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Structures of the Dimeric and Monomeric Chromanones, Gonytolides A–C, Isolated from the Fungus *Gonytrichum* sp. and Their Promoting Activities of Innate Immune Responses

Haruhisa Kikuchi,* Masato Isobe, Mizuki Sekiya, Yuko Abe, Tsuyoshi Hoshikawa, Kazunori Ueda, Shoichiro Kurata, Yasuhiro Katou, and Yoshiteru Oshima

Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-yama, Aoba-ku, Sendai 980-8578, Japan

hal@mail.pharm.tohoku.ac.jp

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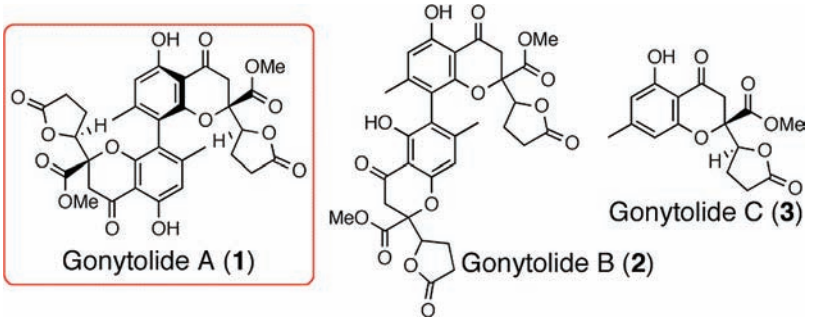
ABSTRACT



Innate immunity is the front line of self-defense against microbial infection. After searching for natural substances that regulate innate immunity using an *ex vivo Drosophila* culture system, we identified a novel dimeric chromanone, gonytolide A, as an innate immune promoter from the fungus *Gonytrichum* sp. along with gonytolides B and C. Gonytolide A also increased TNF-α-stimulated production of IL-8 in human umbilical vein endothelial cells.

Innate immunity is the front line of self-defense against microbial infection,[1,2] and the basic mechanisms of this process, including pathogen recognition and immune response activation, are evolutionarily conserved.[3] In mammals, innate immunity interacts with adaptive immunity and plays a key role in regulating the immune response.[4] Therefore, innate immunity is a good pharmaceutical target for the development of immune regulators to suppress unwanted immune responses, such as septic shock, inflammatory diseases, and autoimmunity. The innate immune system also provides targets for the development of agents that stimulate protective immune responses toward some diseases, such as infectious diseases and cancer.

To screen pharmaceuticals that target innate immunity, we established an *ex vivo* culture system based on the *Drosophila* IMD (immune deficiency) signaling pathway.[5,6] Because of the striking conservation between the mechanisms that regulate insect immunity and mammalian innate immunity,[2,3] *Drosophila* is a model organism for genetic and molecular studies of innate immunity, and our culture system has proven to be useful for identifying immune regulators that act on human innate immunity. We used this system to search for natural substances that regulate

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innate immunity.[7−10] This paper describes the isolation and structure elucidation of the novel dimeric and monomeric chromanones, gonytolides A−C (**1**−**3**), from the fungus *Gonytrichum* sp. The promoting activities of gonytolide A (**1**) and its derivatives on innate immune responses are also described.

Gonytrichum sp. was cultivated in production medium containing cottonseed proteins. The culture broth (10 L) was extracted with butanol at room temperature to yield an extract (21.1 g). In the *ex vivo Drosophila* culture system,[6] 100 μg/mL of the extract increased the innate immune response by 176%. Then, bioactivity-guided fractionation of the extract yielded gonytolide A (**1**) (87 mg). Gonytolide B (**2**) (3 mg) and C (**3**) (2 mg) were also isolated from the extract.

HREIMS (*m*/*z* 638.1616 [M^+]) indicated a molecular formula of gonytolide A (**1**) as $C_{32}H_{30}O_{14}$. The ^{13}C NMR spectrum of **1** showed the presence of the following carbon atoms: one keto carbonyl, two ester carbonyl, five sp^2 quaternary, one sp^2 tertiary, one oxygenated quaternary, one oxymethine, one methoxyl, three methylene, and one methyl (Table S1 in Supporting Information). Because only 16 carbons were observed in the ^{13}C NMR spectrum (150 MHz, $CDCl_3$), compound **1** was deduced to have a symmetrical structure. The signals of five sp^2 quaternary (δ 161.1, 156.1, 150.2, 113.7 and 105.9) and one sp^2 tertiary carbons (δ 111.0) indicated the presence of a pentasubstituted benzene ring. The ^1H NMR signal (Table S2, Supporting Information) at δ 11.45 (1H, s) was assigned to a phenolic proton hydrogen-bonded to a carbonyl group, and the HMBC correlations of this phenolic proton to C-4a, C-5 and C-6 were observed. In addition, the correlations of H-6 to C-5, C-8 and C-14, and of H_3-14 to C-6, C-7 and C-8 in the HMBC spectrum provided evidence for the partial structure **A** (Figure 1). The remaining sp^2 carbon (δ 156.1) was attached to an oxygen atom due to its chemical shift, and was assigned to C-8a. On the other hand, $^1H-^1H$ COSY revealed the connectivity between C-9−C-10, and the HMBC correlations for H-9 to C-12; H-10 to C-11; and H-11 to C-10 and C-12 indicated a γ-lactone moiety. HMBC correlations were observed for H_3-15 to C-13; and H_2-3 to C-2, C-4, C-9 and C-13, which suggested the partial structure B. Due to the HMBC correlation for H-3 to C-4a and the remaining oxygen atom, the partial structures **A** and **B** were connected through the C-4−C-4a bond and the C-2−O−C-8a ether bond, respectively, and the structure of subunit **I** was elucidated. Finally, taking into account the molecular formula $C_{32}H_{30}O_{14}$ and the structural symmetry, two subunits **I** were proposed to be linked through the remaining quaternary carbons C-8 and C-8′ to give the planar structure of gonytolide A (**1**).

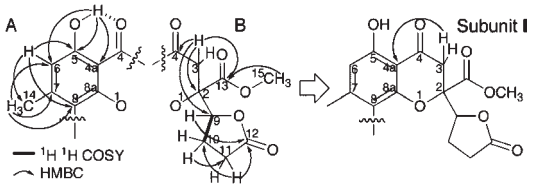


Figure 1. Planar structure of gonytolide A (**1**).

The relative configuration of gonytolide A (**1**) was determined by X-ray single-crystal analysis. The crystals of **1** were obtained by recrystallization from hexane/ethyl acetate. The ORTEP diagram of **1** indicated that the relative configuration was *S**a, 2*R**, 9*S**, 2′*R**, 9′*S** (Figure 2).

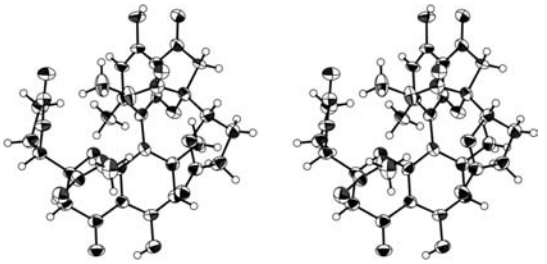
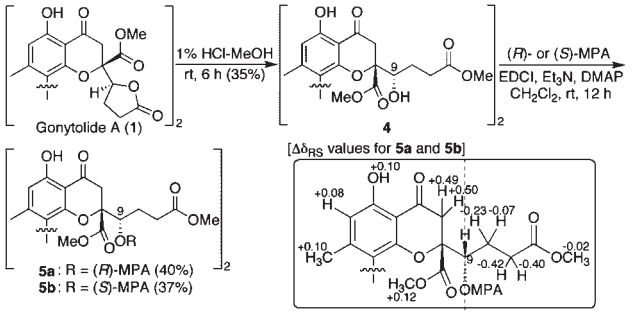


Figure 2. ORTEP stereo diagram of gonytolide A (**1**).

To determine the absolute configuration of **1**, the following transformation was conducted (Scheme 1). γ-Lactone ring opened compound **4** was obtained by the treatment of **1** with HCl in methanol. Esterification of **4** with (*R*)-α-methoxyphenylacetic acid in the presence of EDCI and DMAP yielded 9*O*,9′*O*-(*R*)-MPA diester **5a**. In a similar manner, (*S*)-MPA diester **5b** was afforded. The $\Delta\delta_{RS}$ value of each proton was calculated from the difference in the chemical shifts of **5a** and **5b** in ^1H NMR spectra (600 MHz, $CDCl_3$), and the structure of **4** was fit into the proposed model of α-methoxyphenylacetate[11] in accordance with the sign of $\Delta\delta_{RS}$. As a result, C-9 and C-9′ had *S*-configurations, and the absolute configuration of **1** was determined to be *S*a, 2*R*, 9*S*, 2′*R*, 9′*S*.

Scheme 1. Conversion of Gonytolide A (**1**) into MPA Diesters **5a** and **5b**



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The HREIMS of gonytolide B (**2**) (*m*/*z* 638.1613 [M^+]) gave the molecular formula, $C_{32}H_{30}O_{14}$, which was identical to that of **1**. The ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of **2** showed two sets of 16 signals (C-2−C-15 and C-2′−C-15′, respectively), and the chemical shifts of each signal set were nearly identical to those of **1** (Table S1, Supporting Information). The ^1H NMR spectrum of **2** was also similar to that of **1** (Table S2, Supporting Information). As a result, compound **2** was composed of two chromanone moieties, subunits **I** in the structure of **1**, which were bonded asymmetrically. The correlations of C-2−C-15 were the same as those observed for **1** in the HMBC spectrum of **2**, indicating that the subunit **I** was linked to the other subunit through the quaternary carbon C-8 (Figure 3). On the other hand, the HMBC correlations of the C-5′ phenolic proton (δ_H 11.72) to C-4′a, C-5′ and C-6′; H-8′ to C-6′ and C-8′a; and H_3-14 to C-6′, C-7′ and C-8′ suggested that another subunit **I** was connected to the other one through the quaternary carbon C-6′, and the planner structure of **2** was thereby elucidated. Because only a small amount of **2** was isolated, it was difficult to carry out its chemical conversion and characterize its stereochemistry. However, compounds **1** and **2** are likely biosynthesized by the same pathway, suggesting that the absolute configuration of **2** may be the same as that of **1**.

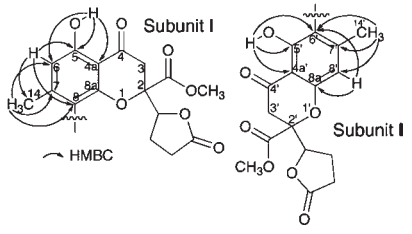

Figure 3. Planar structure of gonytolide B (**2**).

The HREIMS of gonytolide C (**3**) (*m*/*z* 320.0871 [M^+]) gave the molecular formula, $C_{16}H_{16}O_7$, which was equivalent to a half of molecular formula of **1** ($C_{32}H_{30}O_{14}$) plus one hydrogen atom. The ^1H and ^{13}C NMR spectra of **3** were nearly identical to those of **1**, although the signal of H-8 (δ 6.38) emerged in the ^1H NMR of **3** (Tables S1 and S2, Supporting Information). These facts indicated that compound **3** was a monomeric unit of **1**. The absolute configuration of **3** was determined by applying the same transformation as was used to determine the absolute configuration of **1** (Scheme S1 and Figure S1, Supporting Information). Recently, racemic **3** was synthesized[12] as the intermediate compound in a process for the synthesis of (±)-blennolide C.[13]

We evaluated the promoting activities of **1**−**3** on *Drosophila* innate immune response using the *ex vivo Drosophila* culture system.[6] Gonytolide A (**1**), at up to 1 μg/mL dosage, showed immune response-promoting activity, and 10 μg/mL of **1** increased to more than 4.5 times of the *Drosophila* innate immune response (Figure 4). On the other hand, compounds **2** and **3** showed no activity at 10 μg/mL (Figure S2, Supporting Information). These findings indicated that the linkage of the two chromanone moieties (e.g., subunit **I**) through C-8 and C-8′ is important for innate immune-promoting activity.

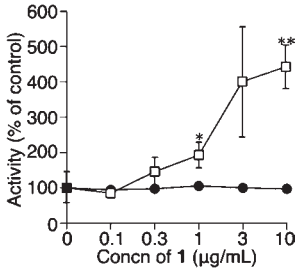

Figure 4. Effect of gonytolide A (**1**) on DAP-type peptidoglycans-mediated activation of *Drosophila Dpt-lacZ*. DAP-type peptidoglycans-mediated activation of *Dpt-lacZ* (□) and *Drosophila* S2 cell viability (●) are represented as the percent relative to the control (DMSO). The bars indicate the standard errors of three independent measurements. *$p < 0.05$ and **$p < 0.01$ vs control (DMSO).

The *Drosophila* IMD signaling pathway resembles the mammalian TNF-α signaling pathway.[2,14] The TNF-α signaling pathway plays a critical role in the host defense against several pathogens, the intrinsic tumor suppression, and the inflammatory response by producing costimulatory molecules, cytokines, chemokines, and adhesion molecules, through activation of NF-κB. We investigated the effect of gonytolide A (**1**) on TNF-α-stimulated production of IL-8, a neutrophil chemotactic factor, in human umbilical vein endothelial cells (HUVECs). As shown in Figure 5, 10 μg/mL of compound **1** increased the production of IL-8 by 70%. Thus, compound **1** promoted the innate immune response through the mammalian TNF-α signaling pathway as well as through the *Drosophila* IMD pathway.

To reveal the structural requirements of gonytolide A (**1**) for the innate immune-promoting activity, we synthesized several derivatives by using natural gonytolide A (**1**). Treatment of **1** with sulfuryl chloride produced 6,6′-dichlorinated derivative **6** (Scheme 2). Methylation of **1** by trimethylsilyldiazomethane in the presence of DIPEA

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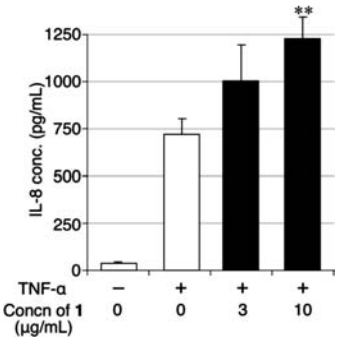
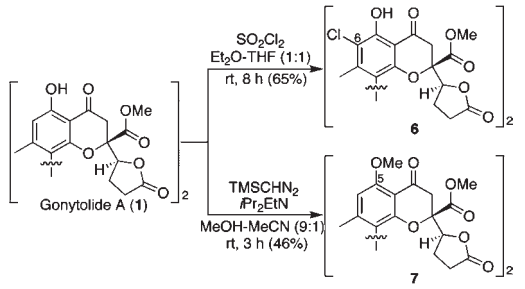


Figure 5. Effect of gonytolide A (**1**) on IL-8 production induced by TNF-α in HUVECs. HUVECs were treated with various concentrations of **1** for 3 h prior to stimulation with 1 ng/mL of TNF-α. The bars indicate the standard errors of four independent measurements. $^{**}p < 0.01$ vs absence of gonytolide A (**1**).

Scheme 2. Conversion of Gonytolide A (**1**) into **6** and **7**



afforded 5*O*,5′*O*-dimethyl compound **7**. The promoting activities of **6**, **7** and γ-lactone ring opened deivative **4** (Scheme 1) on *Drosophila* innate immune response were evaluated (Figure 6). Because compound **6** showed almost the same activity as **1**, introduction of the substituents into benzene rings is tolerant for the activity. Compound **4** also maintained the activity, indicating that the γ-lactone moiety is not crucial. The innate immune-promoting activity of **7** was weaker than that of **1**, because 10 μg/mL of **7** increased the innate immune response by a factor of only 2.5. Thus, the phenolic hydroxy groups at C-5/5′ and the hydrogen bonds between these groups and the carbonyl groups at C-4/4′ are not critical, but may be effective in promoting activity.

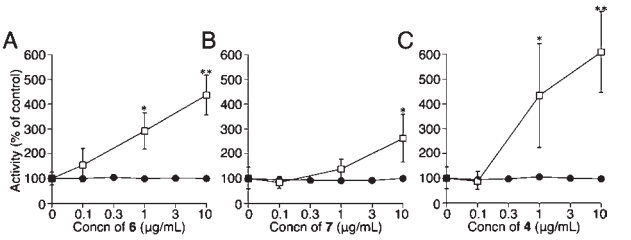


Figure 6. Effects of compounds (A) **6**, (B) **7** and (C) **4** on DAP-type peptidoglycans-mediated activation of *Drosophila Dpt-lacZ*. DAP-type peptidoglycans-mediated activation of *Dpt-lacZ* (□) and *Drosophila* S2 cell viability (●) are represented as the percent relative to the control (DMSO). The bars indicate the standard errors of three independent measurements. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control (DMSO).

A few dimeric and monomeric chromanones substituted with the γ-lactone moiety have been isolated from filamentous fungi,[13,15–23] and showed several biological activities. However, this is the first report of the promoting activity of an innate immune response by chromanone-type compounds. Gonytolide A (**1**) and its biologically active derivatives **4**, **6** and **7** can be used as lead compounds for novel immunostimulating agents against bacterial infections and tumors, specific adjuvants and as probes for innate immune system.

Acknowledgment. We are grateful to the Bloomington Stock Center, *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology, and the Genetic Strain Research Center of the National Institute of Genetics for the fly stocks. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 21249004, 21117005, 21310134, 21117005 and 21710215) from the Ministry of Education, Science, Sports and Culture of Japan; the Uehara Memorial Foundation; the Takeda Science Foundation; the Mitsubishi Foundation; and Program for Promotion of Basic Research Activities for Innovative BioSciences.

Supporting Information Available. Tables, Figures, Schemes, Experimental methods, NMR spectra of new compounds and the cif file for gonytolide A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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