g)]. Fr. 10–2 (1.00 g) and Fr. 10–5 (1.00 g) were further separated by HPLC [YMC-Pack ODS-A, 250 × 20 mm i.d., CH_3CN–H_2O (45:55 or 50:50)] to give tetrahydrodemethoxycurcumin (**3**, 72.4 mg, 0.043%) and tetrahydrobisdemethoxycurcumin (**4**, 93.2 mg, 0.055%) from Fr. 10–2 and 4,5-dihydrobisabola-2,10-diene (**5**, 73.3 mg, 0.021%). Fr. 11 (19.188 g) was separated by ODS column chromatography [510 g, MeOH–H_2O (70:30)] and then purified by HPLC [MeOH–H_2O (70:30)] to give curcumin

(**1**, 9.567 g, 1.40%) and dihydrocurcumin (**2**, 1.380 g, 0.20%). Fr. 12 (12.30 g) was separated by ODS column chromatography [369 g (40:60 → 60:40 → 80:20) → MeOH → acetone] and then purified by HPLC [MeOH–H_2O (50:50)] to give bisacurone (350 mg, 0.046%). The known compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, ^1H NMR, ^{13}C NMR, MS) with reported values,[8–10] or authentic samples.[5a,b,f]

3.4. Hydrogenation of 1

A solution of **1** (1.0 g) in MeOH (20.0 mL) was treated with 10% palladium carbon (Pd–C, 100 mg) and the whole mixture was stirred at room temperature under an H_2 atmosphere for 13 h. The reaction mixture was filtered and then evaporation of the solvent under reduced pressure furnished crude products. The products were separated by HPLC [MeOH–H_2O (65:35)] to give tetrahydrocurcumin (**7**, 385 mg, 38.1%) and hexahydrocurcumin (**8**, 386 mg, 38.0%). Hydrocurcumins **7** and **8** were identified by comparison of the physical data with reported values.[11,12]

3.5. Methylation of 1

A solution of **1** (200 mg) in *N*,*N*-dimethylformamide (DMF, 5.0 mL) was treated with methyl iodide (CH_3I, 1.0 mL) in the presence of sodium hydride (NaH, 40 mg) and the mixture was stirred at room temperature for 13 h. The reaction mixture was poured into ice-water and the whole was extracted with EtOAc. The EtOAc extract was successively washed with saturated aqueous $NaHCO_3$ and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by HPLC [MeOH–H_2O (75:25)] to give monomethylcurcumin (**9**, 107 mg, 51.5%) and dimethylcurcumin (**10**, 22 mg, 10.2%). Methylcurcumins **9** and **10** were identified by comparison of their physical data with reported values.[13]

3.6. Bioassay methods

3.6.1. 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; the dinitrophenylated bovine albumin (DNP-BSA) was prepared as described previously.[17] Other chemicals were from Wako; 24-well and 96-well microplates were from Sumitomo Bakelite Co., Ltd.

3.6.2. Animals. Male ddY mice (25–30 g) were purchased from Kiwa Laboratory Animals (Wakayama, Japan). The animals were maintained at a constant temperature of 23 ± 2 °C and were fed with standard laboratory chow (MF, Oriental Yeast, Japan) for one week. Test samples were suspended with 5% acacia solution, and the solution was administered orally at 10 mL/kg in each experiment, while vehicle was given orally at 10 mg/kg in the corresponding control group.

3.6.3. Effects on ear PCA reaction in mice. The ear PCA reaction was performed according to the method reported previously[6f,18] 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. Forty-seven hours later, test compounds suspended in 5% acacia solution was administered orally. After 1 h, 0.25 mL of PBS, which contains 2.0% Evans blue and 0.25 mg of DNP-BSA, was injected into the vein. Thirty minutes later, mice were killed by cervical dislocation and both ears were 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 (13:5). After centrifugation at 4000 rpm for 10 min, absorbance was measured at 620 nm using a spectrophotometer (Beckmann DU 530). Tranilast was used as a reference compound.

3.6.4. Effects on the release of β-hexosaminidase from RBL-2H3 cells and on enzyme activity of β-hexosaminidase. Inhibitory effects on the release of β-hexosaminidase in RBL-2H3 [Cell no JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] were evaluated by a method reported previously.[6] Briefly, RBL-2H3 cells in 24-well plates [2×10^5 cells/well in MEM containing 10% FCS, penicillin (100 units/mL), streptomycin (100 μg/mL)] were sensitized with anti-DNP IgE (0.45 μg/mL). The cells were washed with 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 (BSA) (incubation buffer) and then 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 antigen (DNP-BSA, final concn 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 incubation buffer (final DMSO concn 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:

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

Control (*C*): DNP-BSA (+), test sample (−); test (*T*): DNP-BSA (+), test sample (+); blank (*B*): DNP-BSA

(−), test sample (+); normal (*N*): DNP-BSA (−), test sample (−).

To clarify that the anti-allergic effects of samples are due to the inhibition on β-hexosaminidase release, but not the false positive from the inhibition of β-hexosaminidase activity. 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 enzyme activity was examined as described above. 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.

3.6.5. Effects on the release of β-hexosaminidase from RBL-2H3 induced by calcium ionophores, A 23187 and ionomycin. The inhibitory effects on A23187 or ionomycin-induced release of β-hexsosaminidase in RBL-2H3 were evaluated similarly to that of antigen-induced degranulation.[6i,19] However, in the case of A23187 and 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 A23187 or ionomycin (final concn 1 μM) at 37 °C for 10 min to stimulate the cells to degranulate. The reactions were 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.

3.6.6. Effects on antigen-induced TNF-α and IL-4 production from RBL-2H3 cells. Inhibitory effects on the release of TNF-α and IL-4 from RBL-2H3 were evaluated by the method reported previously.[6c,f] RBL-2H3 cells (2×10^5 cells/well) were sensitized with anti-DNP IgE as described above. The cells were washed with 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 concn 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 (−).

3.7. 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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