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Excavation of Lead Compounds that Inhibit Mast Cell Degranulation by Combinatorial Chemistry and Activity-Guided

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An allergic reaction ensues after antigen binds to mast cell or basophil high affinity IgE receptor, FcεRI, resulting in degranulation of various inflammatory mediators that produce various allergic symptoms. In this study, i) we isolated the active component for the inhibition of mast cell degranulation from the extract of leaves of *Castanea crenata* and identified it as quercetin; ii) we established the total synthesis procedure of quercetin; iii) using quercetin as positive control, we excavated some lead compounds that possess inhibitory activities for mast cell degranulation by screening the chemical libraries of 1,3-oxazolidine derivatives prepared by solid phase combinatorial chemistry. Some of 1,3-oxazolidine compounds possessing acetyl and 3',4'-dichlorophenyl group displayed strong inhibitory activities on FcεRI-mediated mast cell degranulation, suggesting that they can be used as lead compounds for the development of anti-allergic agents.

Key words: *Castanea crenata*, Quercetin, Mast cell degranulation, Combinatorial chemistry, 1,3-Oxazolidines, Allergy

INTRODUCTION

Immediate hypersensitivity reaction is initiated as allergen cross-links IgE-bound high affinity IgE receptor (FcεRI) on basophil or mast cells. FcεRIs are heterotetrameric receptors composed of four subunits, two disulfide- linked γ-subunits that transduce signals generated by antigen binding, a β-subunit that serves to amplify γ-subunit signaling, and an α-subunit that binds IgE (Blank *et al.*, 1989; Kinet, 1989). Antigenic cross-linking of FcεRIs initiates a series of tyrosine phosphorylation events involving immunoreceptor tyrosine-based activation motifs (ITAMS) of the β and γ-chains (Reth, 1989), finally resulting in the elevation of intracellular calcium levels for the mast cell degranulation (Cambier, 1995; Rivera, 2002). Inhibitory activity for the mast cell degranulation is therefore frequently used to predict the possible anti-allergic activities of lead compounds.

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Activity-guided fractionation from natural products and organic synthesis are representative approaches to obtain lead compounds. Recently more powerful experimental tool, combinatorial chemistry, was introduced to provide virtually unlimited numbers of candidates for lead compounds. Here by employing inhibitory activity on mast cell degranulation as biological guideline, a natural product and chemical library were screened to obtain lead compounds.

Previously we had reported anti-allergic activities from the water extract of leaves of *Castanea crenata* (Fagaceae) (Lee *et al.*, 1999). In this study, using bioassay-directed isolation from the methanol extract of leaves of *Castanea crenata*, quercetin was identified as active component for the inhibition of mast cell degranulation, and the experimental procedure for the total synthesis of quercetin was established. In addition, using quercetin as positive control, lead compounds possessing inhibitory activities for mast cell degranulation were obtained by screening a chemical library composed of 1,3-oxazolidine derivatives, which were synthesized by combinatorial chemistry.

MATERIALS AND METHODS

Materials

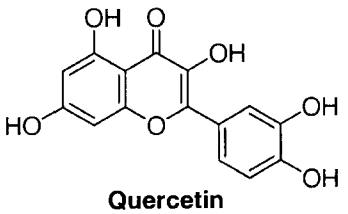
The plant material was collected between May and July, 1995 from Chonnam Province, Korea. The plant was identified by the herbarium of the Faculty of Pharmacy, Chonnam National University, where a voucher specimen (herbarium No. 9512) has been deposited. Chlorinated Wang resin was used as a solid support and all materials were obtained from commercial suppliers and were used without purification.

Antigens and antibodies

DNP-BSA (2, 4-dinitrophenylated bovine serum albumin) was kindly provided by Dr. Koda (College of Pharmacy, Gifu University, Japan). DNP-specific mouse monoclonal IgE was obtained from the hybridoma.

Measurement of β-hexosaminidase release from RBL-2H3 cells

The same procedure was followed as described previously (Cheong *et al.*, 1998; Cheong *et al.*, 1999). RBL-2H3 cells were resuspended in Minimum Essential Medium Eagle supplemented with 10% fetal bovine serum at the density of 5×10^5 cells/well. Cells were dispensed into 24 well plates, and were treated with IgE (0.5 μg/mL) overnight at 37 °C in 5% CO_2 incubator. The next morning, the cells were washed and preincubated in PIPES buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH , 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA) for 10 min at 37°C. The cells were treated with antigen (DNP-BSA, 1 μg/mL) for 10 min at 37 °C. The reaction was stopped in an ice bath for 10 min and the supernatant was used for the enzyme assay. The supernatants were added into 96-well plates and were incubated with substrate (1 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide) for 1 h. Stop solution (0.1 M Na_2CO_3/$NaHCO_3$) was added, and the absorbance at 405 nm was measured with an ELISA reader.



Quercetin

Fig. 1. Structure of quercetin isolated from the leaves of *Castanea crenata*

Total synthesis of quercetin

The total synthesis of quercetin was planned by retrosynthetic analysis. Because quercetin possesses flavone structure, it could be easily synthesized from 2,4,6-trihydroxyacetophenone derivatives and vanillic acid derivatives in the presence of base (Fig. 2).

Synthesis of 1,3-oxazolidine derivatives

Detailed procedure for the synthesis of 1,3-oxazolidinedione derivatives using solid phase was conducted as previously reported (Oh *et al.*, 2000). Properly substituted epoxide was reacted with ammonia in the methanol to synthesize aminoalcohol, and then the product was reacted with aldehyde to produce oxazolidine. Electophile was added to oxazolidine mixtures to produce trisubstituted oxazolidines (Fig. 3).

RESULTS AND DISCUSSION

Isolation of an active component responsible for the inhibition of mast cell degranulation

During allergic reaction, cross linking of IgEs by antigen

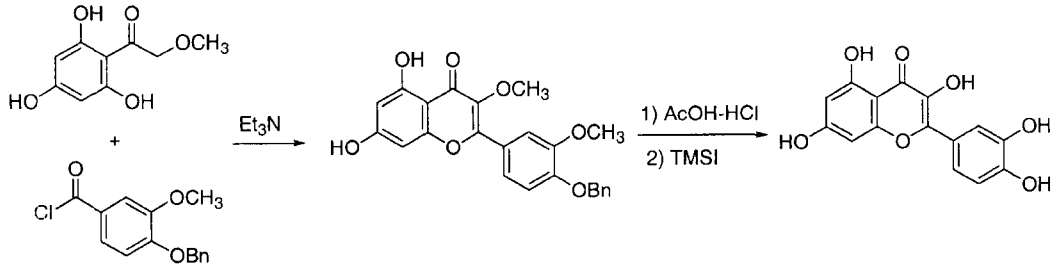


Fig. 2. Diagrammatic procedure for the synthesis of quercetin

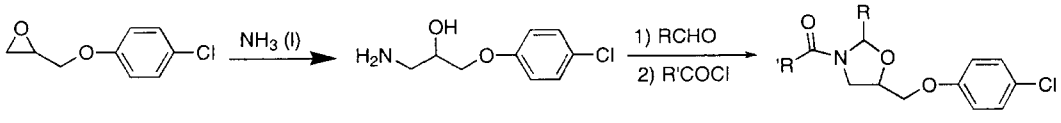


Fig. 3. Synthesis of 1,3-oxazolidine derivatives

causes degranulation of mast cells to release chemical mediators such as histamine and serotonin that play important roles in the pathogenesis of allergic and inflammatory disorders. Therefore, the inhibitory effect of drugs on the histamine release from rat peritoneal mast cells was often rendered to be the anti-allergic activity (Cox, 1967). The β-hexosaminidase is located in the secretory granules of mast cells where histamine is stored, and is released along with histamine when mast cells are immunologically activated (Schwartz *et al.*, 1979; Cheong *et al.*, 1998). Therefore, β-hexosaminidase is designated as 'degranulation marker' and the release of β-hexosaminidase has been used to determine the extent of degranulation and for the evaluation of anti-allergic activities (Fischer *et al.*, 1995). Thus, we employed this bioassay system to evaluate the inhibitory effect on degranulation event *in vitro*.

The activity-guided fractionation method was used. Methanol extract was prepared from 120 g dried leaves of *C. crenata* by extracting with 3 liters of hot 80% methanol, and was consecutively extracted with hexane, methylene chloride, ethyl acetate, and n-butanol. The EC_{50} values for the inhibition of hexosaminidase release were about 30 μg/mL for hexane and ethyl acetate layers, on the other hand, were higher than 200 μg/mL for other layers. The yield and the dose-response relationship are summarized in Table I.

Hexane layer (Fr.1, 3 g) and ethyl acetate layer (Fr.3, 3 g) were loaded onto silica gel column (Φ 2.0×60 cm), and eluted with the mixture of hexane and ethyl acetate (10:1-1:10) (Fr.11-17). As shown in Table II, 30 mg of Fr.165, which inhibits the hexosaminidase more than 70% at 100 μg/mL was isolated after two rounds of flash chromatographic fractionation of hexane layer. After three rounds of fractionations, 26 mg of Fr.3212 was obtained from ethylene acetate layer. Both fractions were yellow crystal of needle type in MeOH and identified as quercetin (Fig. 1) in direct comparison with the authentic sample by spectral analyses (IR, Mass, ^{1}H-NMR). Physicochemical and spectral properties of isolated compound are as follows: mp. >300 °C; IR (KBr, cm^{-1}): 3380, 1670, 1610, 1510, 1240; MS (m/z) 302, 301, 274, 273, 245, 153, 142, 137, 128, 109; ^{1}H-NMR (DMSO-d_6), δ 6.21 (s, 1H), 6.42 (s, 1H), 6.91 (d, 1H), 7.59 (d, 1H), 7.7 (s, 1H), 9.5 (bs, 4H), 12.7 (s, 1H).

Table I. Fractionation of the extracts of Castanea crenata for the inhibitory activities of FceRI-mediated mast cell degranulation

Extract	Amount extracted (g)	Concentration (g/mL)	% Inhibition
Hexanes	6.41	200	79.3
		100	68.2
		30	61.3
Methylene chloride	1.01	200	29.8
Ethyl acetate	3.81	200	92.1
		100	62.3
		30	48.2
n-Butanol	5.75	200	40.7
		100	28.5
		30	25.6

Compounds were dissolved in 100% DMSO and the final concentration of DMSO was adjusted to 0.5%. To exclude the effects of DMSO itself on the degranulation of RBL-2H3 cells, 0.5% DMSO was also added to the control group. Each data point represents mean of triplicate determinations.

% of inhibition = (Treated-Blank-Spontaneous)/(Control-Blank-Spontaneous)

Control : normal allergen-IgE response was evoked with test material not added.

Treated : normal allergen-IgE response was evoked with test material added.

Blank : only test material and substrate were added into ELISA plate.

Spontaneous : allergen-IgE response was not evoked with test material not added.

Total synthesis of quercetin

The best was to corroborate the chemical structure of compound, which was isolated and whose structure was claimed identified, is to synthesize the compound using well-established starting materials and synthetic pathways. As the first step, 5,7-Dihydroxy-4'-benzyloxy-3,3'-dimethoxyflavone was prepared. 2,4,6-Trihydroxy-α-methoxyacetophenone (1.98 g, 0.01 mol) and 4-benzyloxy-3-methoxybenzoyl chloride (2.76 g, 0.01 mol) were refluxed in the presence of triethylamine (5 mL) at 150-160 °C for 4 h then cooled down. The solid product was dissolved in methylene chloride (300 mL) and washed with H_2O (2×50 mL). The organic phase was dried with anhydrous $MgSO_4$, filtered, then concentrated with rotary evaporator to obtain 5,7-dihydroxy-4'-benzyloxy-3,3'-dimethoxyflavone (3.1 g, light green solid). As next step, 5,7-dihydroxy-4'-benzyloxy-3,3'-dimethoxyflavone (2.0 g, 4.8 mmol) was added into acetic acid-HCl, heated for 1 h at 100 °C, then cooled down to produce 4',5,7-trihydroxy-3,3'-dimethoxyflavone (1.3 g, dark yellow solid). 4',5,7-Trihydroxy-3,3'-dimethoxyflavone (1.0 g, 3.0 mmol) was dissolved in chloroform and cooled down, and then trimethylsilyl iodide was added and reacted at 0 °C for 1 h. Reactants were dissolved in methylene chloride (100 mL), and washed with saturated $NaHCO_3$ solution (2×50 mL) and H_2O. Organic layer was dried with anhydrous $MgSO_4$, filtered, and vacuum concentrated, and the solid was recrystalized in methanol to obtain yellow quercetin (0.6 g). Synthesized quercetin was identical with the quercetin isolated from

Table II. Isolation of active component for the inhibition of FcεRI-mediated mast cell degranulation from hexane (F-1) and ethyl acetate (F-3) extracts of Castanea crenata

Inhibition of mast cell degranulation (% Control)							
		F-11	22.0				
		F-12	30.8				
		F-13	46.2				
		F-14	66.0				
		F-15	67.5				
F-1	68.2			F-161	34.0		
				F-162	71.7		
		F-16	70.8	F-163	52.5		
				F-164	24.7		
				F-165	74.7		
		F-17	47.9				
		F-31	23.9				
				F-321	88.8	F-3211	28.0
						F-3212	70.9
						F-3213	23.9
						F-3214	23.0
		F-32	81.9	F-322	61.7		
				F-323	66.8		
				F-324	56.7		
F-3	62.3			F-325	29.5		
		F-33	61.8				
				F-341	32.2		
				F-342	23.3		
		F-34		F-343			
				F-344	28.8		
				F-345	20.7		
		F-35	31.5				
		F-36	18.6				

F-1 and F-3 represent hexane and ethylacetate fraction, respectively. For each fraction, the left column shows the fraction number and the right column represents the percent of inhibition of FcεRI-mediated mast cell degranulation at the concentration of 100 μg/mL.

the leaves of *C. crenata* in terms of physicochemical properties and *in vitro* activities for the inhibition of mast cell degranulation (Fig. 1). Both synthesized and isolated quercetin from *C. crenata* effectively inhibited mast cell degranulation (EC_{50} value was about 5 μg/mL).

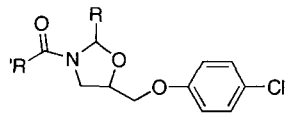
Excavation of lead compounds for the inhibition of mast cell degranulation using combinatorial chemistry approach

Flavonoids including quercetin have been thoroughly studied, for example, numerous flavonoid analogs were synthesized and tested for mast cell degranulation (Cheong *et al.*, 1998). To excavate new class of lead compounds more efficiently rather than through tedious bioactivity-guided assay for natural products, we employed combinatorial chemistry approach to produce various compounds. Previously we have reported the establishment of combinatorial library based on the synthesis of 1,3-oxazolidinedione derivatives using solid phase (Oh *et al.*, 2000). As a random screening strategy as to excavation of anti-allergic lead compounds, we decided to utilize the previously characterized combinatorial library.

Structures and activities of synthesized 1,3-oxazolidine derivatives on mast cell degranulation are shown in Table III. Even though it was not easy to reach a clear conclusion, still we could have certain information regarding their structure-activity relationships. When anti-allergic activities of the compounds **1-15** were compared, acetyl group at position 3 seems to provide better activities than other functional groups tested. In contrast, β-naphthyl group should be avoided at position 2 (compounds **16-21**). *p*-Chlorophenoxymethyl is a better substituent than *p*-hydroxymethylphenoxymethyl at 5 position of 1,3-oxazolidines (from compounds **1-2** vs **22-23**). Overall, 3,4-dichlorophenyl group in R position and methyl group in R position (compounds **1-2**) seems to be the best combinations among the assayed compounds. Their activities were better than quercetin, which is employed as positive control (15 and 60% inhibition at 3 and 10 μM, respectively). Quercetin is one of the strong inhibitors for mast cell degranulation (Lee *et al.*, 1999). Detailed structural information such as cis/trans configuration is described in our previous report (Oh *et al*, 2000).

Combinatorial synthesis specially utilizing solid phase reactions play an important role in building chemical libraries and provide novel guidance to the development of new biologically active compounds. Solid phase reactions provide vast number of compounds within a short time frame, and have been used to find and/or optimize a lead compound in drug discovery processes (Thompson and Ellman, 1996; Hall *et al.*, 2001; Dolle, 2002). In our study, the advantages of combinatorial chemistry in the search of new lead compounds with anti-allergic activities are well demonstrated. We could prepare battery of compounds in a short time frame without tedious isolation and identification processes, which are observed when dealing with natural products. Also these compounds belong to the structural group that is distinct from previously reported anti-allergic compounds, and our

Table III. Dose-response relationship for the inhibitory effects of water extract and quercetin on β-hexosaminidase release from RBL-2H3 cells



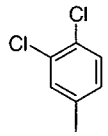
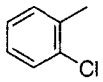
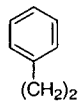
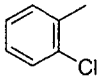
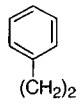
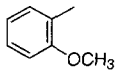
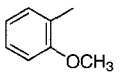
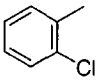
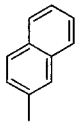
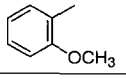
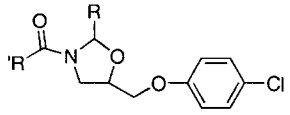
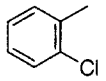
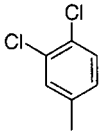
	R	R'	Stereoisomer	Activity (% inhibition at 10/3 μM)
1		CH_3	trans	74/45
2	〃	〃	cis	74/44
3	〃		trans	38/37
4	〃	〃	cis	20/4
5		CH_3	trans	60/16
6	〃	〃	cis	62/19
7	〃		trans	1/4
8	〃	〃	cis	38/34
9			cis	45/2
10		CH_3	trans	45/47
11	〃	〃	cis	45/44
12	〃		trans	53/39
13	〃	〃	cis	17/27
14	〃		trans	42/35
15	〃	〃	cis	59/46
16		CH_3	trans	1/5
17	〃	〃	cis	5/12
18	〃		trans	17/8

Table III. Continued



	R	R'	Stereoisomer	Activity (% inhibition at 10/3 μM)
19	〃	〃	cis	30/21
20	〃		trans	7/5
21	〃	〃	cis	16/29
22		〃	trans	19/26
23	〃	〃	cis	28/37

study suggests new family of compounds to search for the development of anti-allergic compounds. Furthermore, the compounds we tested are structurally related each other, and provided us valuable structure-activity information.

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