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Steroidal Constituents from the Soft Coral *Sinularia dissecta* and Their Inhibitory Effects on Lipopolysaccharide-Stimulated Production of Pro-inflammatory Cytokines in Bone Marrow-Derived Dendritic Cells

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Inflammation is defined as a part of complex biological responses of vascular tissue toward exogenous harmful stimuli.[1] It becomes apparent that inflammation, normally results from an excessive inflammatory response or failure of resolution,[2] is recognized as a causative in various diseases such as atherosclerosis, cancer, asthma, and some neuropathological disorders such as Alzheimer's disease or Parkinson's disease.[3]

Numerous molecules are involved in the induction and maintenance of the inflammatory response. In addition to pivotal cytokines such as interleukin-1 (IL-1), -6, -12, and tumor necrosis factor-α (TNF-α), prostaglandins, and nitric oxide are important chemical mediators of inflammation. TNF-α is a well-characterized pro-inflammatory cytokine released primarily from monocytes and macrophages upon invasion of the host by a wide variety of pathogens. It plays a crucial role in host defense and in the inflammatory response. Although it has numerous beneficial roles in immune regulation, it has also been implicated in the pathogenesis of both acute and chronic inflammatory disease.[4] IL-6 is a particularly interesting molecule because it has both pro- and anti-inflammatory effects. It has been implicated in many inflammatory diseases in both adults and neonates.[5] Whereas, IL-12 plays a central role in the initiation and regulation of cellular immunity. It is involved in type-1 helper T-cell-mediated inflammation as part of the normal immune response, as well as inflammatory diseases, including rheumatoid arthritis, asthma, psoriasis, and Crohn's disease.[6-8]

Marine organisms have been found to be storehouses of sterols, particularly in terms of unique side-chain structures and unusual functionalization.[9] Marine sterols are often found in oxygenated forms, and such sterols sometimes shows a variety of biological and pharmacological activities.[10] A literature survey revealed that the genus *Sinularia* is a rich source of a variety of sesquiterpenes, cembrane-derived diterpenes, and polyhydroxylated steroids.[11,12]

In continuation of our investigation on Vietnamese marine soft corals,[13-17] we report herein the isolation and characterization of a new steroid, dissesterol (**1**), and nine known compounds (**2-10**) from a methanol extract of the soft coral *Sinularia dissecta*. These ten steroids were evaluated for their inhibitory effects on lipopolysaccharide (LPS)-stimulated production of pro-inflammatory cytokines in bone marrow-derived dendritic cells (BMDCs).

Results and Discussion

Using combined chromatographic separations, one new and nine known steroids were isolated from the methanol extract of freeze-dried bodies of the soft coral *S. dissecta*. Their structures were elucidated by physicochemical and spectroscopic methods.

Compound **1** was obtained as a white powder. Its molecular formula, $C_{30}H_{50}O_2$, was defined by a pseudo-molecular ion peak at *m/z* 465.3703 $[M+Na]^+$ (calcd for $C_{30}H_{50}O_2Na$, 465.3708) using high-resolution electrospray ionization mass spectrometry (HRESIMS). Strong absorption band accounting for hydroxyl groups (3366 cm^{-1}) was observed in IR spectrum. The ^{13}C NMR spectrum showed 30 carbon signals including seven methyls, eight methylenes, eleven methines, and four quaternary carbons, as detected by distortionless enhancement by polarization transfer (DEPT) experiments. The signals at δ_C 11.8 (C-18), 21.1 (C-19), 21.1 (C-21), 21.2 (C-26), 21.8 (C-27), 15.2 (C-28), and 14.0 (C-29) indicated the presence of seven methyl groups. Moreover, two oxymethine groups [δ_C 67.3 (C-3) and 73.6 (C-6)] and a trisubstituted double bond [δ_C 128.1 (CH, C-4)/147.1 (C, C-5)] were also identified. In the ^1H NMR spectrum, the presence of four high-field protons at δ_H 0.10 (1H, m, H-22), 0.15 (1H, m, H-24), −0.21 (1H, dd, J = 4.5, 5.5 Hz, H_β-30), and 0.36 (1H, dd, J = 4.5, 9.0 Hz, H_α-30) is characteristic of a gorgosterol-type side chain possessing a cyclopropane ring.[18] In addition, three tertiary methyl [δ_H 0.61 (H-18), 1.14 (H-19), and 0.82 (H-29); each 3H, s)], three secondary methyl [δ_H 0.77 (H-26), 0.87 (H-27), and 0.86 (H-28); each 3H, d, J = 7.0 Hz], one olefinic [δ_H 5.37 (1H, br s, H-4)], and two

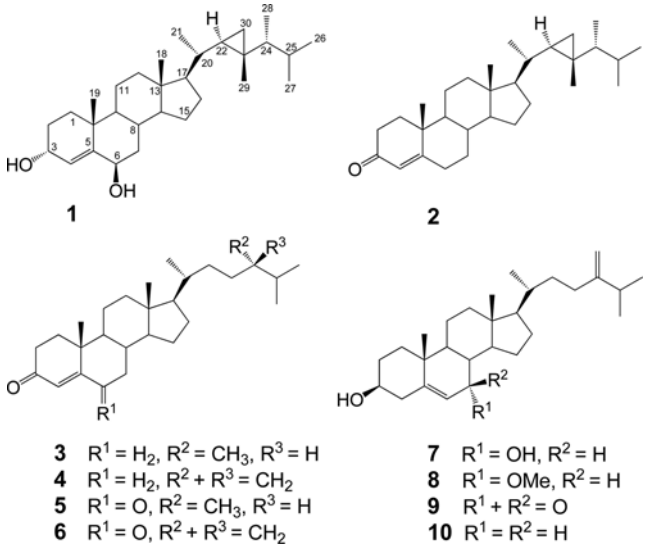

Figure 1. Structures of compounds **1**-**10**.

oxymethine [δ_H 4.03 (1H, m, H-3) and 4.07 (1H, t(2.5), H-6)] protons were also determined. A secondary methyl signal appeared as a broad singlet at δ_H 0.92, which was assigned to H-21 by HSQC, HMBC, and COSY experiments, is also typical for sterols possessing a gorgosterol-type side chain.[18,19] The ^{1}H-^{1}H COSY experiment allowed to assign the proton-proton correlations of H_2-1/H_2-2/H-3/H-4, H-6/H_2-7/H-8/H-9/H_2-11/H_2-12, H-8/H-14/H_2-15/H_2-16/H-17/H-20/H-22/H_2-30, and H_3-28/H-24/H-25/H_3-26. These data together with HMBC cross peaks between H-19 (δ_H 1.14) and C-1 (δ_C 36.5)/C-5 (δ_C 147.1)/C-9 (δ_C 54.1)/C-10 (δ_C 36.7), H-3 (δ_H 4.03) and C-5 (δ_C 147.1), and H-6 (δ_H 4.07) and C-4 (δ_C 128.1)/C-8 (δ_C 30.1)/C-10 (δ_C 36.7) confirmed positions of the double bond at C-4/C-5 and two hydroxy groups at C-3 and C-6. Detailed analyses of other HMBC correlations clearly identified the planar structure of **1** (see Fig. 2).

The proton signals of H-3 at δ_H 4.03 (1H, m) and H-6 at δ_H 4.07 (1H, t, J = 2.5 Hz) are representative of H_β-3 and H_α-6, respectively. In addition, the configurations at C-3 and C-6 were further confirmed by an agreement of the ^{13}C NMR chemical shifts for C-3 (δ_C 67.3), C-4 (δ_C 128.1), C-5 (δ_C 147.1), and C-6 (δ_C 73.6) of **1** compared with those of cholest-4-en-3α,6β-diol[20] at δ_C 66.5 (C-3), 127.6 (C-4), 147.6 (C-5), and 73.1 (C-6), respectively. The relative configuration of compound **1** was also suggested by nuclear overhauser effect spectroscopy (NOESY, see Fig. 2). Thus, the structure of **1** was elucidated as gorgost-4-en-3α,6β-diol and named dissesterol.

In addition to the above new compound, nine known steroids were also isolated from the methanol extract of the soft coral *S. dissecta*. By detailed analyses and comparison of their spectroscopic data with those published in the literatures, the known compounds were characterized as (24*R*)-gorgost-4-en-3-one (**2**),[18,21] (24*S*)-ergost-4-en-3-one (**3**),[22] (24 methylene)-ergost-4-en-3-one (**4**),[23] (24*S*)-ergost-4-en-3,6-dione (**5**),[23] 24-methylenecholest-4-en-3,6-dione (**6**),[23] 3β,7α-dihydroxyergosta-5,24(28)-dien (**7**),[24] 7α-methoxy-ergosta-5,24(28)-dien-3*b*-ol (**8**),[25] cholesta-5,24(28)-dien-3β-ol-7-one (**9**),[26] and ergosta-5,24 dien-3β-ol (**10**).[27]

Continuing with our interest in the evaluation of the biological potential of natural diterpenes and to search novel anti-inflammatory agents from natural products, we have evaluated the effects of ten sterols (**1**-**10**) in the inflammatory response by BMDCs (see Supplementary data). We first used a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, MO, U.S.A.) to confirm that these compounds have no or little effect on the cell viability (data not shown).

Compound **1** showed a strong suppression of LPS-stimulated IL-12 p40 production with an inhibition rate up to 100.0% at 10 μM and IC_{50} values of 4.0 ± 0.1 μM (see Fig. 3). However, other compounds showed negligible effects with inhibition values less than 50% activity even at 50 μM. Compound **9** exhibited the most potent inhibitory activity on the IL-6 production, with 54.0% inhibition at 10 μM and IC_{50} 9.4 ± 1.2 μM (see Fig. 3). SB203580, an inhibitor of cytokine suppressive binding protein/p38 mitogen-activated protein kinase, was used as a positive control.[28] SB203580 inhibited IL-12 p40, IL-6, and TNF-α production with IC_{50} values of 5.2 ± 0.1, 3.5 ± 0.1, and 7.5 ± 0.2 μM, respectively.

IL-12 is an inducible, heterodimeric disulfide-linked cytokine composed of p35 and p40 subunits. Expression of the p35 subunit of IL-12 is constitutive and ubiquitous. Therefore, the biological activity of IL-12 is regulated mainly by induction of the p40 subunit and is regulated primarily at the level of transcription. Since IL-12 is a key cytokine in Th1-mediated autoimmune responses, down regulation of IL-12 production by the steroid compounds, especially compound **1** from *S. dissecta*, may ameliorate autoimmune diseases.

To our knowledge, the present study is the first report for anti-inflammatory activities of the chemical components isolated from soft coral *S. dissecta* and warrants further studies concerning the potential of the extract of *S. dissecta* for medicinal purpose.

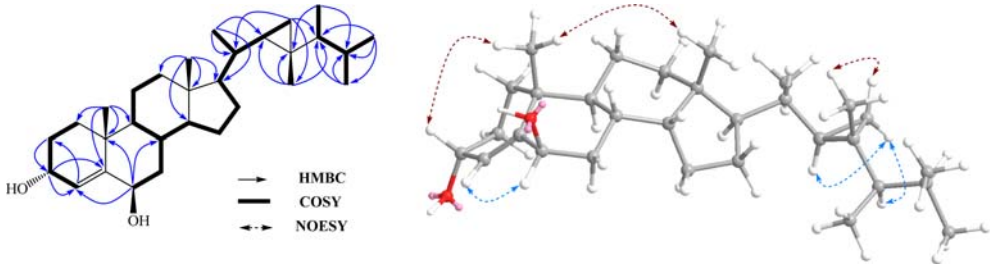

Figure 2. Key HMBC, COSY, and NOESY correlations of compound **1**.

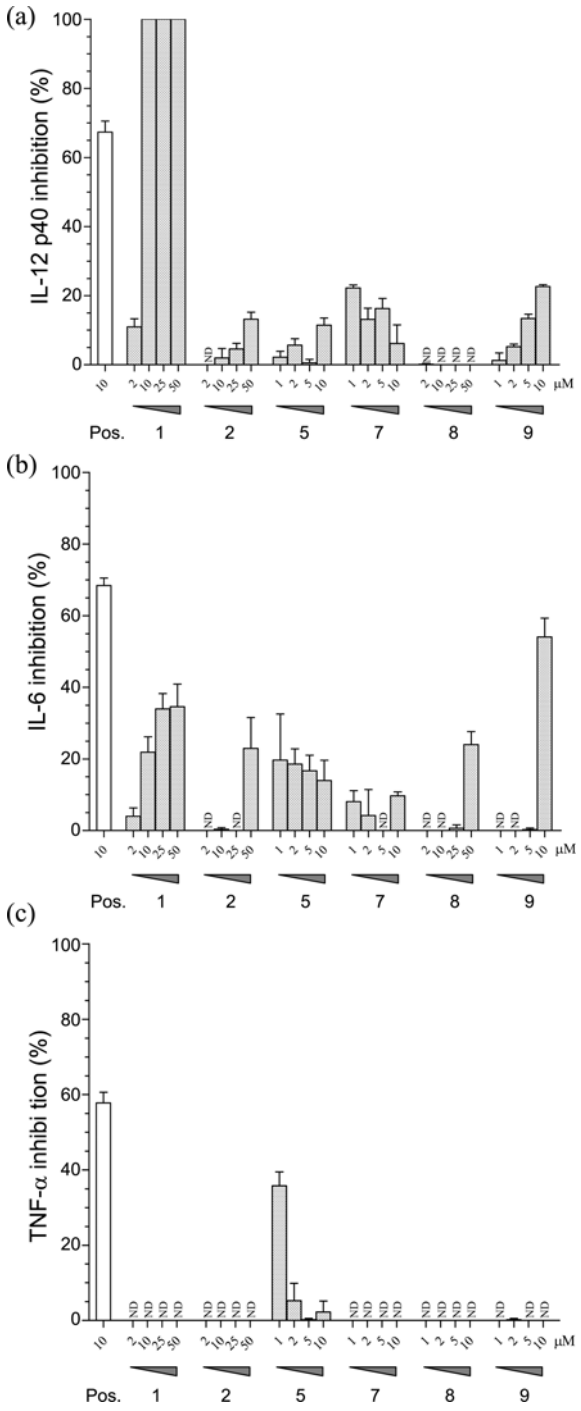

Figure 3. Effect of sterols (**1**, **2**, **5**, **7-9**) on IL-12 p40 (a), IL-6 (b), and TNF-α (c) production by LPS-stimulated BMDCs. DCs were treated with the compounds at the indicated concentrations for 1 h before stimulation with LPS (10 ng/mL). Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA. The data were presented as inhibition rate (%) compared to the value of vehicle-treated DCs. SB203580 was used as positive control (Pos.). ND-Not detected.

Experimental Section

General Procedures. Optical rotations were determined on a JASCO P-2000 polarimeter (Hachioji, Tokyo, Japan). IR spectra were obtained on a Bruker TENSOR 37 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany). The high resolution mass spectra were gained using a Shimadzu LCMS-IT-TOF (Tokyo, Japan). The ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AM500 (Bruker, Billerica, MA, USA) and JEOL ECA 600 (Tokyo, Japan) FT-NMR spectrometers. TMS was used as an internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck, Darmstadt, Germany) and YMC RP-18 resins (30-50 μm, Fuji Silysia Chemical Ltd., Kasugai, Aichi, Japan). Thin layer chromatography (TLC) used pre-coated silica gel 60 F_{254} (1.05554.0001, Merck, Darmstadt, Germany) and RP-18 F_{254S} plates (1.15685.0001, Merck, Darmstadt, Germany) and compounds were visualized by spraying with aqueous 10% H_2SO_4 and heating for 3-5 minutes.

Biological Material. The sample of soft coral *S. dissecta* was collected during April 2010 at Hai Van-Son Cha, Hue, Vietnam and identified by Prof. Do Cong Thung (Institute of Marine Environment and Resources, VAST). A voucher specimen (SD042010_01) was deposited at the Institute of Marine Biochemistry and Institute of Marine Environment and Resources, VAST.

Extraction and Isolation. Fresh frozen samples of the soft coral *S. dissecta* (1.5 kg) were well grinded and extracted three times with hot MeOH (at 50 °C for 5 h each time). The obtained solutions were filtered, combined, and concentrated under reduced pressure to yield a dark brown viscous residue (9.15 g, A). This residue was suspended in water (0.5 L) and partitioned in turn with *n*-hexane (2 × 0.5 L) and CH_2Cl_2 (3 × 0.5 L). The combined dichloromethane soluble portions were evaporated under reduced pressure to afford CH_2Cl_2 extract (1.83 g, B). Extract B was crudely separated by silica gel CC using gradient concentrations of ethyl acetate in *n*-hexane from 0 to 100% to yield four fractions, B-1 to B-4. Fraction B1 (647 mg) was further separated on silica gel CC using *n*-hexane-EtOAc (25:1) as eluents, to give three subfractions, B1.1 to B1.3. Subfraction B1.1 (253 mg) was then chromatographed over silica gel CC using eluent of *n*-hexane-acetone (14:1), and further purified by YMC RP-18 CC eluting with MeOH-acetone-H_2O (4:2:0.2) to afford **4** (110 mg). Compound **3** (20 mg) was purified from subfraction B1.2 (158 mg) by silica gel CC eluting with *n*-hexane-EtOAc (15:1) and followed by Saphadex LH-20 CC (MeOH-acetone 1:1). Subfraction B1.3 (230 mg) afforded **2** (52 mg), after subjecting it to silica gel CC eluting with dichloromethane-acetone (21.5:1), followed by YMC RP-18 CC with MeOH-acetone (6.5:1). Fraction B2 (80 mg) was separated by YMC RP-18 CC, using eluent of MeOH-acetone-H_2O (95:3:2) to yield three subfractions, B-2.1 to B-2.3. Subfraction B2.2 (51 mg) was further separated by YMC RP-18 CC, eluting with MeOH-acetone-H_2O (5:1:0.2) to yield **10** (36 mg). Subfraction B2.3 (28 mg) afforded compound **6** (17 mg), after subjecting it to silica gel CC eluting with *n*-hexane-EtOAc (8.5:1). Fraction B3 (60 mg) was fractionated into five subfractions, B3.1 to B3.5, by silica gel CC using *n*-hexane-acetone (12:1) as eluent. Subfraction B3.2

Table 1. The NMR spectroscopic data ($CDCl_3$ + CD_3OD) of compound **1**

C	δ_C[a]	δ_H[b] mult. (*J* in Hz)
1	36.5	1.16 m/1.60 m
2	28.5	1.45 m/1.85 m
3	67.3	4.03 m
4	128.1	5.37 br s
5	147.1	-
6	73.6	4.07 t (2.5)
7	38.9	1.02 m/1.83 m
8	30.1	1.76 m
9	54.1	0.65 m
10	36.7	-
11	20.8	1.32 m/1.47 m
12	39.7	1.05 m/1.95 m
13	42.8	-
14	55.8	0.90 m
15	24.2	1.05 m/1.52 m
16	28.0	1.25 m/1.94 m
17	57.8	1.15 m
18	11.8	0.61 s
19	21.1	1.14 s
20	35.1	0.93 m
21	21.1	0.92 br s
22	31.8	0.10 m
23	25.6	-
24	50.6	0.15 m
25	32.0	1.49 m
26	21.2	0.77 d (7.0)
27	21.8	0.87 d (7.0)
28	15.2	0.86 d (7.0)
29	14.0	0.82 s
30	20.9	β −0.21 dd (4.5, 5.5) α 0.36 dd (4.5, 9.0)

[a]125 MHz. [b]500 MHz. Assignments were confirmed by HSQC, HMBC, and COSY experiments.

(37 mg) was separated by CC over silica gel eluting with CH_2Cl_2-EtOAc (20:1), followed by YMC RP-18 CC with MeOH-acetone (3.5:1), to give **8** (7 mg). Fraction B4 (740 mg) was passed through Sephadex LH-20 with MeOH-acetone (1:1) to yield five subfractions, B4.1 to B4.5. Subfraction B4.1 (78 mg) afforded compound **7** (24 mg) after subjecting it to silica gel CC eluting with *n*-hexane-acetone (4:1). Subfraction B4.2 (46 mg) was further separated by silica gel CC eluting with CH_2Cl_2-MeOH (25:1), followed by Sephadex LH-20 with MeOH-acetone (70:30) to yield compounds **1** (9 mg), and **5** (10 mg). Compound **9** (17 mg) were obtained from subfraction B4.3 (120 mg) by silica gel CC using CH_2Cl_2-MeOH (24:1) as eluent and further purified by YMC RP-18 CC with MeOH-acetone-H_2O (10:1.1.5).

Dissesterol (1): Amorphous white powder, $[\alpha]_D^{25}$ −27.2 (*c* 0.30, CH_2Cl_2); HRESIMS *m/z* 465.3703 $[M+Na]^+$ (calcd for $C_{30}H_{50}O_2Na$, 465.3708); IR (KBr) ν_{max} 3366, 2902, 1647, 1558, 1073, and 982 cm^{-1}; ^1H NMR ($CDCl_3$+CD_3OD, 500 MHz) and ^{13}C NMR ($CDCl_3$ + CD_3OD, 125 MHz) are given in Table 1.

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