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RESEARCH ARTICLE

A new sulfonic acid derivative, (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid, isolated from the cold water sea urchin inhibits inflammatory responses through JNK/p38 MAPK and NF-κB inactivation in RAW 264.7

Dong-Sung Lee · Xiang Cui · Wonmin Ko · Kyoung-Su Kim · Il Chan Kim · Joung Han Yim · Ren-Bo An · Youn-Chul Kim · Hyuncheol Oh

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Abstract In this study, we isolated a new sulfonic acid derivative, (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**), from the sea urchin collected from the Sea of Okhotsk. We established the structure of this new compound by analysis of NMR and HRMS data, along with comparison of the data with those of the related compounds reported in the literature. In addition, we investigated its anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages. Compound **1** inhibited the production of NO, iNOS, PGE_2, and COX-2, and it also suppressed the production of pro-inflammatory cytokines, such as TNF-α and IL-1β. It inhibited the translocation of the NF-κB subunit p65 into the nucleus by interrupting the phosphorylation and degradation of IκB-α. In addition, compound **1** significantly decreased the phosphorylation of JNK and p38 in LPS-stimulated RAW264.7 macrophages, suggesting that suppression of the inflammation process by compound **1** was mediated through the MAPK pathway. Taken together, this study showed that the anti-inflammatory effects of a new sulfonic acid derivative, (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid were mediated through the inhibition of NF-κB and JNK/p38 MAPK signaling pathways.

Keywords Sea urchin · A new sulfonic acid derivative · (*Z*)-4-Methylundeca-1,9-diene-6-sulfonic acid · Anti-inflammation · Nuclear factor-κB · Mitogen-activated protein kinase

Dong-Sung Lee and Xiang Cui contributed equally to this work.

D.-S. Lee · Y.-C. Kim · H. Oh
Hanbang Body-Fluid Research Center, Wonkwang University, Iksan 570-749, Republic of Korea

X. Cui · R.-B. An
Key Laboratory of Natural Resources and Functional Molecules of the Changbai Mountain, Affiliated Ministry of Education, Yanbian University College of Pharmacy, 977 Gongyuan Road, Yanji 133002, Jilin, People's Republic of China

X. Cui · W. Ko · K.-S. Kim · Y.-C. Kim · H. Oh (✉)
Institute of Pharmaceutical Research and Development, College of Pharmacy, Wonkwang University, Iksan 570-749, Republic of Korea
e-mail: hoh@wku.ac.kr

W. Ko · K.-S. Kim · Y.-C. Kim (✉) · H. Oh
Standardized Material Bank for New Botanical Drugs, College of Pharmacy, Wonkwang University, Iksan, Republic of Korea
e-mail: yckim@wku.ac.kr

I. C. Kim · J. H. Yim
Korea Polar Research Institute, KORDI, 7-50 Songdo-dong, Yeonsu-gu, Inchon 406-840, Republic of Korea

Introduction

Lipopolysaccharide (LPS), a main component of the outer membrane of gram-negative bacteria, is a well-known and important proinflammatory factor that can cause endotoxemia, shock, and finally multiple organ dysfunction syndromes (Sachithanandan et al. 2011). Macrophages play an important role in regulating inflammatory responses by generating proinflammatory mediators. In macrophages, stimulation with LPS can induce the expression of proinflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and chemokines (Lee et al. 2012). In addition, activated macrophages may themselves secrete pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Karpurapu et al. 2011). It is well known that the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways play key roles in

controlling proinflammatory mediators and cytokines. NF-κB is arrested in the cytoplasm by inhibitor kappa B (IκB) in normal conditioned cells. Upon activation by various stimuli, including TNF-α and LPS, the IκB protein is phosphorylated and degraded resulting to free NF-κB, which than translocates to the nucleus (Klemm and Ruland 2006). The translocation of NF-κB into the nucleus triggers the expression of pro-inflammatory genes, such as iNOS and COX-2 (Paul et al. 2006). In addition, activation of the MAPK pathway also plays an essential role in the initiation and development of inflammatory processes that are transmitted by sequential phosphorylation events. There are three major groups of MAPK cascades, extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. Each MAPK is activated by its upstream activation of MAPK kinase (MKK) and MAPK kinase kinase (MKKK). Once activated by kinases, MAPK can phosphorylate transcription factors or other downstream kinases that produce the expression of pro-inflammatory mediators of extracellular stimuli (Kwon et al. 2002; Christman et al. 1998).

In recent years, a number of bioactive compounds have been isolated from marine organisms such as sea urchins, algae, sponges, coelenterates, tunicates, echinoderms, bryozoans, sea slugs, and mollusks (Donia and Hamann 2003; Haefner 2003). In the course of our ongoing search for marine bioactive compounds from cold-water habitats, we directed our attention to the sea urchin collected from the Sea of Okhotsk, resulting in the isolation of a new sulfonic acid derivative, (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**). Here, we report the isolation and structure determination of compound **1** and its anti-inflammatory effects in the LPS-induced inflammatory response in RAW 264.7 macrophages.

Materials and methods

General experimental procedures and materials

HRESIMS data were obtained using a hybrid Q-TOF mass spectrometer (SYNAPT G2, Waters, MS Technologies, Manchester, UK) located at the Korea Basic Science Institute, Chungbuk, Korea. Optical rotations were recorded using a JASCO P-2000 digital polarimeter. NMR spectra (1D and 2D) were recorded in CD_3OD with a JEOL JNM ECP-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C), and the chemical shifts were referenced relative to the residual solvent peaks (δ_H/δ_C 3.30/49.0). HSQC and HMBC experiments were optimized for $^1J_{CH} = 140$ Hz and $^nJ_{CH} = 8$ Hz, respectively. The solvents for the extraction and flash column chromatography were reagent grade without further purification, while the solvents used for HPLC were analytical grade. Flash column chromatography was performed using YMC octadecyl-functionalized silica gel (C_{18}). HPLC (YOUNGLIN-YL9100) separations were performed using a Shiseido Capcell Pak® C_{18} column (20 × 150 mM, 5-μm particle size) with a flow rate of 5 mL/min. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise. Primary antibodies, including those against COX-2, iNOS, IκB-α, p-IκB-α, p50, p65, p-ERK, ERK, p-JNK, JNK, p-p38, and p38, as well as the appropriate secondary antibodies used for western blotting analysis, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enzyme-linked immune sorbent assay (ELISA) kits for PGE_2, TNF-α, and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

Extraction and isolation

The sea urchin *Brisaster latifrons* (09C-ST8-DR2), identified by Dr. Joung Han Yim, was collected at the Sea of Okhotsk (N 53° 27.179′ E 144° 27.382′) on January 28, 2009. The frozen sample (108 g) was extracted with 100 % MeOH (2 × 500 mL). After solvent removal, the MeOH extract (3.34 g) was subjected to C_{18}-functionalized silica gel flash column chromatography and eluted with a stepwise gradient of 20, 40, 60, 80, and 100 % (v/v) of MeOH in H_2O (500 mL each). The fraction (17.4 mg) eluted with 60 % MeOH was further purified by using semi-preparative reversed phase HPLC (SHISEIDO, 20 × 150 mM, 5 μm, 5 mL/min), with a gradient from 40 % to 80 % MeOH in H_2O over 40 min to afford compound **1** (8.5 mg; $t_R = 20$ min).

*(Z)-4-Methylundeca-1,9-diene-6-sulfonic acid (**1**)*

Yellow oil (MeOH); $[\alpha]_D^{21} = -1.3°$ (*c* 0.61, MeOH); ^1H, ^{13}C NMR, and HMBC data, Table 1; HRESIMS *m/z* 245.1204 $[M–H]^-$ (calcd for $C_{12}H_{21}O_3S$, 245.1211).

Cell culture and viability assay

RAW264.7 cells were maintained at 37 °C under a humidified atmosphere containing 5 % CO_2 and 95 % air. The suspension consisted of RAW264.7 cells (5×10^5 cells/mL) in DMEM medium, supplemented with heat-inactivated FBS (10 %), penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM). The effects of various experimental conditions on cell viability were evaluated by determining mitochondrial reductase function with an

Table 1 NMR data for (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**) in CD_3OD

Position	^{13}C	^{1}H (mult. *J* in Hz)	HMBC (H→C#)
1	116.3	4.97 br d (10.0)	1, 3
		5.00 br d (17.1)	
2	138.4	5.81 ddt (17.1, 10.0, 7.1)	1, 3, 4
3	42.2	1.88 m	1, 2, 4, 5, 12
		2.16 m	
4	31.8	1.90 m	3, 5, 12
5	39.0	1.32 m	3, 4, 6, 12
		1.96 m	
6	58.6	2.73 m	5, 8, 7
7	32.1	1.55 m	5, 6, 8, 9
		2.02 m	
8	25.5	2.22 m	6, 7, 9, 10
		2.31 m	
9	131.2	5.39 m	7, 8, 11
10	125.3	5.46 br dq (10.6, 6.6)	8, 11
11	13.0	1.61 d (6.6)	9, 10
12	20.1	0.91 d (6.0)	3, 4, 5

^{13}C Recorded at 100 MHz. ^{1}H Recorded at 400 MHz

assay based on the reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), to formazan crystals. The formation of formazan from MTT is proportional to the number of functional mitochondria in the living cells. For the determination of cell viability, 50 μL of MTT (2.5 mg/mL) was added to cell suspension (1×10^5 cells/mL in each well of the 96-well plates) at a final concentration of 0.5 mg/mL, and the mixture was further incubated for 3–4 h at 37 °C. The cells were then dissolved in acidic 2-propanol and the optical density measured at 590 nm to detect formazan. The optical density of the control (untreated) cells was considered an indication of 100 % viability.

Measurement of NO production

Production of nitrite, a stable end-product of NO oxidation, was used to measure iNOS activity. The nitrite in conditioned media was determined by a method based on Griess reaction. An aliquot of each supernatant (100 μL) was mixed with an equal volume of Griess reagent [0.1 % (w/v) *N*-(1-naphthyl)-ethylenediamine and 1 % (w/v) sulfanilamide in 5 % (v/v) phosphoric acid] for 10 min at room temperature. The absorbance of the final product was measured spectrophotometrically at 525 nm with an ELISA plate reader, and the nitrite concentration of the sample was determined using a standard curve of sodium nitrite in phenolred-free DMEM.

PGE_2, TNF-α, and IL-1β assays

Levels of PGE_2, TNF-α, or IL-1β, as appropriate, were determined using a commercially available kit from R&D Systems (Abingdon, UK). The assay was performed according to the manufacturer's instructions. Briefly, RAW264.7 cells were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of compound **1**, and then stimulated for 18 h with LPS. The culture medium was collected immediately after treatment and spun at 13,000 g for 2 min to remove particulate matter. The medium was then added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for PGE_2 and mouse TNF-α or IL-1β. An enzyme-linked polyclonal antibody specific for PGE_2, mouse TNF-α, or IL-1β was added to the wells, incubated for 20 h, followed by a final wash to remove any unbound antibody. A substrate solution was added, and the absorbance was measured at 450 nm (with the correction wavelength set at 540 or 570 nm). This absorbance was proportional to the amount of PGE_2, TNF-α, or IL-1β present.

Preparation of cytosolic and nuclear fractions

The cells (1×10^7 cells/mL in 60-mM dishes) were collected and washed with phosphate-buffered saline (PBS) and suspended in 200 μL of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail]. After the cells were allowed to swell on ice for 15 min, an aliquot of 12.5 μL of 10 % NP-40 was added. The tubes were agitated on a vortex for 10 s and then centrifuged for 5 min. The resulting supernatant represented the cytosolic extract. The nuclear pellets were re-suspended in 50 μL of ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and a protease inhibitor cocktail] and incubated on ice for 1 h with intermittent vortexing. This nuclear extract was centrifuged for 10 min at $15{,}000 \times g$; the resulting supernatant represented the nuclear fraction. After centrifugation at $15{,}000 \times g$ for 10 min, the supernatant was decanted and stored at −70 °C until further use. The protein content was determined using a bicinchoninic acid protein assay kit.

Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors (0.1 mM PMSF, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). The protein concentration was determined using a Lowry protein assay kit (P5626, Sigma). An equal amount of protein for each sample

was separated by 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5 % skim milk and sequentially incubated with primary antibodies (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibodies, followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

DNA-binding activity of NF-κB

Cells were pre-treated for 12 h with the indicated concentrations of compound **1** and then stimulated for 1 h with LPS (1 μg/mL). The DNA-binding activity of NF-κB in nuclear extracts was measured using the Trans AM kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, 30 μL of complete binding buffer (DTT, herring sperm DNA, and binding buffer AM3) and 20 μL of the sample, which consisted of 20 μg of nuclear extract in complete lysis buffer, were added to each well. The mixture was incubated for 1 h at room temperature with mild agitation (100 rpm on a rocking platform). After each well was washed with the washing buffer, 100 μL of diluted NF-κB antibody (1:1,000 in 1 × antibody binding buffer) was added, followed by incubation for 1 h on the rocking platform. After the wells were washed with the buffer, 100 μL of diluted HRP-conjugated antibody (1:1,000 in 1 × antibody binding buffer) was added to each and incubated for 1 h on the rocking platform. Developing solution was added to each well and allowed to react for 5 min, and then the wells were washed to remove the supernatant. Within 5 min, absorbance at 450 nm was recorded using a spectrophotometer.

Statistical analysis

The data are expressed as mean ± S.D. values for at least three independent experiments. One-way analysis of variance and Newman–Keuls post hoc test was used to compare three or more groups. The statistical analysis was performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc, San Diego, CA, USA).

Results

Isolation of (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**) and its cytotoxic effects on RAW264.7 macrophage cells

The compound **1** was isolated from the 100 % MeOH extract of the sea urchins collected from the Sea of Okhotsk by several fractionation and purification steps. The molecular formula of **1** was assigned to be $C_{12}H_{22}O_3S$ on the basis of HRESIMS data (*m/z* 245.1204 $[M–H]^-$), which was fully supported by the ^1H and ^{13}C NMR data (Table 1). The ^1H, ^{13}C, and DEPT NMR spectra in CD_3OD showed the presence of two methyl groups, one olefinic methylene group, four sp^3 methylene groups, three sp^2 methines, and two sp^3 methine carbons. A detailed analysis of COSY, HSQC, and HMBC data revealed the entire carbon framework in the molecule accounting for 12 carbon and 21 hydrogen atoms. Therefore, it was suggested that the compound possesses sulfonic acid functional group to account for the molecular formula. The attachment of sulfonic acid group at C-6 was supported by chemical shift comparison with those in the previously reported compound, (*Z*)-8-methylundec-2-ene-6-sulfonic acid (Kita et al. 2002), to provide the planar structure of **1** as shown. The geometry of the double bond at C9-C10 was assigned to be *cis* based on the coupling constant (10.6 Hz), and this was supported by chemical shifts comparisons with those in the related compounds, hedathiosulfonic acids A and B. Hedathiosulfonic acids A and B were isolated from sea urchin, and the structures were assigned as (6*R*,8*S*,*Z*)-8-methylundec-2-ene-6-sulfonothioic acid and (4*S*,6*R*,*Z*)-4-methylundeca-1,9-diene-6-sulfonothioic acid, respectively (Kita et al. 2002). Therefore, the planar structure of **1** was determined to be (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (Fig. 1a). However, the absolute configurations at C-4 and C-6 were not determined.

We determined the cytotoxic potential of compound **1** by measuring its effect on the viability of the RAW264.7 macrophages, following with the MTT assay. As shown in Fig. 1b, cell viability was not significantly altered up to 250 μM of **1**. Therefore, for all subsequent experiments, the concentration range of compound **1** was maintained between 25 and 200 μM.

Effects of compound **1** on the production of pro-inflammatory mediators and cytokines in RAW264.7 macrophages stimulated with LPS

Macrophage activation by immunological stimuli plays a pivotal role in the inflammatory condition through the overproduction of inflammatory mediators and cytokines, including NO, PGE_2, TNF-α, IL-1β, IL-6, IL-12, and interferon-γ (Fujiwara and Kobayashi 2005). In addition, increased production of the inflammatory mediators and cytokines may lead to severe tissue damage and multiple organ failure (Kao et al. 2007). Therefore, to investigate the effects of compound **1** on the productions of NO, PGE_2, TNF-α, and IL-1β in LPS-stimulated macrophages, the cells were stimulated with LPS (1 μg/mL) for 18 h in the presence or absence of non-cytotoxic concentrations of

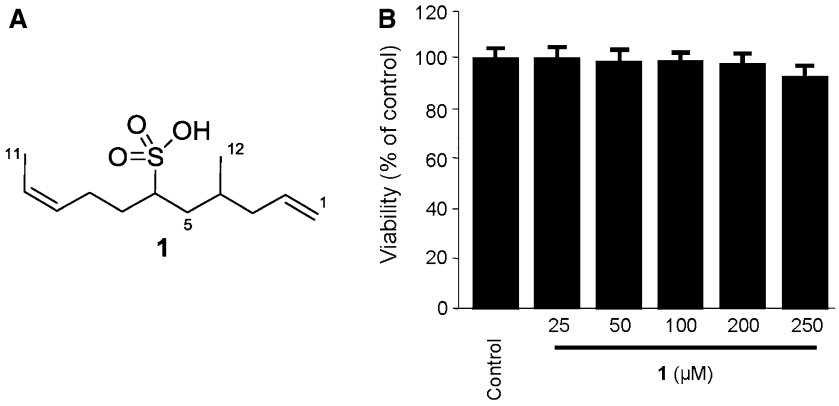

Fig. 1 Chemical structure of compound **1** and its effects on the viability of RAW264.7 macrophage cells. RAW264.7 macrophages were incubated for 24 h with various concentrations of compound **1** (25–250 μM). Cell viability was determined as described under Materials and methods. The data represent the mean values of three experiments ± SD

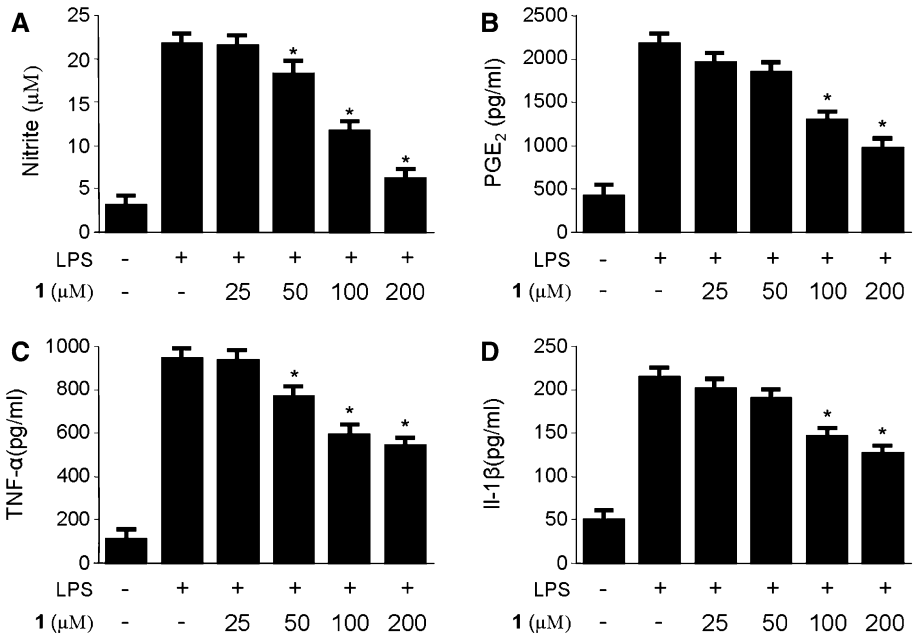

Fig. 2 The effects of compound **1** on nitrite (**a**), PGE_2 (**b**), TNF-α (**c**), and IL-1β (**d**) production in RAW264.7 macrophages stimulated with LPS. The cells were pre-treated for 12 h with compound **1** and stimulated for 18 h with LPS (1 μg/mL). The concentrations of nitrite (**a**), PGE_2 (**b**), TNF-α (**c**), and IL-1β (**d**) were determined as described under Materials and methods. The data represent the mean ± SD values of three experiments. *$p < 0.05$ compared to the group treated with LPS alone

compound **1**. When RAW264.7 macrophages were pre-treated with compound **1** for 12 h and subsequently stimulated with LPS, compound **1** was shown to decrease the production of NO, PGE_2, TNF-α, and IL-1β (Fig. 2) in a dose-dependent manner, as determined by enzyme immunoassays.

Effects of compound **1** on the expression of iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophages

The expression of pro-inflammatory enzymes, including COX-2 and iNOS, plays an important role in immune-activated macrophages via the production of iNOS-derived NO, COX-2-derived PGE_2, and cytokines, including TNF-α and IL-1β (Fujiwara and Kobayashi 2005). Our next finding suggests that compound **1** suppresses LPS-induced expression of pro-inflammatory enzymes such as iNOS and COX-2 in LPS-stimulated macrophages. RAW264.7 macrophages were stimulated with LPS (1 μg/mL) for 18 h in the presence or absence of non-cytotoxic concentrations of compound **1**. The LPS treatment significantly increased the expression of iNOS and COX-2, but pre-treatment of RAW264.7 with compound **1** for 12 h resulted in decrease of iNOS and COX-2 expression (Fig. 3).

Effects of compound **1** on IκB-α levels, NF-κB nuclear translocation, and DNA-binding activity of NF-κB in LPS-stimulated RAW264.7 macrophages

The expression of pro-inflammatory mediators and enzymes is attributed to upregulated NF-κB, the activation of which induces the transcription of inflammatory cytokines, as well as iNOS and COX-2 genes (Cogswell et al. 1994). Therefore, the phosphorylation and degradation of IκB-α, an inhibitor of NF-κB nuclear translocation, were evaluated to determine the mechanisms by which compound **1** suppressed the production of LPS-induced pro-

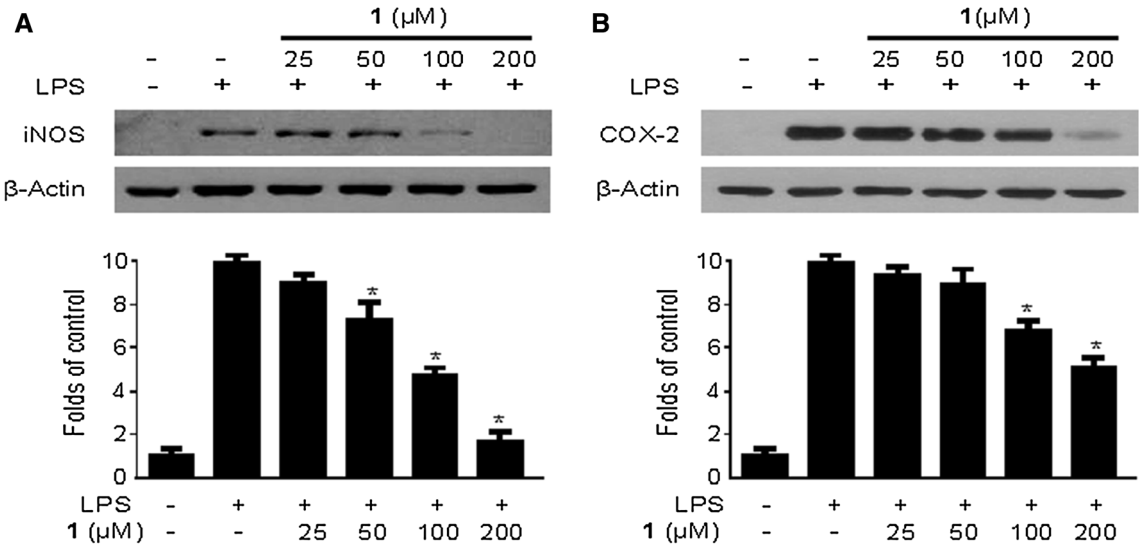

Fig. 3 The effects of compound **1** on iNOS (**a**) and COX-2 (**b**) protein expression in RAW264.7 macrophages stimulated with LPS. The cells were pre-treated for 12 h with compound **1** and challenged for 18 h with LPS (1 μg/mL). Western blot analyses (**a**, **b**) were performed as described under Materials and methods, and representative blots of three independent experiments are shown. The data represent the mean ± SD values of three experiments. $*p < 0.05$ compared to the group treated with LPS alone

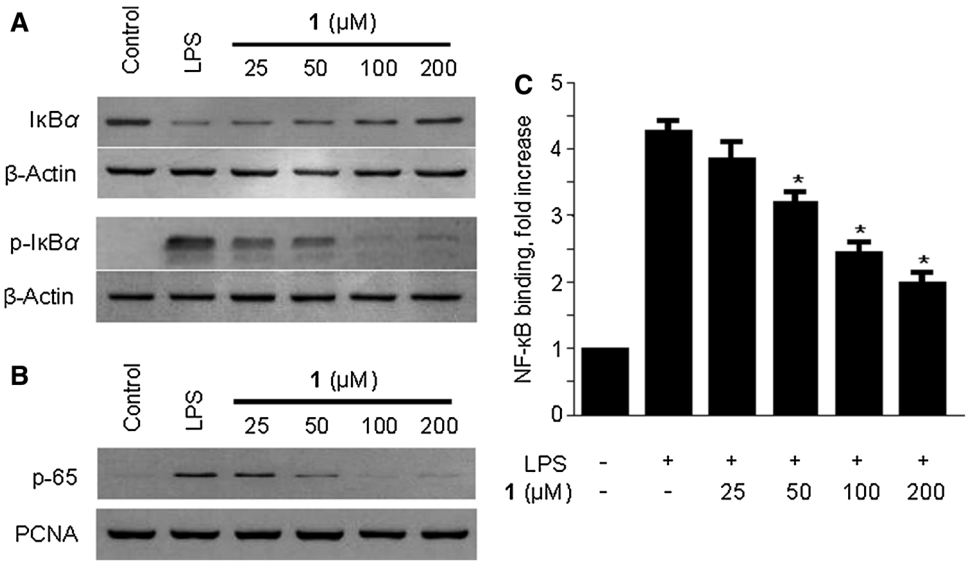

Fig. 4 The effects of compound **1** on the protein expression level of IκB-α phosphorylation, degradation of IκB-α (**a**), NF-κB activation (**b**), and DNA-binding activity of NF-κB (**c**) in RAW264.7 macrophages stimulated with LPS. The cells were pre-treated for 12 h with compound **1** and stimulated for 1 h with LPS (1 μg/mL). The western blot analysis of IκB-α and p-IκB-α in the cytoplasm and NF-κB in the nucleus (**a**, **b**) was performed as described under Materials and methods. A commercially available NF-κB ELISA (Active Motif) was then used to test the nuclear extracts and determine the degree of NF-κB binding (**c**). The data represent the mean ± SD values of three experiments. $*p < 0.05$ compared to the group treated with LPS alone

inflammatory enzymes and mediators. As shown in Fig. 4, IκB-α was degraded after RAW264.7 macrophages were exposed to LPS for 1 h. However, pretreatment with compound **1** markedly inhibited the LPS-induced phosphorylation and degradation in a dose-dependent manner, thereby preventing p65 translocation to the nucleus (Fig. 4a, b). We also investigated the DNA-binding activity of NF-κB in nuclear extracts from RAW264.7 macrophages stimulated with LPS for 1 h. Treatment with LPS increased the DNA-binding activity of NF-κB by approximately threefold. However, compound **1** impaired this activity in a dose-dependent manner (Fig. 4c).

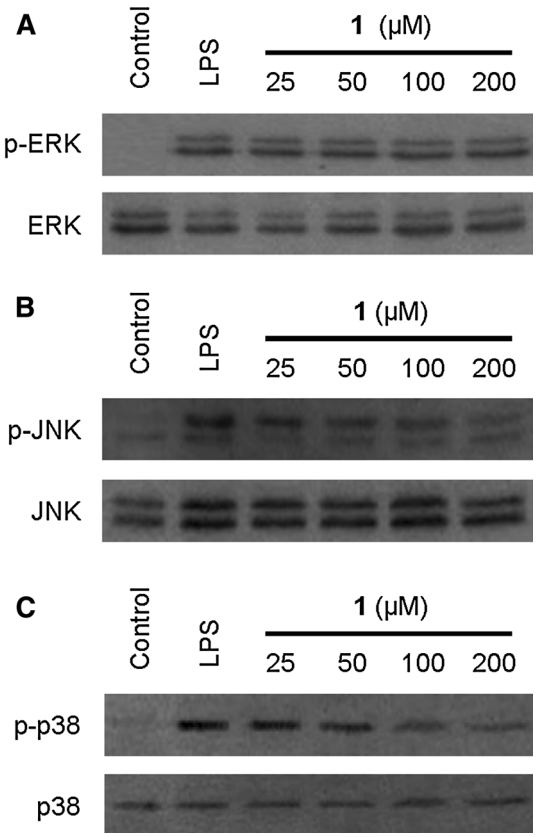

Fig. 5 The inhibitory effect of compound **1** on the protein levels of ERK, JNK, and p38 in RAW264.7 macrophages stimulated with LPS. The cells were pre-treated for 12 h with compound **1** and stimulated for 1 h with LPS (1 μg/mL). The levels of p-ERK (phosphorylated-ERK), ERK (**a**), p-JNK (phosphorylated-JNK), JNK (**b**), p-p38 (phosphorylated-p-38), and p38 (**c**) were determined by western blot analysis. The western blot analyses were performed as described under Materials and methods; representative blots of three independent experiments are shown

Effects of compound **1** on the phosphorylation of MAPKs in LPS-stimulated RAW264.7 macrophages

MAPK is a crucial regulator of inflammatory mediators, including NO and pro-inflammatory cytokines (Feng et al. 2002). It also plays critical roles in cell growth and differentiation, and it participates in the control of cellular responses to stress and NF-κB activity in macrophages (Surh et al. 2001). In order to determine whether the suppression of inflammatory reactions by compound **1** was mediated through a MAPK pathway, we assessed the effects of compound **1** on the LPS-induced phosphorylation of ERK, JNK, and p38 MAPKs in LPS-stimulated RAW264.7 macrophages. As shown in Fig. 5, the phosphorylation of ERK, JNK, and p38 decreased after treatment with LPS (1 h) in RAW264.7 macrophages. However, 3 h of pre-treatment with compound **1,** at 25–100 μM, significantly inhibited the LPS-induced phosphorylation of JNK and p38, in a dose-dependent manner (Fig. 5b, c), but not that of ERK (Fig. 5a). On the other hand, the expression levels of ERK, JNK, and p38 MAPKs were unaffected by LPS or compound **1**.

Discussion

The ocean is the primary source of life. As life has evolved, marine organisms provided a steady stream of very unusual new structures with biological activity. Recently many studies began to produce powerful drugs to treat human diseases and used biotechnology to manufacture new products by marine organisms (Swathi et al. 2012). It has been suggested that marine organisms from cold-water habitats have evolved with unique chemistry under physical conditions different from those of terrestrial plants and animals (Moon et al. 2000). In the course of chemical investigation on marine organisms from the Sea of Okhotsk, the MeOH extract of sea urchin *Brisaster latifrons* was selected for further study to yield a new sulfonic derivative named (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**). Two closely related metabolites hedathiosulfonic acids A and B have been isolated from sea urchin *Echinocardium cordatum*, and the compounds were shown to exhibit low acute toxicity against mice (Kita et al. 2002). However, anti-inflammatory effects of this type of metabolites have not been previously reported. Therefore, we attempted to unravel the mechanisms of the anti-inflammatory effects of (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**).

The inflammation response is a complex reaction of the immune system regulated by many inflammatory mediators, such as NO, prostaglandins, and cytokines. An overproduction of iNOS-derived NO and COX-2-derived PGE_2 can have cytotoxic effects in pathological processes, especially in inflammatory and autoimmune disorders (Palmer et al. 1988); these mediators play a regulatory role in a variety of physiological and pathological processes following an immune response and inflammation (Griswold and Adams 1996). Therefore, the inhibition and control of inflammatory mediators are useful targets for research on new anti-inflammatory agents. In the present study, we showed that the pre-treatment of LPS-stimulated RAW264.7 macrophages with compound **1** (25–200 μM) inhibited COX-2 and iNOS expression, thereby suppressing COX-2-derived PGE_2 and iNOS-derived NO production. Moreover, compound **1** suppressed LPS-induced TNF-α and IL-1β production in RAW264.7 macrophages. These results suggest that compound **1** exerts its anti-inflammatory effects via the suppression of inflammatory enzymes and mediators such as iNOS, COX-2, NO, PGE_2, TNF-α, and IL-1β.

The key anti-inflammatory targets include enzymes, cytokines, and transcription factors, such as NF-κB and MAPKs (Gautam and Jachak 2009). Several studies have demonstrated that NF-κB has a significant role in regulating the expression of inflammation-associated enzymes and cytokine genes that contain NF-κB binding motifs within their respective promoters (Thanos and Maniatis 1995). The transcription factor NF-κB is implicated in the regulation of many genes that code for mediators of immune, acute-phase, and inflammatory responses, including iNOS and COX-2 (Surh et al. 2001; Lowenstein et al. 1993). The p50/p65 heterodimer is the most common dimer found in the NF-κB signaling pathway (Verma et al. 1995). In basal conditions, NF-κB is sequestered in the cytoplasm by inhibitor proteins, usually IκB, but when released, NF-κB dimers can translocate to the nucleus to activate target genes by binding with high affinity to κB elements in their promoters. We examined the effects of compound **1** on IκB-α phosphorylation and NF-κB (p65) nuclear translocation in RAW264.7 macrophages. As shown in Fig. 4, the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 induced by LPS were significantly reduced after pre-treatment with compound **1**. In addition, the enhanced DNA-binding activity of NF-κB was also suppressed in RAW264.7 macrophages after treatment with compound **1**. These results suggested that compound **1** regulated inflammatory reactions through the inhibition of NF-κB signaling.

The MAPKs perform critical roles in controlling cellular responses to stress and activating NF-κB (Carter et al. 1999; Nakano et al. 1998). NF-κB is activated by the phosphorylation of IκB via activation of the MAPKs, as well as NF-κB-inducing kinase (NIK) and IKK. The MAPKs are a family of protein serine/threonine kinases, including ERK, JNK, and p38 MAPK. The MAPKs react to extracellular stimuli and control a variety of cellular activities, including gene expression, differentiation, apoptosis, and inflammation. MAPKs regulate inflammatory and immune responses (Ajizian et al. 1999), and the MAPK signaling pathways are known to be involved in expressing iNOS and COX-2, as well as producing pro-inflammatory cytokines in LPS-induced macrophages (Uto et al. 2005). The inhibition of any one of the ERK, JNK, and p38 MAPK pathways is sufficient to block the induction of pro-inflammatory mediators by LPS (Yoon et al. 2010). The ERK pathway plays a key role in transducing chronic inflammatory articular pain (Cruz et al. 2005). Since ERK is known to be involved in the regulation of IL-6, IL-12, IL-23, and TNF-α synthesis (Goodridge et al. 2003), these results suggest a possible involvement of ERK in joint damage associated with pro-inflammatory cytokine production by macrophages. The p38 MAPK is generally considered as the most promising MAPK therapeutic target for inflammatory disease, because p38 MAPK isoforms have been implicated in the regulation of many of the inflammatory processes, such as the production of cytokines and pro-inflammatory mediators (Korb et al. 2006). JNK is a member of the MAPK family and is activated by environmental stress and some pro-inflammatory cytokines; it also has an important role in immune system signaling (Dong et al. 2002). To investigate whether the suppression of inflammatory reactions by compound **1** was mediated through the MAPK pathway, we assessed the effect of compound **1** on the LPS-induced phosphorylation of ERK, JNK, and p38 in RAW264.7 macrophages. Compound **1** significantly suppressed the LPS-induced phosphorylation of JNK and p38 in a dose-dependent manner, but it did not suppress the phosphorylation of ERK (Fig. 5).

In conclusion, a new secondary metabolite, (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**) was isolated from the organic extract of the sea urchin collected from the Sea of Okhotsk, and we demonstrated its anti-inflammatory effects in RAW264.7 macrophages, which were mediated through the inhibition of NF-κB and JNK/p38 MAPK signaling pathways. These finding suggested that (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**) may be a potential therapeutic agent for the treatment of inflammatory disease. Future studies are expected to confirm the anti-inflammatory effects of (*Z*)-4-methylundeca-1,9-diene-6-sulfonic acid (**1**) in a representative anti-inflammatory animal model.

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