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INHIBITION OF NF-KAPPA B ACTIVATION BY PENICILLIC ACID AND DIHYDROPENICILLIC ACID ISOLATED FROM FUNGI

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Abstract – In the course of our screening of NF-κB inhibitors from microbial secondary metabolites, we isolated two 2(5*H*)-furanones, penicillic acid and dihydropenicillic acid, from the culture filtrate of *Penicillium*. Both penicillic acids inhibited LPS-induced NO production and NF-κB activation. They did not inhibit protein synthesis. Interestingly, penicillic acid inhibited advanced glycation end product (AGE)-induced NO production.

INTRODUCTION

NF-κB is the transcription factor that promotes expressions of cytokines including interleukin 1 (IL-1), IL-2, IL-6, IL-8, IL-10, IL-12, and TNF-α; cell adhesion molecules including E-selectin, ICAM-1, and VCAM-1; and anti-apoptotic proteins including IAPs, FLAP, survivin, and Bcl-XL. In inflammatory sites NF-κB is often activated in inflammatory cells such as macrophages and mast cells. NF-κB is usually located in the cytoplasm as an inactive form. Stimulants such as TNF-α and lipopolysaccaride (LPS) induce nuclear translocation and activation of NF-κB. Activation of NF-κB is involved in the etiology of various inflammatory diseases such as rheumatoid arthritis, retinal inflammation, cachexia, and also in cancer and leukemia. In one hand, protein glycation may cause complications in diabetes mellitus. Advanced glycation end products were suggested to activate NF-κB and NO production.[1] NO is produced in inflammation by inducible NO synthase (iNOS) that is up-regulated by NF-κB.

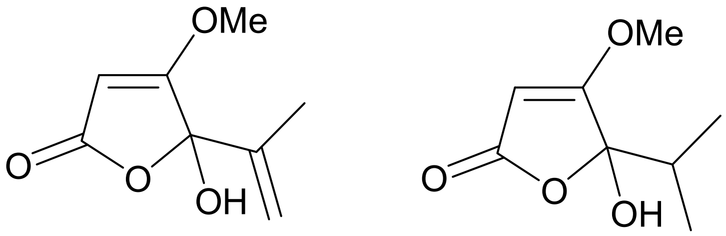
We previously designed and synthesized a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ).[2,3] It showed potent anti-inflammatory and anticancer activities in animals.[4] We also isolated a piperidine compound, 9-methylstreptimidone, from the culture filtrate of *Streptomyces* as an inhibitor of NF-κB.[5] However, simpler and more effective inhibitors of NF-κB may be further expected. Therefore, we looked for NF-κB inhibitors among microbial culture filtrates. As a result, we isolated 2 known compounds,

compounds, penicillic acid and dihydropenicillic acid from the culture filtrate of a fungus *Penicillium*.

RESULTS AND DISCUSSION

Screening system for NF-κB inhibitors. To obtain selective NF-κB inhibitors, we employed mouse macrophage-like cell line RAW264.7 cells that produce nitrogen monoxide (NO) when they were stimulated with lipopolysaccharide (LPS). LPS-induced NO production is mediated by NF-κB activation.[6] Firstly we tested the inhibitory activity of the culture filtrate samples on LPS-induced NO production. The toxicity was also evaluated by MTT assay. Secondly the samples were evaluated by electrophoresis mobility shift assay for NF-κB. To exclude protein synthesis inhibitors, the samples were also tested whether they inhibit leucine incorporation.

Purification and structure determination of penicillic acid. During the course of our screening for microbial secondary metabolites possessing NF-κB inhibitory activity, we isolated penicillic acid from the culture filtrate of a fungus. The producing organism was identified as *Penicillium* by the morphological characteristics. The culture filtrate (1L) provided by Meiji Seika Co. Ltd. was mixed with 1L of ethyl acetate and the ethyl acetate phase was obtained (× 2). The active ethyl acetate elutes were concentrated to dryness under reduced pressure to a brown residue (342 mg), which was subjected to preparative TLC with silica gel and $CHCl_3$-MeOH (5:1) as the developing solvent. The active fraction (54 mg) was further purified with preparative TLC with silica gel and hexane-acetone (1:1). The active fraction (25 mg) was dissolved in water pH9.0, washed with EtOAc, adjusted to pH7.0, then extracted with EtOAc. The active compound was eluted in HW-40 column with MeOH to give 9.0 mg crystal of penicillic acid. In order to define the structure of the active substance, ^{1}H-NMR, ^{13}C-NMR, and mass spectrum (FAB mode) were used. By the FAB-MS (m/z) spectrum, its molecular mass was found to be 170. The ^{1}H and ^{13}C spectral data were identical to penicillic acid **1** (Figure 1). Penicillic acid was first reported as an antibiotic in 1936.[7]



1 **2**

Figure 1. Structure of penicillic acid **1** and dihydropenicillic acid **2**

Purification and structure determination of dihydropenicillic acid. During the course of our further screening, we isolated dihydropenicillic acid from the culture filtrate from the fungus. The producing organism was a again identified as *Penicillium*. The culture filtrate (2 L) again provided by Meiji Seika Ltd. was mixed with 2L of ethyl acetate and the ethyl acetate phase was obtained (× 2). The active ethyl acetate elutes were concentrated to dryness under reduced pressure to a black residue (14.6 g), which was subjected to preparative TLC with silica gel and CH_3Cl-MeOH (200:1～MeOH) as the developing The active fraction (2.90 g) was further purified with preparative TLC with silica gel and hexane-acetone (3:1～3:2). Furthermore, the active fraction (1.71 g) was purified with preparative TLC with silica gel toluene-acetone (10:1～acetone). Then, the active fraction (0.704 g) was left at rest in hexane-CH_3Cl for 2 weeks, and the crystal was collected. In order to define the structure of this crystal, ^1H-NMR and ^{13}C-NMR were used. The ^1H and ^{13}C spectral data were identical to dihydropenicillic acid **2** (Figure 1). Dihydropenicillic acid was first reported as antibiotic in 1994.[8] Both penicillic acid and dihydropenicilic acid easily tautomerize forming the ring cleaved carboxylic acids (Please see the attached fugure). Therefore, there is no description on the optical rotation of these compounds in the previous literature. then could not provide the OD values.

Penicillic acid and dihydropenicillic acid inhibit LPS-induced NO production and iNOS expression. We assessed the effect of penicillic acid and dihydropenicillic acid on the production of NO in LPS-stimulated cells. LPS increased NO production over the basal level at 20 h. When RAW264.7 cells were pretreated with these 2 compounds for 2 h prior to LPS stimulation, the NO content in the conditioned media was decreased (Figure 2A). Both chemicals showed similar inhibitory activities without toxicity. The effect was comparable to that of (-)-DHMEQ. NO is produced by inducible NO synthase (iNOS) L-arginine. Treatment with these 2 compounds also led to a significant decrease of iNOS protein (Figure 2B) in a dose-dependent manner. As shown in Figure 2A, these compounds did not decrease the cell viability in terms of MTT assay.

Penicillic acid and dihydropenicillic acid inhibit LPS-induced NF-κB activation. The EMSA results indicated that LPS activated NF-κB in RAW264.7 cells in 30 min and that this activation was inhibited by penicillic acid and dihydropenicillic acid (Figure 3A). Penicillic acid was slightly stronger than dihydropenicillic acid to inhibit NF-κB. As shown in Figure 3B, both compounds inhibited the NF-κB-dependent IκB-α reactivation. We found that penicillic acid and dihydropenicillic aid inhibited the p65/NF-κB DNA binding *in vitro* (Figure 3C). In one hand, they did not inhibit the protein synthesis in terms of leucine incorporation at the concentration to inhibit NF-κB (data not shown). Thus, penicillic acid and dihydropenicillic acid were found to be inhibitors of NF-κB that inhibit the DNA binding.

Penicillic acid inhibits AGE-induced NO production in RAW264.7 cells. Commercially available AGE-BSA induced NO production in RAW264.7 cells at 500 μg/ml (Figure 4A). Addition of penicillic acid reduced the effect of AGE-BSA as shown in Figure 4B. Using biotinylated AGE-BSA, the binding between AGE-BSA and its receptor on the RAW264.7 cell surface was not influenced by penicillic acid even at 30 μg/ml (data not shown). Therefore, penicillic acid should inhibit the intracellular signaling.

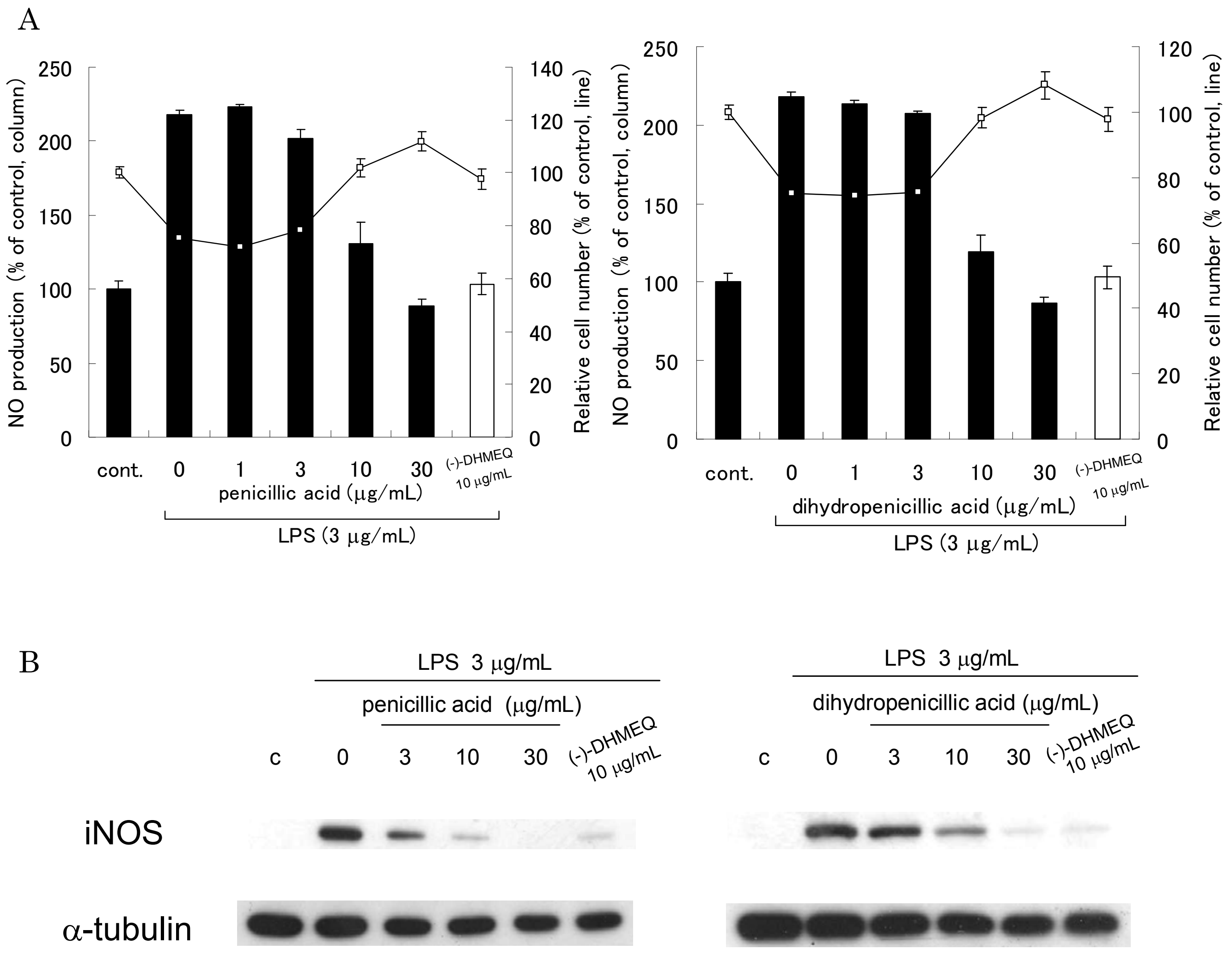


Figure 2. Inhibitions of NO production and iNOS expression by penicillic acid and dihydropenicillic acid. (A) Effect of NO production. RAW264.7 cells in 96-wells plate were pretreated with each chemical at the indicated concentrations for 2 h, then stimulated with LPS (3 μg/mL) for 20 h. The nitrite contents in the conditioned media were determined by the Griess method. The cell viability was also assessed by the MTT method. All data are mean ± S.D. of three independent determinations. (B) Effect of iNOS expression. The expression of iNOS protein in total cell extracts was analyzed by Western blotting.

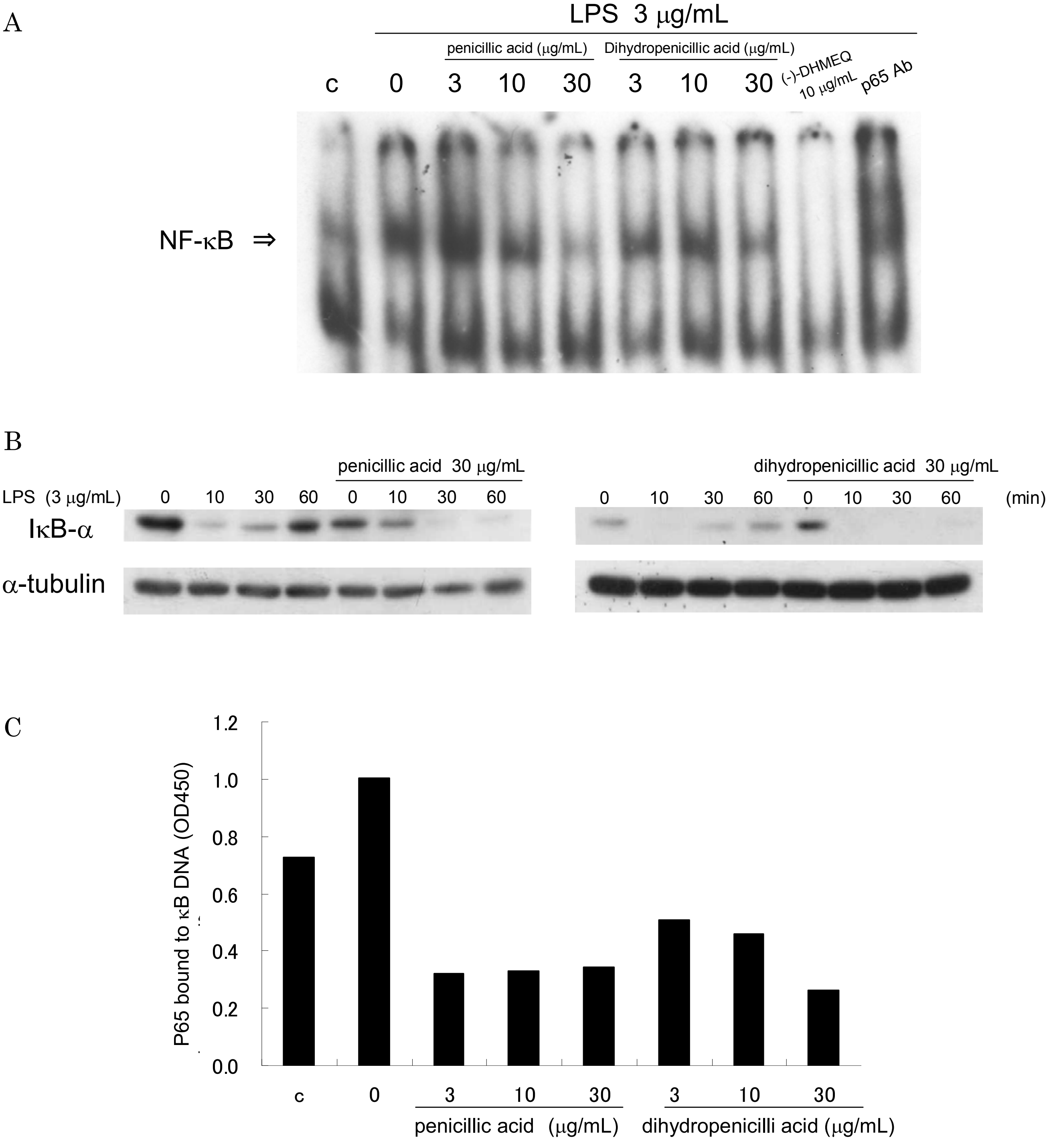


Figure 3. Inhibitions of LPS-induced NF-κB activation by penicillic acid and dihydropenicillic acid in RAW264.7 cells. (A) Electrophoresis mobility shift assay. RAW264.7 cells were pretreated with each chemical at the indicated concentrations for 2 h, then stimulated with LPS (3 μg/mL) for 30 min, and extracted for nuclear proteins. Nuclear proteins were mixed with a ^{32}P-labeled NF-κB probe. (B) IκB-α reactivation. The amount of IκB-α protein in total cell extracts was analyzed by Western blotting. (C) Inhibition of NF-κB binding to DNA *in vitro*. The chemicals were added to the nuclear extract after the preparation of nuclear extracts. NF-κB (p65) that bound to DNA was measured by ELISA. The data are the representative of 3 independent experiments.

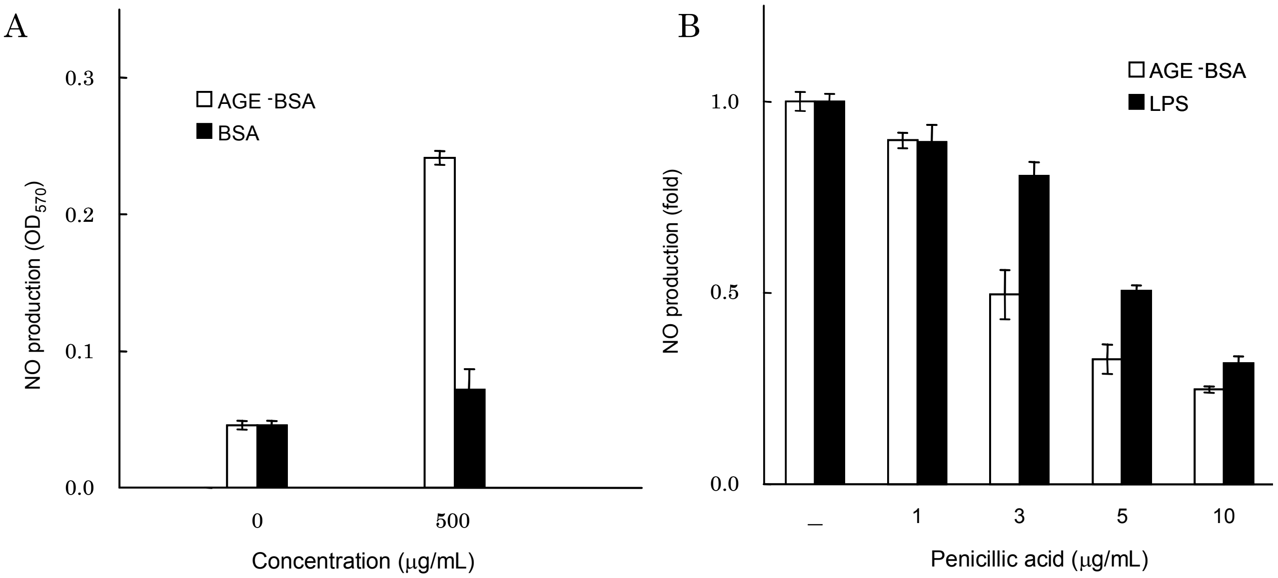


Figure 4. Inhibition of AGE-induced NO production by penicillic acid in RAW264.7 cells. (A) Induction of NO production by AGE-BSA. RAW264.7 cells in 96-wells plate were pretreated with the chemical at the indicated concentrations for 2 h, then, stimulated with AGE-BSA or BSA for 18 h. The nitrite contents in the conditioned media were determined by the Griess method. All data are means ± S.D. of three independent determinations. (B) Inhibition by penicillic acid. The cells were added by penicillic acid prior to the addition of AGE-BSA. The cells were pretreated with the chemical, then, stimulated with AGE-BSA or LPS.

DISCUSSION

We isolated penicillic acid and dihydropenicillic acid from the culture filtrate of *Penicillium* as inhibitors of LPS-induced NO production. They were then shown to inhibit NF-κB. These compound were found to inhibit the NF-κB/DNA binding. Penicillic acid was first isolated in 1936 from *Penicillium verrucosum*.[7] It showed antibacterial, antifungal, and herbicidal activities. It induces DNA strand breaks,[10] and inhibits Na^+, K^+-dependent Ca^{2+} channel.[11] It also protects neural cells by inhibiting FasL-induced activation of caspase 8.[12] Dihydropenicillic acid was isolated in 1994 from a fungus as a DNA-damaging mycotoxin.[8] It was also isolated in 1996 from *Aspergillus melleus* as a nemacidal compound.[13] However, inhibition of NF-κB and NO production by these compounds have not been reported. Penicillic acid or its analogues may be useful as anti-inflammatory or anticancer agents, since another NF-κB inhibitor, DHMEQ, showed potent anti-inflammatory and anticancer activities in animal experiments.[4] Advanced glycation end product (AGE) is considered to be involved in the etiology of diabetes mellitus. Interestingly penicillic acid inhibited AGE-induced NO production.

EXPERIMENTAL

Materials. Anti-iNOS and anti-tublin antibodies were purchased from Amersham Bioscience, Tokyo. Anti-mouse-IgG-antibody derived from rabbit was also purchased from Amersham Bioscience.

AGE-BSA was purchase from Sigma-Aldrich, St. Louis, MO.

Cell culture. Mouse macrophage RAW264.7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 μg/mL kanamycin, 100 units/mL penicillin G, 600 μg/mL L-glutamine, and 2.25 g/L $NaHCO_3$.

Preparation of screening samples. Culture fluids of microorganisms were provided by Meiji Seika Co. Ltd. The culture filtrate (500 μL) was mingled with *n*-BuOH in equal proportions to extract second metabolites from the fluid. After violently shaking, this mixture was centrifuged for 5 min at 13,000 rpm. Organic solvent phase was divided and evaporated in vacuo. The extract was dissolved in 200 μL MeOH.

NO production assay.[14] Cell suspension (100 μL) of RAW264.7 cells at concentration of 50% confluence was seeded into the 96-wells plate. Then chemicals were added into the well plates. After 2 h, the cells were stimulated with LPS at 3 μg/mL and incubated for 20 h. Then 100 μL of the Griess reagent solution was added into each plate. The concentration of NO was obtained by measuring an absorbance of 570 nm.

MTT assay.[15] Cell suspension (100 μL) of RAW264.7 cells at concentration of 50% confluence was seeded into the 96-wells plate. Then the samples were added into the well plates and incubated for 20 h. Then 10 μL of the MTT (3-[4,5-dimethylthiazol-2–yl]-2,5-diphenyltetrazolium bromide) solution was added into each plate and incubated for 4 h at 37℃, 5% CO_2. Subsequently, the culture supernatant was replaced with 100 μL DMSO to dissolve formazan crystal made from succinic dehydrogenase in the mitochondria and its substrate MTT. An absorbance of 570 nm was measured with a microplate reader.

Western Blotting. The cells were lysed with lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μM Na_3VO_4, 1% Nonidet P-40, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 1 mM PMSF). Each extract (30 μg of protein) was fractionated on a polyacrylamide-SDS gel and then transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 30 min at rt for blocking in TBS buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl) containing 7.5% skim milk. After having been washed for 1 h with 0.1% Tween 20 in TBS buffer, the membrane was incubated for 1 h at rt with each antibody in TBS buffer. After 1 h wash with the TBS-Tween buffer, the membrane was incubated for 1 h at rt with anti-IgG rabbit or anti-IgG mouse antibody linked to horseradish peroxidase. Immunoreactive proteins were visualized by the ECL detection system.

Electrophoresis mobility shift assay. The cells were extracted with a buffer consisting of 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, 0.2 mM EDTA and 0.5 mM DDT. Each nuclear extract (5 μg of protein) was incubated with a binding buffer containing 1 μg poly dI・dC and ^{32}P-labeled probe for 20 min at room temperature. Then, DNA-protein complexes were separated from free DNA on 4% native polyacrylamide gel in 0.25 mM TBE buffer (225 mM Tris, 2.5 mM boric acid, 5 mM EDTA). The DNA probe used for NF-κB binding was the double-stranded oligonucleotide containing the κB site from the mouse κB light chain enhancer (5'-ATGTGAGGGGACTTTCCCAGGC-3').

Binding of NF-κB to κB DNA. Binding of NF-κB to κB DNA *in vitro* was measured with the TransAM NF-κB p65 Transcription Factor Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

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