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Three diketopiperazines from *marine-derived bacteria* inhibit LPS-induced endothelial inflammatory responses

Hyejin Kang [a,†], Sae-Kwang Ku [b,†], Hyukjae Choi [c], Jong-Sup Bae [a,*]

[a] College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, BK21 Plus KNU Multi-Omics Based Creative Drug Research Team, Kyungpook National University, 80 Dahak-ro, Buk-gu, Daegu 41566, Republic of Korea
[b] Department of Anatomy and Histology, College of Korean Medicine, Daegu Haany University, Gyeongsan 38610, Republic of Korea
[c] College of Pharmacy, Yeungnam University, Gyeongsan 38541, Republic of Korea

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ABSTRACT

Diketopiperazine is a natural products found from bacteria, fungi, marine sponges, gorgonian and red algae. They are cyclic dipeptides possessing relatively simple and rigid structures with chiral nature and various side chains. Endothelial dysfunction is a key pathological feature of many inflammatory diseases, including sepsis. In the present study, three (**1**–**3**) of diketopiperazines were isolated from two strains of marine-derived bacteria. The compounds were investigated for their effects against lipopolysaccharide (LPS)-mediated endothelial inflammatory responses in vitro and in vivo. From 1 μM, **1**–**3** inhibited LPS-induced hyperpermeability, adhesion, and migration of leukocytes across a human endothelial cell monolayer and in mice in a dose-dependent manner suggesting that **1**–**3** may serve as potential scaffolds for the development of therapeutic agents to treat vascular inflammatory disorders.

The systemic vascular inflammatory response in endotoxemia can lead to rapid organ failure, shock, and death.[1,2] It represents a major burden to the health care system, with more than 400,000 cases/year in the United States alone.[1] Presence of lipopolysaccharide (LPS), a bacterial endotoxin, ranks the highest among the risk factors contributing to lethal endotoxemia.[3] Endotoxins are known to activate innate immune responses, resulting in the production of a vast spectrum of inflammatory cytokines.[1,4] These pro-inflammatory cytokines are known to trigger vascular endothelial activation.[2] The integrity of vascular endothelium is essential for controlling the flux of proteins, fluid, and immune cells across vessels into tissues, and vascular endothelial dysfunction is an established event in acute inflammation.[5,6] Systemic accumulation of LPS triggers leukocyte infiltration within the vascular wall and promotes vascular permeability.[7] Therefore, maintenance of vascular integrity is crucial for vascular and tissue homeostasis.

Sepsis is an uncontrolled inflammatory response to a pathogen[8] that lacks effective drug treatments.[9] The mortality rate of sepsis is approximately 80% in developing countries,[10] while treatment can reduce this rate to approximately 33%.[11] In patients with sepsis, interaction of LPS with endothelial cells results in increased endothelial permeability, which is mediated at least in part by activated Src family kinases[12] and phosphorylated zonula adherence proteins,[12] as well as by nuclear factor (NF)-κB, which enhances production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1.[13] Pro-inflammatory cytokines further exacerbate the inflammatory response[8] and induce expression of adhesion molecules such as ICAM-1 and VCAM-1[8] on the endothelial cell surface, leading to attachment and diapedesis of leukocytes across the membrane. These vascular inflammatory responses often constitute the cardinal and initial events in sepsis, as well as in the development of atherosclerosis.[14–16] Resolution of endothelial dysfunction and restoring the barrier function of the endothelium is thus crucial during early management of sepsis and other vascular inflammatory disorders.

Diketopiperazine is a natural products found from bacteria, fungi, marine sponges, gorgonian and red algae.[17] They are cyclic dipeptides possessing relatively simple and rigid structures with chiral nature and various side chains. The compounds in this structure class have been known to possess diverse bioactivities including antibiotic activity, anti-cancer activity, neuroprotective activity, and anti-inflammatory activity.[18] In the course of screening for anti-LPS natural products, the ethyl acetate extracts of two independently fermented bacterial strains derived from marine sources showed potent inhibition on LPS-mediated inflammatory

* Corresponding author. Tel.: +82 53 950 8570; fax: +82 53 950 8557.
E-mail address: baejs@knu.ac.kr (J.-S. Bae).
† These authors contributed equally to this work.

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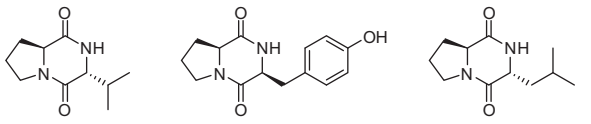

Figure 1. Compounds **1**–**3** isolated from the fermented *Bacillus* sp. HC001 (**1**–**2**) and *Piscicoccus* sp. 12L081 (**3**).

responses in human umbilical vein endothelial cells (HUVECs) and in mice. Repeated chromatographic separation gave three of diketopiperazines, *cyclo* (L-Pro-D-Val) (**1**), *cyclo* (L-Pro-L-Tyr) (**2**) and *cyclo* (L-Pro-D-Leu) (**3**), and these proline-containing compounds have been reported from the fermented bacterial strains,[19] fungal strains,[20] and the other macroorganisms.[21] Although several of diketopiperazines have been reported to have diverse biological activities including antibiotic and anti-inflammatory activities,[18] anti-LPS-mediated inflammatory responses are not known. Based on previous Letters, which demonstrated the potential effects of LPS on vascular inflammatory responses and antibiotic and anti-inflammatory activities of diketopiperazines,[18] we hypothesized that treatment with diketopiperazines would suppress LPS-induced vascular inflammatory responses in human endothelial cells and in mice.

The extracts of two independently fermented bacterial strains of *Bacillus* sp. HC001 and *Piscicoccus* sp. 12L081 were prepared by ethyl acetate extraction. Each extracts were fractionated by silica gel VLC and preparative RP-HPLC, and three known diketopiperazines (**1** and **2** from of *Bacillus* sp. HC001; **3** from *Piscicoccus* sp. 12L081) were obtained (Fig. 1).

In vitro assays involving LPS-induced inflammatory responses elicited in human umbilical vein endothelial (HUVEC) cells were employed to mimic inflammatory phenotype changes in the endothelium and to assess the potential of the isolated compounds to mitigate such changes.

Compound **1** (Fig. 1) was obtained as a white amorphous solid. The structure of **1** was identified to be *cyclo* (L-Pro-D-Val), a known diketopiperazine, based on the spectroscopic data analyses.[21] LR-ESI-MS showed a protonated ion of compound **1** at *m*/*z* $[M+H]^+$ 197.2, and ^{13}C NMR spectrum (63 MHz, CD_3OD, Supplementary data Table S1) exhibited 10 carbon signals including two amide carbonyl carbons (δ_C 172.6 and 167.6), two α carbons of amino acids (δ_C 61.5 and 60.0), a nitrogen connected carbon (δ_C 46.2), and five sp^3 carbons with no attachment to heteroatoms (δ_C 29.8, 29.5, 23.3, 18.9 and 16.6). Additionally, two distinctive doublet methyl signals at δ_H 1.11 (3H, d, J = 6.9 Hz) and 0.96 (3H, d, J = 6.9 Hz), and two α protons at δ 4.22 (1H, m) and 4.05 (1H, m) were observed in ^1H NMR spectrum (CD_3OD, 250 MHz, Supplementary data Table S1) of compound **1**. The de-replication with these spectroscopic information enable to identify the *cyclo* (Pro-Val) as the planar structure of compound **1**. Then the structure was confirmed by the comparison of MS and NMR data of the literature.[21] The absolute configurations of compound **1** were determined as *cyclo* (L-Pro-D-Val) by comparison of specific rotation of compound **1** to that in the literature (Table S2).[21,22]

The structures of compounds **2** and **3** (Fig. 1) were also identified to be *cyclo* (L-Pro-L-Tyr) (**2**) and *cyclo* (L-Pro-D-Leu) (**3**) with the same strategy (Table S2).[21–23]

Initially, a permeability assay was used to determine the effects of each compound on the barrier integrity of a HUVEC monolayer. Flux of an Evans blue dye–albumin complex across the HUVEC

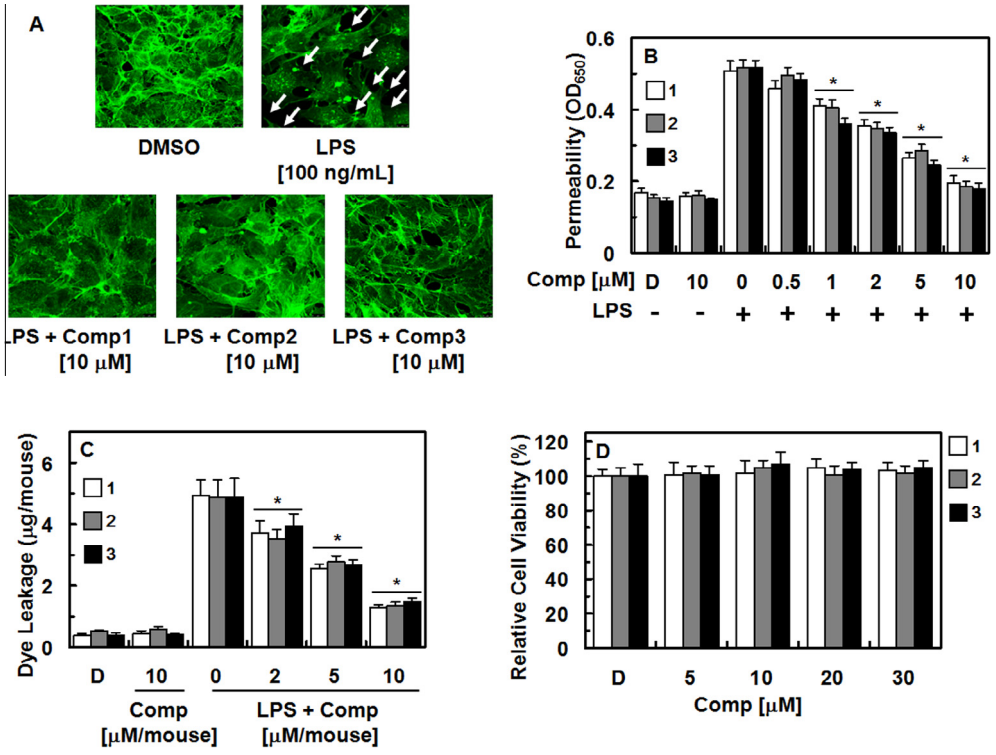

Figure 2. Effects of compounds **1**–**3** on LPS-induced barrier disruption in vitro and in vivo. (A) Staining for F-actin. HUVEC monolayers grown on glass coverslips were stimulated with LPS (100 ng/mL, 4 h), followed by treatment with each compound (10 μM) for 6 h and immunofluorescence staining for F-actin. The arrows indicate intercellular gaps. (B) The effects of various concentrations of **1** (white bar), **2** (gray bar), and **3** (black gray bar) on LPS-induced (100 ng/mL, 4 h) barrier disruption were monitored by the flux of Evans blue-bound albumin across HUVECs. (C) The effects of **1**–**3** at 2, 5 or 10 μM/mouse on LPS-induced (15 mg/kg, ip) vascular permeability in mice were examined by measuring the amount of Evans blue in peritoneal washings (expressed μg/mouse, n = 5). (D) The effects of **1**–**3** on cellular viability were measured using Microculture tetrazolium (MTT) assays. Results are expressed as the mean ± SD of three independent experiments. $^*p$ <0.05 versus LPS.

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