Inhibitory Effect of Norditerpenes on LPS-Induced TNF-α Production from the Okinawan Soft Coral, *Sinularia* sp.

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An ethanol (EtOH) extract of the soft coral (*Sinularia* sp.), collected in Okinawa, demonstrated a potent inhibitory effect on lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) production by murine macrophage-like cells (RAW264.7). The activity-guided purification of the EtOH extract resulted in the isolation of two norditerpenes, norcembrenolide (1) and sinuleptolide (2). These structures were identified from the spectroscopic data. Norcembrenolide (1) and sinuleptolide (2) inhibited TNF-α production in a dose-dependent manner, and showed a more potent effect than prednisolone at the concentration of 33 μg/ml. They also exhibited an inhibitory effect on nitric oxide (NO) production not influenced by cytotoxicity.

**Key words** tumor necrosis factor-α (TNF-α); nitric oxide (NO); RAW264.7; norditerpene; norcembrenolide; sinuleptolide

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Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is normally released from the activated macrophages, mast cells, and many other cells in immunologic and inflammatory responses, and is beneficial to the host. On the other hand, unregulated TNF-α release plays important roles in chronic inflammatory diseases such as asthma, multiple sclerosis, and rheumatoid arthritis. Thus, inhibitors of TNF-α production will contribute to the treatment of many chronic inflammatory diseases.

Some natural products have been reported to be inhibitors of TNF-α production. Sesquiterpene lactones are also known to have inhibitory effects on TNF-α production. For example, cynaropicrin, reynosin, and Saussurea Lappa (NEAA) (Gibco BRL). Raw264.7 cells (105 cells/ml) were maintained in DMEM (IW AKI, Chiba, Japan) and supplemented with 10% fetal bovine serum (FBS) (IW AKI), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.), and 1% MEM non-essential amino acid solution (NEAA) (Gibco BRL). Raw264.7 cells (1 × 105 cells/ml) were stimulated with LPS (E. coli 0111:B4, Sigma, St. Louis, MO, USA).

**Materials and Methods**

**Marine Natural Materials**

*Sinularia* sp. was collected by hand at depths of 10 m off Hedo Point, Okinawa, Japan, in June 2001.

**Extraction and Isolation of Norditerpenes**

The soft coral *Sinularia* sp. (wet weight 4.7 kg) was extracted three times with EtOH and the combined extract was evaporated under reduced pressure. The residue was partitioned between diethyl ether (Et2O) and H2O. The aqueous layer was then extracted with normal butanol (n-BuOH). The residue (5.8 g) of n-BuOH was chromatographed on Sephadex LH-20 with methanol (MeOH). A total of three fractions were obtained.

The active fraction (B-2, 2.31 g) was flash chromatographed on Si gel with a solvent consisting of chloroform (CHCl3), MeOH=98:2. A total of five fractions were obtained. The most active fraction (B-2-2, 230.6 mg) was chromatographed on a C-18 reversed phase HPLC with MeOH:H2O=4:6 (COSMOSIL, 5C18-AR-II, φ10×250 mm, flow rate 2.0 ml/min, UV 218 nm), and gave norcembrenolide (1, 48.0 mg) and sinuleptolide (2, 7.6 mg).

Norcembrenolide (1): [α]D20 +95.2° (c=0.3, MeOH), mp 233–234 °C. UV λmax (EtOH) nm (log ε): 210 (9.10). Electrom impact (EI)-MS m/z: 348 (M+) 1H-NMR (600 MHz, C5D5N) δ: 3.10 (1H, m, H-1), 2.49 (1H, dd, J=18.3, 18.0 Hz, H-2), 2.81 (1H, m, H-2), 2.72 (1H, dd, J=15.6, 10.2 Hz, H-4), 2.88 (1H, dd, J=16.2, 2.4 Hz, H-4), 4.59 (1H, dd, J=9.6, 2.4 Hz, H-5), 2.55 (2H, d, J=18.0 Hz, H-7), 2.21 (1H, d, J=15.0 Hz, H-9), 2.46 (1H, dd, J=15.3, 7.5 Hz, H-9), 4.91 (1H, s, H-10), 4.99 (1H, s, H-11), 6.66 (1H, dd, J=11.1, 4.0 Hz, H-13), 2.27 (1H, m, H-14), 4.24 (1H, ddd, J=15.9, 11.1, 6.6 Hz, H-14), 4.81 (1H, s, H-16), 4.83 (1H, s, H-16), 1.69 (1H, s, H-17), 1.45 (1H, s, H-18).

Sinuleptolide (2): [α]D20 +39.2° (c=0.8, MeOH), mp 207–208 °C. UV λmax (EtOH) nm (log ε): 210 (9.10). EI-MS m/z: 348 (M+), 1H-NMR (600 MHz, C5D5N) δ: 3.01 (1H, m, H-1), 2.45 (d, J=12.6 Hz, 2.87–2.88 (m, H-2), 2.61 (d, J=13.8 Hz, H-4), 2.89–2.92 (m, H-4), 4.68 (dd, J=11.4 Hz, H-5), 2.43 (d, J=18.0 Hz, H-7), 2.59 (dd, J=18.0 Hz, H-7) 2.15 (d, J=15.6 Hz, H-9), 2.29 (dd, J=15.3, 7.5 Hz, H-9), 4.99 (d, J=7.8 Hz, H-10), 4.93 (s, H-11), 6.66 (dd, J=11.1, 4.5 Hz, H-13), 2.26 (ddd, J=14.5, 5.3, 5.3 Hz), 4.13 (ddd, J=14.9, 11.1, 4.5 Hz, H-14), 4.78 (s, H-16), 4.82 (s, H-16), 1.59 (s, H-17), 1.71 (s, H-18).

**Reagents**

Prednisolone, lipopolysaccharide (LPS), Griess–Romeijn nitrite reagent, and other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**TNF-α Production from RAW264.7 Cells Stimulated by LPS**

Macrophage-like cell Raw264.7 cells were obtained from Riken Cell Bank (Saitama, Japan). Cells were maintained in DMEM (IWAKI, Chiba, Japan) and supplemented with 10% fetal bovine serum (FBS) (IWAKI), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.), and 1% MEM non-essential amino acid solution (NEAA) (Gibco BRL). Raw264.7 cells (1 × 105 cells/ml)
The actual amount of NO was estimated to be 1301.2 pg/ml for LPS and 179.5 pg/ml for control.

**NO Production from RAW264.7 Cells Stimulated by LPS** Raw264.7 cells (1 x 10^6 cells/ml) (1 ml) were incubated for 24 h in 24-well plates in DMEM with 10% FBS, penicillin, streptomycin, and 1% NEAA. The medium was then replaced with DMEM without phenol red. The various concentrations of test samples were added to the well and the plates incubated for 3 h. The cells were then stimulated by LPS (1 μg/ml) and incubated for another 20 h. Supernatants were then collected and NO production was assessed by measuring the accumulation of nitrite using Griess reagent according to a previous report. The actual amount of NO was estimated to be 5.6 μM for LPS.

**Cytotoxicity Assay** The cytotoxicity of the test compounds was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation for 16 h in an inhibitory assay, 50 μl of MTT solution (2 mg/ml in phosphate buffered saline) was added to cell suspensions (1 x 10^5 cells/ml) containing various concentrations of test samples and LPS and the cells incubated for another 4 h. The optical density (OD) was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 540 nm.

**RESULTS AND DISCUSSION**

In this study, we investigated natural inhibitors of LPS-induced TNF-α production that was isolated from marine invertebrates found in Fukuoka and Okinawa, Japan. The EtOH extract of the Okinawan soft coral *Sinularia* sp. showed potent inhibitory activity. After being partitioned into Et2O, n-BuOH, and aqueous layers, the active compounds were concentrated to the n-BuOH extract. The n-BuOH extract was chromatographed on Sephadex LH-20 with MeOH. One fraction showed the highest inhibitory activity. Bioassay guided the separation of the active fraction by Si-gel and reversed phase HPLC to give two active norditerpenes, norcembrenolide (1) as the major compound and sinuleptolide (2) as the minor compound (Fig. 1).

Nocembranolide (1) was first reported as the cembranolide lacking a methyl group at C-4 from the soft coral *Sinularia leptoclados* in 1978. This structure was confirmed by single-crystal X-ray in1985. Sinuleptolide (2) was subsequently isolated as the 5-epimer from the same specimens in 1993. Recently, these cembranoids were isolated from the soft coral *Sinularia gardineri* as the heptacyclic nocembranoid dimers singardin and sesquiterpene guaianediol in 1996.

Nocembranolide (1) and sinuleptolide (2) inhibited LPS-induced TNF-α production by RAW264.7 cells. These norditerpenes showed inhibitory activity in a dose-dependent manner (Fig. 2). The 50% inhibitory concentrations (IC_{50}) for LPS-induced TNF-α production of 1 and 2 were 20 μg/ml (57.4 μM) and 15 μg/ml (43.1 μM), respectively. The IC_{50} values of 1 and 2 were higher than that of prednisolone 33 μg/ml (86.7 μM). Prednisolone is known to be an anti-inflammatory compound, and inhibits TNF-α production at the translation level.

The inorganic free radical NO has also been produced in LPS-activated macrophages. We tested the ability of the norditerpenes to enhance NO production when RAW264.7 cells were seeded on 24-well plates at 1 x 10^5 cells in 1 ml of DMEM medium containing 10% FBS and 1% MEM non-essential amino acids. After 24 h incubation, the various concentrations of test samples were added to the well and the plates incubated for 1 h. The cells were then stimulated by LPS (10 ng/ml) and incubated for another 16 h. Supernatants were then collected and total TNF-α production by RAW264.7 cells was evaluated by ELISA kit according to the manufacturer’s protocol. The actual amount of TNF-α was estimated as the relative inhibition rate against LPS stimulation (% of LPS). Each value represents the mean of duplicate determinations.

**Fig. 1. Structure of Nocembranolide (1) and Sinuleptolide (2) from the Okinawan Soft Coral, *Sinularia sp.*

**Fig. 2. Inhibitory Effect of Nocembranolide (1) and Sinuleptolide (2) on LPS-Induced TNF-α Production by RAW264.7 Cells**

Raw264.7 cells were seeded on 24-well plates at 1 x 10^5 cells in 1 ml of DMEM medium containing 10% FBS and 1% MEM non-essential amino acids. After 24 h incubation, the various concentrations of test samples were added to the well and the plates incubated for 1 h. The cells were then stimulated by LPS (10 ng/ml) and incubated for another 16 h. Supernatants were then collected and total TNF-α content was determined using an ELISA kit according to the manufacturer’s protocol. The activity was evaluated as the relative inhibition rate against LPS stimulation (% of LPS). Each value represents the mean of duplicate determinations.

**Fig. 3. Determination of IC_{50} Values of Test Compounds** for LPS-induced TNF-α Production by RAW264.7 Cells

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**Fig. 4. Cytotoxicity Assay** The cytotoxicity of the test compounds was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation for 16 h in an inhibitory assay, 50 μl of MTT solution (2 mg/ml in phosphate buffered saline) was added to cell suspensions (1 x 10^5 cells/ml) containing various concentrations of test samples and LPS and the cells incubated for another 4 h. The optical density (OD) was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 540 nm.
Some terrestrial natural anti-TNF compounds have been reported thus far, however, this is the first report indicating that norditerpenes originating from marine invertebrates have inhibitory effects on LPS-induced TNF-α production. Many sesquiterpene lactones are known to possess anti-inflammatory activities. In 1998, Cho et al. reported that sesquiterpene lactones, cynaropicrin, reynosin, and santamarine from Sausurea lappa have inhibitory activity towards TNF-α production. Moreover, Merfort et al. reported that sesquiterpene lactones from numerous genera of Compositae suppressed transcription factor NF-κB, regulating the transcription of TNF-α. They also suggested that the most active sesquiterpene lactones have reactive centers in the form of an α-methylene-γ-lactone group and an α,β- or α,β,γ,δ-unsaturated carbonyl group. Since norcembrenolide and sinuleptolide also have a structure analogous to the α-methylene-γ-lactone group, the mode of inhibitory action on TNF-α production for the norditerpenes may be the same as that of the sesquiterpene lactones.

In the present study, we identified two norditerpenes, norcembrenolide (1) and sinuleptolide (2), from the Okinawan soft coral, Sinularia sp. which have potent inhibitory effects on LPS-induced TNF-α production. These norditerpenes inhibited TNF-α production in a dose-dependent manner like that of positive control, prednisolone, and also inhibited subsequent NO production. The inhibitory mechanisms of these compounds should be studied in greater detail. It is hoped these norditerpenes will contribute to the development of therapies for various diseases involving TNF-α.

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REFERENCES


Fig. 3. Inhibitory Effect of Norcembrenolide (1) and Sinuleptolide (2) on LPS-Induced NO Production by RAW264.7 Cells

Raw264.7 cells were seeded on 24-well plates at 1×10⁶ cells in 1 ml of DMEM medium containing 10% FBS and 1% MEM non-essential amino acids. After 24 h incubation, the medium was replaced with DMEM free from phenol red and the various concentrations of test samples were added to each well, and the plates incubated for 3h. The cells were then stimulated by LPS (1 μg/ml) and incubated for another 20 h. Supernatants were collected, and NO production was assessed by measuring the accumulation of nitrite using Griess reagent. The activity was evaluated as the relative inhibition rate against LPS stimulation (% of LPS). Each value represents the mean±S.E.M. (n=8).

Fig. 4. Cytotoxicity of Norcembrenolide (1) and Sinuleptolide (2) in RAW 264.7 Cells

Cytotoxicity was measured by MTT assay. The cell suspension after inhibitory assay was used. Each value represents the mean±S.D. of three experiments.