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Anti-allergic property of 4,8-sphingadienine stereoisomers *in vivo* and *in vitro* model

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Abbreviations

GlcCer, glucosylceramides; IEC, intestinal epithelial cell; SB, Siraganian buffer; SD,

sphingadienine.

Abstract

4,8-Sphingadienines (SD), metabolites of glucosylceramides (GlcCer), are sometimes determined as key mediators of the biological activity of dietary GlcCer, and *cis*/*trans* geometries of 4,8-SD have been reported to affect its activity. Since regulating excessive activation of mast cells seems an important way to ameliorate allergic diseases, this study was focused on *cis/trans* stereoisomeric-dependent inhibitory effects of 4,8-SD on mast cell activation. Degranulation of RBL-2H3 was inhibited by treatment of 4-*cis*-8-*trans*- and 4-*cis*-8-*cis*-SD, and their intradermal administrations ameliorated ear edema in passive cutaneous anaphylaxis reaction, but 4-*trans*-8-*trans*- and 4-*trans*-8-*cis*-SD did not. Although the activation of mast cells depends on the bound IgE contents, those stereoisomers did not affect IgE contents on RBL-2H3 cells after the sensitization of anti-TNP IgE. These results indicated that 4-*cis*-8-*trans*- and 4-*cis*-8-*cis*-SD directly inhibit the activation of mast cells. In conclusion, it was assumed that 4,8-SD stereoisomers with *cis* double bond at C4-position shows anti-allergic activity by inhibiting downstream pathway after activation by the binding of IgE to mast cells.

Key words

38 Anti-allergic activity, glucosylceramide, mast cell, sphingoid base, type I allergy

39

40 **Introduction**

41 Allergic diseases, a group of immune-mediated disorders, are classified into
42 four types from I to IV. Type I allergic diseases, such as food allergies, rhinitis and
43 asthma, are major health problems in the world [1]. In the mechanism of type I allergy,
44 the contribution of IgE-activated mast cells is well defined. IgE bind to the high affinity
45 IgE receptor I (FcεRI), and the resulting IgE crosslinking induces mast cell activation.
46 Mast cell-derived mediators, such as histamine, lipid mediators, cytokines, chemokines
47 and β-hexosaminidase, induce allergic symptoms. Therefore, it is considered that
48 allergic symptoms are mitigated by inhibiting mast cell activation.

49 Glucosylceramides (GlcCer) from plants have recently been recognized as
50 functional food components. It has been reported that dietary GlcCer improve skin
51 barrier functions [2], have anti-inflammatory effect against atopic dermatitis [3] and
52 colitis [4], and suppress mRNA expression of the pro-inflammatory cytokines IL-1β and
53 IL-6 [5]. GlcCer in the food are absorbed directly from intestinal epithelial cells (IECs),
54 or as catabolized form of ceramide, sphingoid base and fatty acid [6, 7]. It was
55 demonstrated that oral administration of GlcCer improved skin barrier function by

56 restoring diminished TGF-β in the skin [8]. Furthermore, it was indicated that 4,8-
57 sphingadienine (SD), a metabolite of GlcCer, may be the active compound mediating
58 the improvement of skin barrier function [9].
59 4,8-SD is one of the major sphingoid bases of plant-derived GlcCer. 4,8-SD is
60 sometimes determined as a key mediator of the biological activity of dietary GlcCer [2].
61 4,8-SD specifically refers to a C18 aliphatic chain with an amino group at C2, two
62 hydroxyl groups at C1 and C3, and two double bonds at C4 and C8. Usuki *et al*.
63 reported the relationship between *cis*/*trans* geometries of konjac-derived GlcCer
64 components and activation of the semaphorin 3A signaling pathway [10]. Therefore,
65 *cis*/*trans* geometries of 4, 8-SD were assumed to be important when biological activity
66 of 4,8-SD is assessed. This study focused on the *cis/trans* stereoisomeric-dependent
67 inhibitory effects of 4,8-SD on mast cell activation using four kinds of synthetic 4,8-SD
68 stereoisomers.
69
70 **Materials and Methods**
71 *Reagents*. GlcCer from pineapple was kindly gifted by Maruzen Pharmaceuticals Co.,
72 Ltd (Hiroshima, Japan). GlcCer from rice, konjac and soybean was purchased from
73 Nagara Science Co., Ltd. (Gifu, Japan). Eagle's minimum essential medium (MEM)

and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide was purchased from Sigma (St Louis, MO, USA). Trypsin and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). L-glutamine, Blocking One, and Chemi-Lumi One L were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Anti-mouse IgE and anti-goat IgG-HRP conjugate antibodies were purchased from Bethyl Laboratories (Montgomery, AL, USA). Anti-trinitrophenyl (TNP) IgE was purchased from BD Biosciences (Franklin Lakes, NJ, USA). TNP-BSA was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Picryl chloride (PiCl) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Anti-mouse β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents were ordinary commercial and guaranteed products.

Mice. Female 4-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were housed and bred in a specific pathogen-free condition, with temperature- and humidity-controlled, at Kobe University Life Science Laboratory. All animal experiments were approved by the Animal Experiment Ethnics Committee of

92 Kobe University and performed according to the guidelines. Permission number was
93 2020-01-02.
94
95 *Cell culture.* Rat basophilic leukemia cell line, RBL-2H3 cells, were cultured in dishes
96 in Eagle's MEM supplemented with 10% (v/v) heat-inactivated FBS (57 °C, 30 min),
97 100 μg/ml streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine. Cell cultures
98 were incubated at 37 °C in a 5% CO_2 incubator. Passage numbers 14–32 were used.
99 Human intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm^2 plastic
100 flask in DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100
101 μg/ml streptomycin, and 100 U/mL penicillin, and incubated at 37 °C in a 5% CO_2
102 incubator. Passage numbers 48–64 were used. When either cell line reached 80%
103 confluence, cells were recovered from the culture dish or flask by trypsin digestion after
104 washing with phosphate-buffered saline (PBS). The cells were replated in a new dish or
105 flask.
106
107 *β-Hexosaminidase assay*. RBL-2H3 cells were adjusted to $2x10^6$ cells/mL by a
108 hemocytometer calculation and dispensed into 96-well plates at $2x10^5$ cells/well. Cells
109 were incubated overnight in the humidified 5% CO_2 incubator at 37 °C. Cells were

110 sensitized with anti-TNP IgE for 2 h. Cells were washed twice with Siraganian buffer
111 (SB: 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 40 mM NaOH, 25 mM
112 PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) before adding test sample solution in
113 RPMI1640 medium to each well and incubated for 1 h at 37 °C. After incubation, the
114 cells were washed twice with SB and then were challenged with TNP-BSA dissolved in
115 SB for 1 h at 37 °C. The reaction was stopped by cooling in an ice bath for 10 min. SB
116 solution (50 μl) was transferred into 96-well microplate and incubate with 50 μl of
117 substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.2 M citrate
118 buffer at pH 4.5) for 1 h at 37 °C. The reaction was stopped by adding stop solution (0.2
119 M glycine-NaOH at pH 13). The absorbance was measured at 405 nm with a microplate
120 reader (SH-9000, Corona Electric Co., Hitachi, Japan). The β-hexosaminidase release
121 rate was calculated as a percentage of the release amount in the degranulation group.
122
123 *Co-culture system composed of Caco-2/RBL-2H3.* The Caco-2/RBL-2H3 cells co-
124 culture system was done in accordance with a previous study [11], with some
125 modifications. Transwell inserts on which Caco-2 cells had been cultured were added
126 into the plate wells preloaded with RBL-2H3 cells. In an experiment to evaluate the
127 anti-allergic effect of test samples, 0.2 ml of SB or test sample solution was applied into

the apical side. After incubation for 6 h, Caco-2 inserts were removed and RBL-2H3 cells were sensitized with anti-TNP IgE for additionally more 4 h. RBL-2H3 were challenged with TNP-BSA for 1 h. The remaining procedure was the same as with the β-hexosaminidase activity assay.

Western blot of IgE on mast cells. RBL-2H3 cells (6.0×10^5 cells/1500 μL) were seeded in a 6-well tissue culture plate and incubated in RPMI 1640 medium overnight. The cells were sensitized with 200 ng/mL anti-TNP IgE for 4 h. The anti-TNP IgE-sensitized RBL-2H3 cells were pre-incubated for 2 h with each 4,8-SD stereoisomer. The cells were then washed twice with SB and lysed in 50 μL Radioimmunoprecipitation buffer (RIPA buffer; 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 7.4, 50 mM glycerophosphate, 20 mM NaF, 10.5 mM leupeptin, aprotinin, 10 mM pepstatin A, 1 mM DTT and 100 μM PMSF). Collected proteins were quantified by Lowry assay. Cell lysates containing 30 μg of proteins were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred onto polyvinylidene fluoride membranes (General Electric, Fairfield, CT, USA). The membranes were blocked using Blocking One at room temperature for 30 min. After four washes of Tris-buffered saline with Tween-20 (TBST; 20 mM Tris, 150 mM NaCl,

146 pH7.6, 0.05% Tween), the membranes were incubated with the primary antibody, anti-
147 mouse IgE or anti-β-actin antibodies, at 4 °C overnight. Following four washes of
148 TBST, the membranes were incubated with the second antibody, anti-goat or anti-mouse
149 IgG-HRP conjugate antibodies, at 4 °C for 1 h. The target signals were detected with an
150 enhanced chemiluminescence kit, Chemi-Lumi One L, and the intensity of bands was
151 evaluated using Image J.
152
153 *Passive cutaneous anaphylaxis (PCA) reaction*. PCA reaction was performed as
154 described previously [12], with minor modification. Each stereoisomer (0.85
155 mg/mouse) was intradermally injected into the ear under isoflurane anesthesia. After 2
156 h, mice were passively sensitized by intravenous injection of 2 μg anti-TNP IgE. After
157 30 min, the ear thickness was measured using thickness micrometer (Peacock G-1A,
158 OZAKI MFG. Co. Ltd, Japan) and mice were challenged by single application of 1.6%
159 (w/v) picryl chloride in acetone:olive oil (1:1) as an antigen. After 2 h, the ear thickness
160 was measured again. Ear edema was calculated from the difference in ear thickness
161 between before and after challenge.
162
163 *Stereoisomers of 4,8-SD.* Four stereoisomers of 4,8-SD (4*t*,8*t*-SD, 4*t*,8*c*-SD, 4*c*,8*t*-SD,

4*c*,8c-SD) were synthesized according to the reported methods [13, 14]. The detail of the synthesis will be reported elsewhere.

Statistical analysis. All the data were presented as mean ± standard deviation. Statistical significances among each group were evaluated by analysis of variance (ANOVA) and Tukey-Kramer test to determine differences between groups. Statistical significance was defined as $p < 0.05$.

Results and discussion

Anti-allergic effects of GlcCer from pineapple, rice, konjac and soybean on degranulation of RBL-2H3 cells in Caco-2/RBL-2H3 cells co-culture system

To check whether GlcCer derived from plants possessed ant-allergic property, GlcCer from pineapple, rice, konjac and soybean was applied to the apical side of co-culture system composed of Caco-2 and RBL-2H3 cells. As shown in Fig. 1, GlcCer from pineapple inhibited significantly IgE-mediated degranulation of RBL-2H3 cells through Caco-2 cells by approximately 84% compared with the positive control which was sensitized by TNP-IgE and challenged by TNP-BSA. However, the other plants did not. It was reported that 4,8-SD of GlcCer in pineapple is an active compound

182 contributing the improvement of skin barrier function [9]. It was demonstrated that the
183 sphingoid bases in GlcCer from higher plants can be desaturated at the C8-position by a
184 stereo-unselective Δ8-*cis/trans*-sphingolipid desaturase [15] and there are nine different
185 types [16]. Therefore, the differences in the structure of 4,8-SD might affect anti-
186 allergic activity of GlcCer.
187
188 *Inhibitory effects of 4,8-SD stereoisomers on degranulation in RBL-2H3*
189 To confirm whether 4,8-SD stereoisomers affected the degranulation of mast
190 cells, RBL-2H3 cells were treated with the four stereoisomers of 4,8-SD and evaluated
191 β-hexosaminidase activity as an index of anti-allergic activity. *Cis*-stereoisomers at C4
192 of 4,8-SD inhibited β-hexosaminidase release from RBL-2H3 sensitized by IgE and
193 challenged by antigen, on the other hand, *trans*-stereoisomers at C4 did not (Fig. 2A).
194 Moreover, it was demonstrated that 4-*cis*-8-*cis*-SD inhibited stronger in its release than
195 4-*cis*-8-t*rans*-SD (Fig. 2B). It was checked by MTT assay that each 4,8-SD
196 stereoisomer did not show any cytotoxicities to RBL-2H3 (data not shown).
197
198 *4,8-SD stereoisomers suppress directly β-hexosaminidase release from RBL-2H*
199 There are many stages in the development of type I allergy. In particular, the central to

200 the initiation and propagation of the immediate hypersensitivity reaction is the
201 production of antigen-specific immunoglobulin E (IgE) and the subsequent fixation of
202 IgE on FcεRI receptors on mast cells [17]. Previously, the sphingoid base 4,8-SD of
203 GluCer from *Arisaema amurense* Maxim. inhibited the inflammatory responses induced
204 by TNF-α and LPS [18]. We thus measured the amount of IgE bound to sensitized
205 RBL-2H3 cells by Western blotting in order to investigate the effects of each
206 stereoisomer toward FcεRI expression on the surface of RBL-2H3 cells. As shown in
207 Fig. 3, the amount of IgE bound to RBL-2H3 remained constant without any effect of
208 stereoisomers treatment. Although this result did not directly prove that each
209 stereoisomer does not affect FcεRI expression, it was inferred that the amount of FcεRI
210 on RBL-2H3 is not altered by each stereoisomer, indicating that *cis*-forms of 4,8-SD
211 directly affected RBL-2H3 to inhibit β-hexosaminidase release.
212
213 *Anti-allergic activity of 4,8-SD stereoisomers in PCA reaction*
214 It was ascertained that 4,8-SD stereoisomers suppressed the degranulation of mast cells
215 in Fig. 2. PCA reaction was adapted to confirm their activities *in vivo*. PCA reaction is a
216 common animal model for evaluating type I hypersensitivity. As shown in Fig. 4, PCA
217 reaction significantly exacerbated edema on mouse ear (59.0 ± 3.3 μm) compared with

control mice (5.2 ± 1.8 μm). Intradermal administration of 4-*cis*-8*trans*- and 4-*cis*-8*cis*-SD significantly ameliorated ear edema to be 26.0 ± 5.0 and 35.0 ± 3.1 μm, respectively compared with PCA positive group (59.0 ± 3.3 μm). On the other hand, intradermal administration of 4-*trans*-*8trans*- and 4-*trans*-8*cis*-SD seemed to inhibit ear edema (38.0 ± 7.7 and 43.6 ± 6.4 μm, respectively), but they did not show significance. This result suggested that *cis* stereoisomers at C4 position in 4,8-SD possessed inhibitory effects on mast cell activation *in vivo* as well as *in vitro*.

Researches in the inhibition of allergy by food factors have often been focused on restoring the Th1/Th1 balance disrupted by allergen. However, 4,8-SD was a novel and attractive food factor that acts directly on mast cell to suppress allergy. We conclude that *cis* stereoisomers of 4, 8-SD may be an effective treatment of Type I hypersensitivity.

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299 Figure legends

300

301 Figure 1. Inhibition of mast cell degranulation by GlcCer from plants in the Caco-

302 2/RBL-2H3 cells co-cultured system.

303 GlcCer (160 μg/mL) from each plant was applied to the apical side of Caco-2/RBL-

304 2H3 cells co-culture system. After 6 h, Transwell inserts were removed and then RBL-

305 2H3 cells were sensitized with anti-TNP IgE for 4h. After all media were replaced with

306 SB, degranulation of RBL-2H3 cells was evoked by TNP-BSA. Different superscript

307 letters indicate the statistical significance between groups ($p < 0.05$). Values are shown

308 as means ± SD (n = 3).

309

310 Figure 2. Inhibitory effects of 4,8-sphingadienine stereoisomers on β-hexosaminidase

311 release from RBL-2H3/

312 A: Structures of four geometry isomers of sphingadienines (SD). The nomenclature of

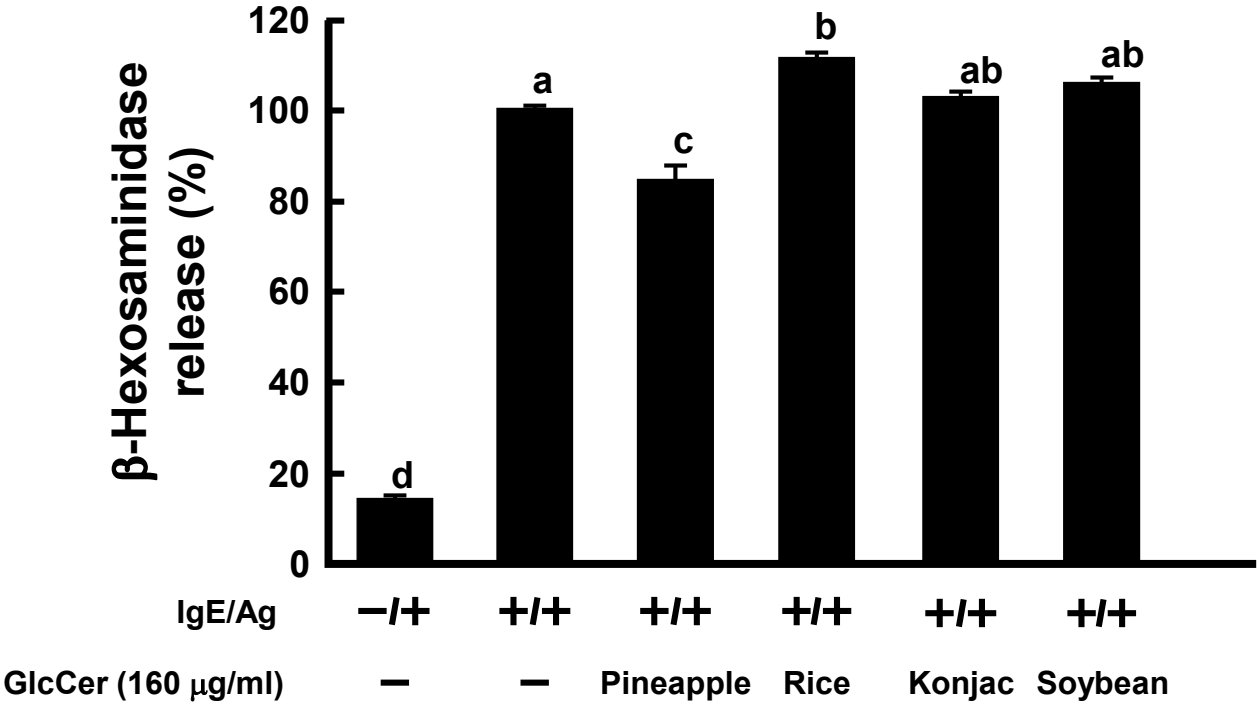
313 the compounds followed by the recommendation of IUPAC-IUBMB Joint Commission.

314 B, C: RBL-2H3 cells were sensitized with anti-TNP IgE for 4 h. The anti-TNP IgE-

315 sensitized RBL-2H3 cells were pre-incubated for 2 h with 4,8-sphingadienine

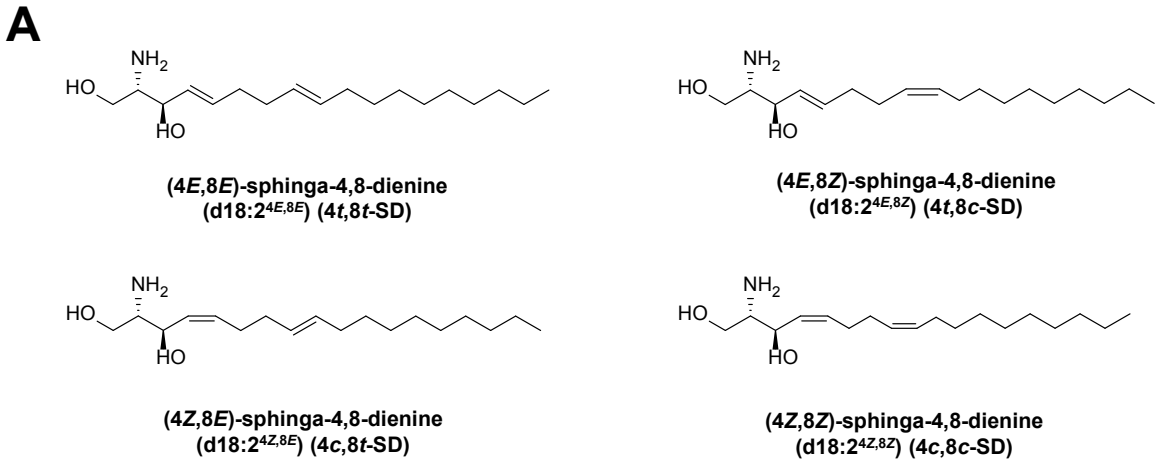
316 stereoisomers. The cells were challenged with TNP-BSA. The β-hexosaminidase release

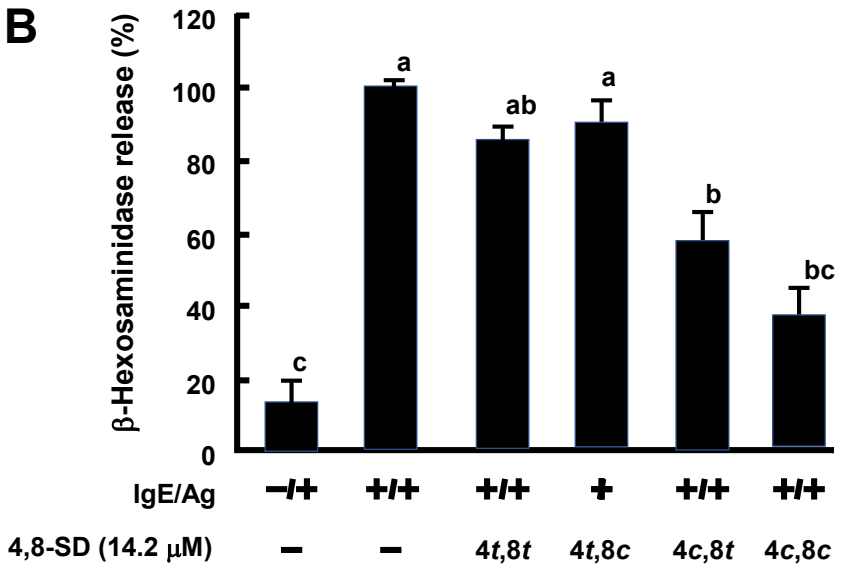
317 rate was calculated as a percentage of the release amount in the degranulation group.
318 Data are shown as means ± SE (n = 3). Values with different letters are significantly
319 different ($P < 0.05$).
320
321 Figure 3. 4,8-Sphingadienine stereoisomers did not affect IgE contents in RBL-2H3
322 cells. RBL-2H3 cells were sensitized with anti-TNP IgE for 4 h after pretreatment with
323 each 4,8-sphingadienine stereoisomer for 2 h. IgE levels in the samples were measured
324 by SDS-PAGE and western blotting analysis. Data are shown as means ± SE (n = 3).
325
326 Figure 4. Inhibitory effects of 4,8-sphingadienine stereoisomers on PCA reaction.
327 4,8-Sphingadienine stereoisomers (0.85 mg/mouse) were intradermally injected into the
328 ear. After 2 h, mice were passively sensitized by intravenous injection of 2 μg anti-TNP
329 IgE. After 30 min, the ear thickness was measured using thickness micrometer and mice
330 were challenged by single application of 1.6% (w/v) picryl chloride. After 2 h, the ear
331 thickness was measured again. Ear edema was calculated from the difference in ear
332 thickness between before and after challenge. Data are shown as means ± SE (n = 5).
333 Values with different letters are significantly different. ($P < 0.05$)

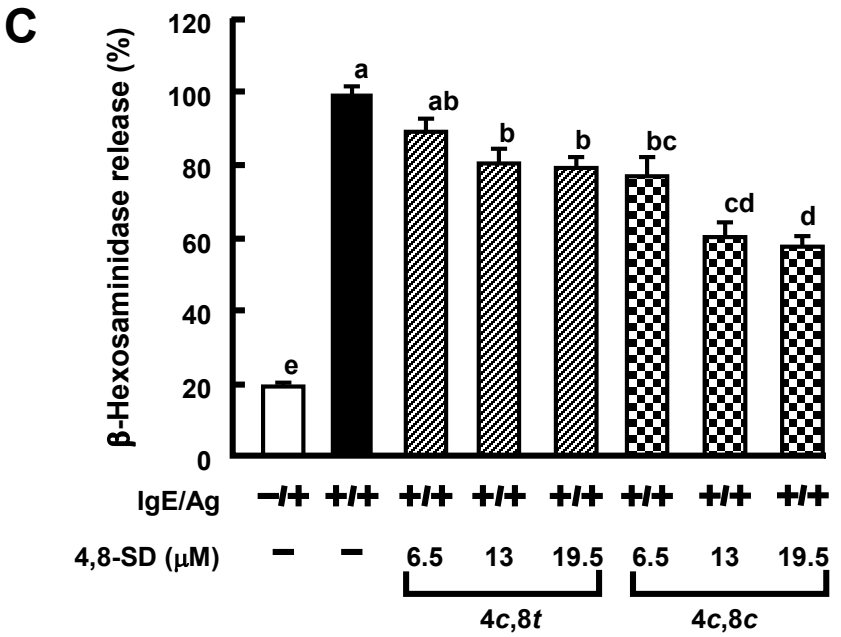


Up

Shimizu *et al.*, Fig. 1







Up

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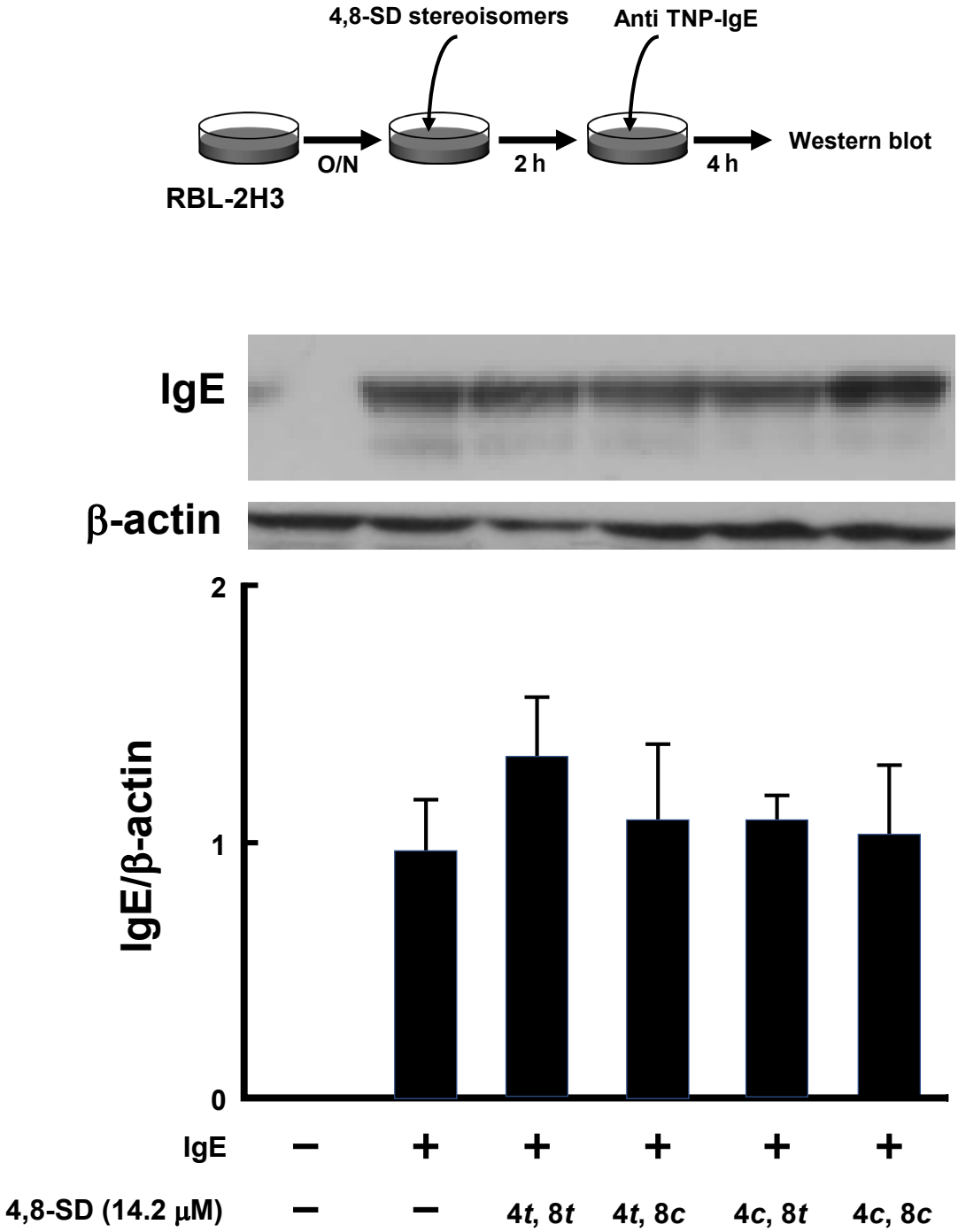
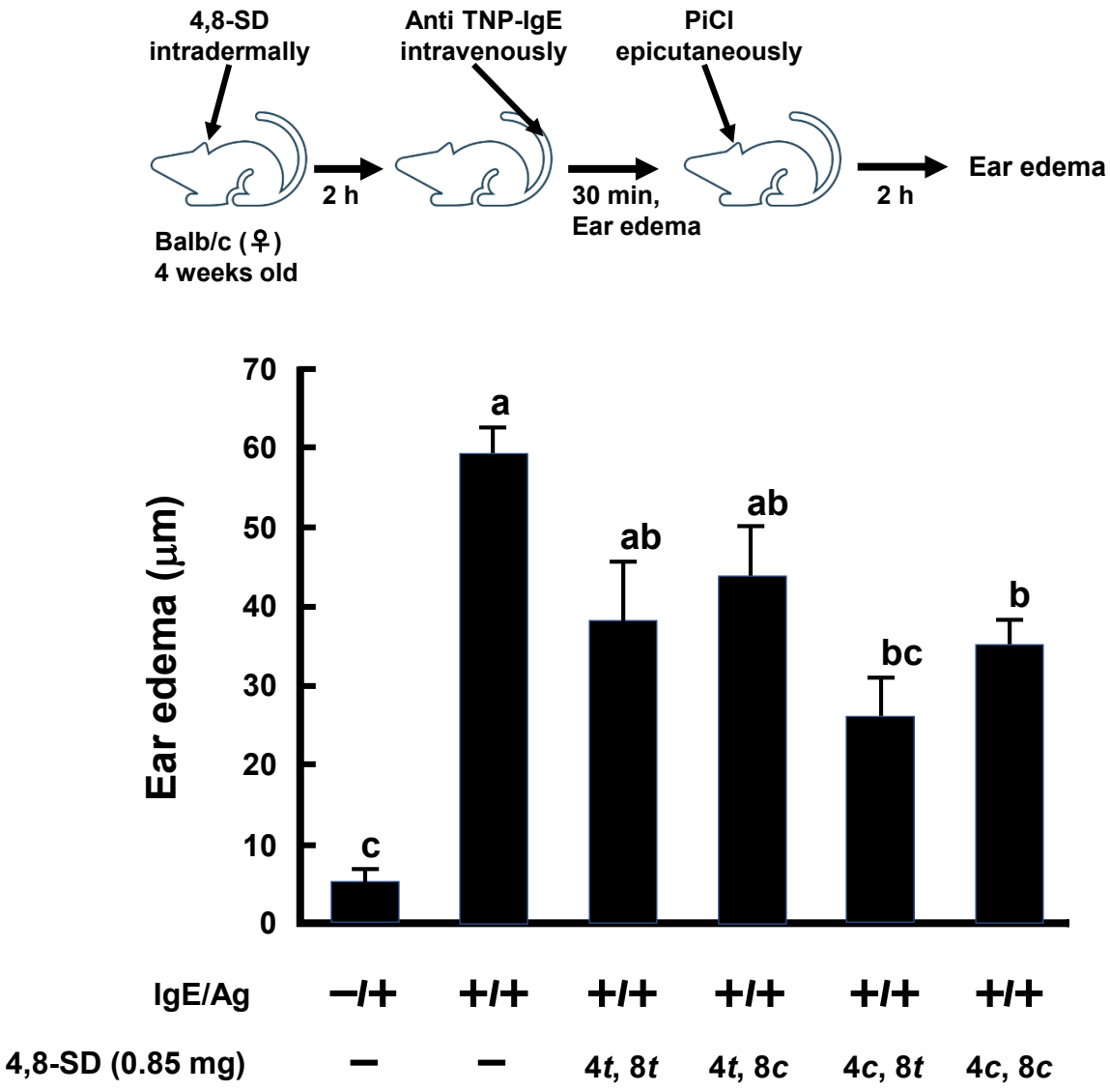


Up

Shimizu *et al.*, Fig. 3



Up

Shimizu *et al.*, Fig. 4