

Inhibitory activities of compounds from the marine actinomycete *Williamsia* sp. MCCC 1A11233 variant on IgE-mediated mast cells and passive cutaneous anaphylaxis

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16 Running title: The anti-allergic activity of compounds from the MCCC 1A11233.

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ABSTRACT

The compounds of the deep-sea-derived marine *Williamsia* sp. MCCC 1A11233 (CDMW) were isolated, which are secondary metabolites of the actinomycetes. In this study, seven kinds of CDMW were found to decrease degranulation and histamine release in immunoglobulin E (IgE)-mediated rat basophilic leukemia (RBL)-2H3 cells. The production of cytokines (tumor necrosis factor- α , interleukin-4) was inhibited by these CDMW in RBL-2H3 cells, and their chemical structures were established mainly based on detailed analysis of their NMR spectra. CDMW-3, CDMW-5, and CDMW-15 were further demonstrated to block mast cell-dependent passive cutaneous anaphylaxis in IgE-sensitized mice. Bone marrow mononuclear cells (BMMCs) were established to clarify the effect of CDMW-3, CDMW-5, and CDMW-15 on mast cells. The seven kinds of CDMW decreased the degranulation and histamine release of BMMCs. Furthermore, flow cytometry results indicated that CDMW-3, CDMW-5, and CDMW-15 increased the annexin⁺ cell population of BMMCs. In conclusion, CDMW-3, CDMW-5, and CDMW-15 have obvious anti-allergic activity due to induction of the apoptosis of mast cells.

KEYWORDS: deep-sea-derived compounds; anti-allergy; RBL-2H3; BMMCs; passive cutaneous anaphylaxis; apoptosis of mast cells

INTRODUCTION

Marine microorganisms, as new sources of active materials, have been the focus of much attention from ocean researchers at home and abroad, in particular chemical researchers and biological medicine researchers ^[1-3]. Deep-sea extreme conditions include no or little light, low concentrations of oxygen and intensely high pressures. Thus, deep-sea organisms all require a diverse array of biochemical and physiological adaptations for survival ^[4]. Deep-sea microorganisms have been considered an important source of bioactive leading compounds ^[5]. At present, some researchers from the United States, Japan, Germany, and France have made contribution to the research of the deep-sea microbial. ^[6] Professor Fenical from the United States discovered a novel structure, a unique active antitumor lead compound (salinosporamide A) from a new deep-sea actinomycete, which has entered preclinical studies as a proteasome inhibitor ^[7]. However, the anti-allergic activities of compounds isolated from deep-sea actinomycetes have seldom been studied.

Allergic disease encompasses four types that are of global public health concern. Type I is the major type, including food allergy, allergic rhinitis, asthma and atopic dermatitis. Type I allergic disease is induced by food, dust and pollen ^[8]. Allergy is an acquired hypersensitivity reaction, manifesting in various forms ranging from allergic rhinitis and conjunctivitis, urticarial and asthma to severe anaphylaxis. These may all cause an enormous socioeconomic burden, however, there are no fundamental approaches to therapy for type I allergic diseases.

In allergic reaction, allergens are captured by antigen-presenting cells (APCs),

64 especially dendritic cells (DCs), and internalized by APCs via phagocytosis,
65 pinocytosis or endocytosis ^[9]. The degraded allergens are presented by the major
66 histocompatibility complex class II (MHC II) to T cell receptors (TCRs) on naïve
67 CD4⁺ T cells. The activated CD4⁺ T cells can be divided into two types of T helper
68 cells (Th1 or Th2), which are classified by the type of cytokines they produce ^[10-11].
69 Allergen-specific Th2 cells secrete Th2 cytokines such as interleukin (IL)-4 and
70 IL-13, which enhance class switching of immunoglobulins from immunoglobulin M
71 (IgM) to allergen-specific immunoglobulin E (IgE) in B cells ^[12]. B cells produce
72 many allergen-specific IgE antibodies, which bind to the high affinity IgE receptor
73 (FcεRI) on the surface of mast cells ^[13]. Upon a second exposure to an allergen
74 recognized by an allergen-specific IgE on FcεRI, cross-linking of IgE-FcεRI with the
75 allergen induces the activation of mast cells, leading to degranulation and the release
76 of inflammatory mediators including histamine, β-hexosaminidase, lipid mediators,
77 Th2 cytokines and chemokines ^[14].

78 Mast cells play an important role in IgE-mediated allergic responses ^[15].
79 Activated mast cells generate several biologically active products, such as
80 cytoplasmic granule-derived mediators, lipid-derived mediators and
81 proinflammatory cytokines ^[16]. Mast cells are activated by cross-linking of
82 allergen-IgE bound to FcεRI on the cell surface ^[17]. Cytoplasmic granule-derived
83 mediators, like histamine, can increase vascular permeability. Lipid-derived
84 mediators, such as leukotrienes (LTs) and prostaglandins (PGs), can stimulate
85 erythema and vasodilation ^[8]. Proinflammatory chemokines and cytokines have the

86 capability to gather other immune cells either directly or indirectly. Therefore, the
87 activation of mast cells leads to both acute inflammation and chronic inflammation
88 ^[16]. In most research studies, rat basophil cells (RBL-2H3) have been used as a
89 model of IgE-mediated allergic responses *in vitro* ^[18]. The surface of RBL-2H3 cells
90 has FcεRI receptors for IgE. Antigens induce formation of the IgE-FcεRI complex,
91 and then initiate several cascades of intracellular events that lead to degranulation or
92 secrete proinflammatory mediators of allergic responses ^[19].

93 In previous studies, our team isolated various compounds from the
94 deep-sea-derived actinomycetes *Nesterenkonia flava* MCCC 1K00610 ^[20],
95 *Microbacterium* sp. MCCC 1A11207 ^[21] and the fungus *Penicillium granulatum*
96 MCCC 3A00475 ^[22]. Then the anti-allergic bioactivities of these compounds were
97 verified using the RBL-2H3 cells model. We previously isolated 24 compounds from
98 the deep-sea-derived actinomycete *Williamsia* sp. MCCC 1A11233 (CDMW) ^[23].
99 However, the activities of these CDMW have not been explored. In this study, we
100 assessed the anti-allergic properties of these CDMW by investigating their effects on
101 IgE-stimulated mast cells *in vitro* and mast cell-dependent passive cutaneous
102 anaphylaxis (PCA) *in vivo*.

103

104 MATERIALS AND METHODS

105 Reagents

106 RPMI 1640, Eagle's minimum essential medium (EMEM),
107 penicillin-streptomycin solution and fetal bovine serum (FBS) purchased from
108 Hyclone (Logan, UT, USA). Anti-dinitrophenyl (DNP)-IgE, Evans blue,

109 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and
110 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide purchased from Sigma (St Louis,
111 MO, USA). DNP-BSA purchased from Biosearch (Petaluma, CA, USA). Goat
112 anti-mouse IgE, IgG1, IgG2a antibodies purchased from Abcam (Cambridge, UK).
113 TNF- α , IL-4 ELISA kits purchased from eBioscience (San Diego, CA, USA.).

114 Strain MCCC 1A11233 was isolated from a sediment sample (depth 1654 m; E
115 49.81, S 37.86) from the southwestern Indian Ocean in February 2014. There are 24
116 kinds of compounds were obtained and numbered CDMW-1~CDMW-24 as
117 described by Xie et al^[23].

118 **RBL-2H3 assay**

119 RBL-2H3 cells were obtained from the American Type Culture Collection
120 (ATCC, Manassas, VA, USA). The RBL-2H3 were cultured in EMEM and
121 supplemented with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin at
122 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere (Forma 3111,
123 Thermo, Waltham, MA, USA).

124 Cell viability was checked using a
125 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric
126 assay (Sigma-Aldrich). RBL-2H3 cells were grown in 96-well plates (5×10^5
127 cells/mL) overnight. After treatment with 20 μ g/mL of CDMW for 24 h, the cells
128 were washed and then treated with 100 μ L of MTT (0.2 mg/mL) and then incubated
129 for an additional 4 h. Cells were then washed, and the insoluble formazan products
130 were dissolved in 100 μ L of DMSO. Absorbance was measured by

131 spectrophotometry at 550 nm using a microplate reader (Bio-Tek Instruments,
132 Winooski, VT, USA).

133 β -Hexosaminidase and histamine were measured using the model of the
134 IgE-mediated mast cell allergic reaction. RBL-2H3 cells were sensitized by
135 anti-DNP-IgE (0.1 $\mu\text{g/mL}$) in 48-well plates (1×10^6 cells/mL) overnight, and the
136 cells were washed with PBS buffer (pH 7.4). After treatment with 20 $\mu\text{g/mL}$ of the
137 CDMW for 1 h in Tyrode's buffer at 37 °C, the cells were then stimulated by
138 DNP-BSA (0.1 $\mu\text{g/mL}$) for 1 h in Tyrode's buffer at 37 °C. To measure the total
139 β -hexosaminidase activity of the RBL-2H3 cells, they were lysed with 0.1% Triton
140 after removing the supernatant. The activity was quantified by measuring the
141 fluorescence intensity (360 nm excitation and 450 nm emission). After stimulation
142 with DNP-BSA (0.1 $\mu\text{g/mL}$) for 15 min in Tyrode's buffer at 37 °C, histamine was
143 measured in the supernatant using an enzyme immunoassay kit (IBL, Hamburg,
144 Germany).

145 Degranulation inhibition rate were calculated as $100 \times [(\text{DNP-BSA}_{\text{release rate}} -$
146 $\text{Sample}_{\text{release rate}}) / (\text{DNP-BSA}_{\text{release rate}} - \text{PBS}_{\text{release rate}})]$. Histamine inhibition rate
147 were calculated as $100 \times [(\text{DNP-BSA}_{\text{concentration}} - \text{Sample}_{\text{concentration}}) / (\text{DNP-BSA}$
148 $\text{concentration} - \text{PBS}_{\text{concentration}})]$.

149 RBL-2H3 cells were sensitized by anti-DNP-IgE (0.1 $\mu\text{g/mL}$) overnight. The
150 cells were treated with seven kinds of CDMW (20 $\mu\text{g/mL}$) for 1 h and stimulated
151 with DNP-BSA for 4 h or 6 h. Interleukin (IL)-4 and tumor necrosis factor
152 (TNF)- α in the cell supernatant were quantified by sandwich immunoassays using

153 the protocol supplied by Perprotech (Princeton, NJ, USA).

154 **Chemical structure assays of the CDMW**

155 The chemical structures of the CDMW-2, CDMW-3, CDMW-5, CDMW-6,
156 CDMW-8, CDMW-15, and CDMW-21 were confirmed by comparing the NMR and
157 MS data with literature references ^[24-26].

158 **Passive cutaneous anaphylaxis in mice**

159 Female BALB/c mice, at 6-8 weeks, were purchased from the Shanghai
160 Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China),
161 and housed in an SPF environment maintained at 22 ± 1 °C with a relative humidity
162 of $55 \pm 10\%$. Experiments were performed in conformity with the laws and
163 regulations for treatment of live animals of Jimei University, SCXK 2012-0005.

164 An IgE-dependent PCA reaction was performed in accordance with a previous
165 study^[46]. Anti-DNP-IgE (0.1 µg) was injected subcutaneously into the right ear of
166 each BALB/c mouse. After 24 h, the mice were orally administered CDMW-3,
167 CDMW-5, and CDMW-15 (20 mg/kg). After 1 h, the mice were injected
168 intravenously with DNP-BSA (0.2 mg) in 200 µL PBS containing Evans blue (5
169 mg/mL). After 30 min, mice were photographed and sacrificed. For subsequent
170 measurements, the pigmented areas of the ears were collected and mixed in 250 µL
171 of 3 M KOH at 37 °C overnight. Acetone and phosphoric acid (13:5) were mixed
172 and added (225 µL), after which the samples were centrifuged (3000 rpm, 15 min).
173 The supernatants were used for absorbance measurements ($A_{620\text{ nm}}$).

174 **Bone marrow mononuclear cells (BMMCs) assay**

175 BMMCs were obtained as described by Jung-Hwan Kim^[27]. Bone marrow cells
176 from the thighbone of BALB/c mice were grown in RPMI 1640 medium and
177 supplemented with IL-3 (10 ng/mL; R&D Systems, Minneapolis, MN, USA) and
178 M-SCF (50 ng/mL, R&D Systems) for about 4 weeks, and the BMMCs were sorted
179 by c-Kit⁺ FcεRIα⁺ using FACS.

180 To detect the IgE-activated BMMCs, cells were sensitized with 0.1 μg/mL
181 anti-DNP-IgE overnight. CDMW-3, CDMW-5, and CDMW-15 were added to the
182 medium at a final concentration of 20 μg/mL for 1 h, followed by stimulation with
183 0.5 μg/mL DNP-BSA for 1 h. Then the cells were washed with staining buffer. To
184 detect apoptotic cells, annexin V and propidium iodine (PI) (Biolegend Pharmingen,
185 San Diego, CA, USA) staining was used. All FACS experiments were performed
186 with a Guava easyCyte 6-2L system and analyzed with GuavaSoft 3.1.1 software
187 (Millipore, Billerica, MA, USA).

188 **Statistical analysis**

189 Results are expressed as the mean ± standard deviation (SD); differences
190 between means were analyzed by Analysis of Variance (ANOVA) of
191 Least-Significant Difference test results in Statistical Product and Service Solutions.
192 A $p < 0.05$ was considered statistically significant. In the animal model study, samples
193 from individual mice were processed and analyzed separately. Each experiment was
194 repeated at least three times.

195

196

197 **RESULTS**

198 **Effects of CDMW on degranulation and histamine release in IgE-sensitized** 199 **RBL-2H3 cells**

200 CDMW are obtained as described by Chunlan Xie ^[23], numbered as
201 CDMW-1~CDMW-24. To determine the anti-allergic activities of the CDMW, we
202 examined their potential bioactivities in suppressing degranulation and histamine
203 release in IgE-sensitized mast cells. The release of β -hexosaminidase is an index of
204 degranulation. DNP-BSA can induce the degranulation in IgE-sensitized RBL-2H3
205 cells. At a concentration of 20 μ g/mL, there were seven kinds of CDMW that could
206 significantly inhibit antigen-induced degranulation, CDMW-2, CDMW-3, CDMW-5,
207 CDMW-6, CDMW-8, CDMW-15, and CDMW-21 (Table 1). The inhibition rates
208 were greater than 15%, among which the CDMW-3, CDMW-5, CDMW-8, and
209 CDMW-15 showed the greatest inhibition rates of 25.29%, 25.07%, 27.63% and
210 31.06%, respectively. The effects of the CDMW on histamine release were also
211 assessed to investigate potential anti-allergic activities. The seven kinds of CDMW
212 also had an apparent effect of inhibiting antigen-induced histamine release in
213 IgE-sensitized RBL-2H3 cells (Table 1). The inhibition rates were greater than 30%,
214 among which CDMW-2, CDMW-3, CDMW-5, and CDMW-15 showed the greatest
215 inhibition rates of 49.33%, 50.10%, 73.38%, and 54.51%, respectively. Thus, among
216 all of the CDMW, the CDMW-3, CDMW-5, and CDMW-15 had the most significant
217 effects on degranulation and histamine release.

218 **Effects of CDMW on IL-4 and TNF- α release in IgE-sensitized RBL-2H3 cells**

219 Allergic inflammatory responses are induced by proinflammatory cytokines
220 from activated mast cells. Mast cells play an important role in initiating and
221 perpetuating the inflammatory response in allergic reactions by secreting large
222 amounts of cytokines such as IL-4 and TNF- α ^[34]. Consistent with a previous report
223 ^[28], increases in the production of TNF- α and IL-4 were obvious upon DNP-BSA
224 stimulation in mast cells. The seven kinds of CDMW could all decrease the
225 production of IL-4 and TNF- α (Figures 1A and 1B). In particular, CDMW-5 and
226 CDMW-15 (20 μ g/mL) significantly diminished the release of IL-4 by 29.18% and
227 50.02% and TNF- α by 31.21% and 31.80%, respectively, in IgE-sensitized RBL-2H3
228 cells. These results showed that the seven kinds of CDMW, especially CDMW-5,
229 and CDMW-15, had inhibitory effects on the production of proinflammatory
230 cytokines in RBL-2H3 cells. None of the seven kinds of CDMW (20 μ g/mL) had
231 significant cytotoxic effects on RBL-2H3 cell viability (data not shown).

232 Identification of the chemical structures of the CDMW of MCCC 1A11233

233 As seven kinds of CDMW displayed possible anti-allergic activity, we further
234 analyzed their structures. As shown in Figure 2 and Table 2, there are four
235 diketopiperazines: cyclo-(Pro-Pro) (CDMW-2), cyclo-(L-prolyl-L-leucine)
236 (CDMW-3), cyclo-(D-Trp-L-Pro) brevianamide F (CDMW-5), cyclo-(L-Val-L-Pro)
237 (CDMW-6); one indole: *N*-[2-(1*H*-indol-3-yl)ethyl]-propanamide (CDMW-8); and
238 two phenolics: 2-(acetylamino)-benzoic acid (CDMW-15), 2-phenyl-2,3-butanediol
239 (CDMW-21).

240 Effects of the CDMW on IgE-mediated PCA in mice

241 In this study, we investigated whether three of the CDMW (CDMW-3,
242 CDMW-5, and CDMW-15) could inhibit IgE-mediated PCA, an immediate
243 hypersensitivity reaction in the dermis, and allergic reactions in mast cells. IgE was
244 injected subcutaneously into mouse ear tissue, followed by injection of DNP-BSA
245 containing 5% Evans blue. Oral administration of CDMW-3, CDMW-5, and
246 CDMW-15 at doses of 20 mg/kg significantly suppressed IgE-induced PCA, which
247 was observed by the impaired extravasation of Evans blue dye in the ear (Figures 3A
248 and 3B). The quantity of Evans blue in the ears decreased by 18.86%, 33.35%, and
249 37.26% after administration of CDMW-3, CDMW-5, and CDMW-15, respectively.

250 **Effects of CDMW on degranulation and histamine release in IgE-sensitized** 251 **BMMCs**

252 Mast cells play prominent roles in allergic responses and anaphylaxis. To further
253 verify the effects of CDMW on mast cells, we introduced the IgE-mediated BMMC
254 model. As shown in Figure 5A, 93.54% of the cells were c-Kit⁺FcεRI⁺ mast cells after
255 culturing for 4 weeks with IL-3 and M-SCF. The degranulation and histamine release
256 of IgE-mediated BMMCs were determined. As shown in Table 2, the seven kinds of
257 CDMW that could inhibit the degranulation of RBL-2H3 cells. It also demonstrated
258 suppression effects on the degranulation and histamine release in BMMCs. The
259 CDMW-3, CDMW-5, and CDMW-15 especially displayed more efficient suppression
260 according to the degranulation inhibition percentages of 18.52%, 27.36%, and 29.68%
261 and the histamine inhibition percentages of 41.43%, 38.08%, and 43.94%,
262 respectively.

263 **Effects of CDMW on the apoptosis of activated BMMCs**

264 In order to clarify the mechanism of the inhibitory effect of the three kinds of
265 CDMW (CDMW-3, CDMW-5, and CDMW-15) on mast cells, the apoptosis of
266 BMMCs was measured by flow cytometry though staining with annexin V and PI.
267 BMMCs were sensitized by anti-DNP-IgE for 16 h, followed by dividing them into
268 un-stimulated BMMCs and DNP-BSA-stimulated BMMCs to culture with or
269 without CDMW-3, CDMW-5, and CDMW-15. The three kinds of CDMW showed
270 no significant effects on the high-affinity receptor FcεRI of either un-activated
271 BMMCs or activated BMMCs. They also showed no significant effects on the
272 annexin V⁺ cell population in un-stimulated BMMCs (data not shown). In
273 DNP-BSA-stimulated BMMCs, the CDMW-3, CDMW-5, and CDMW-15
274 interestingly increased the annexin V⁺ cell population. As shown in Figure 5B, the
275 total annexin V⁺ cell population was 22.03% in DNP-BSA-stimulated BMMCs,
276 while after culturing with the CDMW-3, CDMW-5, and CDMW-15, the annexin V⁺
277 cell population rose to 35.34%, 36.47%, and 34.50%, respectively. To eliminate the
278 effects of dead cells, the PI⁺ annexin V⁺ cell population was counted (Figure 5C), and
279 the three compounds, especially CDMW-15, could significantly up-regulate the early
280 apoptosis of DNP-BSA-stimulated BMMCs.

281

282 **DISCUSSION**

283 Due to the harsh environment, the secondary metabolites of deep sea microbes
284 have great value in research and applications and are the basis of numerous drug
285 development resources. However, research on the activities of secondary metabolites

that have been isolated from deep sea microbes is still very scarce. Approximately 75% of known antibiotics are derived from secondary metabolites produced by filamentous actinomycetes ^[28]. For example, rapamycin which is an immune inhibitor comes from actinomycetes ^[29]. It was reported that the compounds isolated from the actinomycetes *Nesterenkonia flava* MCCC 1K00610^[20] and *Microbacterium* sp. MCCC 1A11207 ^[21] show anti-allergy activity in the RBL-2H3 cell model. Under the concentration of 20 µg/mL, nine compounds (1, 4–10, and 13) which isolated from the actinomycetes *Nesterenkonia flava* MCCC 1K00610 showed the positive effects in degranulation of RBL-2H3 cells, but the inhibitory effects were not very well. Microindolinone A (1) which isolated from *Microbacterium* sp. MCCC 1A11207 did not show any positive effects under the concentration of 20 µg/mL. Now Xie et al. isolated the 24 kinds of compounds, CDMW-1~CDMW-24, from actinomycete *Williamsia* sp. MCCC 1A11233. It is the first report on chemical constituents of *Williamsia* species about the 24 kinds of compounds^[23]. The yield of the compounds is high in the fermentation. CDMW-15 is one of the major compounds, which has efficient activity of anti-allergy. The yield of compounds also provide the potential to prevent or treat IgE-sensitized allergic disorders

A recent study revealed that mast cells release chemical mediators, such as histamines, cytokines and proteases, which play a crucial role during allergy-mediated inflammation ^[30]. There were many studies in which factors involved in anti-allergic activities have been reported. Various factors examined as potential resources for anti-allergic therapeutics ^[31-32]. Almost all of them were

308 applied directly to RBL-2H3 cells ^[32-33]. The RBL-2H3 cell monoculture system was
309 widely used as a tool for investigating the effects of factors on the activation of mast
310 cells, especially degranulation ^[34-35], histamine release and cytokine release ^[17].
311 Ishida found that a water spinach extract can inhibit mast cell degranulation and has
312 a certain anti-allergic activity in the RBL-2H3 cell model ^[36]. It was reported that
313 chitosan can inhibit IgE-mediated activation of RBL-2H3 cell function,
314 degranulation and histamine release levels upon treatment with 1000 µg/ml of COS I
315 and COS II reduced obviously. ^[37] In addition, ethyl acetate extracts of *Cordyceps*
316 *sinensis* could inhibit an IgE-mediated allergic response using the RBL-2H3 cell
317 model ^[38]. IL-4 and TNF-α are essential participants in allergy-related diseases ^[39].
318 Consistently with the previous report, the increase of the production and expression
319 of TNF-α and IL-4 was obvious by DNP-BSA stimulation in mast cell ^[40]. The
320 recent study reported that the RBL-2H3 cell model can prove the anti-allergy activity
321 of sulfated polysaccharide from *Gracilaria lemaneiformis* (GLSP). This study
322 revealed that GLSP can inhibit the release and production of pro-inflammatory
323 mediators and cytokines in RBL-2H3 cells. The levels of IL-4 and TNF-α were
324 significantly reduced by GLSP (100-200 µg/mL) in a concentration dependent
325 manner. ^[41] In Table 1 and Figure 1, our results indicated that the seven kinds of
326 CDMW can inhibit the release of β-hexosaminidase, histamine, IL-4 and TNF-α in
327 RBL-2H3 cells (20 µg/mL). They all had no effects on cell viability. The CDMW-3,
328 CDMW-5, and CDMW-15 especially had more obvious inhibitory effects on the
329 release β-hexosaminidase, histamine, IL-4 and TNF-α in RBL-2H3 cells. These all

330 indicated, that CDMWs have stronger inhibition effect which compared with COS
331 and GLSP in 20 µg/mL.

332 Diverse bioactive compounds, including polyketides, alkaloids, fatty acids,
333 peptides, and terpenes ^[42], all displayed a wide range of biological activities,
334 including antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory,
335 anti-inflammatory, antioxidant and anticancer activities ^[43]. We further analyzed the
336 chemical structures of the seven compounds and named them (Figure 2 and Table 2).
337 Cyclo-(D)-Pro-(D)-Leu of *Nesterenkonia flava* MCCC 1K00610 exhibited
338 significant anti-allergic activities. The degranulation inhibition rate of cyclo
339 (D)-Pro-(D)-Leu was 16.99% ^[20]. As can be seen in Table 2, CDMW-3 and
340 CDMW-5 are also diketopiperazines. But the degranulation inhibition rate of
341 CDMW-3 was 25.29%, which was 1.49-fold that of cyclo-(D)-Pro-(D)-Leu in 20
342 µg/mL. Comparing the structures of cyclo-(D)-Pro-(D)-Leu and CDMW-3, this may
343 because the strength of the hydrogen bond is different. It was reported that the
344 phenolic fraction of mint had anti-inflammatory activity ^[44]. CDMW-15 and
345 Cyclomarin A are phenolic. Cyclomarin A, isolated from *Streptomyces* sp., has
346 anti-inflammatory and antiviral effects ^[45]. CDMW-15 also can inhibit the release of
347 β-hexosaminidase and histamine in RBL-2H3 cells (Table 1) and BMMCs (Table 2).
348 CDMW-15, which is a part of the phenolic fraction, also exhibited anti-allergic
349 activity in IgE-mediated mast cells and PCA.

350 PCA is generally used to measure local allergic responses *in vivo* ^[46]. It was
351 reported that *Enterococcus faecalis* IC-1 and FK-23 have anti-allergic effects in an *in*

352 *in vivo* PCA model ^[30]. *Cassia tora* seed extract (CTE) and its active component
353 aurantio-obtusin could suppress the IgE-sensitized PCA response, but the
354 concentration was 200 mg/kg and 50 mg/kg ^[47]. As we can see from Figures 3A and
355 3B, the CDMW-3, CDMW-5, and CDMW-15 can reduce the quantity of Evans blue
356 in the ears of mice in 20 mg/kg. Thus, it was demonstrated that the CDMW-3,
357 CDMW-5, and CDMW-15 can exert more efficient anti-allergic effects in an *in vivo*
358 PCA model.

359 Mast cells are often associated with human allergic disease and implicated in
360 mouse models of the autoimmune diseases multiple sclerosis and rheumatoid
361 arthritis ^[48-49]. Activation of FcεRI receptors in mast cells binding and aggregating
362 IgE, leads to degranulation and production of inflammatory mediators. Inflammatory
363 mediators include inflammatory cytokines, lipid-derived mediators and histamine ^[50].
364 It is thought that allergic symptoms are mitigated by inhibiting mast cell activation
365 ^[30]. We explored the effect of CDMW about the activated mast cells. The mast cells
366 may significantly contribute to immunity by shifting the balance of protective and
367 pathologic responses. Although mast cells exert a protective function, activation of
368 mast cells can result in deleterious effects. To detect the effects of CDMW on mast
369 cell function, we cultured the BMMCs with IL-3 and M-SCF for 4 weeks (Figure
370 5A). The seven kinds of CDMW inhibited the release β-hexosaminidase and
371 histamine in BMMCs (Table 2). The CDMW-3, CDMW-5, and CDMW-15
372 especially had more obvious inhibitory effects in 20 μg/mL. It was demonstrated that
373 the CDMW-3, CDMW-5, and CDMW-15 have inhibitory effect in activated

374 BMMCs. It is reported that ESO, which has anti-allergy activity, can induce the
375 apoptosis of activated BMMCs, but the proportion is very small in 100 $\mu\text{g/mL}$ [51]. In
376 order to further characterize the anti-allergic effect of the CDMW, we also used flow
377 cytometry to detect the apoptosis of BMMCs. The CDMW-3, CDMW-5, and
378 CDMW-15 up-regulated the PI⁺ annexin V⁺ cells. This had significant effects on the
379 apoptosis of activated BMMCs. As can be seen in Figures 5B and 5C, the CDMW-3,
380 CDMW-5, and CDMW-15 can induce the early apoptosis of activated BMMCs,
381 especially CDMW-15. It indicated that the compounds may have potential to prevent
382 or treat IgE-sensitized allergic disorders.

383 Seven compounds (four cyclic dipeptides, CDMW-2, CDMW-3, CDMW-5, and
384 CDMW-6; three phenol derivatives, CDMW-8, CDMW-15, and CDMW-21) showed
385 a significant inhibitory effect on the IgE-mediated allergic reaction. As shown in
386 Table 1, the inhibition rate of degranulation followed the order: cyclo-(L-Pro-D-Trp)
387 (CDMW-5) > cyclo-(L-Pro-L-Leu) (CDMW-3) > cyclo-(L-Pro-L-Pro) (CDMW-2) >
388 cyclo-(L-Pro-L-Val) (CDMW-6), which was consistent with the inhibition rates for
389 histamine release. Interestingly, these four cyclic dipeptides all contain a proline
390 moiety, except that CDMW-5 contains a tryptophan unit, while the CDMW-2,
391 CDMW-3, and CDMW-6 contain a leucine, proline and valine, respectively. This
392 indicates that the number of carbons and cyclization are important for bioactivity:
393 the longer the carbon chain, the stronger the bioactivity (CDMW-5 > CDMW-3 >
394 CDMW-2). For the same carbon chain, more residues with ring systems produced
395 stronger bioactivity (CDMW-2 > CDMW-6). The CDMW-15 is a benzoic acid

396 derivative. The structure of the carboxylic group may enhance the inhibitory effect
397 of CDMW-15.

398 Taken together, the present study demonstrated that seven kinds of CDMW
399 could suppress degranulation and the generation of histamine and proinflammatory
400 cytokines (IL-4 and TNF- α) in IgE-sensitized RBL-2H3 cells and BMMCs. The
401 CDMW-3, CDMW-5, and CDMW-15 had greater suppression effects. Furthermore,
402 the CDMW-3, CDMW-5, and CDMW-15 could induce the early apoptosis of
403 activated BMMCs and had anti-allergic effects in the PCA model. Our results
404 suggest that CDMW-3, CDMW-5, and CDMW-15 can ease the IgE-mediated
405 allergic reaction. The three kinds of compounds have the potential to prevent or treat
406 IgE-sensitized allergic disorders.

407

408 **Abbreviations Used:** ANOVA, Analysis of Variance; APC, antigen-presenting cells;
409 BMMCs, bone marrow-derived mast cells; CDMW, compounds of deep-sea-derived
410 marine *Williamsia sp.* MCCC 1A11233; DCs, dendritic cells; DNP, Dinitrophenyl;
411 FACS, flow cytometry; FBS, fetal bovine serum; Fc ϵ RI, high affinity IgE receptor I;
412 IgE, immunoglobulin E; IL-4, interleukin-4; LTs, leukotriene; MHC II, major
413 histocompatibility complex class II; MTT,
414 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA, passive
415 cutaneous anaphylaxis; PGs, prostaglandins; TCRs, T cell receptors; Th, T helper
416 cells; TNF- α , tumor necrosis factor- α ;

417

418 Conflict of interest

419 The authors declare that there is no conflict of interests.

420

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426

427 Author contributions

428 Guangming Liu designed the study, participated in data analysis and extensively
429 reviewed the manuscript. Yuanyuan Gao performed the experiments, analyzed the
430 data and drafted the manuscript. Xianwen Yang and Chunlan Xie provided the
431 compounds and reviewed the manuscript. Other authors participated in the
432 experiments and reviewed the manuscript.

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Table and Figure captions**Table 1** Effects of CDMW on degranulation and histamine release in RBL-2H3 cells

The RBL-2H3 cells were incubated overnight in 48-well plates with 100 ng/mL of DNP-specific IgE in the medium. The medium was replaced by Tyrode's buffer containing the indicated concentrations of CDMW (20 µg/mL) followed by stimulation with 500 ng/mL DNP-BSA for 1 h and 15 min; β -hexosaminidase and histamine release was measured.

Table 2 Names of the CDMW and effects of CDMW on degranulation and histamine release in BMDCs

The BMDCs were incubated overnight in 48-well plates with 100 ng/mL of DNP-specific IgE in the medium. The medium was replaced by Tyrode's buffer containing the indicated concentrations of CDMW (20 µg/mL) followed by stimulation with 500 ng/mL DNP-BSA for 1 h and 15 min; β -hexosaminidase and histamine release was measured.

Figure 1 Effects of CDMW on IL-4 and TNF- α production in RBL-2H3 cells

RBL-2H3 cells were incubated overnight in 48-well plates with 100 ng/mL of DNP-specific IgE in the medium, and treated with the seven compounds for 1 h, then stimulated with DNP-BSA for 4 h and 6 h. IL-4 (A) and TNF- α (B) production was determined by enzyme-linked immunosorbent assay kit.

Results are expressed as the mean $\pm$ SD of three independent experiments. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01, significantly different from DNP-BSA group.

Figure 2 Chemical structures of the CDMW

By analysis and comparison of the NMR and MS data, the chemical structures of the compounds

581 were determined.

582 **Figure 3** Effects of CDMW-3, CDMW-5, and CDMW-15 on IgE-mediated PCA

583 A, Mice were sensitized with IgE for 1 h and then orally administered CDMW-3, CDMW-5, and

584 CDMW-15 (20 mg/kg) for 1 h. The ears of mice with Evans blue absorbed were photographed.

585 B, The absorbed dye was extracted with 3 M KOH and acetone and phosphoric acid (13:5).

586 Data are expressed as the mean $\pm$ SD ($n \geq 3$). Statistical differences are indicated by *P* values

587 (one-way ANOVA). **P* < 0.05, ***P* < 0.01, significantly different from DNP-BSA group.

588 **Figure 4** Identification of BMMCs by flow cytometry and effects of CDMW-3, CDMW-5, and

589 CDMW-15 on the apoptosis of BMMCs.

590 BMMCs from the thighbones of BALB/c mice were incubated with the CDMW-3, CDMW-5,

591 and CDMW-15 (20 μ g/mL).

592 A, Bone marrow cell were cultured for 4 weeks with IL-3 (10 ng/mL) and M-SCF (50 ng/mL)

593 for getting c-Kit⁺Fc ϵ RI⁺ mast cells. The results were analyzed by flow cytometry.

594 B, Effects of CDMW-3, CDMW-5, and CDMW-15 on the annexin V⁺ cell population.

595 C, Effects of CDMW-3, CDMW-5, and CDMW-15 on the PI⁺ annexin V⁺ cell population.

596 The flow cytometric chart on the left is the result of a single experiment, and the histogram on

597 the right is the average of three independent experiments.

598 Results are expressed as the mean $\pm$ SD of three independent experiments. Statistical differences

599 are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01, significantly different

600 from DNP-BSA group.

601

602 **Table 1**
603

Compound	Inhibition rate of degranulation (%)	Inhibition rate of histamine (%)
CDMW-2	19.74 ± 2.97	49.33 ± 1.88
CDMW-3	25.29 ± 2.29	50.10 ± 2.79
CDMW-5	25.07 ± 2.59	73.38 ± 5.24
CDMW-6	15.87 ± 1.91	39.94 ± 2.32
CDMW-8	27.63 ± 1.97	46.09 ± 3.97
CDMW-15	31.06 ± 0.71	54.51 ± 2.98
CDMW-21	23.28 ± 3.69	31.46 ± 1.22
Other 17 compounds	N	N

604 Note: N- inhibition rate less than 5.00 %

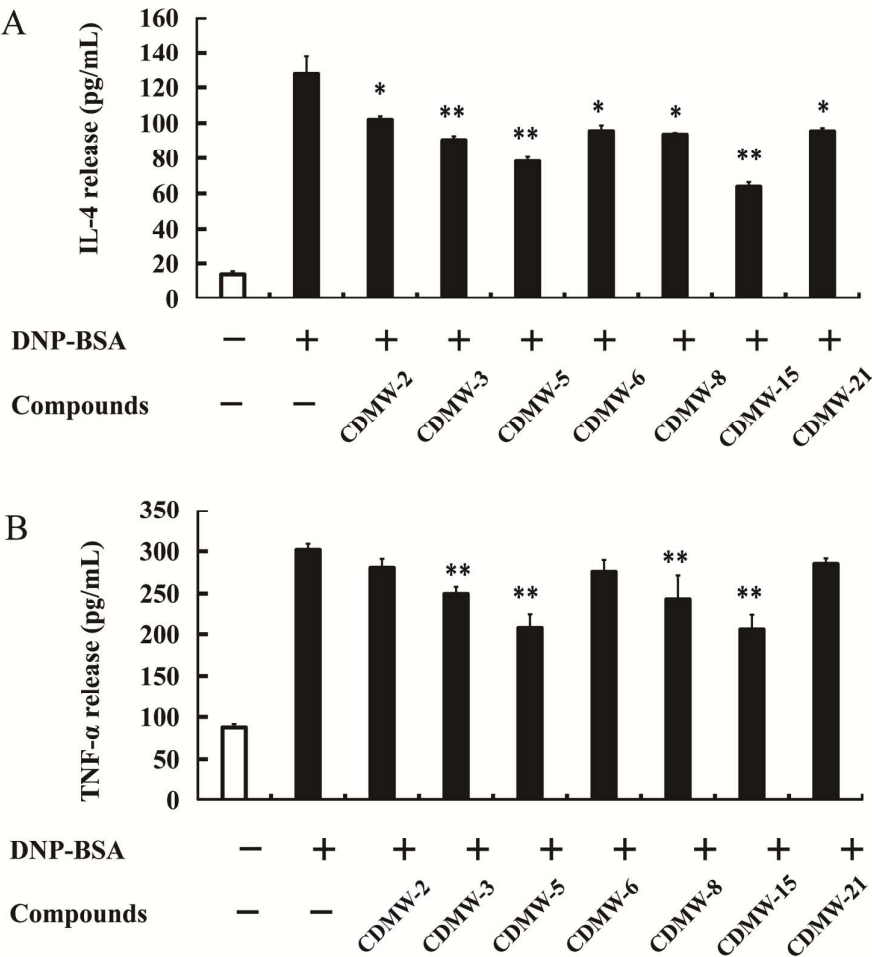
605 **Table 2**

606

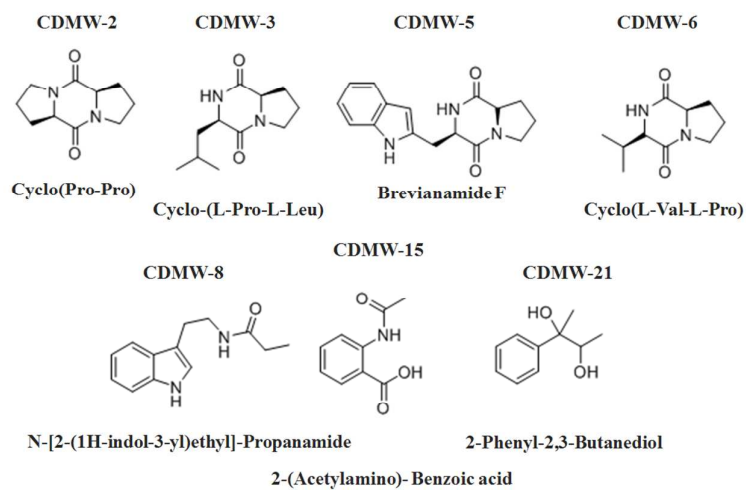
Compound	Name	Inhibition rate of Degranulation (%)	Inhibition rate of Histamine (%)
CDMW-2	Cyclo (Pro-Pro)	12.00 ± 2.75	17.15 ± 2.75
CDMW-3	Cyclo (L-Pro-L-Leu)	18.52 ± 1.84	41.43 ± 2.38
CDMW-5	Brevianamide F	27.36 ± 3.53	38.08 ± 1.83
CDMW-6	Cyclo (L-Val-L-Pro)	10.27 ± 2.59	34.84 ± 3.08
CDMW-8	N-[2-(1 <i>H</i> -indol-3-yl)eth yl]-Propanamide	16.44 ± 2.68	36.96 ± 1.35
CDMW-15	2-(Acetylamino)-benzoi c acid	29.68 ± 3.61	43.94 ± 2.49
CDMW-21	2-Phenyl-2,3-butanediol	16.27 ± 1.35	20.27 ± 1.29

607

608 **Figure 1**

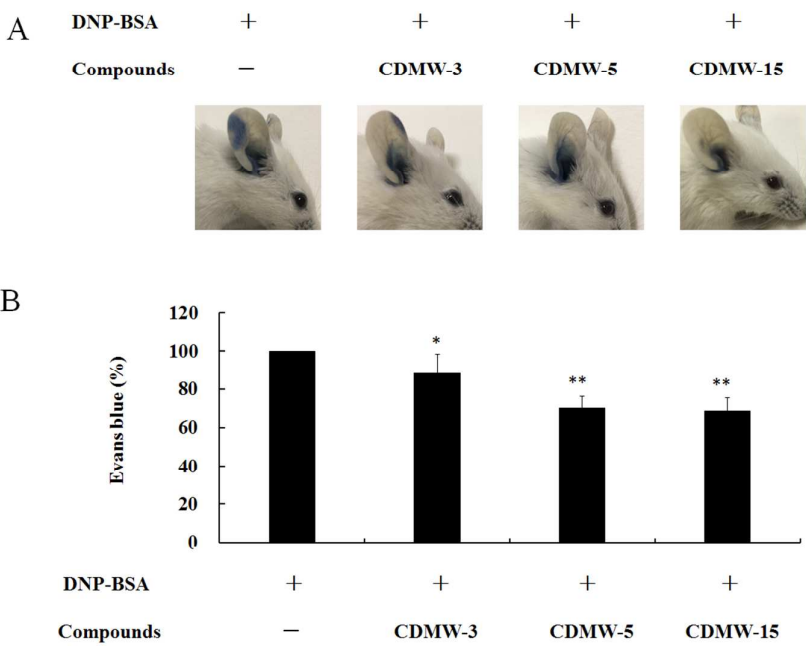


609
610

Figure 2

615 **Figure 3**

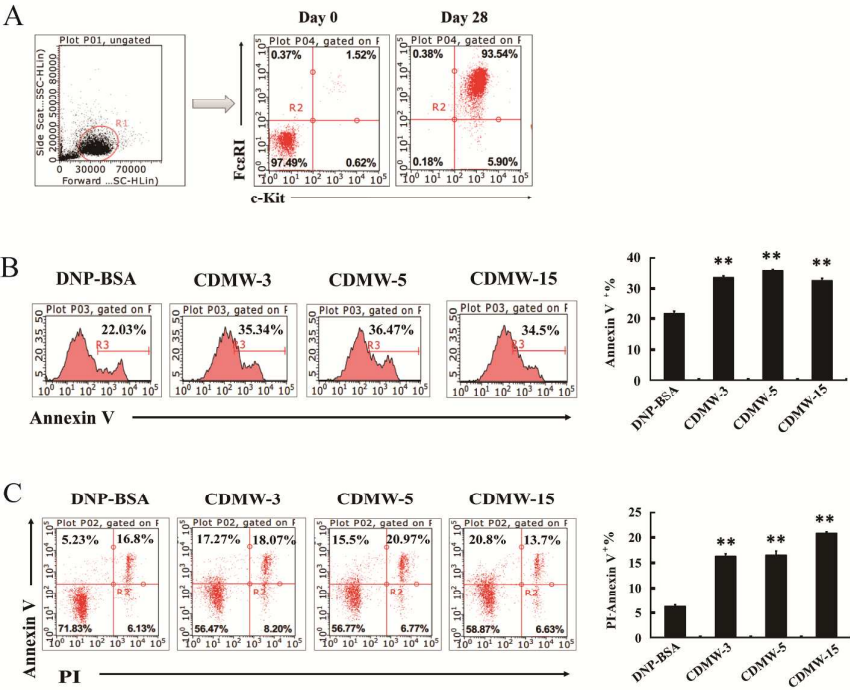
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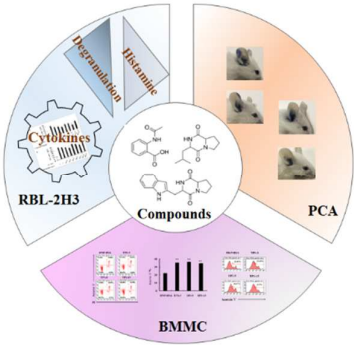
618

619 **Figure 4**



620

621 **Graphic for table of contents**



622