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Homoisoflavonoid derivatives from the roots of *Ophiopogon japonicus* and their in vitro anti-inflammation activity

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ABSTRACT

Three new homoisoflavonoids (**1**–**3**) were isolated from the roots of *Ophiopogon japonicus* (Liliaceae). The structures of new metabolites were determined on the basis of spectroscopic analyses including 2D NMR. The anti-inflammatory activities of new compounds (**1**–**3**) were investigated by their effects on the release of the inflammatory chemokine eotaxin, stimulated by IL-4 and the combination of IL-4 and TNF-α in BEAS-2B cells, which mimics the in vivo conditions in bronchial allergic asthma.

Asthma is an allergic inflammatory disease of the airways. The interaction between bronchial epithelial cells and eosinophils is an important feature of an asthma attack. Most evidence suggests that eosinophilic infiltration and activation may account for the unique, spasmodic, and cyclic nature of hyperreactive airways.[1] The mechanisms underlying the selective recruitment of eosinophils are complex, and include multistep processes, probably mediated by the cooperative action between cytokines that cause eosinophil priming and increased survival interleukin [IL]-3, IL-5, granulocytemacrophage colony stimulating factor, and those that activate the endothelium IL-1, tumor necrosis factor (TNF)-α, IL-4, IL-13, and eosinophilselective chemoattractant molecules, especially C-C chemokines.[2] Eotaxin is a C–C chemokine implicated in the recruitment of eosinophils in a variety of inflammatory disorders and, unlike all other eosinophil chemoattractants, is eosinophil-specific.[3] This characterizes eotaxin as a key mediator in allergic diseases of which eosinophilic infiltration is characteristic.[3,4] Cultured bronchial epithelial cells, including normal bronchial epithelium, have been observed to produce eotaxin after stimulation with TNF-α and T helper (Th) type 2 cytokines.[5]

During a screening procedure on higher plants to find novel candidates as anti-inflammatory agents, the 70% EtOH extract of the roots of *Ophiopogon japonicus* Ker-Gawler (Liliaceae) was shown to exhibit considerable inhibitory activity. *O. japonicus* is an evergreen perennial. Its tuber is sweet with a slightly bitter aftertaste and have been employed in traditional Chinese medicine as an expectorant, antitussive, and tonic agent as well as showing pharmacological effects on the cardiovascular system.[6] It is recommended for latent heat in the lungs due to 'yin'-asthenia, fever in consumptive disease or general debility, dehydration of febrile disease, and dry mouth.[7] In the folk medicine of Vietnam it serves as expectorant, anti-cough and tonic agent.[8] Previous phytochemical studies of the tuber derived from *O. japonicus* resulted in the isolation of homoisoflavonoids,[9,10] saponins,[11] and amides,[12] as well as monoterpene glycosides.[10b,13] Homoisoflavonoids have been shown to potential anti-oxidation activities in vitro.[14] Recently, the anti-inflammatory effect of the aqueous extract from radix *O. japonicus* was examined in mouse and rat models, and results demonstrate that the aqueous extract presents remarkable anti-inflammatory activity, ruscogenin and ophiopogonin D are two of its active components, which supported its traditional use in the

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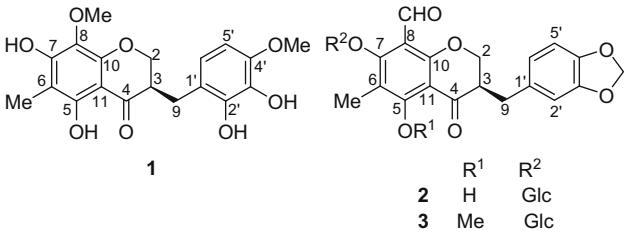

Figure 1. Chemical structures of isolated compounds **1–3**.

treatment of various diseases associated with inflammation.[15] However, no data is available on the influence of the components on eotaxin expression in bronchial epithelial cells, BEAS-2B, in vitro. In our present study, three compounds including a new homoisoflavonoid (**1**), and two new homoisoflavonoid glycosides (**2** and **3**) were isolated from the ethyl acetate fraction of the roots of *O. japonicus*. This paper reports the isolation, structure elucidation of the new isolated metabolites. In addition, the ability to suppress eotaxin expression stimulated by the Th2 cytokine IL-4 alone, and/or in combination with TNF-α in BEAS-2B of these homoisoflavonoids were determined.

Repeated chromatography of the EtOAc-soluble fraction of the 70% EtOH extract of *O. japonicus* on silica gel, YMC gel, Sephadex LH-20, and C_{18} columns led to the isolation of three new compounds (**1**–**3**).[16]

Compound **1** was obtained as colorless needles with the molecular formula $C_{19}H_{20}O_8$, as established by positive HR-FAB-MS with a $[M+Na]^+$ ion at *m*/*z* 399.1058. The IR spectrum indicated the presence of a phenolic hydroxy at 3395 and carbonyl group at 1640 cm^{-1}. In the UV spectrum, λ_{max} value at 280 nm was observed.[17a] The ^1H NMR signals at δ 4.25 (1H, dd, *J* = 7.8, 11.5 Hz, H-2ax), 4.43 (1H, dd, *J* = 5.0, 11.5 Hz, H-2 eq), and 3.14 (1H, m, H-3ax) showed the protons of the γ-dihydropyrone moiety of a homoisoflavanone.[9,10] The ^1H NMR spectrum of **1** also indicated the presence of a methyl group at δ 2.05 (3H, s), and two methoxy groups attached to an aromatic nucleus at δ 3.85 (3H, s) and 3.76 (3H, s). In addition, two benzylmethylene protons appeared at δ 2.84 (1H, dd, *J* = 10.5, 14.5 Hz, H-9a) and 3.89 (1H, dd, *J* = 4.5, 14.5 Hz, H-9b), and a pair of proton signals at δ 6.54 (1H, d, *J* = 8.2 Hz, H-5′) and 6.81 (1H, d, *J* = 8.2 Hz, H-6′) was evidence for two aromatic protons. The ^{13}C NMR and DEPT spectrum of **1** showed 19 carbon signals in the molecule, which supported for the homoisoflavanone structure. The full NMR assignments and connectivities of **1** were determined by the HMQC and HMBC spectroscopic data analysis. The HMBC spectrum confirmed the correlations between methyl protons (δ_H 2.05, s) and carbon signals at δ_C 103.5 and 158.2 indicated for the location of this methyl group at position C-6. The diagnostic long-range correlations were observed for two methoxy protons (δ_H 3.85, s) and (δ_H 3.76, s) to δ_C 128.1, and 159.8, which confirmed the location of these methoxy at C-8, and C-4′, respectively. In addition, the diagnostic long-range correlations were observed for the protons of the γ-dihydropyrone moiety and C-4 (δ 195.8), the benzylmethylene protons and C-3 (δ 46.7), C-4 (δ 195.8), and C-1′ (δ 117.5) (Fig 2). Based on the above analysis, the structure of compound **1** was elucidated as 5,7,2′,3′-tetrahydroxy-6-methyl-8-methoxy-3-(4′-methoxybenzyl)chroman-4-one, a new compound named ophiopogonanone G (Table 1).

Compound **2** was obtained as colorless needles. Its positive HR-FAB-MS showed a $[M+Na]^+$ ion at *m*/*z* 541.1320, which established the molecular formula $C_{25}H_{26}O_{12}$ of **2**. The IR spectrum showed absorption bands at 3440 (OH) and 1618 (C=O) cm^{-1}.[17b] The ^1H NMR spectrum of **2** indicated the presence of a methylenedioxy group at δ 5.95 (2H, s), an aldehyde group at δ 10.05 (1H, s), and a methyl group attached to an aromatic nucleus at δ 2.10. Also in the ^1H NMR spectrum, the proton signals at δ 4.28 (1H, dd, *J* = 8.0, 11.0 Hz, H-2ax), δ 4.45 (1H, dd, *J* = 4.8, 11.0 Hz, H-2 eq), and δ 3.20 (1H, m, H-3ax) were assigned for the γ-dihydropyrone moiety of a homoisoflavanone.[9,10] In addition, two benzylmethylene protons appeared at δ 2.67 (1H, dd, *J* = 10.5, 14.0 Hz, H-9a), and δ 3.82 (1H, dd, *J* = 4.5, 14.0 Hz, H-9b), and three ABX aromatic protons appeared at δ 6.75 (1H, d, *J* = 1.4 Hz, H-2′), 6.72 (1H, d, *J* = 8.0 Hz, H-5′), and 6.82 (1H, dd, *J* = 1.4, 8.0 Hz, H-6′). Furthermore, the ^1H NMR spectrum of **2** showed the presence of signals corresponding to an anomeric proton of a sugar moiety appeared at δ 4.95 (1H, d, *J* = 7.8 Hz, H-1″). The ^{13}C NMR and DEPT spectrum of **2** showed 25 carbon signals in the molecule. Among them, six signals at δ 105.4, 75.5, 77.4, 71.8, 78.3 and 62.9 belonged to a glucose unit, and the other 19 signals appearing belonged to a chroman-4-one skeleton. These data suggested that compound **2** was a monoglucoside of ophiopogonanone C.[9,10] All ^1H and ^{13}C NMR signal assignments of **2** were confirmed by the present study from the HMQC and HMBC spectra (Fig. 2). The sugar was assigned as glucopyranose on the basis of NMR data and the R_f value compared with authentic glucose after enzymatic (naringinase) hydrolysis of **2**.[18] The $J_{H,H}$ value (7.8 Hz) of the anomeric proton (H-1″) indicated that glucose was linked via a β-linkage. In addition, the position of the glucose linkage in **2** was established at the C-6 of the homoisoflavonoid moiety by the HMBC technique (Fig. 2). Thus, the structure of the **2** was established as 5-hydroxy-6-methyl-7-[*O*-β-D-glucopyranoside]- 8-aldehydo-3-(3′,4′-methylenedioxybenzyl)chroman-4-one, a new homoisoflavonoid glycoside named as ophiopogoside A.

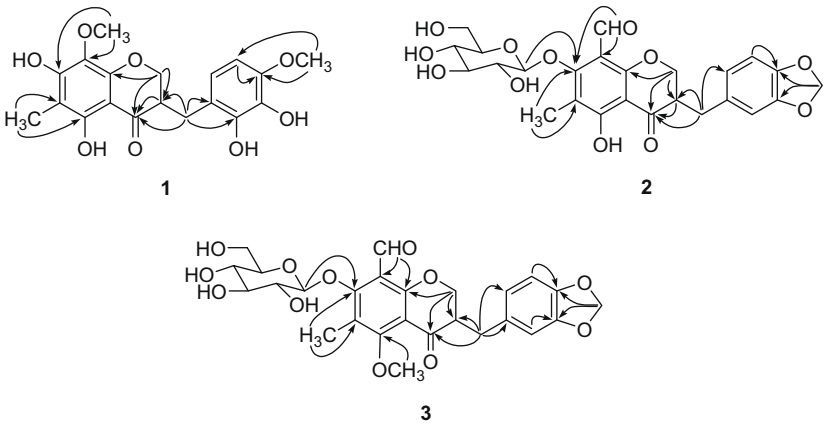

Figure 2. Selected HMBC correlations of **1–3**.

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds **1-3** (pyridine-d_5)

Position	1		2		3	
	δ_H[a]	δ_C	δ_H	δ_C	δ_H	δ_C
2	4.43, dd, 5.0, 11.5 4.25, dd, 7.8, 11.5	69.5	4.45, dd, 4.8, 11.0 4.28, dd, 8.0, 11.0	70.2	4.40, dd, 5.0, 11.5 4.24, dd, 8.0, 11.5	70.1
3	3.14, m	46.7	3.20, m	33.1	3.18, m	32.7
4		195.8		196.1		195.7
5		158.2		167.1		168.4
6		103.5		102.9		114.7
7		157.4		169.7		169.5
8		128.1		105.4		104.6
9	3.89, dd, 4.5, 14.5 2.84, dd, 10.5, 14.5	32.5	3.82, dd, 4.5, 14.0 2.67, dd, 10.5, 14.0		3.85, dd, 5.0, 14.5 2.74, dd, 10.5, 14.5	
10		152.7		165.2		165.0
11		102.4		102.4		107.7
1′		117.5		131.7		131.5
2′		156.7	6.75, d, 1.4	109.6	6.76, d, 1.8	108.5
3′		155.3		148.4		148.2
4′		159.8		146.9		146.7
5′	6.54, d, 8.2	108.8	6.72, d, 8.0	108.3	6.74, d, 7.9	108.5
6′	6.81, d, 8.2	131.0	6.82, dd, 1.4, 8.0	125.4	6.85, dd, 1.8, 7.9	126.1
5-OMe					3.68, s	60.7
6-Me	2.05, s	7.8	2.10, s	7.5	2.08, s	8.1
8-OMe	3.85, s	55.7				
8-CHO			10.05, s	190.7	10.1, s	191.4
4′-OMe	3.76, s	58.4				
–OCH_2O–			5.95, s	101.7	5.92, s	101.3
1″			4.95, d, 7.8	105.4	5.08, d, 7.8	104.7
2″			3.82, m	75.5	3.85, m	74.5
3″			3.75, m	77.4	3.72, m	78.6
4″			3.85, m	71.8	3.81, m	72.8
5″			4.00, m	78.3	3.90, m	78.5
6″			3.92, dd, 2.2, 12.0 3.57, m	62.9	4.02, dd, 2.0, 12.2 3.57, m	62.4

[a] Mult, *J* in hertz.

Compound **3** was obtained as a colorless powder. The positive HR-FAB-MS spectrum showed the $[M+Na]^+$ peak at *m*/*z* 555.1477, which established a molecular formula of $C_{26}H_{28}O_{12}$[17c] The ^{1}H and ^{13}C NMR spectra of **3** also showed seven and six characteristic signals of a sugar moiety in the region ranging from δ_H 3.57 to 5.08 and from δ_C 62.4 to 104.7, respectively. The sugar was identified as glucose and its β-glycosidic linkage was revealed on the basis of the large vicinal coupling constant at δ 5.08 (1H, d, J = 7.8 Hz, H-1″). The linkage position of glucose was confirmed by HMBC correlation between H-1″and C-6 (Fig. 2). The spectral feature demonstrated its close similarity to compound **2**, except for the addition of a methoxy group at δ_H 3.68 (3H, s) and δ_C 60.7. The attachment of methoxy group was confirmed by the correlation signal between δ_H 3.68 (-OCH_3) and δ_C 168.4 (C-5) in HMBC spectrum (Fig. 2). Thus, compound **3** was deduced to be 5-methoxy-6-methyl-7-[*O*-β-D-glucopyranoside]-8-aldehydo-3-(3′,4′-methylenedioxybenzyl)chroman-4-one, named ophiopogoside B.

Asthma is defined as an obstructive disease of the pulmonary airways resulting from spasms of airway muscle, increased mucus secretion, and inflammation.[5] In our experiments, we investigated the ability of isolated compounds to regulate cytokine-induced eotaxin expression in the human bronchial epithelial cell line BEAS-2B. In a preliminary study, the cytotoxic effects of isolated compounds were evaluated using the MTT assay,[19] these compounds did not affect the cell viability of BEAS-2B cells in either the presence or absence of IL-4 even at a dose of 25 μM after a period of 48 h.[20] There was less significant obvious difference between the control and treated cells. In the next study, ophiopogonanone G (**1**), ophiopogoside A (**2**), and ophiopogoside B (**3**) were tested whether they suppressed IL-4-induced eotaxin production in BEAS-2B cells. During incubation time of 48 h, BEAS-2B cells produced 10.7 pg/mL in the resting stage. However, after IL-4 stimulation (20 ng/mL), eotaxin production increased dramatically to 60.5 pg/mL. Compounds (**1**–**3**) (1.0, 5.0, 10.0 and 25.0 μM) significantly downregulated IL-4-induced eotaxin production in a dose-dependent manner (Fig. 3A). At the concentration of 25 μM, compounds **1**–**3** reduced eotaxin production to 30.8, 28.5 and 25.5 pg/mL, respectively. Osthol, a positive control,[20,21] significantly inhibited IL-4-induced eotaxin production to 20.1 and 12.4 pg/mL at the concentration of 10.0, and 25.0 μM, respectively. In this experiment, ophiopogonanone G (**1**) with no glucose moiety in the structure showed somewhat weaker activity than two others. To examine whether the tested compounds could inhibit eotaxin production in the combination of IL-4 and TNF-α stimulation, the cell were pre-incubated with compounds (**2** and **3**) 2 hour and then activated with IL-4 (10 ng/mL) and TNF-α (10 ng/mL). The BEAS-2B cells produced 28.1 pg/mL eotaxin in the resting stage, however, after stimulation, eotaxin production increased to 456.2 pg/mL. Ophiopogoside A (**2**) (5.0, 10.0 and 25.0 μM) suppressed IL-4- and TNF-α-induced eotaxin expression of BEAS-2B cells in a dose-dependent manner with the production from 435.7 to 215.2 pg/mL. In the same manipulation, ophiopogoside B (**3**) reduced eotaxin production to 198.4 pg/mL at the concentration of 25.0 μM (Fig. 3B). In this experiment, osthol, at the concentration of 25.0 μM, decreased eotaxin production to 80.5 pg/mL. Eosinophils including eotaxin are important inflammatory effectors cells that accumulate at the site of allergic inflammation, such as the airway submucosa.[22,23] The activated eosinophils release cytotoxic molecules such as major basic proteins, eosinophil peroxidase, eosinophilic cationic protein, lipid mediators and cytokines that cause tissue damage and consequently result in the manifestations of allergic diseases, such as allergic asthma. The interaction of bronchial epithelium with eosinophils represents a crucial mechanism in the initiation of lo-

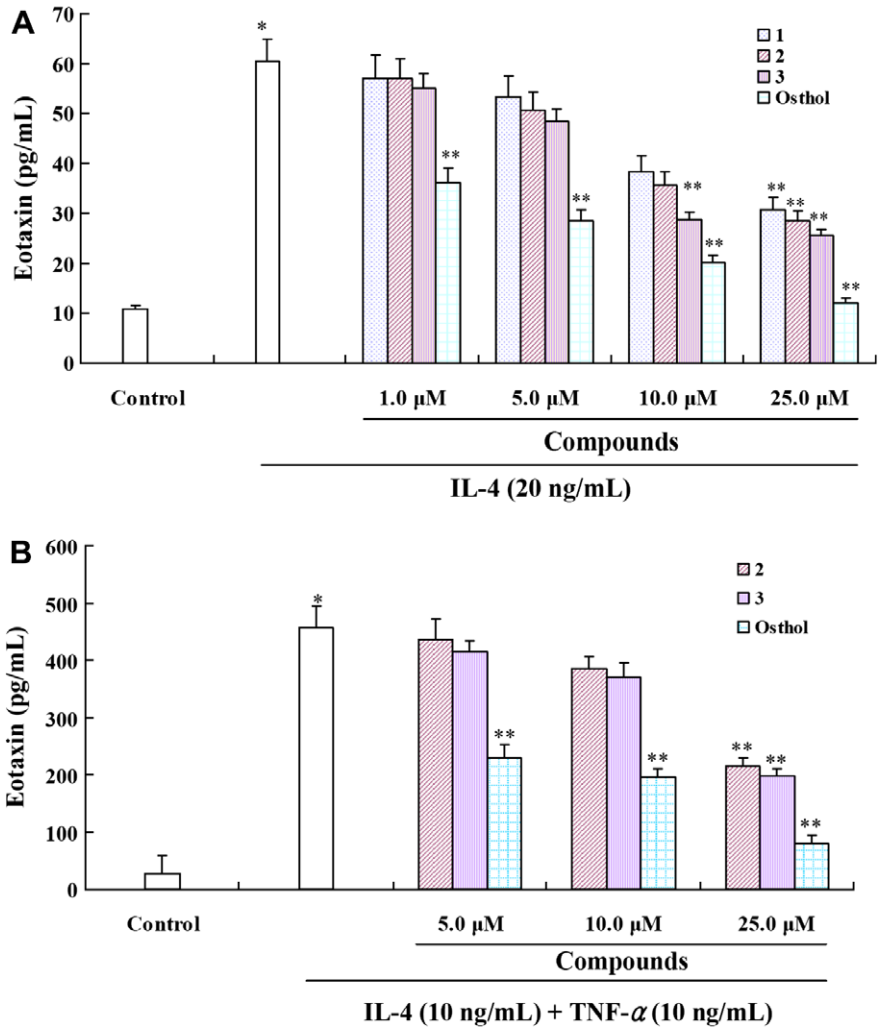

Figure 3. BEAS-2B cells were pretreated with isolated compounds and positive compound, osthol, 2 hs before IL-4 stimulation (20 ng/mL) (A) and before IL-4 (10 ng/mL) plus TNF-α (10 ng/mL) stimulation (B). Cell supernatants were collected for measurement of eotaxin concentration after 48 h incubation. Control value was obtained in the absence of stimulation and tested compounds. These data represent the average values of three repeated experiments. $^{*}p < 0.05$ versus control, $^{**}p < 0.001$ versus stimulated groups.

cal inflammation in allergic asthma.[5] In this study, we examined the release of the inflammatory chemokine eotaxin, stimulated by IL-4 and the combination of IL-4 and TNF-α in BEAS-2B cells, which mimics the in vivo conditions in bronchial allergic asthma. The suppressive effect of the new isolated metabolites on IL-4-induced eotaxin expression demonstrated in our study suggests that the *O. japonicus* and its homoisoflavonoids components may be of benefit in allergic diseases.

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16. The roots of plant were collected from Hoa Binh province, North of Vietnam, in July 2006 and identified by Professor Pham Thanh Ky, Department of Pharmacognosy, Hanoi College of Pharmacy. A voucher specimen (HN-00148) was deposited in the herbarium of the Hanoi College of Pharmacy. The dried roots of *Ophiopogon japonicus* (0.8 kg) were extracted with 2 L of 70% ethanol, three times. The 70% EtOH extract was combined and concentrated to yield a residue which was suspended in water and then successively partitioned with $CHCl_3$, EtOAc, and BuOH to afford $CHCl_3$-, EtOAc-, and BuOH-soluble fractions. The EtOAc fraction (11.3 g) was separated by silica gel column chromatography using a gradient of $CHCl_3$/MeOH (from 100:1 to 1:1) to yield ten fractions (E1–E10) according to their TLC profiles. The fraction E8 (0.82 g) was chromatographed over silica gel column using a gradient of $CHCl_3$/MeOH (from 20:1 to 5:1), to yield three subfractions E8.1–E8.3. Fraction E8.2 was further purified by semi preparative HPLC [RS Tech Optima Pak C_{18} column (10×250 mm, 10 μm particle size); mobile phase acetonitrile (CAN)/H_2O (75:25) with 0.1% formic acid; flow rate 2 mL/min; UV detection at 205 nm], resulted in the isolation of compound **1** (6.8 mg; t_R = 46.1 min). The E9 fraction was also purified by semi preparative HPLC [RS Tech Optima Pak C_{18} column (10×250 mm, 10 μm particle size); mobile phase MeOH/H_2O (80:20) with 0.1% formic acid; flow rate 2 mL/min; UV detection at 205 nm] resulted in the isolation of compound **2** (5.1 mg; t_R = 21.7 min) and **3** (5.5 mg; t_R = 32.4 min) (Fig. 1).
17. (a) Physicochemical of new compounds: compound **1**: yellow glue-like solid, $[\alpha]_D^{25}$ −14.4 (*c* 0.10, MeOH), UV λ_{max} (MeOH) nm: 280; IR (KBr) cm^{-1}: 3395, 2922, 1640; FAB-MS *m*/*z* 399.1 $[M+Na]^+$; HR-FAB-MS *m*/*z* 399.1058 $[M+Na]^+$ (calcd for $C_{19}H_{20}O_8Na$, 399.1062); for ^1H and ^{13}C NMR spectral data, see Table 1.; (b) Compound **2**: yellow amorphous powder, $[\alpha]_D^{25}$ −81.4 (*c* 0.10, MeOH), UV λ_{max} (MeOH) nm: 285, 320; IR (KBr) cm^{-1}: 3440, 2920, 2860, 1618; FAB-MS *m*/*z* 541.1 $[M+Na]^+$; HR-FAB-MS *m*/*z* 541.1320 $[M+Na]^+$ (calcd for $C_{25}H_{26}O_{12}Na$, 541.1326); for ^1H and ^{13}C NMR spectral data, see Table 1.; c Compound **3**: yellow amorphous powder, $[\alpha]_D^{25}$ −127.7 (*c* 0.10, MeOH), UV λ_{max} (MeOH) nm: 290, 320; IR (KBr) cm^{-1}: 3451, 2917, 2857, 1620; FAB-MS *m*/*z* 555.1 $[M+Na]^+$; HR-FAB-MS *m*/*z* 555.1477 $[M+Na]^+$ (calcd for $C_{26}H_{28}O_{12}Na$, 555.1480); for ^1H and ^{13}C NMR spectral data, see Table 1.
18. (a) (a) Naringinase (100 mg, from *Penicillium decumbens*) was added to a suspension of **2** and **3** (3 mg) in 50 mM acetate buffer (pH 5.5), and the mixture was stirred at 37 °C for 5 h. The reaction mixture was extracted with EtOAc (10 mL × 3), and the water layer was checked by silica gel TLC (EtOAc–MeOH–H_2O–AcOH, 65:20:15:15). The spot on the TLC plate was visualized by an anisaldehyde-H_2SO_4 reagent. The configuration of glucose was determined by a GC method, and the sugar derivative thus obtained showed a retention time of 21.30 min, identical with that of authentic D-glucose; (b) Min, B. S.; Na, M. K.; Oh, S. R.; Ahn, K. S.; Jeong, G. S.; Li, G.; Lee, S. K.; Joung, H.; Lee, H. K., *J. Nat. Prod*. **2004**, *67*, 1980; (b) Min, B. S.; Na, M. K.; Oh, S. R.; Ahn, K. S.; Jeong, G. S.; Li, G.; Lee, S. K.; Joung, H.; Lee, H. K. *J. Nat. Prod.* **2004**, *67*, 1980.
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20. Human bronchial epithelial BEAS-2B cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI-1640 medium (Invitrogen, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Gibco) at 37 °C in 5% CO_2 in a humidified incubator. Cells were centrifuged and resuspended in fresh media in 12-well plates at a concentration of 5×10^5/mL for 12 h before experimental use. When BEAS-2B cells reached 80% confluence in the 12-well plates the culture medium was replaced with RPMI-1640 without FBS. The recombinant human IL-4 and TNF-α were purchased from R&D systems (Minneapolis, MN, USA). The cells were then pretreated with different concentrations of isolated compounds for 2 h before stimulation with IL-4 (20 ng/mL) alone, or IL-4 (10 ng/mL) combined with TNF-α (10 ng/mL). Cell supernatants were collected after 48 h for ELISA of eotaxin. Culture supernatants were used to detect the production of eotaxin using sandwich ELISA from R&D Systems, performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).
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