Anti-inflammatory Diterpene from *Thyrsanthera suborbicularis*

Piseth KHIEV,[a] Sei-Ryang OH,[b] Hee-Sung CHAE,[b] Ok-Kyoung KWON,[b] Kyung-Seop AHN,[b] Young-Won CHIN,[c] and Hyeong-Kyu LEE*,[a,b]

[a] *University of Science & Technology; Daejeon 305–333, Korea:* [b] *Immune Modulator Research Center, Bio-Therapeutics Research Institute, Korea Research Institute of Bioscience & Biotechnology; 685–1 Yangcheong-ri, Ochang-eup, Cheongwon-gun, ChungBuK 363–883, Korea: and* [c] *College of Pharmacy, Dongguk University-Seoul; 26, Pil-dong-3-ga, Jung-gu, Seoul 100–715, Korea.*

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Bioactivity-guided isolation on a *n*-hexane-soluble fraction of *Thyrsanthera suborbicularis* led to the isolation of a new rosane-type diterpene, 19-hydroxy-1(10), 15-rosadiene (1), along with three known compounds, taraxerol, acetyl aleuritolic acid, and spathulenol. The structures of isolated compounds were determined by interpretation of NMR spectroscopic data and mass spectrometry. Compound 1 demonstrated significantly inhibitory activity on nitric oxide production in RAW264.7 lipopolysaccharide (LPS)-activated mouse macrophages with an IC_{50} value of 2.91 μg/ml *via* the suppression of inducible nitric oxide synthase (iNOS) mRNA expression.

Key words *Thyrsanthera suborbicularis*; Euphorbiaceae; rosane diterpenoid; nitric oxide; inducible nitric oxide synthase

Thyrsanthera suborbicularis PIERRE ex GAGNEP. (Euphorbiaceae), distributed in Cambodia, Vietnam and Thailand, is the sole member of the genus *Thyrsanthera*.[1)] The roots of *T. suborbicularis* have been used to treat malaria locally in Cambodia.[1)] As a part of our ongoing search for anti-inflammatory agents of plant origin,[2)] *T. suborbicularis* was selected for phytochemical investigation since its *n*-hexane extract was found to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in the initial screening test. There has been no report of phytochemical and pharmacological study for *T. suborbicularis* so far. Thus, the present study described the isolation and biological evaluation of a new 19-hydroxy-1(10),15-rosadiene (**1**) and three known compounds from *T. suborbicularis*, using a bioactivity-guided fractionation method.

Results and Discussion

Compound **1** was obtained as white amorphous powder and its molecular formula was assigned as $C_{20}H_{32}O$ by a molecular ion peak at *m/z* 288.2441 $[M]^+$ (Calcd 288.2453) in the high resolution-electron ionization-mass spectra (HR-EI-MS). The ^{1}H-NMR spectroscopic data of **1** displayed three singlet methyl signals at δ_H 0.90 (3H, H-20), 0.97 (3H, H-17), and 0.99 (3H, H-18), an oxygenated methylene signal at 3.56 (1H, d, J=10.7 Hz, H-19a) and 3.35 (1H, d, J=10.7 Hz, H-19b), an olefin proton signal at δ_H 5.53 (1H, m, H-1), and vinyl proton signals at δ_H 5.79 (1H, dd, J=17.5, 10.7 Hz, H-15), 4.90 (1H, dd, J=17.5, 1.3 MHz, H-16a), and 4.84 (1H, dd, J=10.7, 1.3 Hz, H-16b). The ^{13}C-NMR spectrum (Table 1) exhibited 20 carbon signals consisting of three methyls, nine methylenes, four methines, and four quarternary carbons, sorted by distortionless enhancement by polarization transfer (DEPT) and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectroscopic data. The observed ^{1}H- and ^{13}C-NMR chemical shifts of **1** were closely similar to those of hugorosediol[3,4)] except the absence of a hydroxyl group at C-3. The ^{1}H–^{1}H correlation spectroscopy (COSY) correlations (Fig. 2) between δ_H 5.53 (H-1) and δ_H 2.01 (H-2a), as well as between δ_H 2.01 (H-2a) and 1.28 (H-3b) permitted the position of H-1 in the proposed structure **1**. Furthermore, the heteronuclear multiple bond coherence (HMBC) correlations (Fig. 2) of both H-19 and H-18 to C-3, as well as both H-1 and H-19 to C-5 confirmed the location of the double bond as shown in Fig. 1. The location of a methyl group (C-20) was determined on C-9 by the observed HMBC correlations of H-20 to C-9, C-10, and C-11, and H-1 to C-9.

The relative configuration of compound **1** was resolved by the interpretation of nuclear Overhauser effect spectroscopy (NOESY) correlations. Nuclear Overhauser effect (NOE) correlations of H-8 (δ_H 1.73) to both CH_3-17 (δ_H 0.97) and H-5 (δ_H 2.24), and H-5 (δ_H 2.24) to CH_3-18 (δ_H 0.99) pro-

Table 1. ^{1}H- (800 MHz) and ^{13}C-NMR (200 MHz) Chemical Shifts of Compound **1** (in $CDCl_3$; δ in ppm; J in Hz)

Position	^{1}H	^{13}C
1	5.53 (m)	118.2 (d)
2a	2.01 (m)	22.9 (t)
2b	1.02 (m)	
3a	1.70 (m)	32.0 (t)
3b	1.25 (m)	
4	—	35.8 (s)
5	2.24 (m)	42.3 (d)
6a	1.75 (m)	19.1 (t)
6b	1.20 (m)	
7a	1.55 (m)	26.0 (t)
7b	1.21 (m)	
8	1.73 (m)	31.4 (d)
9	—	37.2 (s)
10	—	148.7 (s)
11a	1.65 (dt, J=13.4, 3.2 Hz)	35.6 (t)
11b	1.43 (td, J=13.4, 3.2 Hz)	
12a	1.50 (td, J=13.6, 3.4 Hz)	33.0 (t)
12b	1.23 (m)	
13	—	36.5 (s)
14a	1.18 (m)	39.9
14b	1.09 (m)	
15	5.79 (dd, J=17.5, 10.7 Hz)	151.5 (d)
16a	4.90 (dd, J=17.5, 1.3 Hz)	108.8 (t)
16b	4.84 (dd, J=10.7, 1.3 Hz)	
17	0.97 (s)	22.5 (q)
18	0.99 (s)	24.3 (q)
19a	3.56 (d, J=10.7 Hz)	65.5 (t)
19b	3.35 (d, J=10.7 Hz)	
20	0.90 (s)	21.1(q)

* To whom correspondence should be addressed. e-mail: hykylee@kribb.re.kr

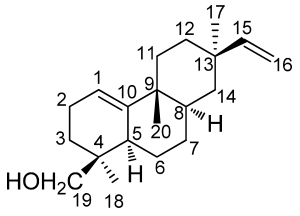

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Fig. 1. Structures of Isolated Compounds from *T. suborbicularis*

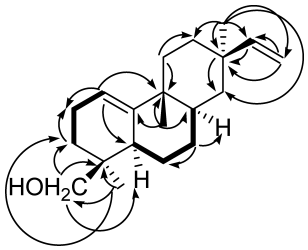

Fig. 2. Key ^{1}H–^{1}H COSY (▬) and HMBC (H→C) Correlations of Compound **1**

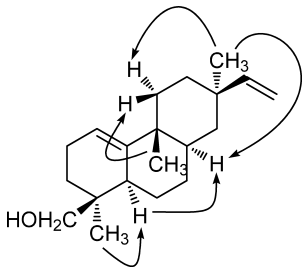

Fig. 3. Key ^{1}H–^{1}H NOESY (↔) Correlations of Compound **1**

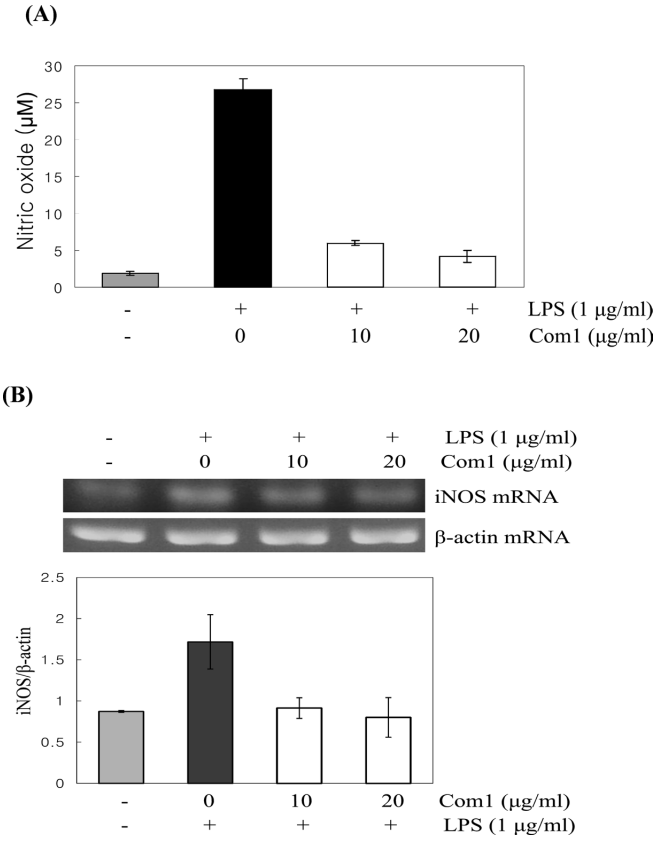

Fig. 4. NO Production and iNOS Protein Level in RAW264.7 Cells Treated with Compound **1**

Cells incubated with compound **1** (10, 20 μg/ml) in the present and absent of LPS for 24 h. (A) NO production was measured by Griess reagent. Data are shown as the mean±S.E.M. of values obtained from triplicate cultures and are representative of three experiments with similar results. (B) iNOS mRNA level was determined by reverse transcription-polymerase chain reaction. Data are presented as mean±S.E.M. from three experiments performed in triplicate for iNOS/β-actin.

vided these groups were positioned in the same direction (Fig. 3). A methyl signal at δ_H 0.90 (CH_3-20) did not exhibited NOE correlations with both H-5 and H-8 instead NOE correlations of CH_3-20 to H-11a (δ_H 1.65), and CH_3-17 to H-11b (δ_H 1.43), implying that CH_3-20 may be positioned in the opposite direction in contrast to those of H-5 and H-8. Therefore, the structure of compound **1** was determined as 19-hydroxy-1(10),15-rosadiene in Fig. 1.

The three known compounds were identified as taraxerol,[5] acetyl aleuritolic acid,[6,7] and spathulenol[8] by comparing their spectroscopic data with the published data.

All the isolates were evaluated for their inhibitory activity against NO production in LPS-induced RAW264.7 cells. Of the compounds tested, compound **1** exhibited the most potent inhibitory activity against NO production in LPS-induced RAW264.7 cells with IC_{50} value of 2.91 μg/ml while other compounds deemed inactive (>20 μg/ml) in this assay system. Also, it was found that compound **1** suppressed inducible nitric oxide synthase (iNOS) mRNA expression at doses of 10 and 20 μg/ml by reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 4B).

Experimental

Procedures Melting points were determined on a Kofler micro-hostage (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. Optical rotation were measured with a Jasco P-1020 polarimeter (Jasco Corp., Japan), UV using UV–VIS Spectrophotometer 2400 (Shimadzu Co., Ltd., Japan), and Fourier transform (FT)-IR spectra using a Jasco FT/IR-4200 (Jasco Corporation, Japan). NMR spectra were recorded on a Varian UNITY 400 (Varian, Inc., Palo Alto, CA, U.S.A.) and Brucker DMX-800 MHz FT-NMR spectrometer with the tetramethylsilane as an internal standard. High resolution-electrospray ionization (HR-ESI)-MS (Micromass U.K. Ltd., Manchester, U.K.) and HR-EI-MS (JEOL, Tokyo, Japan) were performed with on a Waters Q-Tof Premier spectrometer and JEOL JMS AX505W spectrometer, respectively. Sephadex LH-20 (25—100 μm, Sigma-Aldrich, Steinheim, Germany), silica gel (230—400 mesh, SiliCycle Inc., Quebec, Canada), RP-C18 (Cosmosil 40C_{18}-PREP, Kyoto, Japan) were used for column chromatography. TLC was performed on precoated Kiesel-gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and Kiesel-gel 60 RP-18F_{254s} (0.25 mm, Merck, Steinheim, Germany). Reagents $NaHCO_3$ and Na_2CO_3 were purchased from Daejung, Korea. Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Hyclone (Hyclone Labs Logan, UT, U.S.A.). Bovine serum albumin (BSA), LPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-thiazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). iNOS, β-actin, monoclonal antibodies and peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.).

Plant Material Whole plants of *T. suborbicularis* were collected in Kandal province, Cambodia in February 2007. This plant samples were identified by Prof. Yok Lin, Department of Biology, Faculty of Science, and Royal University of Phnom Penh, Cambodia. A voucher specimen (KRIBB 023087) has been deposited at herbarium of Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea.

Extraction and Isolation Air-dried whole plants of *T. suborbicularis* (6.0 kg) were extracted with MeOH at room temperature three times to obtain 366.0 g of solid extract. The MeOH extract was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, successively, to give 70.0 g of *n*-hexane-soluble extract, 23.4 g of $CHCl_3$-soluble extract,

8.7 g of EtOAc-soluble extract, and 35.0 g of *n*-BuOH-soluble extract, respectively. The *n*-hexane-soluble extract inhibited NO production with IC_{50} value of 12.9 μg/ml and was subjected to a silica gel column chromatography (10×80 cm, 230—400 mesh) with a stepwise gradient mixture of *n*-hexane–EtOAc (70 : 1 to 1 : 1, v/v) to yield ten fractions (TS8-1—TS8-10). Active fraction TS8-6 (5.8 g, IC_{50}=16.3 μg/ml) was crystallized to yield taraxerol (82 mg) and two fractions (TS8-6-2 and TS8-6-3). Fraction TS8-6-2 (300 mg) was chromatographed on medium pressure liquid chromatography (MPLC) column of RP C-18 (3.5×40 cm, $40C_{18}$-PREP), eluted with 100% MeCN to obtain acetyl aleuritolic acid (203 mg). Fraction TS8-6-3 (5.3 g) chromatographed on MPLC column of RP C-18 (7.0×40 cm, $40C_{18}$-PREP), eluted with MeOH–H_2O (4 : 1 to 9 : 1, v/v) to yield five fractions (TS8-6-3-A—TS8-6-3-E). Fraction TS86-3-D (870 mg) was further fractionated using Sephadex LH-20 column chromatography (2.5×170 cm) eluted with 75% of MeOH in H_2O to give spathulenol (20 mg), and compound **1** (177 mg).

19-Hydroxy-1(10),15-rosadiene (**1**): White amorphous powder; mp 98—99 °C; $[\alpha]_D^{18}$ +13.3 (c=3.1, $CHCl_3$); UV (EtOH): λ_{max} nm (log ε): 205 (4.13); IR (film) cm^{-1}: 3347, 1636, 908; HR-EI-MS m/z 288.2441 $[M]^+$ (Calcd for $C_{20}H_{32}O$, 288.2453). The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data see Table 1.

Cell Culture The RAW264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine (1 mM), 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 μg/ml) and incubated at 37 °C in humidified 5% CO_2 incubator. For stimulation, the medium was replaced with fresh RPMI 1640, and then cells were stimulated with LPS in the presence or absence of compound **1** for the indicated periods.

MTT Assay for Cell Viability The cell viability was examined by MTT assay. RAW264.7 cells were seeded at 1×10^5/ml densities in 96 well plates (Nunc, Denmark). Each group had non-treated group as control. Compound **1** (10, 20 μg/ml) was added to each well and incubated for 24 h at 37 °C, 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-thiazolium bromide (MTT) solutions (5 mg/ml) were added to each well and then cells were cultured for another 4 h. The supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The optical density was read at 590 nm. Cytotoxicity was calculated by subtracting from one the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells.

Measurement of Nitric Oxide (NO) Production NO production was assayed by measuring nitrite in supernatants of cultured RAW264.7 cells. Cells were seeded at 1×10^6/ml in 96 well culture plates. After pre-incubation of RAW264.7 cells for 18 h, cells were pretreated with compound **1** (10, 20 μg/ml) and stimulated LPS (1 μg/ml) for 24 h. The supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 min. The concentration of nitrite was measured by reading at 570 nm. Sodium nitrite ($NaNO_2$) was used as a standard curve.

RNA Extraction and Reverse Transcription PCR (RT-PCR) Total cellular RNA was isolated using an easy-BLUETM RNA extraction kit according to the manufacturer's instructions. Briefly, total RNA (2 μg) was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 1 mM deoxyribonucleotide triphosphates (dNTPs) at 42 °C for 1 h. The reaction was then stopped by incubating the solution at 70 °C for 15 min, after which 3 μl of the cDNA mixture was used for enzymatic amplification. PCR was then performed using a reaction mixture comprised of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of *Taq* DNA polymerase, and 0.1 μM each of primers specific for iNOS and β-actin. The amplification conditions were as follows: denaturation at 94 °C for 3 min for the first cycle and then 35 cycles of 94 °C for 45 s, annealing of iNOS at 56 °C for 45 s or annealing of β-actin at 53 °C for 45 s with a final extension at 72 °C for 7 min. The PCR products were then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The primers used were 5′-AGCCCAACAATACAAATGACCCTA-3′ (sense) and 5′-TTCCTGTTGTTTCTATTTCCTTTGT-3′ (antisense) for iNOS, 5′-ATGAAGATCCTGACCGAGCGT-3′ (sense) and 5′-AACGCAGCTCAGTAACAGTCCG-3′ (antisense) for β-actin.

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