A Novel (S)-(+)-Decursin Derivative, (S)-(+)-3-(3,4-Dihydroxy-phenyl)-acrylic Acid 2,2-Dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]-chromen-3-yl-ester, Inhibits Ovalbumin-Induced Lung Inflammation in a Mouse Model of Asthma

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(S)-(+)-Decursin is a coumarin compound present in herbal extracts that has various biological activities. (S)-(+)-Decursin attenuates pathophysiologic progression in cancer, bacterial infection and neuropathy. Asthma is an inflammatory disease associated with increased infiltration of leukocytes, especially eosinophils, and secretion of mucus into the airways. Although (S)-(+)-decursin, as well as (S)-(+)-decursin analogues, have various pharmacological properties, the effect of these compounds on asthma is not known. In the present study, we synthesized (S)-(+)-3-(3,4-dihydroxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]-chromen-3-yl-ester (compound 6, C6) from (S)-(+)-decursin and examined if C6 had any inhibitory effects on lung inflammation in a mouse model of ovalbumin-induced asthma. C6 significantly inhibited the leukocytosis ($p < 0.01$) and eosinophilia ($p < 0.05$) in bronchoalveolar lavage (BAL) fluid. Examination of lung tissues stained with hematoxylin and eosin and periodic acid Schiff reagents showed that C6 suppressed the increased infiltration of inflammatory cells and elevated mucus hypersecretion. Protein levels of interleukin (IL)-5 ($p < 0.05$) and eotaxin ($p < 0.01$) were significantly reduced in BAL fluid by C6. C6 also significantly reduced total and ovalbumin-specific immunoglobulin E (IgE) levels in BAL fluid ($p < 0.01$) as well as that in serum ($p < 0.05$). C6 may have pharmacological effects for asthma and may be a potent therapeutic agent for the treatment of allergic airway diseases.

Key words (S)-(+)-decursin derivative; asthma; eosinophil; immunoglobulin E

A variety of herbal extracts consist of different chemicals with reported novel therapeutic properties. (S)-(+)-Decursin isolated from *Angelica gigas* NAKAI (*A. gigas* NAKAI) is a coumarin compound with various biological activities. *A. gigas* NAKAI, also known as Cham Dang Gui, belongs to the *Umbelliferae* family. Its root has been used as a traditional medicine for hemopoietic disease and enervation in Korea.^{1,2} (S)-(+)-Decursin has been studied for its anti-cancer, anti-bacterial and neuro-protective properties.^{3–6} Recently, the (S)-(+)-decursin analogue, decursinol angelate, also received attention for its potent pharmacological effects in various diseases.^{6–8} T helper (Th1) and Th2 cytokines play important roles in inflammatory responses after bacterial infection. Th2 cytokines including interleukin (IL)-4, IL-10 and IL-13 are increased in mycobacterial infection.⁹ Infection with *Helicobacter pylori* increases IL-4 and IL-6 productions.¹⁰ Asthma is also involved in alternation of Th2 cytokines.¹¹ We hypothesized that anti-bacterial property of (S)-(+)-decursin is associated with the suppression of cytokine release and the activity is effective on airway inflammation.

Asthma is an inflammatory disease of the airways whose prevalence is increasing yearly.¹² Asthma is characterized by airway remodeling and bronchial hyperresponsiveness induced by diverse stimuli; it results in wheezing, coughing, chest tightness and shortness of breath.^{13,14} The inflammation is associated with increased infiltration of leukocytes, especially eosinophils, and considerable secretion of mucus into the airways.¹⁵ Eosinophils associated with allergic inflammation are effector cells and play pathogenic roles in

asthma by their release of cytotoxic granule proteins.¹⁶

The recruitment of eosinophils to the airways is caused by various mediators, including chemokines. Eotaxin, a CC chemokine, is a potent chemoattractant for eosinophils and is generally elevated after asthma induction.¹⁷ Also, cytokines and inflammatory mediators released by Th2 lymphocytes increase in various allergic diseases and participate in the development of asthma.¹⁵ Among Th2 cytokines, IL-5 promotes the proliferation and activation of eosinophils and eosinophil infiltration into the airways.¹⁸ In the asthmatic reaction, immunoglobulin E (IgE) produced by plasma cells induces the degranulation of mast cells, which results in structural remodeling of the airways, including mucus hypersecretion and goblet cell metaplasia.^{19,20}

In the present study, to investigate a new agent with anti-inflammatory effects that might have more effective pharmacological properties than (S)-(+)-decursin, we synthesized (S)-(+)-3-(3,4-dihydroxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]-chromen-3-yl-ester (compound 6, C6) from (S)-(+)-decursin. We examined if this compound had any inhibitory effects on leukocytes infiltration into the airways, mucus secretion and the expressions of Th 2 cytokines and IgE in a mouse model of ovalbumin (OVA)-induced asthma.

MATERIALS AND METHODS

Preparation of (S)-(+)-3-(3,4-Dihydroxy-phenyl)-acrylic Acid 2,2-Dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]-chromen-3-yl-ester C6 was synthesized from (S)-(+)-

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Fig. 1. Structure of (S)-(+)-Decursin

Chart 1. Synthesis of (S)-(+)-3-(3,4-Dihydroxy-phenyl)-acrylic Acid 2,2-Dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]chromen-3-yl-ester (6)

(1) (S)-(+)-decursin as shown in Fig. 1, (2) caffeic acid, (3) 3-(3,4-diacetoxy-phenyl)-acrylic acid, (4) of 3-(3,4-diacetoxy-phenyl)-acryloyl chloride, (5) (S)-(+)-3-(3,4-diacetoxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]chromen-3-yl-ester, (6) (S)-(+)-3-(3,4-diacetoxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]chromen-3-yl-ester.

decursin (Fig. 1) in 4 steps as shown in Chart 1. In the first step, to a solution of caffeic acid (compound 2) (1 equivalent; eq) in 10 ml of pyridine was added acetic anhydride (10 eq) at room temperature. The reaction mixture was stirred for 1 d and concentrated *in vacuo*, and the desired compound 3 was obtained by a silica gel column chromatography with a 74.8% yield. In the second step, thionyl chloride (5 eq) was added to a solution of compound 3 (1 eq) in 18 ml of anhydrous benzene and a catalytic amount of *N,N*-dimethylformamide (1 drop) was added. The reaction mixture was refluxed for 6 h and then allowed to cool to room temperature. After removal of solvent by rotary evaporation, the residue was used for the next reaction. In the third step, to a solution of (S)-(+)-decursin (compound 1 in Fig. 1, 0.8 eq) and pyridine (2.4 eq) in 30 ml of anhydrous methylene chloride was added a solution of compound 4 in 20 ml of anhydrous methylene chloride. After stirring for 5 h at room temperature, the solvent was removed *in vacuo*. The desired compound 5 was obtained by a silica gel column chromatography with an 84.5% yield. In the final step, to a solution of compound 5 (1 eq) in 60 ml of acetone was added 20 ml of 30 N HCl. The mixture was refluxed for 5 h. After cooling to room temperature, the reaction mixture was evaporated, extracted with EA/H₂O and washed with brine. The washed solution was dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The desired compound 6 (C6) was obtained by silica gel column chromatography with a 93.2% yield.

Induction of Asthma in BALB/c Mice and Drug Administration Six-week-old female BALB/c mice were purchased from Daehan Biolink Co., Ltd. (Seoul, Korea). They were maintained at room temperature (about 22±1 °C) and humidity (about 55±10%) in an air-conditioned room. The mice were divided into 4 groups (*n*=10 per group), and 3 groups were induced for airway inflammation. Each mouse was actively sensitized as described in our previous publications.^{21,22} Briefly, mice were immunized by intraperitoneal (i.p.) injection of 20 µg of chicken ovalbumin (OVA) (Grade III; Sigma-Aldrich, Korea) with 1 mg aluminum hydroxide (Sigma-Aldrich) on days 1 and 14. OVA-challenged mice were immunized by inhalation with aerosolized 5% OVA so-

lution using an ultrasonic nebulizer (ME-U12, Omrom, Tokyo, Japan). Inhalation of OVA was continued for 1 h per day from days 21 to 27 after the second sensitization. Two groups of asthma-induced mice, C6 and dexamethasone (Dex) groups, were treated by i.p. injection with 1 mg/kg of C6 or Dex (Sigma-Aldrich) between days 14 and 27. C6 dissolved in dimethyl sulfoxide (DMSO) was diluted to less than 1/100 with phosphate buffered saline (PBS). Dex was used as a positive control for its repressive effects on asthma. The normal group was sensitized and given inhaled PBS without drug administration.

Bronchoalveolar Lavage (BAL) Fluid After days 14 from the second sensitization, the mice were sacrificed and BAL fluid was collected by lavage of the lung *via* the trachea with 1 ml of PBS. After 3 lavages, approximately 0.7 ml of recovered BAL fluid was centrifuged (400×*g*, 4 °C, 5 min). The supernatant was stored at -70 °C until analysis of cytokines and IgE. Cells in the BAL fluid were resuspended in PBS for total cell counts and differential counts of leukocytes. Total cell number was determined using a Neubauer hemocytometer, and cell suspensions were applied to a slide by cytospin and stained with Wright's stain for differential counts. The leukocytes were divided into neutrophil, eosinophil, lymphocyte and macrophage and these cells were differentiated by general leukocyte morphology. The percentage of each leukocyte was determined by 300 cells counting per cytospin slide.

Histological Analysis of Airway Inflammation Lung tissues were excised from the mice and fixed with Carnoy's solution for 3 h at 4 °C. Fixed tissues were embedded in paraffin, and cut into 5 µm sections with a microtome (Leica, Nussloch, Germany). Paraffin sections were placed on glass slides, deparaffinized, rehydrated and stained with hematoxylin and eosin (Sigma) to evaluate the infiltration of inflammatory cells into the peribronchial connective tissues. Periodic acid-Schiff stain (Sigma) was used to examine mucus production in lung. After staining of lung tissue, histological features for asthma were observed using a microscope.

Enzyme-linked Immunosorbent Assay (ELISA) of the BAL Fluid or Serum To determine the concentrations of cytokines in the BAL fluid or serum, we performed a sandwich ELISA using Opt EIA Set mouse IL-4, IL-5, interferon (IFN)-γ (BD Biosciences, San Diego, CA, U.S.A.) and Quantikine kit IL-13 and eotaxin (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. To measure the concentrations of IgE and OVA-specific IgE, the microwells were coated with anti-mouse IgE and 100 µg/ml of OVA per well, respectively, and were incubated overnight at 4 °C. After the preparation of anti-mouse IgE and OVA-coated well, we carried out a sandwich ELISA using Opt EIA Set mouse IgE (BD Biosciences) according to the manufacturer's instructions.

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Detection in Serum The concentrations of ALT and AST were measured by a Reitman-Frankel method using the serum of asthma-induced mice using ALT and AST set (Asan Pharm., Korea) according to the manufacturer's instructions. The data was expressed as the relative ratio to the normal group, which was set 100%.

Statistical Analysis The results are expressed as the

means $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA followed by least significance difference (LSD) test. SPSS statistical software, Version 10.0 (SPSS, Inc., Chicago, IL, U.S.A.) was used for the statistical analyses. A significant value was defined as $p < 0.05$.

RESULTS

C6 Has an Inhibitory Effect on Leukocytes Infiltration of Airways, Especially Eosinophils We induced asthma in BALB/c mice using OVA for examination of possible suppressive effects of synthesized compound C6 in asthma. To evaluate the total number of leukocytes and eosinophils in BAL fluid, we collected the cells from BAL fluid and stained them with Wright's stain. Total leukocytes and eosinophils in asthma-induced mice were remarkably increased compared to normal mice ($p < 0.01$; Fig. 2). However, C6 significantly inhibited the leukocytosis ($p < 0.01$) and eosinophilia ($p < 0.05$). From the results for differential leukocyte counts, C6 decreased the number of eosinophils ($p < 0.05$) and increased the proportion of macrophages compared with the asthmatic control ($p < 0.05$; Fig. 2B). Dex blocked the infiltration of total leukocytes, particularly eosinophils ($p < 0.01$), into the BAL fluid as described in previous studies.^{21,22} These results indicate that C6 has inhibitory effects on cellular components of lung inflammation in an asthma model.

C6 Blocks the Pathologic Changes of Lung Tissue in Asthmatic Mouse Model To determinate if C6-induced alterations of histological features in airways inflammation, we stained lung tissues using hematoxylin and eosin and periodic acid-Schiff stain. The lung tissues in the asthmatic mouse model had dense infiltrates of leukocytes around bronchi and significant mucus secretion by goblet cells within the bronchi compared to normal tissue (Fig. 3). C6 suppressed the increased leukocytes infiltration (Fig. 3A) and the hypersecretion of mucus in lung tissue (Fig. 3B). This is

in agreement with the results shown in Fig. 2, which suggest that C6 diminishes airway inflammation in asthma.

C6 Regulates Cytokine Release into BAL Fluid of the Asthmatic Mouse Model To investigate possible inhibitory mechanisms of C6 in airway inflammation, we examined the levels of cytokines associated with asthma in BAL fluid. In the asthmatic control mouse, IL-5 ($p < 0.05$)

Fig. 2. Effects of C6 on the Recruitment of Inflammatory Cells into BAL Fluid

The OVA-induced asthma model mice were divided into 3 treatment groups: untreated (Con), treated between the sensitization and inhalation periods with 1 mg/kg of compound 6 (C6) or dexamethasone (Dex). The negative control group (Normal) was sensitized and treated with PBS without drug administration. The recovered cells in BAL fluid were used for total cell counts and differential counts after Wright's staining (A and B). The results are the means $\pm$ S.E.M. of 10 independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate statistically significant differences between the normal group and asthma control group, or between the asthma control group and drug-treated group.

Fig. 3. Effects of C6 on Cell Infiltration and Mucus Hypersecretion in Lung Tissues

To confirm the pathologic changes in lung tissues, we performed hematoxylin and eosin staining (A) and periodic acid-Schiff staining (B) as described in Materials and Methods. Magnification $\times 200$.

Fig. 4. Effects of C6 on the Levels of Cytokines and Immunoglobulin E (IgE)

BAL fluid was collected and the cells were eliminated. (A) Levels of IL-4, IL-5, IL-13, eotaxin and IFN- γ in BAL fluid were analyzed by ELISA as described in Materials and Methods. Total and OVA-specific IgE levels in (B) BAL fluid and (C) serum were analyzed by ELISA. Results are the means $\pm$ S.E.M. of 10 independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate statistically significant differences between the normal group and asthma control group, or between the asthma control group and drug-treated group.

and eotaxin ($p < 0.01$) levels were significantly increased in BAL fluid compared with normal mice, and IFN- γ level was significantly decreased ($p < 0.01$) (Fig. 4A). C6 significantly suppressed the elevated expressions of IL-5 ($p < 0.05$) and eotaxin ($p < 0.01$) in BAL fluid (Fig. 4A). In contrast, IL-4, IL-13 and IFN- γ levels in BAL fluid were similar in the C6 and control groups (Fig. 4A). These results suggest that the suppressive effects of C6 on eosinophilia and airway inflammation are associated with the modulation of cytokine and chemokine release, such as IL-5 and eotaxin.

C6 Decreases the Levels of Total IgE and OVA-Specific IgE in BAL Fluid and Serum Because IgE is associated with Th2 cell-mediated immune responses in the pathogenesis of allergic inflammation, we investigated the effects of C6 on total IgE and OVA-specific IgE levels in BAL fluid and serum. Total and OVA-specific IgE in BAL fluid were significantly increased following asthma induction ($p < 0.05$) (Fig. 4B). C6 significantly inhibited both total ($p < 0.01$) and OVA-specific IgE ($p < 0.05$) in BAL fluid. Similarly, both total and OVA-specific IgE levels in serum were significantly elevated in asthma induction, and decreased by C6 treatment ($p < 0.01$, $p < 0.05$, respectively) (Fig. 4C).

C6 Has No Cytotoxicity in an Asthmatic Mouse Model

Fig. 5. Effects of C6 on a Liver Toxicity

The alterations of AST (A) and ALT levels (B) in serum were measured by Reitman–Frankel methods. Data are expressed as the means $\pm$ S.E.M., and the data are presented in relation to the negative control, which was set at 100%.

To confirm the cytotoxicity of C6, we investigated the alteration of ALT and AST levels in serum. The ALT and AST levels are known as indicators of cellular damage in liver. The levels of ALT and AST were detected by Reitman–Frankel methods, and were not altered by administration of C6 (Fig. 5). These results indicate that C6 may have not the cytotoxic effect on an asthmatic mouse model.

DISCUSSION

The increasing prevalence of asthma is a worldwide problem, and there is a pressing need for the development of new agents for the treatment of asthma. In this study, we focused on the possibility of a chemical compound derived from (*S*)-(+)-decursin as an anti-allergic drug for asthma therapy using an asthmatic mouse model induced by ovalbumin. We first prepared the new compound from (*S*)-(+)-decursin: (*S*)-(+)-3-(3,4-dihydroxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2*H*,8*H*-pyrano[3,2-*g*]chromen-3-yl-ester (compound **6**, C6). We then demonstrated that: (1) C6 significantly inhibits total leukocytosis and eosinophilia in BAL fluid; (2) C6 diminishes the infiltration of leukocytes and mucus hypersecretion in the lung tissue; (3) C6 decreases the levels of IL-5 and eotaxin in BAL fluid; and (4) C6 reduces the levels of total IgE and OVA-specific IgE in serum and BAL fluid.

(*S*)-(+)-Decursin is a coumarin compound that inhibits the progression of various diseases, including cancer, inflammation and neurologic disorders.^{3–6} A decursin analogue is also effective for the modulation of various tumors.^{7,8} However, the effects of these chemical compounds with anti-inflammatory effects have not been reported for airways inflammatory diseases, such as asthma. An inflammatory response is important during the development and pathogenesis of asthma. The associated cytokines or chemokines activate hemopoiesis in the bone marrow and induces movement of

matured eosinophils in peripheral blood into lung tissues.^{17,23)} In our study, we determined the anti-inflammatory effects of a novel (S)-(+)-decursin derivative (C6).

As shown in Figs. 2 and 3A, the number of leukocytes, especially eosinophils, in BAL fluid and peribronchiolar areas of lung tissue were increased following the induction of asthma in mice, and these were decreased by C6. Other chemicals have been reported for their pharmacological effects on allergic disease. An adenosine receptor antagonist, C0036E08, blocked histamine release by a human mast cell line (HMC-1 cell).²⁴⁾ A novel capsazepine analogue, capsazepinoid, prevented leukotriene D₄-induced contractions of human small airways.²⁵⁾ However, these chemicals were not effective on the recruitment of eosinophils in the airway. Eosinophils release cytotoxic granule proteins, including major basic protein, eosinophilic cationic protein and eosinophil peroxidase in allergic diseases and they correlate with asthma severity.^{17,18)}

In addition, mucus hypersecretion in lung tissue was reduced by C6 (Fig. 3B). In asthma, hypertrophy of goblet cells and mucus production are increased and accompany airway obstruction.²⁶⁾ These results indicate that C6 may affect inflammatory cells and inhibit their activities in the development of asthmatic responses. Mucus hypersecretion in the airway inflammation is associated with IL-4 and IL-13 and it is also involved in other cytokines and growth factors, such as epidermal growth factor (EGF) and transforming growth factor (TGF)- β . Increased tumor necrosis factor- α (TNF- α) in the airways induces EGF receptor expression in the airway epithelium and a ligand for EGF receptor, such as EGF and TGF- β , binds to EGF receptor and the activated receptor finally causes mucin gene and protein expressions, resulting in mucus secretion.^{27,28)} TGF- β also increases the goblet cell proliferation. Because IL-4 and IL-13 levels in BAL fluid are not altered after the C6 treatment, we think that the inhibitory effect of C6 on mucus hypersecretion may be caused by the complex regulation of other factors, such as TGF- β and EGF. Further experiments will be needed to better understand the underlying mechanisms and it is currently under investigation. Inflammatory cells produce and release many cytokines and mediators of inflammation that play important roles in the initiation and development of asthma. The protein levels of IL-5 and eotaxin were increased in positive asthmatic control mice, but were inhibited by C6 (Fig. 4A). Eotaxin is important for the early derivation of eosinophils from the bone marrow, and these cells are matured by IL-5. Mature eosinophils are recruited by IL-5 into the airways.²⁹⁾ Eosinophils in the airways mediate the release of transforming growth factor (TGF)- β 1, which increases fibroblast proliferation and maturation, followed by airway remodeling.³⁰⁾

In contrast, IFN- γ levels in BAL fluid were decreased following induction of asthma (Fig. 4A). IFN- γ is a Th1 cytokine that counteracts Th2 immune responses, and has inhibitory effects on the allergic response and various inflammatory responses.^{31,32)} However, C6 had no effect on the inhibition of IFN- γ . These results show that the inhibition of eosinophilia and mucus production by C6 is associated with reduced IL-5 and eotaxin, but that C6 is not affective on IFN- γ modulation.

In addition, C6 blocked the increased total and OVA-specific IgE in serum and BAL fluid after the induction of

asthma (Figs. 4B, C). Elevated IgE in serum or BAL fluid is associated with the development and the pathogenesis of asthma.³³⁾ IgE binds to high-affinity receptors on mast cells and receptor cross-linkage induces the degranulation of mast cells. In asthma, mast cells migrate into the lung tissue and secrete proinflammatory mediators, such as histamine, prostaglandin, and cytokines.¹⁸⁾ They play important roles in the development of asthma. Because C6 blocked the leukocytes migration into airways, we also examined the inhibitory effect of C6 on chemotaxis in a human mast cell line (HMC-1). In chemotaxis assay for HMC-1 cells, we used stem cell factor (SCF) that has been known as a crucial chemoattractant in the migration of mast cells.³⁴⁾ After treatment with 10 μ g/ml of C6 for 24 h, HMC-1 cell migration in response to SCF was inhibited up to 20% (data not shown). This result shows that C6 may block the infiltration of mast cells and then it may decrease the receptor cross-linkage with IgE on mast cells.

To examine the cytotoxicity of C6, we studied alterations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum. AST and ALT are members of the transaminase family that are increased in serum following hepatocellular damage,³⁵⁾ and they have been used for confirmation of drug liver toxicity. Both AST and ALT were not altered by the i.p. injection of C6 (Fig. 5). We also investigated the cytotoxic effects of C6 on mouse splenocytes. Splenocytes were isolated from mouse spleens and splenocyte survival was measured by MTT assay after addition of C6 to cell suspensions. C6 had no cytotoxic effects on the splenocytes (data not shown). These results indicate that the concentration of C6 used in this study had no apparent cytotoxic effects on the mice.

In conclusion, we found that C6 inhibited leukocytosis and eosinophilia in lung tissue, and that C6 alleviated the pathogenesis of lung tissue damage in the asthmatic mouse model. Although the modulation mechanism by C6 in asthma is not known, these results suggest that the anti-allergic effects of C6 may be associated with the inhibition of IL-5, eotaxin and IgE production. We suggest that C6 may have pharmacological effects for asthma and that it may be beneficial for the treatment of allergic airways disease.

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