JOURNAL OF NATURAL PRODUCTS

Article

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Oscarellin, an Anthranilic Acid Derivative from a Philippine Sponge, *Oscarella stillans*, as an Inhibitor of Inflammatory Cytokines in Macrophages

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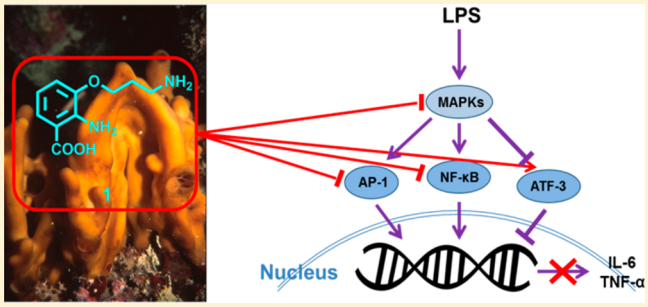
Supporting Information

ABSTRACT: A new anthranilic acid derivative (**1**) was isolated from a Philippine sponge, *Oscarella stillans* (Bergquist and Kelly). The structure of compound **1**, named oscarellin, was determined as 2-amino-3-(3′-aminopropoxy)benzoic acid from spectroscopic data and confirmed by synthesis. We examined the immunomodulating effect of compound **1** and its mechanism in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Our data indicated that the expression of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were significantly reduced by the pretreatment of **1** (0.1−10 μM) for 2 h. In addition, compound **1** suppressed activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun NH_2-termimal kinase (JNK), but not p38 mitogen-activated protein kinase (MAPK) in LPS-stimulated RAW 264.7 cells. Compound **1** abrogated LPS-induced nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activities, whereas the induction of activating transcription factor-3 (ATF-3) was increased. Taken together, our results suggest that compound **1** attenuates pro-inflammatory cytokines via the suppression of JNK, ERK, AP-1, and NF-κB and the activation of the ATF-3 signaling pathway.



Marine organisms are worthwhile sources of bioactive and structurally diverse compounds. Marine sponges of the family Plakinidae include seven genera, *Corticum*, *Oscarella*, *Placinolopha*, *Plakina*, *Plakinastrella*, *Plakortis*, and *Pseudocorticium*.[1] Several secondary metabolites including sterols,[2,3] alkylpyrrole carboxaldehyde,[4,5] sesterterpene glycosides,[6] and lysophospholipids[7] have been isolated from the sponge genus *Oscarella*. A sponge collected at Honda Bay in Philippines was reported as a new species, *Oscarella stillans*, of the family Plakinidae (phylum Porifera, class Demospongiae, order Homosclerophorida).[8] The chemical constituents and biological activity of *O. stillans* have not been previously studied.

Inflammation is an essential defensive response to harmful stimuli including pathogens, irritants, and inflammatory cytokines, which interact with other cells.[9,10] Although the inflammatory process is a beneficial physiologic reaction, excessive or persistent inflammation causes serious inflammatory diseases including septic shock, rheumatoid arthritis, and autoimmune diabetes.[9] Among the immune cells, macrophages play an essential role in regulation of host defense mechanisms.[11] Lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria, is known to induce the activation of macrophages.[12] Upon LPS exposure, macrophages are activated and subsequently release various inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and nitric oxide (NO).[13−15] Therefore, inhibition of macrophage activation is considered a desired therapeutic strategy for treatment of various inflammatory diseases.[11]

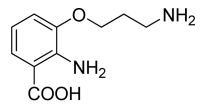
In continuation of our search for bioactive compounds, we investigated the chemical constituents of the sponge *O. stillans* and also evaluated immunomodulatory activity of the isolated compound **1** on inflammatory mediators, such as cytokines and NO, in LPS-treated RAW 264.7 cells. Herein, we report the isolation and structure of a new anthranilic acid derivative isolated from the sponge *O. stillans* and its immunomodulatory effect through the regulation of c-Jun NH_2-termimal kinase (JNK), extracellular signal-regulated kinase (ERK), activating transcription factor-3 (ATF-3), activator protein-1 (AP-1), and nuclear factor-κB (NF-κB) in LPS-induced RAW 264.7 macrophages.

RESULTS AND DISCUSSION

Isolation and Identification of Oscarellin (1). Extracts of the sponge *O. stillans* were combined, and an aqueous MeOH solution thereof was subjected to solvent partitioning with hexane, CH_2Cl_2, and *n*-BuOH. The *n*-BuOH fraction was

Received: August 28, 2016

DOI: 10.1021/acs.jnatprod.6b00787
J. Nat. Prod. XXXX, XXX, XXX−XXX



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fractionated on a Sephadex LH-20 column. A selected fraction was rechromatographed on RP-C_{18} and subjected to RP-C_{18} HPLC using different combinations of solvents to obtain compound **1**.

Compound **1** was obtained as an amorphous solid. The molecular formula of **1** was deduced to be $C_{10}H_{14}N_2O_3$ by HRESIMS data. The IR spectrum implied the presence of NH and/or COOH (3500−2500 cm^{-1}) and carbonyl (1696.6 cm^{-1}) groups. The ^1H NMR spectrum of **1** revealed signals for a 1,2,3-trisubstituted benzene ring at δ_H 6.64 (t, J = 8.0 Hz), 7.01 (d, J = 8.0 Hz), and 7.49 (d, J = 8.0 Hz) and a 3-aminopropoxy group at δ_H 2.18 (p, J = 6.5 Hz), 3.19 (t, J = 7.5 Hz), and 4.15 (t, J = 6.0 Hz). The ^{13}C NMR spectrum of **1** revealed 10 carbons, and its data confirmed the presence of a trisubstituted benzene ring (δ_C 113.8, 116.3, 117.7, 125.1, 141.5, and 148.6) and a 3-aminopropoxy group (δ_C 28.8, 38.9, and 67.3) together with a carboxylic acid (δ_C 171.9). The locations of the carboxylic acid and 3-amino propoxy groups were established from HMBC correlations shown in Figure 1.

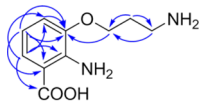

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Figure 1. Selected HMBC correlations of **1**.

The carboxylic acid and 3-aminopropoxy moieties were assigned to C-1 and C-3 of the benzene ring, respectively, because of the HMBC correlations of H-6/C-7 and H-1′/C-3. Thus, the structure of **1** was determined as 2-amino-3-(3′-aminopropoxy)benzoic acid and was designated oscarellin.

For the purpose of structure confirmation, compound **1** was synthesized via selective protection, O-alkylation, and deprotection reactions of 3-hydroxyanthranilic acid consecutively, according to the synthetic route illustrated in Scheme 1. All synthetic details and experimental data, including ^1H NMR and ^{13}C NMR, are described in the Supporting Information (Figures S10−S15). The spectroscopic data of the synthetic compound were identical with those of oscarellin.

Effect of Oscarellin (1) on Proliferation of RAW 264.7 Cells. To investigate the cytotoxic effect of oscarellin (**1**), RAW 264.7 cells were treated with **1** (0.1, 1, 10, 50, and 100 μg/mL) for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell proliferation. Oscarellin (**1**) did not affect cell proliferation, because a significant inhibitory effect was not seen at concentrations up to 100 μg/mL (Figure S16). In subsequent experiments, RAW 264.7 cells were treated with various concentrations (0.1, 1, and 10 μg/mL) of **1** to examine its anti-inflammatory effect.

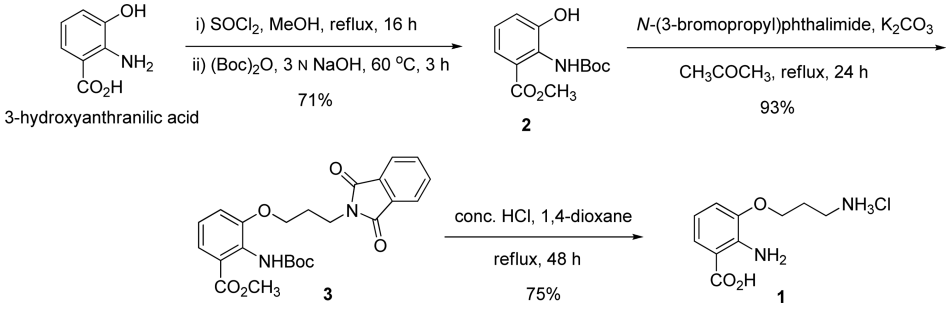
Effect of Oscarellin (1) on Production of Cytokines and NO in LPS-Treated RAW 264.7 Cells. LPS has been suggested to activate macrophages.[12] The inflammatory mediators, such as TNF-α, IL-6, and NO, are produced by activated macrophages.[16,17] These inflammatory mediators cause tissue and cell damage, leading to various pathological systemic effects. Inhibiting these mediators by preventing their production could be used as therapy for treatment of various inflammation-mediated diseases.

We evaluated the effect of oscarellin (**1**) on the expression levels of inflammatory mediators in LPS-treated RAW 264.7 cells. Dexamethasone (10 and 20 μM) was used as a positive control to compare the anti-inflammatory effect in this experiment. As shown in Figure 2, production of TNF-α, IL-6, and NO was markedly elevated after treatment with LPS (1 μg/mL) for 24 h. However, pretreatment of cells with **1** (0.1, 1, and 10 μg/mL) 2 h before LPS exposure caused a concentration-dependent decrease in TNF-α and IL-6 production, whereas NO production was not inhibited.

Oscarellin (1) Inhibits AP-1 and NF-κB Activation in LPS-Stimulated RAW 264.7 Cells. NF-κB and AP-1 are critical in the regulation of pro-inflammatory mediator production.[18−20] NF-κB bound to IκB is present in the cytosol, forming an inactive trimeric complex. Upon exposure of cells to LPS, IκB is rapidly phosphorylated, and then activated NF-κB translocates into the nucleus.[21,22] Because this process is required for the activation of pro-inflammatory gene transcription, we determined whether oscarellin (**1**) influences NF-κB activation by a luciferase reporter assay. LPS induced an almost 4-fold increase in luciferase activity, and this increase was reduced by treatment with **1** (Figure 3A). As shown in Figure 3B, **1** concentration-dependently suppressed p65 expression, whereas pretreatment of cells with **1** decreased p65 translocation to the nucleus. Pretreatment with **1** suppressed LPS-induced IκB degradation (Figure 3C). These data indicate that **1** suppresses LPS-stimulated NF-κB activation.

We next investigated whether oscarellin (**1**) is able to alter the activation of another transcription factor, AP-1, by a luciferase reporter assay and a Western blot. Pretreatment with

Scheme 1. Synthetic Route for Compound 1



DOI: 10.1021/acs.jnatprod.6b00787
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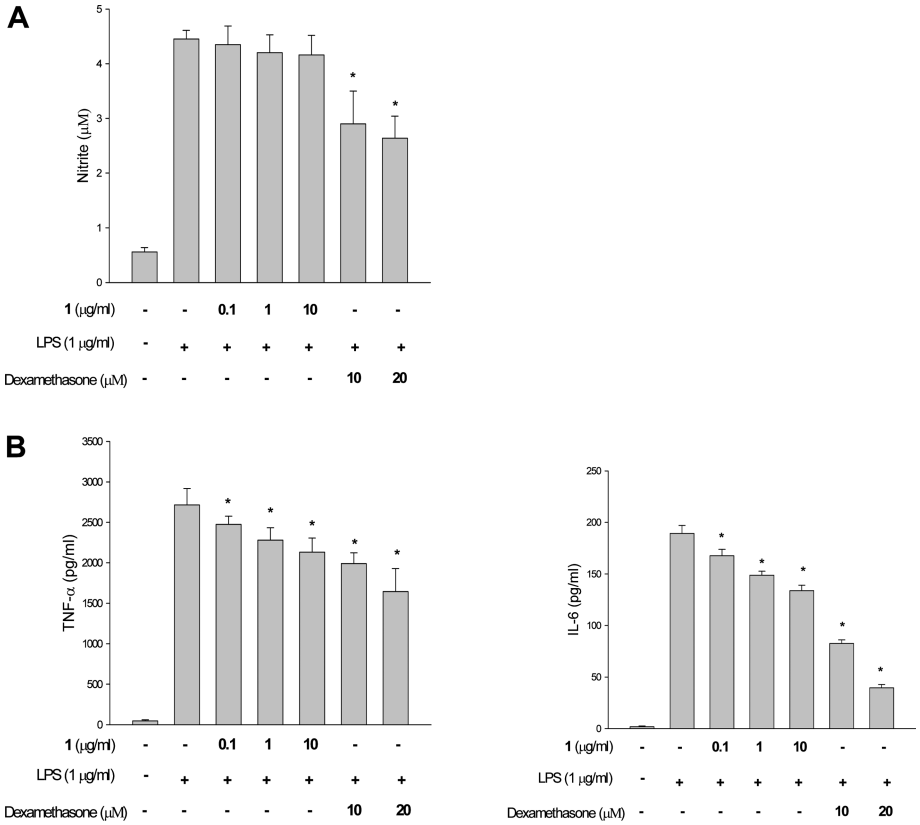

Figure 2. Effects of compound **1** on the production of NO and cytokines in LPS-stimulated RAW 264.7 cells. Cells were pretreated with **1** [0.1 (0.476 μM), 1 (4.76 μM) and 10 (47.6 μM) μg/mL) or dexamethasone (10 and 20 μM] for 2 h followed by the stimulation with LPS (1 μg/mL) treatment in RAW 264.7 cells. The amounts of nitrite (A) and cytokines (B) in the medium were measured. Data are expressed as the means ± SEM of quintuplicate replicates from a representative experiment. *$p < 0.05$, significantly different from the group treated with LPS alone.

1 concentration-dependently inhibited the LPS-induced AP-1 activity (Figure 4A). This pretreatment also attenuated the LPS-induced nuclear translocation of c-Jun and c-Fos (Figure 4B). Collectively, the data indicate that oscarellin (**1**) inhibits LPS-induced pro-inflammatory cytokines by blocking the activation of NF-κB and AP-1.

Oscarellin (1) Increases the Activation of ATF-3 in LPS-Treated RAW 264.7 Cells. LPS induces activation of ATF-3 in various cells including macrophages.[21−23] ATF-3 has a crucial role as a negative regulator of LPS-induced inflammatory responses.[22,23] To investigate the effect of **1** on ATF-3 expression, cells were preincubated with various concentrations (0.1−10 μg/mL) of **1** for 4 h followed by stimulation with LPS. The expression of ATF-3 was significantly enhanced by LPS, and **1** additively increased concentration-dependent ATF-3 expression (Figure 5A). To ascertain the involvement of ATF-3 in **1**-treated cells, the effect of ATF-3 short hairpin (sh)RNA on **1** action was examined. ATF-3 shRNA partially but significantly suppressed the inhibitory effects of **1** on TNF-α and IL-6 production (Figure 5B). These data indicate that inhibitory effects of **1** on pro-inflammatory cytokine production are due to the induction of ATF-3.

Effect of Oscarellin (1) on the LPS-Stimulated MAP Kinases. LPS treatment results in activation of MAPK pathways followed by inducing the expression of pro-inflammatory cytokines in macrophages.[13,24−26] The effect of oscarellin (**1**) on the activation of MAP kinases was examined in LPS-treated cells. LPS caused an increase of MAPK phosphorylation, while **1** inhibited the activation of JNK and ERK1/2 not but p38 MAPK (Figure 6). These results suggest that JNK and ERK1/2 pathways are important for inhibitory activity of **1**, but p38 MAPK did not seem to be involved in its action.

In summary, a new anthranilic acid derivative (**1**) was isolated from a Philippine sponge, *O. stillans*. The structure of oscarellin (**1**) was determined as 2-amino-3-(3′-aminopropoxy)benzoic acid from spectroscopic data and confirmed by synthesis. The present study also indicates that **1** has immunomodulatory activity as a result of the inhibition of TNF-α and IL-6 production in activated macrophages. Moreover, the inhibitory activity of oscarellin (**1**) is associated with inactivation of JNK, ERK AP-1, and NF-κB and activation of ATF-3.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting point was measured on a Electrothermal 9300 (Electrothermal Engineering LTD). UV spectra were obtained on a Hewlett-Packard HP8453 diode array spectrometer. IR spectra were recorded on a Bio-Rad FTS-155 FT-IR spectrometer. NMR experiments were conducted with a Varian VXR-500 spectrometer installed with a 3 mm $^{1}H/^{13}C$ switchable gradient microprobe (MDG-500-3) and a pulsed field gradient driver. NMR signals are reported as ppm (δ), referenced to CD_3OD (δ_H, 3.31; δ_C, 49.2) and DMSO-d_6 (δ_H, 2.50). FABMS and HREIMS, and ESIMS spectra were obtained on a VG ZAB-E and Micromass Q-TOF mass spectrometer, respectively. Column chromatography was carried out on Sephadex LH-20 (Sigma-Aldrich, 25−100 μm) and RP-18 (Merck, 40−63 μm). Preparative HPLC was performed using a Phenomenex ODS-2 (300 × 10 mm) column and a UV detector (254 nm).

DOI: 10.1021/acs.jnatprod.6b00787
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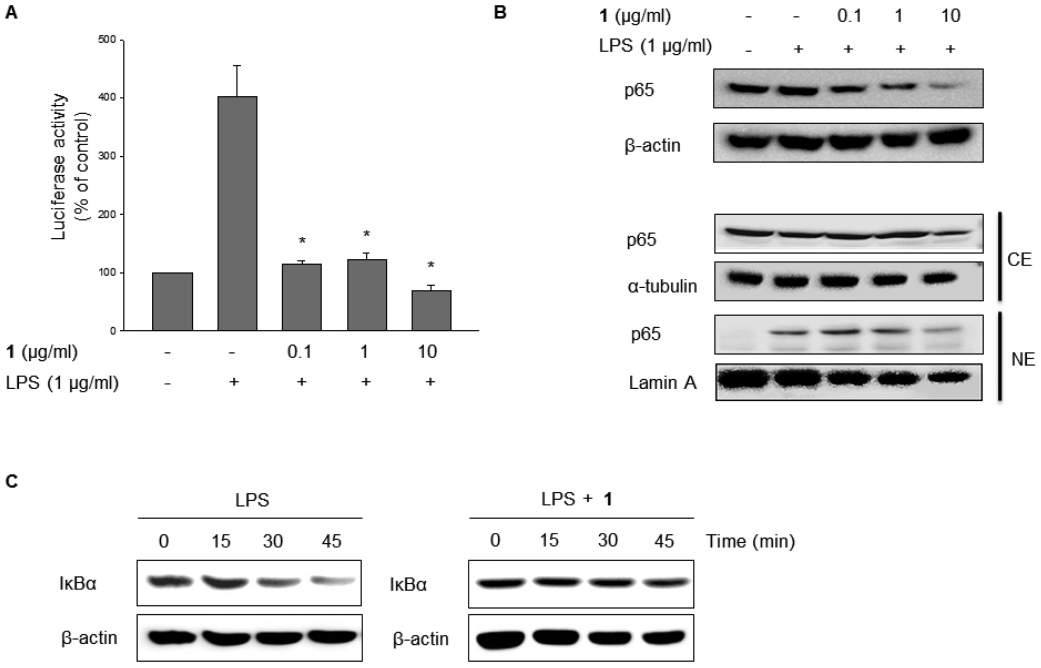

Figure 3. Effects of compound **1** on the NF-κB activation and IκBα degradation in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were transfected with a pGL3-NF-κB-Luc reporter plasmid and Renilla plasmid, pretreated with various concentrations of **1** (0.1, 1, 10 μg/mL) for 2 h, followed by treatment with 1 μg/mL LPS for 4 h. (B) RAW 264.7 cells were pretreated with **1** (0.1, 1, 10 μg/mL) for 2 h followed by treatment with 1 μg/mL of LPS for 4 h. The protein level of p65 was detected by Western blotting to analyze the translocation of NF-κB. (C) RAW 264.7 cells were pretreated with 10 μg/mL of **1** for 2 h, followed by treatment with 1 μg/mL of LPS for different time periods. The β-actin protein level was considered as an internal control. The results illustrated are representative of three separate experiments. *$p < 0.05$, significantly different from the group treated with LPS alone.

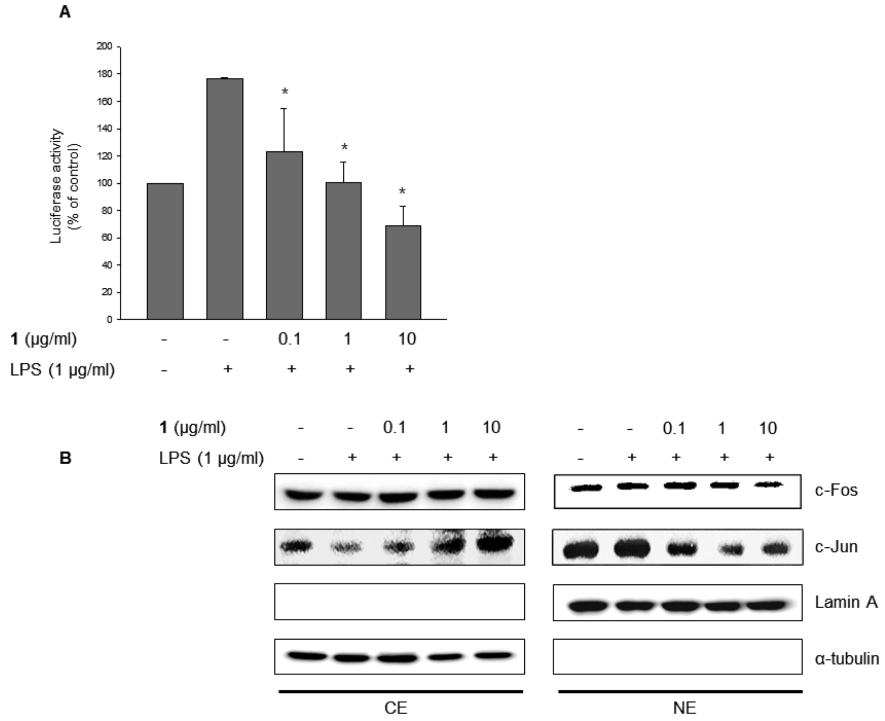

Figure 4. Effects of compound **1** on AP-1 activation and c-Jun and c-Fos expression in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were transfected with a pGL2-AP-1 Luc reporter plasmid and Renilla plasmid, pretreated with various concentrations of **1** for 2 h followed by treatment with 1 μg/mL of LPS for 4 h. (B) RAW 264.7 cells were pretreated with **1** for 2 h, followed by treatment with 1 μg/mL of LPS for 4 h. The protein level of c-Fos and c-Jun was detected by Western blotting to analyze the translocation of AP-1. The levels of lamin A and α-tubulin protein were considered as an internal control. The results are mean ± SEM of quintuplicates from a representative experiment. The intensity of the bands was quantitated by densitometry. The levels of c-Fos and c-Jun expression are in arbitrary units, and data are normalized to the respective amount of lamin A and α-tubulin protein. *$p < 0.05$, significantly different from the group treated with LPS alone.

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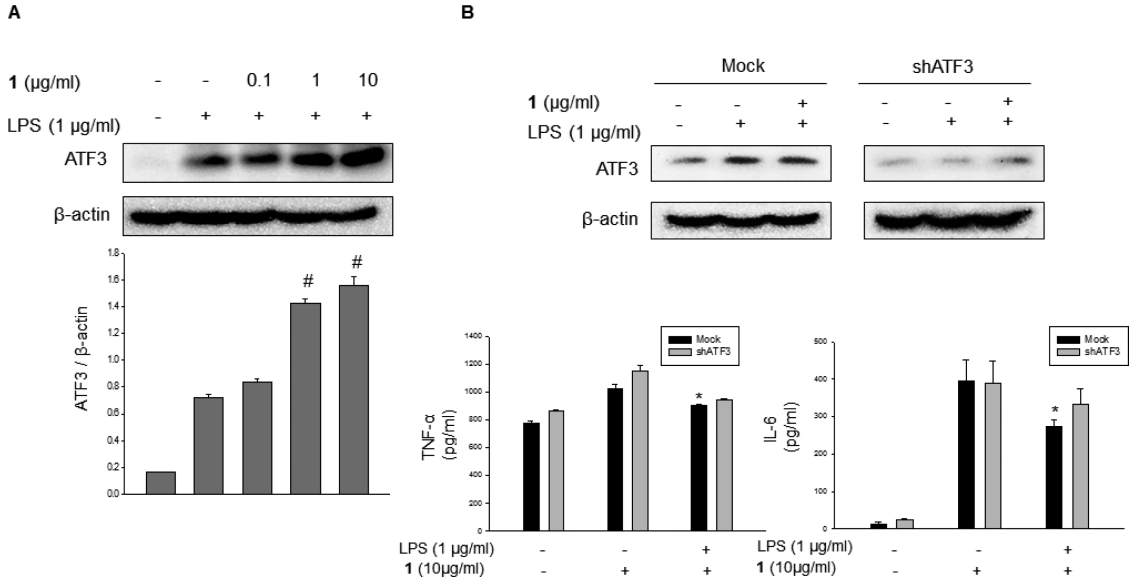

Figure 5. Effects of compound **1** on ATF3 activation in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with **1** for 2 h, followed by treatment with 1 μg/mL of LPS for 4 h. The β-actin protein level was considered as an internal control. The results illustrated are representative of three separate experiments. The intensity of the bands was quantitated by densitometry. The levels of ATF-3 expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. (B) RAW 264.7 cells were stably transfected with vector control or specific knockdown of ATF3 (shATF3) with 10 μg/mL of **1** for 2 h, followed by treatment with 1 μg/mL of LPS for 24 h. The amounts of nitrite and cytokines in the medium were measured. $^{\#}p < 0.05$ versus LPS alone. $^{*}p < 0.05$, significantly different from control shRNA.

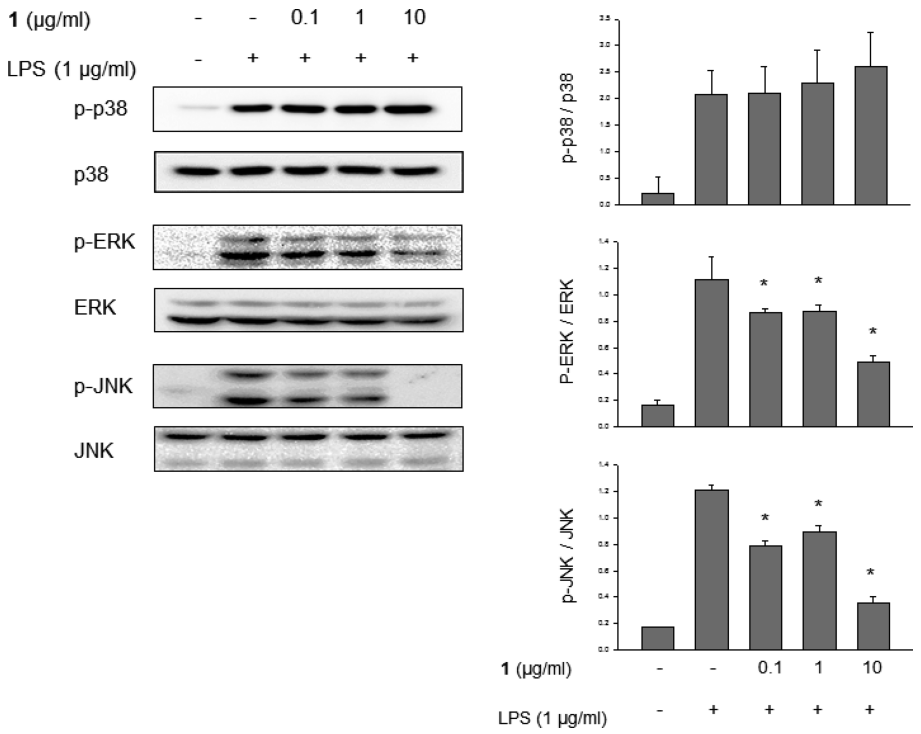

Figure 6. Effects of compound **1** on the activation of MAP kinases on LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with **1** for 2 h, followed by treatment with 1 μg/mL of LPS for 15 min. The β-actin protein level was considered as an internal control. The results illustrated are from a single experiment and are representative of three separate experiments. The intensity of the bands was quantitated by densitometry. The levels of MAPK expression are in arbitrary units, and data are normalized to the respective amount of MAPK protein. $^{*}p < 0.05$, significantly different from the group treated with LPS alone.

Animal Material. The sponge was collected at Honda Bay, Philippines, in 1995. It consists of a series of fused thin tubes (length 3−5 cm and thickness about 2 mm), some with short blind branches. Its surface is very smooth and slippery. The color of the living sponge is dark honey yellow, along with greenish tinges underwater. It is adhered to an overhanging coral substrate on perpendicular planes at ~12 m depths. The sponge is *Oscarella stillans* (class Demospongiae, order Homosclerophorida, family Plakinidae), and it was first reported by Bergquist and Kelly (2004).[8] A voucher specimen (registration code 16PH95) has been deposited in the Department of Chemistry and Biochemistry, University of Oklahoma. Another voucher sample is curated in the Department of Invertebrate Zoology at the Natural

DOI: 10.1021/acs.jnatprod.6b00787
J. Nat. Prod. XXXX, XXX, XXX−XXX

History Museum in Washington DC under the accession number OCDN3255-W.

Extraction and Isolation. The specimens (0.9 kg wet wt) were extracted twice with MeOH and then with MeOH/CH_2Cl_2 (1:1). The combined extracts were evaporated in vacuo to remove the solvent. The extract was subjected to solvent partitioning to give hexane (4.01 g), CH_2Cl_2 (1.44 g), and *n*-BuOH (1.39 g) soluble fractions. The *n*-BuOH fraction was fractionated over a Sephadex LH-20 column (MeOH only) to give fractions F008−F015. Fraction F010 was further purified over RP-18 vacuum flash column chromatography (25−30% MeOH/H_2O with 0.1% TFA) and reversed-phase C_{18} HPLC using 23% MeOH/H_2O with 0.1% TFA as eluent to yield compound **1** (17 mg).

*Oscarellin (**1**):* colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 210 (4.55), 242 sh (3.83), 318 (3.55); IR ν_{max} 3500−2500 (br, NH and/or COOH), 1696.6 (C═O), 1195; ^{1}H NMR (CD_3OD, 500 MHz) δ_H 2.18 (2H, p, J = 6.5 Hz, H-2′), 3.19 (2H, t, J = 7.5 Hz, H-3′), 4.15 (2H, t, J = 6.0 Hz, H-1′), 6.64 (1H, t, J = 8.0 Hz, H-5), 7.01 (1H, d, J = 8.0 Hz, H-4), 7.49 (1H, d, J = 8.0 Hz, H-6), (DMSO-d_6, 500 MHz) δ_H 2.05 (2H, p, J = 6.3 Hz, H-2′), 3.02 (2H, t, J = 7.3 Hz, H-3′), 4.05 (2H, t, J = 6.0 Hz, H-1′), 6.47 (1H, t, J = 7.8 Hz, H-5), 6.92 (1H, d, J = 7.5 Hz, H-4), 7.32 (1H, d, J = 8.0 Hz, H-6), 8.07 (3H, br s, NH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 28.8 (C-2′), 38.9 (C-3′), 67.3 (C-1′), 113.8 (C-1), 116.3 (C-4), 117.7 (C-5), 125.1 (C-6), 141.5 (C-2), 148.6 (C-3), 171.9 (C-7); ESIMS m/z 211.1 $[M + H]^+$, 233.1 $[M + Na]^+$, 421.2 $[2M + H]^+$, 443.2 $[2M + Na]^+$; FABMS m/z 211.1 $[M + H]^+$; HREIMS m/z 210.1001 $[M]^+$ (calcd for $C_{10}H_{14}N_2O_3$, 210.1004).

Synthesis of Compound 1. The mixture of 3-hydroxyanthranilic acid (0.47 g) in anhydrous MeOH (5.1 mL) and $SOCl_2$ (0.11 mL) was refluxed for 16 h to give a methyl ester of 3-hydroxyanthranilic acid (0.52 g), which further reacted with di-*tert*-butyl dicarbonate (0.89 mL) in 3 N NaOH (15 mL) to produce methyl 2-(*tert*-butoxycarbonylamino)-3-hydroxybenzoate (**2**, 0.58 g; 71% yield) as a pale yellow solid. For **3**, an anhydrous acetone solution (5.4 mL) of **2** (0.29 g) was reacted with K_2CO_3 (0.5 g) and *N*-(3-bromopropyl)-phthalimide (0.38 mg). Subsequently, methyl 2-(*tert*-butoxycarbonylamino)-3-[3-(1,3-dioxoisoindolin-2-yl)propoxy]benzoate (**3**, 0.32 g) in 1,4-dioxane (3.5 mL) was subjected to a reaction with concentrated HCl (1.2 mL). The reaction mixture was stirred for 48 h at 100 °C and then concentrated under reduced pressure. The residue was purified by column chromatography and recrystallization (acetone) to afford compound **1** (0.13 g; mp 206.9−207.4; 75% yield) as its hydrochloride salt (a white solid).

Chemicals and Reagents. Unless stated otherwise, all reagents and chemicals were procured from Sigma-Aldrich. pGL3-NF-κB and the luciferase assay system were from Promega. pGL2-AP-1 (PMA)-TA-luciferase was from Clontech Laboratories, Inc. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Lipofetamine Plus were purchased from Gibco BRL Life Technologies. Cytokine DuoSet ELISA kits (TNF-α, IL-6) were from R&D System. Antibodies for anti-mouse IκB-α, anti-mouse p65, anti-mouse Erk1/2, anti-mouse p-Erk1/2, anti-mouse p38, anti-mouse p-p38, anti-mouse JNK, anti-mouse p-JNK, anti-mouse c-Fos, anti-mouse c-Jun, anti-mouse ATF-3, anti-mouse Lamin A, anti-mouse α-tubulin, and anti-mouse β-actin were from Santa Cruz Biotechnology.

Cell Culture. The macrophage cell line RAW 264.7 was procured from ATCC and grown in DMEM with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS in a 5% CO_2 humidified atmosphere at 37 °C.

Cell Proliferation Assay. RAW 264.7 cells were seeded in a 96-well tissue culture plate (5×10^4 cells/well) (Nunc) and treated with compound **1** (0.1, 1, 10, 50, and 100 μg/mL) for 24 h. After treatment, cell proliferation was determined by incubating the cells with a mixture of 125 μL of full DMEM and 25 μL of MTT solution for another 4 h. Then, the MTT-formazan produced by viable cells was dissolved in 150 μL of DMSO. The amount of the resulting formazan was assessed by measuring the optical density at 550 nm using a scanning multiwell plate reader (Molecular Device).

Nitrite Determination. RAW 264.7 cells were treated with indicated concentrations of compound **1** for 2 h and incubated with LPS (1 μg/mL) for a further 24 h. The NO concentration in the culture supernatants was determined as previously described.[27] After 100 μL of supernatant was added to the empty 96-well plate, 100 μL of Griess reagent (1 part 1% sulfanilamide in 5% phosphoric acid plus 1 part 1% α-naphthylamide in H_2O) was added to each well and incubated at room temperature for 15 min. The absorbance at 550 nm was read by a scanning multiwell plate reader. The levels of NO_2^- were used as an indicator of the amount of NO production.

Cytokine Determination by ELISA. RAW 264.7 cells were treated with compound **1** for 2 h followed by treatment of LPS (1 μg/mL) for 24 h. The supernatant was then collected and used to determine the levels of TNF-α and IL-6 secretion by DuoSet ELISA kits obtained from R&D System.

Transfection and Reporter Assays. Cells (5×10^5 cells/mL) were plated into each well of a six-well plate. The cells were transiently cotransfected with pGL3-NF-κB-Luc or pGL2-AP-1-Luc and pCMV-β-gal using Lipofectamine Plus according to the manufacturer's protocol for use. After transfection for 24 h, the cells were pretreated with compound **1** for 2 h and then stimulated with LPS (1 μg/mL) for 4 h. Each well was washed with cold phosphate-buffered salin (PBS). Luciferase activity was measured in the cell lysates using a Luciferase assay system according to the manufacturer's instructions (Promega).

Construction of shRNA-Expressing Plasmid. The pSUPER plasmid was used to synthesize shRNAs. For construction of the ATF3 shRNA-expressing constructs, oligonucleotide shATF3 5′-GGAGGCGGCGAGAAAGAAA-3′ was selected to target ATF3 mRNA (GenBank accession no. NM007498.3). To evaluate the effect of the shRNA, the expression plasmid encoding shRNA was transfected into RAW 264.7 cells. Stably transfected cells expressing shRNA, which were selected using puromycin, were used for subsequent experiments.

Western Blot Analysis. Western blot analysis was performed as described earlier.[28] Briefly, RAW 264.7 cells were preincubated with various concentrations (0.1, 1, and 10 μg/mL) of compound **1** for 2 h. RAW 264.7 cells were then incubated for 15 min or 4 h with 1 μg/mL of LPS. After treatment, PBS was used to wash the cells. The cells were scraped using scrapers and suspended in a lysis buffer. To obtain cytosolic cell extracts, homogenate was centrifuged for 10 min at 4 °C at 1500*g*. Nuclear fraction was recovered by centrifugation at 13000*g* for 10 min at 4 °C. Proteins in the samples were resolved by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes, which were probed with the appropriate antibodies. Enhanced chemiluminescence solution was used to detect the protein bands. In all immunoblotting experiments, membranes wre stripped and reprobed with an anti-β-actin antibody for protein loading correction.

Statistical Analysis. The results were reported as the mean ± standard error of the mean (SEM). For comparisons between groups, the GraphPad program (Software for Science) was used for statistical analyses. Differences between groups were considered significant at $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00787.

All synthetic details and experimental data (R_f, ^{1}H and ^{13}C NMR) for synthetic intermediates and product; NMR (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC) and mass spectra for **1**; and effect of compound **1** on the cell proliferation (PDF)

DOI: 10.1021/acs.jnatprod.6b00787
J. Nat. Prod. XXXX, XXX, XXX−XXX

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a grant from Marine Biotechnology Program (PJT200620, Genome Analysis of Marine Organisms and Development of Functional Applications) funded by Ministry of Oceans and Fisheries, and grants from the Ministry of Oceans and Fisheries' R&D project (PM13030) and the Korea Polar Research Institute (KOPRI) project (PE13040). We thank Dr. P. Colin, Coral Reef Research Foundation, Koror, Palau, for the sponge material used in this research.

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DOI: 10.1021/acs.jnatprod.6b00787
J. Nat. Prod. XXXX, XXX, XXX−XXX