



Inhibitory Effects of Actinidiamide from *Actinidia polygama* on Allergy and Inflammation

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Actinidia polygama Max. was subjected to supercritical fluid extraction (SFE), and the resulting ethanol extract of marc (SFEM) was subjected to sequential fractionation with various solvents. Each extract and fraction was assayed for anti-inflammatory effect. The ethyl acetate fraction (EtOAc) contained the highest level (70.8% inhibition) of anti-inflammatory activity. In order to identify the active constituents, the EtOAc fraction was further fractionated by silica gel and ODS column chromatography. By activity-guided fractionation, an active ceramide was identified as the anti-inflammatory component, and its structure was determined by NMR and MS analysis. The novel ceramide was named actinidiamide, and was found significantly to inhibit nitric oxide (NO) production (30.6% inhibition at 1 µg/mL) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and β -hexosaminidase release (91.8% inhibition at 1 µg/mL) in IgE-sensitized RBL-2H3 cells. Thus the presence of actinidiamide conveys allergy and inflammation treatment ability to *A. polygama*.

Key words: *Actinidia polygama* Max.; actinidiamide; anti-allergy; anti-inflammation; nitric oxide (NO) production

Actinidia polygama Max. (silver vine) is used as a folk medicine in Korea for treating pain, gout, and inflammation. Only a few studies have provided detailed information on the medicinal effects of *A. polygama* fructus extracts. A water extract of *A. polygama* suppressed rat paw edema induced by carrageenan, inhibited the production of nitric oxide (NO) induced by lipopolysaccharide (LPS), and inhibited the expression of inducible NO synthase (iNOS) protein in RAW 264.7 macrophages.¹⁾ Additionally, it demonstrated anti-asthmatic effects in a murine model of asthma.²⁾ Recently, α -linoleic acid was identified as an active component of *A. polygama* fruits. It strongly inhibits NO production, iNOS, cyclooxygenase (COX)-2, and tumor necrosis factor (TNF)- α expression by blocking the activation of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs) in LPS-activated RAW 264.7

cells.³⁾ Moreover, it inhibited the vascular permeability induced by acetic acid and the edema induced by carrageenan in mouse and rat.³⁾ It is not clear, however, whether α -linoleic acid is the main component responsible for reducing inflammation and allergic reactions.

iNOS can be induced to produce NO by bacterial LPS or immunological stimuli such as interferon (IFN)- γ , and sustained NO synthesis mediates inflammatory reactions in macrophages and other cells. Macrophages play a major role in innate immunity, initiating and propagating defensive reactions against pathogens. Excess NO production by macrophages and other cells exposed to endotoxins can contribute to cerebral injury,⁴⁾ myocardial ischemia,⁵⁾ inflammatory disorders, diabetes and other diseases.⁶⁻⁸⁾ Although iNOS activation is not a major event in all of these diseases, the proinflammatory effects of NO generated by iNOS contribute to pathophysiology in each case. Therefore, inhibiting NO synthesis is potentially beneficial in preventing inflammatory reactions. The murine macrophage-like cell line RAW 264.7 has previously been used to characterize the actions of various immunomodulatory compounds at the molecular level.⁹⁻¹¹⁾

Mast cells are distributed throughout the body and play a primary role in acute inflammation and allergic responses.^{12,13)} They are also important in mediating the inflammatory response in allergic reactions by producing cytokines, including IL-4, IL-5, IL-6, and tumor necrosis factor (TNF)- α .¹⁴⁾ The rat basophilic leukemia cell line RBL-2H3 is a mucosal mast cell line, and is the major model system for antigen-induced degranulation.¹⁵⁾ Several studies have shown that allergic inflammation mediated by mast cells is suppressed by phenolics or flavonoids, such as rutin and chlorogenic acid,¹⁶⁾ EGCG,¹⁷⁾ paeonol,¹⁸⁾ fisetin, kaempferol, myricetin, quercetin, rutin,¹⁹⁾ and morin.²⁰⁾

Supercritical fluid extraction (SFE) is used to improve the flavor of *A. polygama*. We investigated the anti-allergic effects of the ethanol extract of marc (SFEM), which results from SFE.²¹⁾ Our aim was to isolate the constituent in the ethanol extract with the greatest anti-inflammatory activity and to determine its structure.

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Abbreviations: SFE, supercritical fluid extraction; SFEM, marc obtained after supercritical fluid extraction of *Actinidia polygama*; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible NO synthase; COX-2, cyclooxygenase-2; TNF- α , tumor necrosis factor; NF- κ B, nuclear factor kappa B; IFN- γ , interferon- γ

Materials and Methods

Plant sample. Dried fruit of *A. polygama* was purchased from a Chinese drug store in Yeongcheon City, Gyeongbuk, Korea. A voucher specimen (KHU-090723) was deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

Instruments and reagents. Melting points were determined using a Fisher-John apparatus (Fisher Scientific, Pittsburgh, PA) and were not corrected. Optical rotation was measured on a Jasco P-1010 digital polarimeter (Jasco, Tokyo). The IR spectrum was collected on a Perkin-Elmer Spectrum One FT-IR Spectrometer (Perkin-Elmer, Norwalk, CT). EIMS was recorded on a JEOL JMS 700 (JEOL, Tokyo). ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were assessed using a Varian Unity Inova AS 400 FT-NMR spectrometer (Palo Alto, CA). CHCl_3-d_1 with TMS as internal standard was purchased from Sigma (St. Louis, MO). Dinitrophenyl (DNP)-specific IgE, Earle's basal salt solution (EBSS), trypsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and LPS were from Sigma (Sigma, St. Louis, MO). Fetal bovine serum (FBS), minimal essential medium (MEM), and other cell culture reagents were from Gibco BRL (Grand Island, NY). DNP-bovine serum albumin was from Alpha Diagnostic International (San Antonio, TX). All other reagents were from Sigma Aldrich (St. Louis, MO).

Preparation of crude extract, marc of the supercritical fluid extract, and ethanol extract of it from *A. polygama*. One kg of dried *A. polygama* was ground and extracted 3 times with a 10-fold excess w/v of 70% ethanol at room temperature over 24 h. After filtering of the extract solutions, the combined filtrate was concentrated by rotary vacuum evaporator (Eyela Uni Trap UT-1000, Tokyo, Japan) at 55 °C and freeze-dried to use as the ethanol extract of *A. polygama* (EtOH, 306.9 g). Essential oils from *A. polygama* were extracted using the Supercritical Fluid Extraction (SFE) apparatus (SFT-100XW, Supercritical Fluid Technologies, Newark, DE). Briefly, 40 g of *A. polygama* were placed in an extraction vessel (100 mL), and SFE was statically conducted with supercritical CO_2 (flow rate, 2.0 mL/min) at 4,500 psi at 45 °C for 1 h. The remaining marc was extracted 3 times with a 10-fold excess w/v of 70% ethanol at room temperature over 24 h. The ethanol extract was concentrated as described above and freeze-dried as the ethanol extract of SFEM (7.6 g).

Separation of the active compound from *A. polygama*. The SFEM of *A. polygama* (5 kg) was extracted with 80% methanol (34 L) at room temperature over 17 h. The concentrated extract was successively partitioned with water (2.5 L), EtOAc (2 L $\times$ 2), and *n*-butanol (2 L $\times$ 2) to yield an H_2O extract (562 g, APW), an EtOAc extract (580 g, APE), and an *n*-BuOH extract (138 g, APB). The EtOAc extract was fractionated with silica gel (70–230 mesh) column chromatography (c.c. 10 $\times$ 17 cm), and eluted with CHCl_3 –MeOH (10:1 $\rightarrow$ 8:1 $\rightarrow$ 6:1 $\rightarrow$ 4:1 $\rightarrow$ 2:1 $\rightarrow$ 1:1, each 2 L). The eluate was collected in 15 fractions (APE-1 to APE-15) monitored by thin layer chromatography (TLC). All the 10 g collected as fraction APE-9 ($V_e/V_t = 0.35$) was subjected to silica gel c.c. (4 $\times$ 18 cm) eluted with *n*-hexane–EtOAc (2:1, 1.5 L). The eluate was collected in 13 fractions (APE-9-1 to APE-9-13). The APE-9-11 ($V_e/V_t = 0.25$, 290 mg) fraction was subjected to ODS c.c. (3 $\times$ 10 cm) eluted with MeOH– H_2O (2:1, 1.5 L) to produce purified compound **1** (APE-9-11-21, $V_e/V_t = 0.51$, TLC(RP-18 F254s) $R_f = 0.4$ in MeOH – water = 5:1, 45 mg).

Cell culture. RAW 264.7 cells, a murine macrophage cell line, and RBL-2H3 cells, a rat basophilic leukemia cell line, were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). RAW 264.7 cells were cultured in DMEM (Gibco-BRL, Rockville, MD) containing 1% antibiotics (penicillin/streptomycin) and 10% heat-inactivated fetal bovine serum (FBS, Gibco). RBL-2H3 cells were grown in MEM (Gibco) with 10% FBS and 1% penicillin/streptomycin. Both cell lines were grown in a 37 °C humidified incubator with 5% CO_2 in air.

Determination of cell viability. To determine the cytotoxicity of the *A. polygama* extract, cell viability was determined by MTT assay,²²⁾ in which active mitochondria reduce MTT into formazan dye. RBL-2H3 cells were seeded at a density of 3×10^4 cells/well in 96-well

microplates. The next day, the cells were treated with various concentrations (0, 100, 200, and 400 $\mu\text{g/mL}$) of *A. polygama* extract at 37 °C. After 48 h, 100 μL of MTT (5 mg/mL) was added to each well, and the plates were incubated at 37 °C for 4 h. One hundred μL of DMSO was added to each well to dissolve the cells. The plates were kept at room temperature for 5 min, and the absorbance was measured at 550 nm using a multiwell spectrophotometer (Molecular Devices, Sunnyvale, CA). The cytotoxicity of the extract in the IgE-sensitized RBL-2H3 cells was also assessed by MTT assay. The cells (3×10^4 cells/well) were seeded and incubated with 450 ng/mL of DNP-specific IgE at 37 °C overnight before treatment with the extract.

Determination of LPS-induced NO production from RAW 264.7 cells. RAW 264.7 cells were seeded in 96-well plates (1×10^5 cells/well) overnight and treated with various concentrations of test samples (in 0.1% DMSO) in the presence of LPS (final concentration, 100 ng/mL) at 37 °C for 24 h. After LPS stimulation for 24 h, NO production in the cell culture medium was measured by the Griess Reagent System.²³⁾ Briefly, the culture supernatant (100 μL) was mixed with same volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% phosphoric acid) for 10 min, and the absorbance was measured at 540 nm. The NO concentration was calculated from a standard curve of NaNO_2 .

Determination of antigen-induced β -hexosaminidase release from RBL-2H3 cells. The inhibitory effect of the extract on the release of β -hexosaminidase in the RBL-2H3 cells was evaluated as reported by Matsuda *et al.*,²⁴⁾ with modifications. In brief, RBL-2H3 cells seeded in 24-well plates (2×10^5 cells/well) were sensitized with anti-DNP IgE (450 ng/mL) at 37 °C overnight. The cells were washed with Siraganian buffer and then incubated in 160 μL of the incubation buffer at 37 °C for 20 min. Then the cells were treated with 20 μL of extract for 10 min, followed by the addition of 20 μL of antigen (DNP-BSA, 10 $\mu\text{g/mL}$) at 37 °C for 10 min to stimulate the cells to granulate. The reaction was stopped by cooling in an ice bath for 10 min. Twenty-five μL of the supernatant was transferred into a 96-well plate and incubated with 25 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 $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0), and the absorbance was measured at 405 nm using a microplate reader. The extract was dissolved in 0.1% DMSO and added to the incubation buffer.

Statistical analysis. All experiments were performed 3–5 times. Data were expressed as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD). A significant difference from the control for each experimental test condition was assessed by Student's *t*-test for each paired experiment. A *p*-value < 0.05 was regarded as indicating statistical significance.

Results and Discussion

Inhibition of allergic inflammation by the EtOH extract and various SFEM fractions of A. polygama

To test the anti-inflammatory effects of the EtOH extract of *A. polygama* and various SFEM fractions, NO production and cytotoxicity were measured in unstimulated and LPS-stimulated RAW 264.7 cells. The cells were incubated with LPS (100 ng/mL) in the presence of various extracts for 24 h. As Table 1 shows, the EtOH and SFEM extracts decreased the NO concentration in the culture media to $81.0 \pm 2.3\%$ and $29.4 \pm 1.5\%$ LPS-stimulated cells at 100 $\mu\text{g/mL}$, respectively. Moreover, the EtOAc fraction from SFEM markedly suppressed NO production, to $29.2 \pm 4.1\%$ LPS-stimulated cells at 25 $\mu\text{g/mL}$. This fraction had a much stronger effect than the BuOH ($83.2 \pm 4.7\%$) and H_2O ($89.1 \pm 7.0\%$) fractions. We tested cell viability by MTT assay to ensure that the decreased NO production was not due to

Table 1. Effects of the EtOH, SFEM, EtOAc, BuOH, and H₂O Fractions from *A. polygama* on LPS-Induced NO Production in RAW 264.7 Cells

Sample	Concentration (mg/mL)	NO production (%) ^a	Cell survival (%)
EtOH	10	95.38 ± 0.91	95.32 ± 5.06
	100	81.01 ± 2.23	102.73 ± 0.28
SFEM	10	63.38 ± 1.83	96.18 ± 4.23
	100	29.42 ± 1.53	98.17 ± 1.71
EtOAc	25	29.16 ± 4.10	103.98 ± 4.58
BuOH	25	83.23 ± 4.71	95.32 ± 3.20
H ₂ O	25	89.06 ± 7.04	88.88 ± 1.44

^aNitrite levels were measured in the supernatants. Data represent mean ± SEM of three independent experiments.

Table 2. Effects of the EtOH, SFEM, EtOAc, BuOH, and H₂O Fractions from *A. polygama* on β -Hexosaminidase Release from RBL-2H3 Cells Induced by IgE with DNP-BSA

Sample	Concentration (μ g/mL)	β -Hexosaminidase release (%) ^a	Cell survival (%)
EtOH	10	87.89 ± 1.18	98.20 ± 2.49
	100	45.59 ± 7.77	108.27 ± 3.68
SFEM	10	76.24 ± 2.79	102.36 ± 1.09
	100	50.27 ± 0.95	106.94 ± 2.14
EtOAc	100	43.06 ± 3.65	90.32 ± 11.50
BuOH	100	42.37 ± 0.87	114.04 ± 34.70
H ₂ O	100	82.20 ± 5.56	123.42 ± 21.59

^aThe levels of β -hexosaminidase released were measured in the supernatants. Data represent mean ± SEM of three independent experiments.

cell death. Neither the tested fractions nor 0.1% DMSO significantly affected cell viability under the conditions imposed.

RBL-2H3 cells were used to evaluate *in vitro* anti-allergic activity. As a marker of RBL-2H3 cell degranulation, the release of β -hexosaminidase into the cell media was measured. RBL-2H3 cells were sensitized with 450 ng/mL of DNP-specific IgE overnight before treatment with DNP-BSA antigen. Previously, we tested various conditions (DNP-specific IgE, 25–450 ng/mL; antigen, 0.01, 1, 10 μ g/mL) to determine the optimal conditions for degranulation. Optimal degranulation occurred following stimulation with 450 ng/mL of IgE overnight and with 10 μ g/mL of antigen for 10 min. The abilities of the various extracts to inhibit β -hexosaminidase release from IgE-sensitized RBL-2H3 cells induced by DNP-BSA are shown in Table 2. β -Hexosaminidase release was significantly inhibited by the EtOH, SFEM, EtOAc, and BuOH fractions (42.4–50.3% at 100 μ g/mL). The H₂O, EtOH, and SFEM fractions had weak inhibitory effects at 10 μ g/mL (82.2 ± 5.6% at 100 μ g/mL). The extracts were not cytotoxic to IgE-sensitized RBL-2H3 cells, and they were not toxic to the cells not treated with IgE (data not shown).

IgE-mediated allergic reactions, such as asthma and allergic rhinitis, result from the activation of signaling pathways downstream of the IgE receptor. These signaling pathways can be activated by the antigen-induced release of β -hexosaminidase, a marker of mast cell degranulation, and by various pharmacological agents.^{25,26)}

Table 3. NMR Data for Compound (**1**) In pyridine-*d*₅ (δ in ppm, *J* in Hz)

δ C (no.)	δ H (coupling pattern)
Ha-C(1)	62.20
Hb-C(1)	4.73 (dd, <i>J</i> = 11.2, 4.8)
H-C(2)	4.39 (dd, <i>J</i> = 11.2, 5.8)
H-C(3)	5.09 (ddd, <i>J</i> = 5.8, 5.2, 9.2)
H-C(4)	76.98
H-C(5)	4.37 (m)
H-C(6)	73.15
H-C(7)	4.26 (m)
H-C(8)	34.34
H-C(9)	2.00 (m)
H-C(10)	29.76–30.21
H-C(11)	2.27–1.29 (m)
H-C(12)	32.28
H-C(13)	1.33 (m)
H-C(14)	23.09
H-C(15)	1.35 (m)
H-C(16)	14.43
NH	0.88 (t, <i>J</i> = 6.4)
H-C(1')	8.55 (d, <i>J</i> = 9.2)
H-C(2')	175.20
H-C(3')	72.59
H-C(4')	4.59 (m)
H-C(5')	35.85
H-C(6')	2.20 (m)
H-C(7')	130.69
H-C(8')	5.55 (dt, <i>J</i> = 16.0, 6.8)
H-C(9')	130.83
H-C(10')	5.46 (dt, <i>J</i> = 16.0, 6.8)
H-C(11')	33.42
H-C(12')	2.16 (m)
H-C(13')	29.76–30.21
H-C(14')	2.27–1.29 (m)
H-C(15')	32.28
H-C(16')	1.33 (m)
	23.09
	1.35 (m)
	14.43
	0.88 (t, <i>J</i> = 6.4)

Histamine is released from mast cells in response to an antigen or a degranulation inducer. β -Hexosaminidase is stored in secretory granules within mast cells and is released with histamine in response to an antigen. Hence β -hexosaminidase is a marker for histamine release from mast cells. There are several inhibitors of β -hexosaminidase release, including flavonoids,²⁷⁾ diarylheptanoids,²⁴⁾ sesquiterpenes,²⁸⁾ anthraquinones,²⁹⁾ and alkaloids.³⁰⁾

Purification and structural elucidation of an anti-inflammatory compound from the EtOAc fraction of *A. polygama* by activity-guided fractionation

The presence of a ceramide in the EtOAc layer of the 80% methanol extract of *A. polygama* was confirmed by silica gel TLC. As shown in Table 1, the EtOAc fraction exhibited the highest inhibitory activity among the fractions against NO-production in LPS-stimulated RAW 264.7 cells. Repeating the silica gel column chromatography of the ethyl acetate layer yielded purified compound **1**.

Actinidiamide (**1**) is a white amorphous powder; m.p. 117–118 °C (CHCl₃–MeOH); [α]_D²⁵ = +30.8° (c 0.01, pyridine); IR ($\nu_{\max}$, KBr, cm^{−1}) 3,321, 3,201, 2,911, 1,643, 1,517, 1,471; HRFAB-MS (pos.) *m/z* 570.5097 [M + H]⁺; EI-MS *m/z* 569 [M]⁺, 316, 301, 277, 253, 197, 195, 181, 169, 155. For the ¹H-NMR and ¹³C-NMR spectral data for the compound, see Table 3.

Compound **1** was isolated as a white amorphous powder from CHCl₃–MeOH. The molecular formula of **1** was established as C₃₄H₆₇NO₅ (calculated 569.5019) based on its positive HRFAB-MS data for the [M + H]⁺ at *m/z* 570.5097. The IR absorption bands at 3,321 cm^{−1}, 2,911 cm^{−1} and 1,643 cm^{−1} are characteristic of the hydroxyl, the amide carbonyl, and the olefinic group respectively.

The ¹H-NMR spectrum in pyridine-*d*₅ of **1** (Table 3) displayed a low-field doublet at δ H 8.55 (1H, *J* = 9.2 Hz, NH), two olefine methine, oxygenated, or other heteroatomized protons, and two methyl protons at δ H

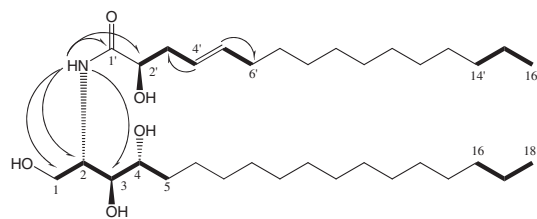


Fig. 1. Structure and Key 2D-NMR (^1H - ^1H COSY and HMBC) Correlations of Compound **1**.

0.88 (6H, t, $J = 6.4$ Hz, H-18, 16') and a strong aliphatic methylene signal at δH 1.29–2.27, which confirms the presence of two long aliphatic chains. Signals of a trans-olefinic bond at δH 5.46 (1H, dt, $J = 16.0, 6.8$ Hz, H-5') and δH 5.55 (1H, dt, $J = 16.0, 6.8$ Hz, H-4'), and four characteristic signals of hydroxyl groups were observed at δH 4.59 (1H, m, H-2'), 4.73 (1H, dd, dd, $J = 11.2, 4.8$ Hz, H-1a), 4.39 (1H, dd, $J = 11.2, 5.8$ Hz, H-1b), 4.37 (1H, dd, $J = 5.2, 3.7$ Hz, H-3), and 4.26 (1H, m, H-4). Another low field signal was observed at δH 5.09 (1H, ddd, $J = 5.2, 5.8, 9.2$, H-2), indicating a methine proton vicinal coupling to the nitrogen atom of an amide linkage representing a sphingolipid skeleton.³¹⁾ The ^{13}C NMR (Table 3) and DEPT spectral data for **1** showed an amide carbonyl carbon at δC 175.20 (C-1'), two olefine methine carbons at δC 130.83 (C-5') and 130.69 (C-4'), and five oxygenated and other heteroatomized carbons at δC 76.98 (C-3), 73.15 (C-4), 72.59 (C-2'), 62.20 (C-1), and 53.16 (C-2), which were assigned on the basis of HSQC data. In the aliphatic legion, methylenes were observed between δC 29.76–30.21 and two terminal methyl groups at δC 14.43 (C-18 and C-16'). The low-field doublet at δH 8.55 (NH) had no correlation with any carbon in the HSQC spectrum of **1**. The signal at δH 8.55 (NH) correlated to δH 5.09 (1H, m, H-2), and that at δH 8.55 (NH) correlated to δC 62.20 (C-1), 53.16 (C-2), 76.98 (C-3), 175.20 (C-1'), and 72.59 (C-2') respectively in the ^1H - ^1H COSY and HMBC spectra of **1**.

The chemical shifts of the allylic methylene carbons in **1** were located at δC 35.85 (C-3') and 33.42 (C-6') based on the correlation between HMBC signals from the olefinic signals at δH 5.46 (H-5') and δH 5.55 (H-4') to these two carbon signals (Fig. 1) respectively. The C=C bond configuration was determined to be *trans* based on the vicinal coupling constants ($J_{4',5'} = 16.0$ Hz). These *trans*-geometries were also deduced from the chemical shifts of allylic carbons C-3' and C-6' (δC 33.85, 33.42 respectively).³²⁾

Further investigation of the EIMS showed important fragment ions at m/z 181 and 155, suggesting a double bond at C-4'. The lengths of the amide chain and the fatty alcohol were determined by EI-MS (Fig. 2). The EI-MS of **1** showed a molecular ion at m/z 569 $[\text{M}]^+$. Significant fragment ions at m/z 253 $[\text{M}]^+$, 312 $[\text{M}]^+$, 197 $[\text{M}]^+$, and 316 $[\text{M} - \text{amide chain}]^+$ in the EI-MS indicated an amino alcohol chain of 16 carbon atoms. The relative stereochemistry of **1** at C-2, C-3, C-4, and C-2' was proposed to be 2*S*, 3*S*, and 4*R*, since the proton and carbon signals were in good agreement with natural and synthetic ceramides reported in the literature.³³⁾ Also, in that because the optical rotation ($[\alpha]_{\text{D}}^{25} = +30.8^\circ$) compared with reported sphingolipids,³⁴⁾ compound **1**

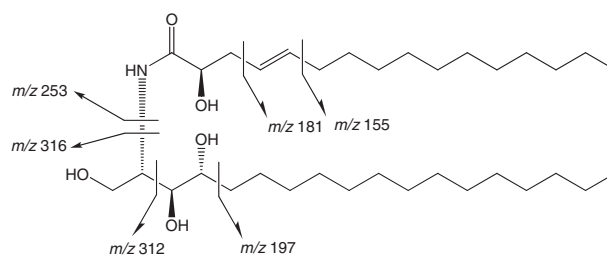


Fig. 2. Important Mass Fragmentation Pattern of Compound **1**.

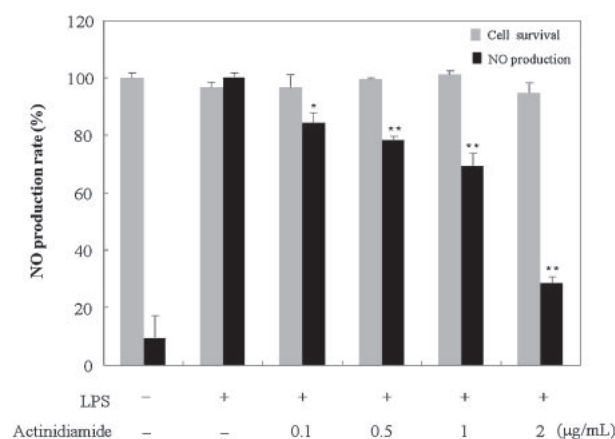


Fig. 3. Cytotoxicity and Inhibitory Effects of Actinidiamide from *A. polygama* on Nitric Oxide (NO) Production by RAW 264.7 Cells Stimulated by LPS.

NO production was assayed in culture media of cells stimulated with LPS (100 ng/mL) over 24 h. Values are mean $\pm$ SEM for three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. LPS alone.

had a sphingosine moiety with a (2*S*,3*S*,4*R*,4'*E*) geometry. Thus the structure was identified as (2*S*,3*S*,4*R*,4'*E*)-2*N*-[(2'*R*)-2'-hydroxy-hexadec-4'-enyl]octadecane-1,3,4-triol, and the chemical was named actinidiamide (Fig. 1).

Inhibitory effects of actinidiamide from A. polygama on allergy and inflammation

Next we evaluated the effects of the novel ceramide actinidiamide on NO production in LPS-stimulated RAW 264.7 cells and on β -hexosaminidase release in RBL-2H3 cells. Ceramides are a family of sphingolipids that have antiproliferative and proapoptotic activities,^{35–37)} but, their role in anti-inflammatory processes is poorly understood.

As shown in Fig. 3, NO production was inhibited by actinidiamide (in 0.08% pyridine) in a dose-dependent manner. At a concentration of 2 $\mu\text{g/mL}$, NO production decreased to 21.7%, and actinidiamide was not cytotoxic. Pyrimidine (0.08%), used as a reference, did not affect cell viability or NO production. The inhibitory effect of actinidiamide on β -hexosaminidase release is shown in Fig. 4. Actinidiamide inhibited β -hexosaminidase release in a dose-dependent manner and had a significant effect at 0.5 ($31.5 \pm 10.3\%$) and 1 $\mu\text{g/mL}$ ($8.2 \pm 3.1\%$) without cytotoxicity. These results suggest that actinidiamide isolated from the EtOAc fraction of SFEM from *A. polygama* potentially regulates allergic inflammation.

In summary, we found that the EtOAc extract of SFEM from *A. polygama* markedly inhibited NO pro-

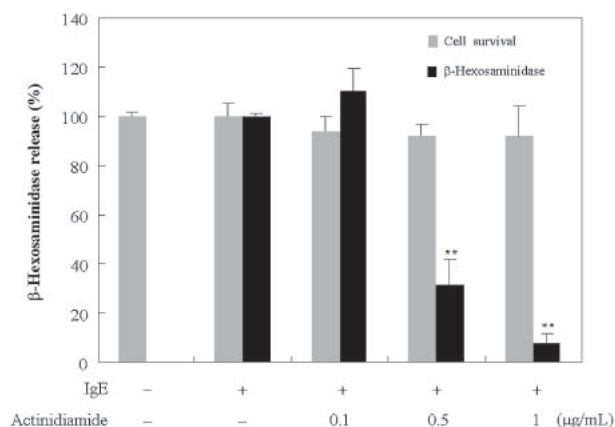


Fig. 4. Cytotoxicity and Inhibitory Effects of Actinidiamide from *A. polygama* on β -Hexosaminidase Release from RBL-2H3 Cells Induced by IgE with DNP-BSA.

RBL-2H3 cells (2×10^5 cells) were sensitized with 450 ng/mL of DNP-specific IgE overnight, and were pretreated with varying doses of actinidiamide over 10 min. Cells were stimulated with 10 μ g/mL of DNP-BSA for 10 min. β -Hexosaminidase release was assayed in the culture media of the cells. Values represent mean $\pm$ SD of three independent experiments. ** $p < 0.01$ vs. IgE alone.

duction without cytotoxicity in LPS-stimulated RAW 264.7 cells. The active component of the EtOAc fraction was isolated by activity-guided fractionation steps, and was identified as the novel ceramide, actinidiamide. Moreover, actinidiamide inhibited NO production in RAW 264.7 cells (0.1–2 g/mL) and degranulation in RBL-2H3 cells induced by DNP-BSA and IgE (0.1–1 g/mL) in a dose-dependent manner. At a concentration of 1 μ g/mL, actinidiamide inhibited NO production and β -hexosaminidase by 31 and 92% respectively. The strong inhibitory activities of actinidiamide might explain the anti-inflammatory effects of the *A. polygama* extract.

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