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Isolation, Synthesis, and Antisepsis Effects of a *C*-Methylcoumarinochromone Isolated from *Abronia nana* Cell Culture

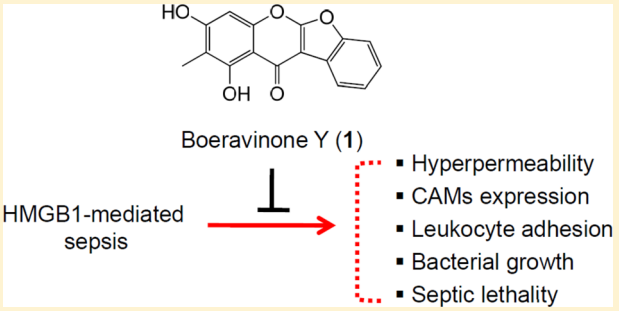
Wonhwa Lee,[†,‡,§] Doohyun Lee,[†,§] Yuri Lee,[†] Taeho Lee,[†] Kyung-Sik Song,*[,†] Eun-Ju Yang,*[,†] and Jong-Sup Bae*[,†]

[†]College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, BK21 Plus KNU Multi-Omics Based Creative Drug Research Team, Kyungpook National University, Daegu 41566, Republic of Korea

[‡]Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea

Supporting Information

ABSTRACT: Only a few isoflavones have been isolated from plants of the genus *Abronia*. The biological properties of compounds isolated from *Abronia* species have not been well established, and their antisepsis effects have not been reported yet. In the present study, a new *C*-methylcoumarinochromone, was isolated from *Abronia nana* suspension cultures. Its structure was deduced as 9,11-dihydroxy-10-methylcoumarinochromone (boeravinone Y, **1**) by spectroscopic data analysis and verified by chemical synthesis. The potential inhibitory effects of **1** against high mobility group box 1 (HMGB1)-mediated septic responses were investigated. Results showed that **1** effectively inhibited lipopolysaccharide-induced release of HMGB1 and suppressed HMGB1-mediated septic responses, in terms of reduction of hyperpermeability, leukocyte adhesion and migration, and cell adhesion molecule expression. In addition, **1** increased the phagocytic activity of macrophages and exhibited bacterial clearance effects in the peritoneal fluid and blood of mice with cecal ligation and puncture-induced sepsis. Collectively, these results suggested that **1** might have potential therapeutic activity against various severe vascular inflammatory diseases *via* inhibition of the HMGB1 signaling pathway.



Sepsis is a systemic inflammatory response that can lead to lethal organ damage. Septic responses result from hyperactivity of the innate immune system and the ensuing proinflammatory cascade in response to severe microbial infections or extensive tissue damage.[1,2] Over the last three decades, considerable progress has been achieved with respect to understanding the pathophysiology of sepsis. Since tumor necrosis factor (TNF)-α mimics septic shock in animal models, several clinical trials have focused on the neutralization of this proinflammatory mediator.[3] However, one trial had very disappointing results.[4,5] Since the early kinetics of systemic TNF-α accumulation in sepsis makes it a difficult therapeutic target in clinical settings,[6] other late proinflammatory mediators may offer a wider therapeutic window. High mobility group box 1 (HMGB1), which is secreted in response to bacterial endotoxins, plays an important role in sepsis.[7−9] When HMGB1 is released from necrotic or activated cells, it triggers inflammation, immune responses, and tissue regeneration.[7−9] In fact, the blockade of HMGB1, even at late stages after the onset of infection, has been shown to rescue mice from lethal sepsis.[10,11] Therefore, HMGB1 acts as a lethal mediator of sepsis, in which serum HMGB1 levels substantially increase.[9,11] In addition, HMGB1 and its receptor axis have been shown to amplify severe vascular inflammatory responses and induce tissue impairment.[12,13] Once released into the extracellular *milieu*, HMGB1 can bind to cell surface receptors, such as toll-like receptors (TLR) 2 and 4, as well as receptor for advanced glycation end-products (RAGE),[9,11,14] to transduce intracellular signals and elicit cellular responses, including chemotaxis and proinflammatory cytokine release.[12,13] Since HMGB1 is considered a late mediator of sepsis, in this study, sepsis was induced by HMGB1 in human endothelial cells.

In a previous study aiming to find antisepsis drug leads from natural sources, boeravinone X was isolated from *Abronia nana* suspension cultures and shown to significantly suppress HMGB1-mediated septic responses in human umbilical vein endothelial cells (HUVECs) and C57BL/6 mice.[15] Further investigation of this plant afforded another promising antisepsis compound. In the current study, we isolated the antisepsis compound **1** from *A. nana* suspension cultures and investigated its potential anti-inflammatory effects in HUVECs and a mouse model of cecal ligation and puncture (CLP)-induced sepsis.

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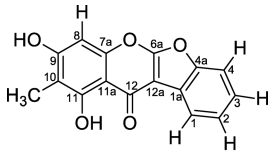

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RESULTS AND DISCUSSION

Compound **1** was isolated as a gray amorphous powder, and its molecular formula was determined by high-resolution fast-atom bombardment mass spectrometry (HRFABMS) to be $C_{16}H_{10}O_5$ based on the protonated molecular ion peak $[M + H]^+$ observed at m/z 283.0609 (calcd for $C_{16}H_{11}O_5$, 283.0606). The ^{1}H NMR spectrum showed a typical methyl signal similar to that of other *C*-methyl isoflavones at δ_H 2.49 (3H, s, CH_3-10).[15,16] An intramolecular hydrogen-bonded proton was observed at δ_H 13.73 (1H, s, OH-11), indicating the presence of a hydroxy group attached to C-11. A low-field-shifted aromatic proton, which resulted from the restricted rotation of ring A and anisotropic effect of the sterically adjacent carbonyl double bond, was observed at δ_H 8.25 (1H, dd, J = 7.5, 1.2 Hz, H-1). Resonances at δ_H 7.58 (1H, dd, J = 7.5, 1.2 Hz, H-4), 7.45 (1H, dt, J = 7.5, 1.2 Hz, H-2), and 7.38 (1H, dt, J = 7.5, 1.2 Hz, H-3) were assigned with this proton, which supported the presence of a 1,2-disubstituted benzene moiety. Acetal and methylene proton signals around δ_H 5−6, which are characteristic of pyran-type rotenoids, were not observed in **1**.[15−17] Therefore, it was deduced that **1** contains a furan ring, instead of a pyran ring. Resonances for 16 carbons, including one carbonyl carbon at δ_C 179.9, five oxygenated aromatic carbons at δ_C 165.7, 164.0, 161.3, 154, and 150.7, and a methyl carbon at δ_C 8.6, were observed. All NMR peaks, including the resonances of two hydroxy groups and one methyl group, were determined using the heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) spectra of **1**. From the HMBC data, it was confirmed that the hydrogen-bonded hydroxy proton signal at C-11 (δ_H 13.73, 1H, s, OH-11) correlated with C-11 (δ_C 161.3) and C-11a (δ_C 103.1). A proton at δ_H 6.83 (1H, s, H-8) correlated with C-7a (δ_C 154.0) and C-9 (δ_C 164). In addition, the methyl group at δ_H 2.49 (1H, s, CH_3-10) correlated with C-9 (δ_C 164.0), C-10 (δ_C 110.1), and C-11 (δ_C 161.3), indicating that a methyl group is present at C-10. From these data, the structure of **1** was deduced as a *C*-methylcoumarinochromone derivative. While this structure was previously reported as boerharotenoid A, major differences were found in the NMR data between **1** and the previously reported compound.[18] In particular, the chemical shifts of six carbons of ring A (C-1-C-4, C-1a, and C-4a) were quite different, despite the use of the same NMR solvent (Table 1). To verify the proposed structure, **1** was chemically synthesized. The ^{1}H and ^{13}C NMR data of the synthetic compound matched very well with those of the isolated compound **1** (Table 1). Consequently, we concluded that the reported boerharotenoid A should have a different structure from that of **1**, indicating that **1** is a new compound. The present compound **1** was given the trivial name boeravinone Y.

Previous studies have shown that lipopolysaccharides (LPS) induce HMGB1 release from murine macrophages and human endothelial cells.[19−21] It was shown that 100 ng/mL LPS was sufficient to induce HMGB1 release.[22] Similarly, in the current study, 100 ng/mL LPS induced the release of HMGB1 from HUVECs (Figure 1A). To investigate the effects of **1** on LPS-mediated release of HMGB1, HUVECs were stimulated with 100 ng/mL LPS for 16 h, followed by treatment with increasing concentrations of **1** for 6 h. As shown in Figure 1A, **1** inhibited the release of HMGB1 from HUVECs at optimal effective concentrations of >0.2 μM. However, in the absence of LPS pretreatment, **1** did not affect HMGB1 release (Figure 1A). To confirm these effects *in vivo*, a CLP-induced sepsis mouse model was used because it closely resembles human sepsis more than LPS-induced endotoxemia.[3] As shown in Figure 1B, treatment with **1** resulted in a marked inhibition of CLP-induced release of HMGB1. The average circulating blood volume for mice is 72 mL/kg.[23] Since the average weight of mice used in this study was 27 g, and the average blood volume was 2 mL, the doses of **1** (28 or 56 μg/kg) used were equivalent to peripheral blood concentrations of 0.5 or 1 μM. Next, the effects of **1** on the expression of HMGB1 receptors (TLR2, TLR4, and RAGE) were investigated in HUVECs. As shown in Figure 1C, treatment with HMGB1 resulted in more than a 2-fold increase in the expression of TLR2, TLR4, and RAGE in HUVECs, which was significantly reduced by **1**. To assess the cytotoxicity of compound **1**, cell viability assays were performed using HUVECs treated with **1** for 48 h. At concentrations up to 5 μM, **1** did not affect cell viability (Figure 1D). High plasma concentrations of HMGB1 in patients with inflammatory diseases are associated with poor prognosis and high mortality. In addition, pharmacological inhibition of HMGB1 was shown to improve survival in animal models of acute inflammation in response to endotoxin challenge.[24] Therefore, we suggest that **1** could be a potential therapy for vascular inflammatory diseases via inhibition of HMGB1 release.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data of the Isolated Compound 1 (Boeravinone Y), Synthesized Compound 1, and Boerharotenoid A (Pyridine-d_5, δ in ppm)

	1		synthesized 1		boerharotenoid A[a]	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	122.1	8.25, dd (7.5, 1.2)	122.2	8.25, dd (7.5, 1.4)	127.4	9.18, d (8)
1a	123.5		123.8		100.5	
2	126.1	7.45, dt (7.5, 1.2)	126.1	7.42, dt (7.5, 1.1)	122.5	7.16, t (8)
3	126.7	7.38, dt (7.5, 1.2)	126.1	7.37, dt (7.5, 1.4)	129.1	7.3, t (8)
4	112.2	7.58, dd (7.5, 1.2)	112.2	7.57, dd (7.5, 1.1)	118	7.32, d (8)
4a	150.7		150.2		157.5	
6a	165.7		165.7		166.2	
7a	154		154		155.2	
8	95.1	6.83, s	95.1	6.8, s	94.8	6.68, s
9	164		163.8		164.1	
10	110.1		110.1		109	
11	161.3		161.4		160.6	
11a	103.1		103.9		105.3	
12	179.9		180		179.7	
12a	98.2		98.3		123	
CH_3-10	8.6	2.49, s	8.6	2.49, s		2.45, s
OH-11		13.73, s		13.73, s		

[a]The NMR data of boerharotenoid A were obtained from a previous study.[18]

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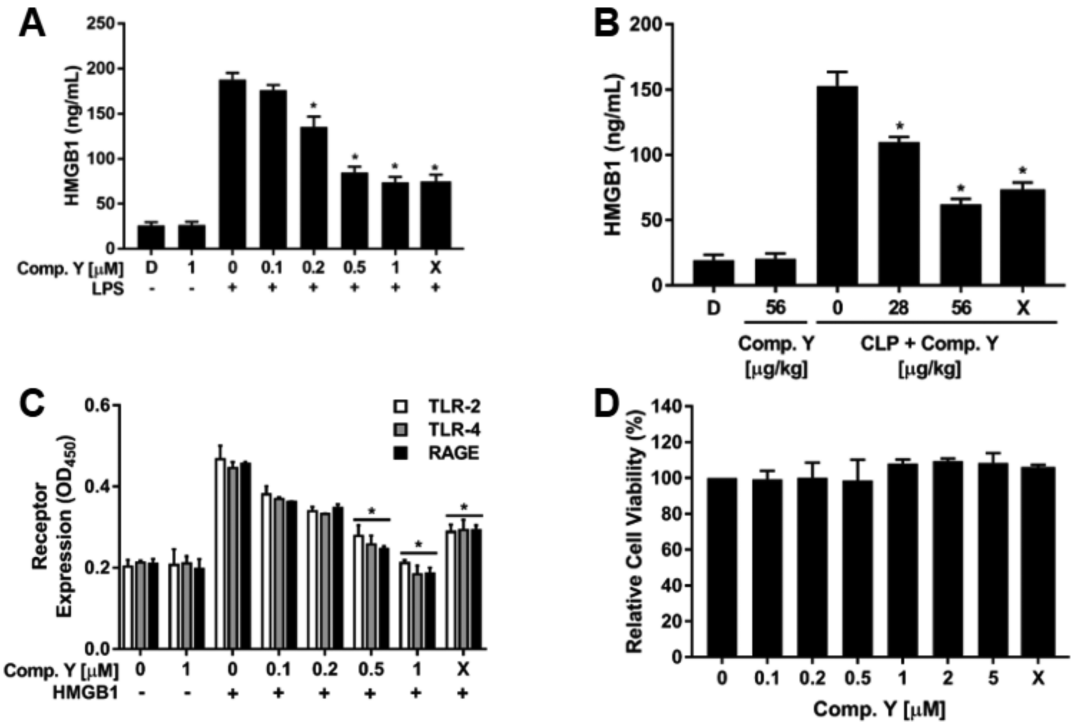

Figure 1. Effects of boeravinone Y (**1**) on HMGB1 release and its receptor expression. (A) HUVECs were treated with the indicated concentrations of **1** for 6 h after stimulation with bacterial LPS (100 ng/mL) for 16 h. HMGB1 release was measured by ELISA. (B) Male C57BL/6 mice underwent cecal ligation and puncture (CLP) and were treated with **1** (28 or 56 μg/kg) intravenously 12 h after CLP (black bar, $n = 5$). Mice were killed 24 h after CLP. Serum HMGB1 levels were measured by ELISA. (C) Confluent HUVECs were activated with HMGB1 (1 μg/mL, 16 h), followed by incubation with **1** for 6 h. The expression of cell surface receptors TLR2 (white bar), TLR4 (gray bar), and RAGE (black bar) was determined by cell-based ELISA. (D) The effects of **1** on cell viability were evaluated using the MTT assay. *$p < 0.05$ vs LPS alone (A), CLP alone (B), or HMGB1 alone (C). X: 1 μM boeravinone X was used as a positive control.

A permeability assay was performed to investigate the effects of **1** on barrier integrity in HUVECs. Treatment with 1 μM **1** alone did not alter the barrier integrity (Figure 2A). In contrast, LPS and HMGB1 have been shown to induce cleavage and disruption of the endothelial barrier integrity.[25−27] Thus, HUVECs were treated with various concentrations of **1** for 6 h after exposure to LPS (100 ng/mL) or HMGB1 (1 μg/mL). As shown in Figure 2A and B, treatment with **1** resulted in a concentration-dependent decrease in LPS- or HMGB1-mediated disruption of barrier integrity. The effects of **1** on HMGB1-induced vascular hyperpermeability in mice were investigated to confirm its vascular barrier protective effects *in vivo*. As shown in Figure 2C, treatment with **1** resulted in marked inhibition of HMGB1-induced peritoneal dye leakage.

HMGB1 has been shown to induce inflammatory responses by promoting the phosphorylation of p38 mitogen-activated protein kinase (MAPK).[28,29] To determine whether **1** can inhibit HMGB1-induced activation of p38 MAPK in HUVECs, cells were activated with HMGB1, followed by incubation with **1** for 6 h. As shown in Figure 2D, HMGB1 increased phosphorylated p38 expression, which was significantly inhibited by treatment with **1**. These findings indicated that **1** could inhibit HMGB1-induced endothelial disruption and maintain human endothelial cell barrier integrity.

The bacterial clearance effects of **1** were evaluated by counting the colony-forming units (CFUs). Twenty-four hours after CLP surgery, bacterial counts in the peritoneal fluid and blood significantly increased. However, **1** significantly decreased the bacterial counts in both the peritoneal fluid and blood (Figure 3). These results suggested that **1** might increase macrophage activity and reduce sepsis.

Several previous studies have shown that HMGB1 mediates inflammatory responses by increasing the expression of cell adhesion molecules (CAMs), such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, on the surface of endothelial cells, thereby promoting the adhesion and migration of leukocytes across the endothelium to the sites of inflammation.[30,31] In the present study, HMGB1 increased the expression of VCAM-1, ICAM-1, and E-selectin on the cell surface, which was reduced by compound **1** (Figure 4A). In addition, the increase in CAM expression corresponded well with enhanced binding and migration of human neutrophils to HMGB1-activated endothelial cells (Figure 4B and C). Treatment with **1** reduced human neutrophil adhesion and migration across the activated endothelial cells in a concentration-dependent manner (Figure 4B and C). These results suggested that **1** not only inhibited endotoxin-induced release of HMGB1 from endothelial cells but also downregulated the proinflammatory signals induced by HMGB1 release, thereby inhibiting the amplification of inflammatory responses by nuclear cytokines. To confirm these effects *in vivo*, the effects of **1** on HMGB1-induced migration of leukocytes were examined in mice. HMGB1 induced the migration of leukocytes into the peritoneal cavity in mice. However, treatment with compound **1** resulted in a significant reduction of the peritoneal leukocyte counts (Figure 4D).

Sepsis is a systemic response to serious infections. It has a poor prognosis when it is associated with organ dysfunction,

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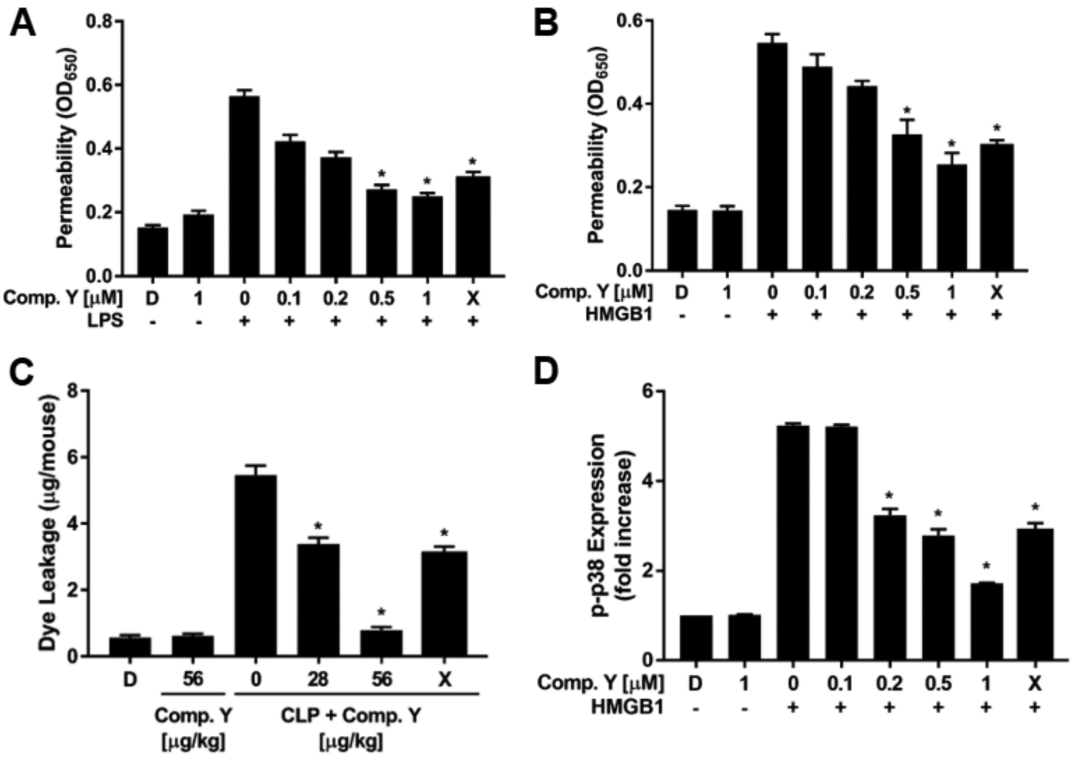

Figure 2. *In vitro* and *in vivo* effects of boeravinone Y (**1**) on HMGB1-mediated hyperpermeability. The effects of **1** treatment at different concentrations for 6 h on barrier disruption induced by bacterial LPS (100 ng/mL, 4 h) or HMGB1 (1 μg/mL, 16 h) were investigated by measuring the flux of albumin-bound Evans blue across HUVEC monolayers. (C) The effects of **1** (28 or 56 μg/kg) on HMGB1 (2 μg/mouse)-induced vascular hyperpermeability in mice were examined by measuring the amount of Evans blue in the peritoneal washings (expressed as μg/mouse, n = 5). (D) HUVECs were activated with HMGB1 (1 μg/mL, 16 h), followed by treatment with **1** at different concentrations for 6 h. The effects of **1** on HMGB1-mediated expression of phospho-p38 were determined by ELISA. *p < 0.05 vs LPS alone (A) or HMGB1 alone (B, C, D). X: 1 μM boeravinone X was used as a positive control.

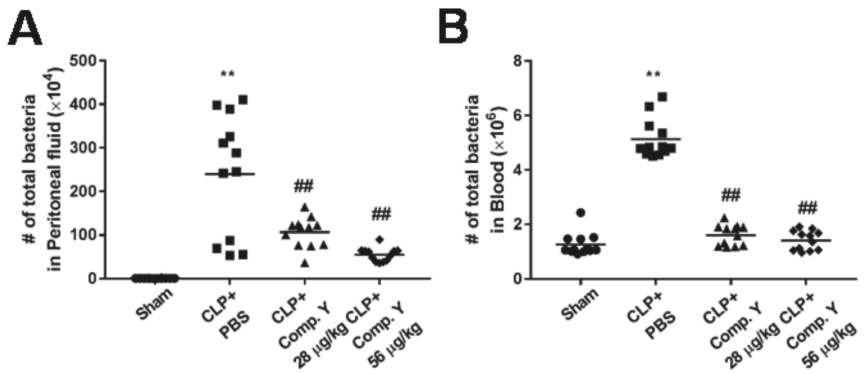

Figure 3. Bacterial clearance effects of boeravinone Y (**1**). Bacterial counts (CFUs) in the peritoneal fluid and blood 24 h after CLP (n = 10). **p < 0.01 vs the sham group; $^{\#\#}p$ < 0.01 vs the CLP group.

hypoperfusion, or hypotension.[12,32] On the bases of our previous findings, it was hypothesized that **1** might reduce mortality in a mouse model of CLP-induced sepsis. To test this hypothesis, **1** was administered to mice after the CLP procedure. Twenty-four hours after the procedure, mice showed signs of sepsis, such as shivering, bristled hair, and weakness. Administration of **1** (56 μg/kg) 12 h after CLP did not prevent CLP-induced death (data not shown); thus, **1** was administered twice (12 and 50 h after CLP). Administration of two doses of **1** resulted in an increase in the survival rate from 40% to 60%, as shown by Kaplan−Meier survival analysis (p < 0.0001; Figure 5A). This marked survival benefit achieved after administration of two doses of **1** suggested that suppression of HMGB1 release and HMGB1-mediated inflammatory responses could be a therapeutic strategy for the management of sepsis and septic shock.

To confirm the protective effects of **1** against CLP-induced death, its effects on CLP-induced pulmonary injury were examined. In the sham group, there were no significant differences between the lungs of the treated and untreated mice under a light microscope (data not shown). In the untreated CLP group, interstitial edema with massive infiltration of inflammatory cells into the interstitium and alveolar spaces was observed, and the pulmonary architecture was severely damaged (Figure 5B). Systemic inflammation during sepsis commonly causes multiple organ failure, wherein the liver and

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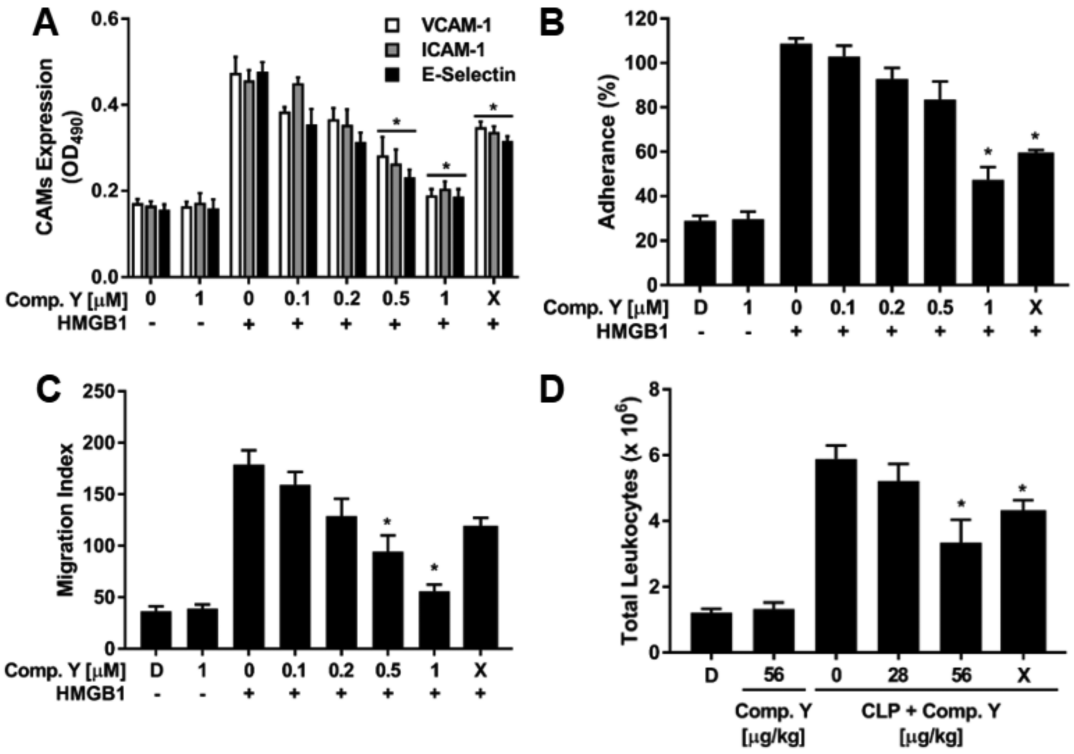

Figure 4. Effects of boeravinone Y (**1**) on HMGB1-mediated inflammatory responses. (A–C) HUVECs were stimulated with HMGB1 (1 μg/mL) for 16 h, followed by treatment with increasing concentrations of **1** for 6 h. (A) HMGB1-mediated expression of cell adhesion molecules VCAM-1 (white bar), ICAM-1 (gray bar), and E-selectin (black bar) in HUVECs. (B) Adherence of human neutrophils to HUVEC monolayers. (C) Migration of human neutrophils through HUVEC monolayers was evaluated by ELISA. (D) The effects of **1** (28 or 56 μg/kg) on CLP-induced leukocyte migration into the peritoneal cavity of mice were analyzed. All results are expressed as the means ± SD of five independent experiments (n = 10). *p < 0.05 vs HMGB1 alone or CLP alone. X: 1 μM boeravinone X was used as a positive control.

kidneys are the major target organs.[33] CLP resulted in a significant increase in the plasma levels of alanine transaminase (ALT) and aspartate transaminase (AST) (markers of hepatic injury; Figure 5C), as well as creatinine and blood urea nitrogen (BUN) (markers of renal injury; Figures 5D and E). Compound **1** was able to reverse all these abnormalities. The levels of another important marker of tissue injury, lactate dehydrogenase (LDH), were also reduced by **1** in CLP-operated mice (Figure 5F). Next, the effects of **1** on the production of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-10, and TNF-α, were examined during sepsis. IL-10 and TNF-α have been considered essential mediators of sepsis-induced vascular inflammation;[34] in addition, IL-6 blockade with neutralizing antibodies was shown to protect against CLP-induced septic mortality.[35] CLP-induced elevation of IL-1β, IL-6, IL-10 (Figure 5G), TNF-α (Figure 5H), and monocyte chemoattractant protein-1 (MCP-1; Figure 5I) levels was significantly inhibited by compound **1** treatment.

Despite decades of intense research, the basic mechanisms of sepsis remain elusive.[36,37] In both experimental animal models of sepsis and human patients, substantial pathological changes have been observed, many of which may result in subsequent organ injury.[36] Variations in age, sex, and medical comorbidities create additional complexities that influence the outcome in septic patients.[38] Insufficient understanding of the pathophysiology of sepsis has resulted in fundamental problems in the design of clinical trials conducted to address a better approach for the management of sepsis. Currently, there are no U.S. Food and Drug Administration (FDA)- or European Medicines Evaluation Agency-approved pharmacological therapeutic interventions directed against specific mediators of sepsis. In the past 30 years, only one FDA-approved therapy was available. However, it was withdrawn in 2011 by the manufacturer because follow-up studies failed to show substantial improvement in the survival of patients with septic shock.[39] The complexity of the disease presents substantial challenges to the understanding of what is aberrant and why the alteration is deleterious.[37,38] Therefore, most antisepsis agents show modest effects in animal models of the disease, which reflects the complexity of the underlying pathogenic mechanisms.[36,38] Simultaneous disruption of multiple pathways, rather than a single mediator, likely account for sepsis mortality.[37,40] Although several compounds have shown modest *in vivo* effects against CLP-induced cell death, the current findings that HMGB1 (a late sepsis mediator)-mediated septic responses may be suppressed by **1** might help to discover other antisepsis agents.

The molecular mechanisms of the anti-inflammatory effects of **1** against HMGB1-mediated septic responses might involve suppression of HMGB1 release (Figure 1A and B), reduction of the expression of HMGB1 receptors (TLR2, TLR4, and RAGE; Figure 1C), and decrease in HMGB1-mediated hyperpermeability (Figure 2A–C) via suppression of p38 activation (Figure 2D). Furthermore, inhibition of the interaction between leukocytes and endothelial cells by **1** might be attributable to the inhibition of the expression of CAMs, such as VCAM, ICAM, and E-selectin (Figure 4). Compound **1** might mainly target the interaction of the released HMGB1 with its receptors in HMGB1-mediated septic responses because binding of HMGB1 to its receptors mediates severe vascular inflammatory

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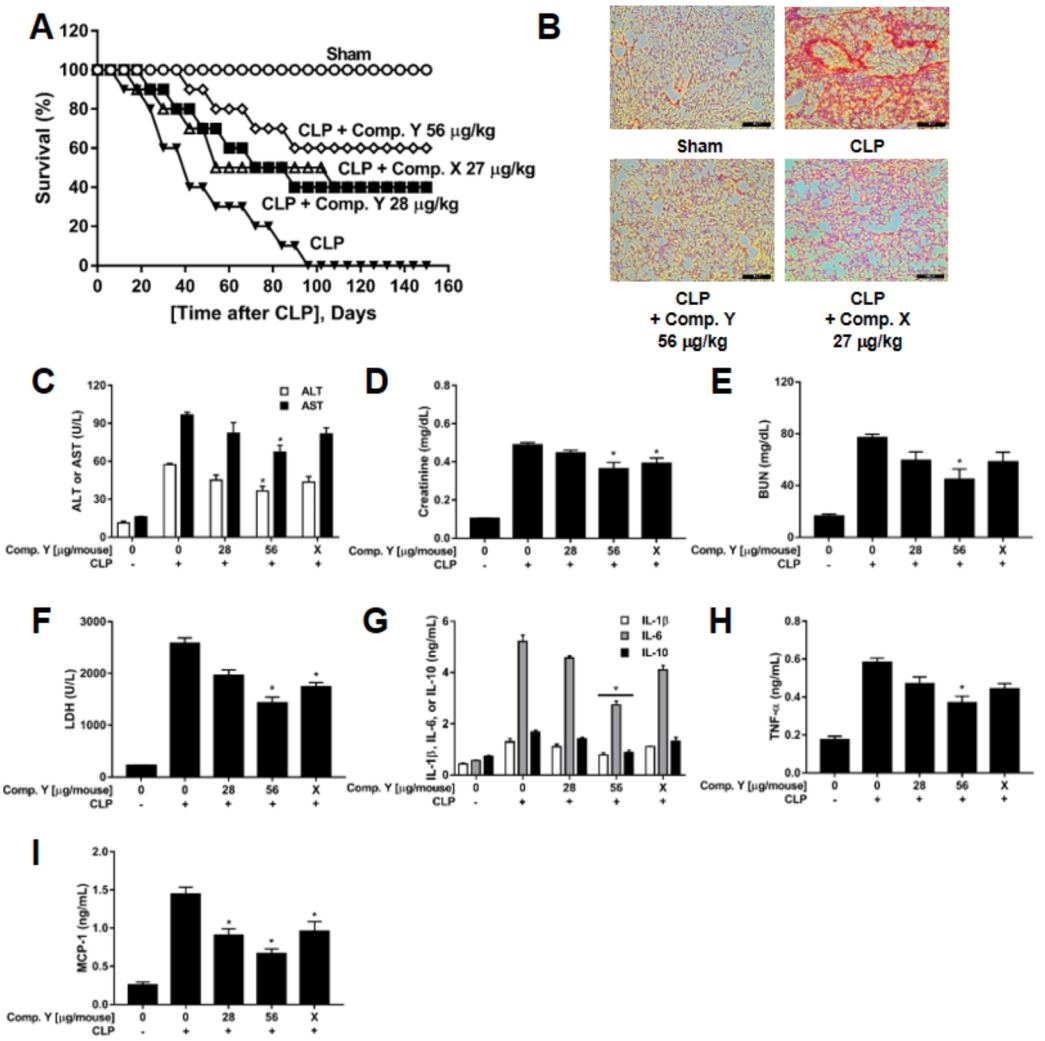

Figure 5. Effects of boeravinone Y (**1**) on CLP-induced mortality and pulmonary injury. (A) Male C57BL/6 mice (n = 20) were treated intravenously with **1** (28 μg/kg, ■ or 56 μg/kg, ◇) at 12 and 50 h after CLP. Animal survival was monitored every 6 h after CLP for 150 h. Untreated CLP mice (●) and untreated sham-operated mice (○) were administered sterile saline (n = 20). Kaplan−Meier survival analysis was performed for evaluation of the overall survival rates. (B) Male C57BL/6 mice underwent CLP and were treated with **1** (28 or 56 μg/kg) intravenously at 12 and 50 h after CLP (n = 5). Mice were killed 96 h after CLP. Photomicrographs of lung tissues (H&E staining, 200×). Untreated sham group (grade 1), untreated CLP group (grade 3), and CLP group treated with **1** (grade 2). The figure shows representative images from three independent experiments. (D−G) Similar to (B) and (C) except that mice were bled to death. AST (C), ALT (C), creatinine (D), BUN (E), and LDH (F) levels in the plasma were measured. Compound **1** decreased the concentrations of IL-1β, IL-6, IL-10, TNF-α, and MCP-1 in the blood of CLP mice (G−I). Cytokine concentrations were determined by ELISA (n = 10 mice/group/experiment). Results are expressed as the means ± SD of five independent experiments (n = 5). *p < 0.05 vs the CLP group. X: 27 μg/kg boeravinone X was used as a positive control.

responses, such as hyperpermeability, adhesion, and migration of leukocytes to the endothelial cells.[9,41−43] Based on the current findings, **1** might be a potential therapeutic agent for treatment of severe vascular inflammatory diseases, such as sepsis and septic shock.

In a previous study, four pyran-type *C*-methylrotenoids were isolated from suspension cultures of *A. nana*. Among them, boeravinone D (IC_{50} = 4.8 μM) and mirabijalone D (IC_{50} = 4.2 μM) were proposed as noncompetitive inhibitors of β-site amyloid precursor protein cleaving enzyme 1 (BACE1, β-secretase), which is a key protease involved in Alzheimer's disease pathogenesis.[17] In addition, a BACE1 inhibitor, boeravinone E (IC_{50} = 5.6 μM), was produced by yeast elicitation of *A. nana* suspension cultures.[16] Furthermore, abronione, boeravinone C, deguelin,[44] and abronisoflavone[45] were isolated from the genus *Abronia*. Although their structures are similar to those of *C*-methylrotenoids, the *C*-methylcoumarinochromones bearing a furan ring instead of a pyran ring have been rarely reported. To the best of our knowledge, boeravinone J,[46,47] triquetrumones A, B, and C,[48] and flemingichromone[49] are the only reported *C*-methylcoumarinochromones so far. *A. nana* belongs to the plant family Nyctaginaceae, which grows in the desert areas of North America and has not been extensively studied yet. This is the first study on the antisepsis effects of a *C*-methylcoumarinochromone isolated from the genus *Abronia*.

EXPERIMENTAL SECTION

General Experimental Procedures. Ultraviolet (UV) and Fourier-transform infrared spectroscopy (FT-IR) spectra were acquired using Shimadzu UV-1800 (Kyoto, Japan) and Cary 630 FTIR (Agilent, Santa Clara, CA, USA) instruments, respectively. NMR analysis was performed using a Bruker Avance Digital 500 NMR spectrometer (Karlsruhe, Germany) with tetramethylsilane (0 ppm) as

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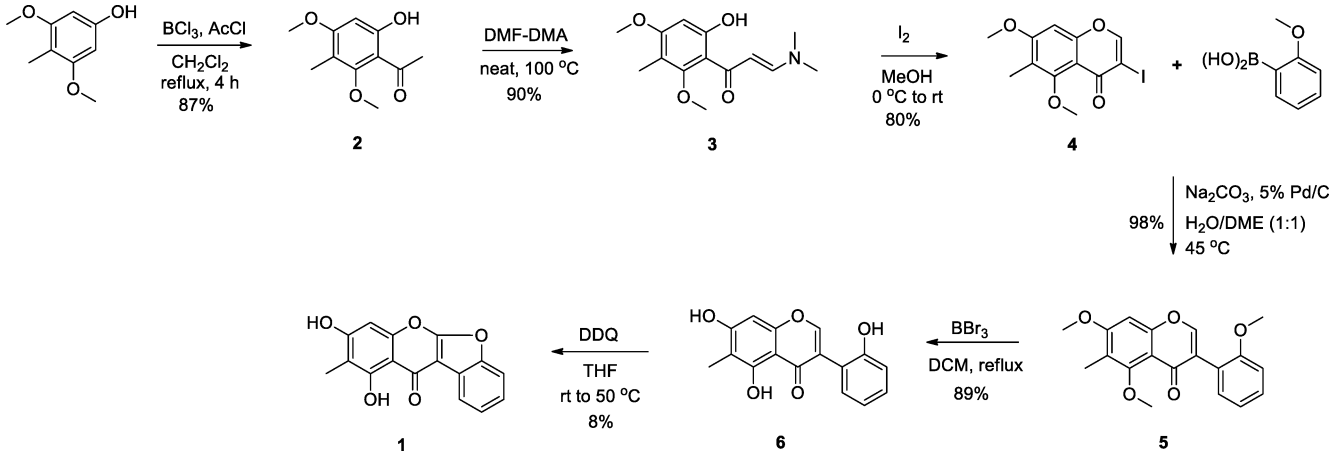
Scheme 1. Synthesis of Boeravinone Y (1)



an internal standard. HRFABMS was conducted using a JMS700 mass spectrometer (JEOL, Tokyo, Japan). For plant extraction and isolation, organic solvents, such as MeOH, EtOAc, and *n*-hexane, were purchased from Duksan Chemical (Anseong, Korea).

Plant Material. Suspension cultures of *A. nana* were prepared as previously described,[16] using the seeds obtained from Alplains (Kiowa, CO, USA). Briefly, the callus of *A. nana* was prepared by placing the seeds onto solid Murashige-Skoog's (MS) medium (pH 5.8) supplemented with 30 g/L sucrose, 1 mg/L 2,4-diphenoxyacetic acid (2,4-D), 3 mg/L 1-naphthaleneacetic acid (NAA), and 2 mg/L kinetin at 25 °C. The induced callus was transferred into 100 mL of liquid MS medium supplemented with 30 g/L sucrose, 1 mg/L 2,4-D, and 0.1 mg/L kinetin. The callus was cultured at 25 °C at 100 rpm in the dark for 25 days. MS medium, sucrose, 2,4-D, NAA, and kinetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The callus was maintained at the Laboratory of Natural Products Medicine, College of Pharmacy, Kyungpook National University (voucher number KNUNPM AN-2009-001).

Extraction and Isolation. The 25-day-old suspension culture was filtered to remove the culture medium (1 kg fresh weight), and fresh *A. nana* cells were extracted with 5 L of MeOH twice for 2 h each. The combined MeOH extract was suspended in 500 mL of deionized water and partitioned with the same volume of EtOAc three times. The EtOAc-soluble fraction was combined and evaporated to dryness (5 g). Five fractions (Fr. 1–Fr. 5) were obtained by medium-pressure silica gel column chromatography (Merck, Art. 9385, 30 × 300 mm; *n*-hexane–[EtOAc–MeOH, 1:1], 50:1 → 5:1; 4 mL/min; 254 nm). Fr. 2 was rechromatographed using a high-performance liquid chromatography (HPLC) system (Vydac C_{18} column, 7.8 × 250 mm, 10 μm; 80% MeOH in water; 2.5 mL/min; 254 nm) to purify compound **1** (13 mg, purity >98% by HPLC under UV light at 280 nm).

*Boeravinone Y (**1**):* gray amorphous powder; UV (MeOH) λ_{max} (log ε) 327 (3.8), 299 (3.7), and 256 (4.2) nm; IR (KBr) ν_{max} 3411, 1618, 1448, 1286, 1117, and 1076 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz), see Table 1; HRFABMS *m/z* 283.0609 $[M + H]^+$ (calcd for $C_{16}H_{11}O_5$, 283.0606; Figure S5, Supporting Information).

Synthesis of 1. The procedure for the synthesis of **1** is shown in Scheme 1. The intermediates were prepared as follows.

*1-(6-Hydroxy-2,4-dimethoxy-3-methylphenyl)ethanone (**2**).* A solution of BCl_3 (1 M in CH_2Cl_2; 8.8 mL, 8.8 mM) was added to a stirred suspension of 3,5-dimethoxy-4-methylphenol (1.34 g, 8 mM) in 50 mL of CH_2Cl_2 at 0 °C. After 5 min, acetyl chloride (AcCl, 0.68 mL, 9.6 mM) was added dropwise. The mixture was heated and kept under reflux for 4 h. Next, it was quenched with 1 N HCl (30 mL), extracted with CH_2Cl_2, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (*n*-hexane–EtOAc, 8:1) to yield the desired acetophenone **2** (1.46 g, 87%) as a light yellow oil: ^1H NMR ($CDCl_3$, 500 MHz) δ 13.44 (1H, s), 6.24 (1H, s), 3.84 (3H, s), 3.73 (3H, s), 2.69 (3H, s), and 2.06 (3H, s).

*(E)-3-(Dimethylamino)-1-(6-hydroxy-2,4-dimethoxy-3-methylphenyl)prop-2-en-1-one (**3**).* Compound **2** (1.45 g, 6.9 mM) was diluted with dimethylformamide–dimethylacetal (7 mL, 4 equiv), and the resulting mixture was stirred at 95 °C for 5 h. The volatile compounds were evaporated *in vacuo*. The mixture was diluted with H_2O and extracted with diethyl ether. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was washed with a solution of *n*-hexane–diethyl ether (4:1) to obtain the desired enamine **3** (1.65 g, 90%) as a yellow solid: ^1H NMR ($CDCl_3$, 500 MHz) δ 14.59 (1H, s), 7.95 (1H, d, J = 12.4 Hz), 6.36 (1H, d, J = 12.4 Hz), 6.23 (1H, s), 3.81 (3H, s), 3.67 (3H, s), 3.17 (3H, s), 2.94 (3H, s), and 2.05 (3H, s).

*3-Iodo-5,7-dimethoxy-6-methyl-4H-chromen-4-one (**4**).* I_2 (0.75 g, 3 mM) was added to a stirred suspension of **3** (0.56 g, 2.1 mM) in 20 mL of MeOH at 0 °C. The reaction mixture was stirred at room temperature (rt) for 4 h. It was quenched with $Na_2S_2O_3$, extracted with CH_2Cl_2, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (*n*-hexane–EtOAc, 3:1) to obtain the desired iodochromenone **4** (588 mg, 80%) as a white solid: ^1H NMR ($CDCl_3$, 500 MHz) δ 8.12 (1H, s), 6.62 (1H, s), 3.91 (3H, s), 3.85 (3H, s), and 2.18 (3H, s).

*5,7-Dimethoxy-3-(2-methoxyphenyl)-6-methyl-4H-chromen-4-one (**5**).* Na_2CO_3 (496 mg, 4.68 mM) and boronic acid (308 mg, 2.03 mM) were added to a solution of **4** (540 mg, 1.56 mM) in dimethoxyethane–H_2O (1:1, 12 mL). Palladium on carbon (5% Pd/C, 166 mg) was then added, and the reaction mixture was stirred at 45 °C for 5 h. It was then filtered through a short Celite pad and washed using CH_2Cl_2. The mixture was diluted with H_2O, extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to yield the isoflavonone **5** (498 mg, 98%) as a white solid: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.79 (1H, s), 7.37–7.3 (2H, m), 7.01 (1H, td, J = 7.5, 1 Hz), 6.97 (1H, d, J = 8.3 Hz), 6.64 (1H, s), 3.92 (3H, s), 3.84 (3H, s), 3.79 (3H, s), and 2.19 (3H, s).

*5,7-Dihydroxy-3-(2-hydroxyphenyl)-6-methyl-4H-chromen-4-one (**6**).* BBr_3 (0.65 mL, 6.75 mM) was added to a solution of **5** (440 mg, 1.35 mM) in CH_2Cl_2 (20 mL) at 0 °C. The mixture was heated and kept under reflux for 10 h. After cooling to rt, the reaction mixture was quenched with $NaHCO_3$, extracted with EtOAc, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (*n*-hexane–EtOAc, 3:1) to obtain the desired isoflavonone **6** (340 mg, 89%) as a light yellow solid: ^1H NMR (DMSO-d_6, 500 MHz) δ 13.15 (1H, s), 10.85 (1H, s), 9.42 (1H, s), 8.21 (1H, s), 7.25–7.16 (2H, m), 6.89 (1H, dd, J = 8, 0.6 Hz), 6.84 (1H, td, J = 7.5, 1 Hz), 6.48 (1H, s), and 2.00 (3H, s).

*9,11-Dihydroxy-10-methylcoumaronochromone (boeravinone Y, **1**).* 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (120 mg, 0.53 mM) was added to a stirred solution of **6** (100 mg, 0.35 mM) in

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tetrahydrofuran (5 mL), and the reaction mixture was heated at 50 °C for 4 h. It was then cooled to rt, and the solvent was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane−EtOAc, 4:1) to obtain the desired **1** (8 mg, 8%) as a white solid: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.63 (1H, s), 10.52 (1H, s), 7.52 (1H, m), 7.32 (1H, m), 7.06−6.99 (2H, m), 6.2 (1H, s), and 1.58 (3H, s).

Cell Culture, Differentiation, and Transfection. HUVECs were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as previously described.[21] Briefly, the cells were cultured in endothelial growth basal medium (EBM-2) containing growth supplements (Cambrex Bio Science) at 37 °C in 5% CO_2 until they reached confluency. All experiments were performed using HUVECs passaged three or five times. U937 cells (an oncogenic human monocyte cell line) were maintained at a density of 2×10^5 to 1×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium containing L-glutamine, 10% heat-inactivated fetal bovine serum, and antibiotics (penicillin G and streptomycin). These monocytes can differentiate into macrophages or dendritic cells,[50] and their differentiation pattern depends on the characteristics of the tissue microenvironment.[51] Phorbol 12-myristate 13-acetate (PMA) is a phorbol ester that commits monocytes to the macrophage lineage. Upon PMA treatment, U937 cells undergo a series of morphological and functional changes. To induce differentiation, U937 cells were resuspended in a fresh medium (4×10^5 cells/mL) for 12 h and treated with PMA (10 ng/mL; Sigma-Aldrich) for up to 24 h, as previously described.[52]

Animals and Husbandry. Male C57BL/6 mice (6−7 weeks old, weighing 27 g) were purchased from Orient Bio Co. (Sungnam, Korea) and acclimatized to the laboratory conditions for 12 days. Five mice were housed per polycarbonate cage under controlled conditions of temperature (20−25 °C), humidity (40−45%), and a 12:12 h light−dark cycle. They were provided with a normal rodent pellet diet and water *ad libitum* during acclimatization. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kyungpook National University (IACUC No. KNU 2017-102).

Cecal Ligation and Puncture. Mice were anesthetized using 2% isoflurane (Forane, JW Pharmaceutical, Seoul, Korea) in oxygen delivered via a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA, USA) first in a breathing chamber and then via a facemask. They were allowed to breathe spontaneously during the procedure. A CLP-induced sepsis model was used as previously described.[53,54] Briefly, a 2 cm midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated with a 3-0 silk suture at 5 mm from the cecal tip and punctured once using a 22-gauge needle for induction of high-grade sepsis.[55] This was gently squeezed to extrude a small amount of feces from the perforation site and then returned to the peritoneal cavity. The laparotomy site was then sutured with a 4-0 silk suture. In the sham control mice, the cecum was exposed, but neither ligated nor punctured, and returned to the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to the study (IRB No. KNU 2017-102).

Expression of Receptors. The same experimental procedures were used for measuring the expression of the cell surface receptors TLR2, TLR4, and RAGE using specific antibodies (A-9, H-80, and A-9, respectively) obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell Viability Assay. Microculture tetrazolium (MTT) was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and treated with compound **1**. After 48 h of incubation, the cells were washed, and 100 μL of MTT (1 mg/mL) was added, followed by incubation for 4 h. Finally, 150 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan salt. The amount of formazan salt was determined by measuring its optical density at 540 nm using a microplate reader (Tecan Austria GmbH, Grödig, Austria).

***In Vitro* Permeability Assay.** Permeability was quantitated by spectrophotometric measurement of the flux of albumin-bound Evans blue across functional HUVEC monolayers using a modified two-compartment chamber model, as previously described.[56] Briefly, HUVECs were plated (5×10^4 cells/well) in Transwells (pore size, 3 μm; diameter, 12 mm) for 3 days. The confluent monolayers were treated with HMGB1 (1 μg/mL) for 16 h, followed by incubation with **1** for 6 h.

***In Vivo* Permeability and Leukocyte Migration Assays.** For the *in vivo* study, mice were anesthetized as described above. Then, they were treated with intravenous HMGB1 (2 μg/mouse) for 16 h, followed by treatment with **1** (28 or 56 μg/kg) for 6 h. For the *in vivo* permeability assay, 1% Evans blue in normal saline was intravenously injected to each mouse. After 30 min, the mice were killed, and their peritoneal exudates were collected by washing the peritoneal cavities with 5 mL of normal saline and centrifuged at 200*g* for 10 min. The absorbance of the supernatants was read at 650 nm. Vascular permeability was determined using a standard curve, as previously described,[57,58] and expressed as μg dye that leaked into the peritoneal cavity per mouse. For assessment of leukocyte migration, mice were killed after 6 h, and their peritoneal cavities were washed with 5 mL of normal saline. Samples (20 μL) of the peritoneal fluid were mixed with 0.38 mL of Türk's solution (0.01% crystal violet in 3% acetic acid), and the number of leukocytes was counted under a light microscope.

Determination of Phosphorylated p38 MAPK Levels. The levels of phosphorylated p38 MAPK were determined in accordance with the manufacturer's instructions, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cell Signaling Technology, Danvers, MA, USA). The absorbance was measured using a microplate reader.

Bacterial Count. Peritoneal lavage fluid and blood were collected 24 h after CLP. Samples were centrifuged at 13 000 rpm for 10 min. Diluted samples were cultured in 5% sheep blood agar (Teknova, Hollister, CA, USA) at 37 °C for 24 h. Bacterial count was determined by counting the CFUs.

Expression of CAMs. The expression of VCAM-1, ICAM-1, and E-selectin in HUVECs was determined by whole-cell ELISA, as previously described.[59,60] Briefly, confluent HUVEC monolayers were treated with HMGB1 (1 μg/mL) for 16 h followed by treatment with **1** (0−1 μM) for 6 h. The medium was then removed, and the cells were washed with phosphate-buffered saline (PBS) and fixed with 50 μL of 1% paraformaldehyde for 15 min at rt. After washing, 100 μL of mouse anti-human monoclonal antibody (anti-VCAM-1, anti-ICAM-1, and anti-E-selectin; Millipore, Temecula, CA, USA; 1:50 dilution each) was added. After 1 h (37 °C, 5% CO_2), the cells were washed three times, and 100 μL of peroxidase-conjugated anti-mouse IgG antibody (Sigma-Aldrich; 1:2000 dilution) was added for 1 h. The cells were washed three times and developed using *O*-phenylenediamene (Sigma-Aldrich) as a substrate. The absorbance was measured at 490 nm.

Cell−Cell Adhesion Assay. Adhesion of the monocytes to endothelial cells was evaluated by fluorescent labeling of the monocytes, as previously described.[61−63] Briefly, human neutrophils (1.5×10^6 cells/mL, 200 μL/well) were labeled with vybrant DiD dye. Then, they were added to washed and stimulated HUVECs. HUVEC monolayers were treated with HMGB1 (1 μg/mL) for 16 h, followed by treatment with **1** (0−1 μM) for 6 h.

***In Vitro* Migration Assay.** The migration assay was performed in Transwell plates (pore size, 8 μm; diameter, 6.5 mm). HUVECs (6×10^4 cells/well) were cultured for 3 days to obtain confluent endothelial monolayers. Before addition of the monocytes to the upper compartment, cell monolayers were treated with HMGB1 (1 μg/mL) for 16 h, followed by treatment with **1** for 6 h. Cells in the upper chamber of the filter were aspirated, and nonmigrating cells on the top of the filter were removed using a cotton swab. Monocytes on the lower chamber of the filter were fixed with 8% glutaraldehyde and stained with 0.25% crystal violet in 20% MeOH (w/v). Each experiment was carried out in duplicate, and within each well, nine randomly selected high-power microscopic fields (200×) were counted and expressed as the migration index.

Hematoxylin and Eosin Staining and Histopathological Examination. Male C57BL/6 mice underwent CLP and were treated with compound **1** (28 or 56 μg/kg) intravenously 12 and 50 h after

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CLP (n = 5). They were sacrificed 96 h after CLP. To investigate the changes in lung morphology, lung samples were removed from each mouse, washed three times with PBS (pH 7.4) to remove any remaining blood, and fixed in 4% formaldehyde (Junsei, Tokyo, Japan) in PBS (pH 7.4) for 20 h at 4 °C. After fixation, the samples were dehydrated using serial dilutions of ethanol, embedded in paraffin, sectioned at 4 μm, and placed on a slide. The slides were deparaffinized in an oven at 60 °C, rehydrated, and stained with hematoxylin (Sigma-Aldrich). To remove the excess stain, the slides were quickly dipped three times in 0.3% acid alcohol and counterstained with eosin (Sigma-Aldrich). The excess stain was removed using serial dilutions of ethanol and xylene, and then the slides were coverslipped. Examination of the lung specimens was conducted by a blinded observer using a light microscope to evaluate the pulmonary architecture, tissue edema, and inflammatory cells' infiltration, as previously defined.[64]

Measurement of Organ Injury Markers. The levels of AST, ALT, BUN, creatinine, and LDH in fresh serum isolated from the plasma of septic mice were measured using biochemical kits (Mybiosource, San Diego, CA, USA). To determine the concentrations of IL-1β, IL-6, IL-10, MCP-1, and TNF-α, commercially available ELISA kits were used according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). The absorbance was measured using a microplate reader.

Statistical Analysis. All experiments were performed at least five independent times. Student's t-test was used for comparison. Values were expressed as the means ± standard deviation (SD). Kaplan–Meier survival analysis was performed to determine the overall survival rates. SPSS for Windows, version 16.0 (SPSS; Chicago, IL, USA) was used to perform all statistical analyses. P values of <0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00826.

^{1}H and ^{13}C NMR, HMQC and HMBC, HRFABMS, IR spectra, and HPLC chromatogram of **1** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: 82-53-950-8565. Fax: 82-53-950-8557. E-mail: kssong@knu.ac.kr (K.-S. Song).

*Tel: 82-53-950-8565. Fax: 82-53-950-8557. E-mail: ejy125@gmail.com (E.-J. Yang).

*Tel: 82-53-950-8570. Fax: 82-53-950-8557. E-mail: baejs@knu.ac.kr (J.-S. Bae).

Author Contributions

§W. Lee and D. Lee contributed equally to this work.

Notes

The authors declare no competing financial interest.

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