Chemical and Biological Evaluation on Scopadulane-Type Diterpenoids from *Scoparia dulcis* of Vietnamese Origin

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From the aerial parts of *Scoparia dulcis* L. (Scrophulariaceae) grown in Vietnam, four scopadulane-type diterpenoids (4—7), of which 7 is new and was given the trivial name scopadulcic acid C, together with nine known compounds were isolated. Their structures were elucidated by spectroscopic analyses. The absolute configurations of 4—7 were ascertained by applying the modified Mosher's method to *iso*-dulcinol (6). The isolation of the lignans nirtetralin and niranthin for the first time from *S. dulcis* is also of chemotaxonomic interest. The cytotoxic activity in KB cells, inhibitory effect on LPS/IFNγ-induced NO production, inhibition of multidrug resistance (MDR), and antibacterial and antifungal activities of the scopadulane-type diterpenoids 4—7 were examined in this study.

Key words *Scoparia dulcis*; Scrophulariaceae; scopadulane-type diterpenoid; multidrug resistance; methicillin-resistant *Staphylococcus aureus*

The tetracyclic diterpenoids scopadulcic acids A (**1**) and B (**2**), and dulcinol/scopadulciol (**3**) were isolated from the perennial herb *Scoparia dulcis* L. (Scrophulariaceae) as the first members of a unique group of labdane-derived diterpenoids,[1—3] which were proposed the name "scopadulane" by Hayashi *et al.*[1] Due to their unique carbon skeleton and interesting biological activities, scopadulane-type diterpenoids have been the focus of continuous studies on different collections of *S. dulcis* from Paraguay, Taiwan, and Bangladesh.[4] In recent years further efforts resulted in the isolation of three new natural metabolites, 4-*epi*-scopadulcic acid B (**4**), dulcidiol (**5**), and *iso*-dulcinol (**6**) from a Bangladeshi collection of *S. dulcis*.[5] We evaluated the chemical composition of *S. dulcis*, which is cultivated for medicinal purposes in Vietnam, by a systematic extraction and isolation procedure and isolated four scopadulane-type diterpenoids, **4**—**7**, of which **7** is new and was given the trivial name scopadulcic acid C, together with nine other known compounds. Antitumor and antitumor promoting activities, and inhibitory activities on the replication of herpes simplex virus type I, on the gastric proton pump, and on bone resorption stimulated by parathyroid hormone were demonstrated by scopadulcic acid B and scopadulciol.[4] Disregarding the cytotoxic effects of **3**—**6** against a panel of six human stomach cancer cell lines,[5] **4**—**7** were tested for their cytotoxicity in KB cells. The inhibitory effect on lypopolysaccharide (LPS)/interferon-γ (IFNγ)-induced nitric oxide (NO) production and calcein efflux studies were carried out to evaluate the potential of **4**—**7** as lead compounds in cancer therapy.[6,7] The antibacterial activities of **4**—**7** against *Escherichia coli*, *Bacillus subtillis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA) as well as antifungal activity against *Candida albicans* were also examined in this study.

The combined *n*-hexane- and CH_2Cl_2-soluble fractions of the MeOH extract from the aerial parts of *S. dulcis* were fractionated by silica gel open column chromatography (CC), reversed-phase octadecyl silica (ODS) gel CC, and preparative ODS HPLC to give diterpenoids **4**—**7**. Moreover, nine known compounds were isolated and determined to be adrenalin, 6-methoxybenzoxazolinone, lupeol, betulinic acid, nirtetralin, niranthin, 4′,5-dihydroxy-3,7-dimethoxyflavone, acacetin, and 3′-hydroxy-4′,5,7-trimethoxyflavone by comparing their physical value ($[\alpha]_D$) and spectroscopic data (NMR, CD) with those reported in the literature.[8—14] It is worthy of note that lupeol, nirtetralin, niranthin, 4′,5-dihydroxy-3,7-dimethoxyflavone, and 3′-hydroxy-4′,5,7-trimethoxyflavone were isolated for the first time from *S. dulcis*. Since triterpenoids[9,15] and flavonoids[16,17] have been isolated from *S. dulcis*, the presence of the lignans nirtetralin and niranthin is of chemotaxonomic interest.

Compound **7** was isolated as an amorphous powder and its molecular formula was determined to be $C_{27}H_{36}O_5$ by negative-ion high-resolution (HR)-FAB-MS. The IR spectrum indicated the presence of carboxylic acid (3592—2651 cm^{-1}), hydroxyl (3430 cm^{-1}), and ester (1706 cm^{-1}) functional groups. The ^{1}H- (Table 1) and ^{13}C-NMR (Table 2) signals of **7** were found to be similar to those of 4-*epi*-scopadulcic B (**4**).[5] Significant differences were detected for C-8 (δ_C 29.4, **4**: 35.9), C-11 (δ_C 37.8, **4**: 45.5), C-12 (δ_C 43.6, **4**: 52.5), C-14 (δ_C 36.2, **4**: 42.6), and C-17 (δ_C 23.9, **4**: 19.9). This was in agreement with the reduction of the carbonyl group at C-13 in **4** (δ_C 213.4) to a hydroxyl group in **7** [δ_H 3.33 (br s), δ_C 74.7]. NOE correlations in the nuclear Overhauser effect spectroscopy (NOESY) of **7** between H_3-18 (δ_H 0.93) and H-5 (δ_H 1.20) and H-6 (δ_H 5.44) established the axial (β)-orientations of the carboxylic acid at C-19 and the benzoyl group at C-6. The β-axial orientation of the benzoyl group at C-6 was in good agreement with the observed axial-equatorial coupling constant between H-5 and H-6 (J=1.7 Hz). The small coupling constant of H-13 [δ_H 3.33 (br s)], which gave a NOE with H_3-17 (δ_H 0.94), determined the axial (β) orientation of the hydroxyl group at C-13. Thus, the structure of **7** was determined as shown in Fig. 1 and named scopadulcic acid C.

It is worthwhile noting that the absolute configurations of diterpenoids **4**—**6**, which were previously isolated from Bangladeshi *S. dulcis*,[5] have not yet been determined. The absolute configurations have to be ensured with respect to the proper correlations between structures and their biological

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Table 1. ^{1}H-[a)] and ^{13}C-NMR Spectroscopic Data of **7** (δ in ppm, J in Hz, $CDCl_3$)

	C/H	**7**
1	34.1	1.75 m
		1.71 m
2	19.3	2.15 m
		1.41 m
3	38.9	1.85 br d (10.1)
		0.98 m
4	42.4	
5	52.3	1.20 br s
6	69.3	5.44 d (1.7)
7	34.8	1.75 m
		1.71 m
8	29.4	
9	53.0	
10	39.1	
11	37.8	1.51 d (11.7)
		1.14 d (11.7)
12	43.6	
13	74.7	3.33 br s
14	36.2	1.35 m
		1.21 m
15	36.3	1.35 m
		1.21 m
16	22.1	1.72 m
		1.26 m
17	23.9	0.94 s
18	29.3	0.93 s
19	181.2	
20	20.8	1.42 s
C=O	165.9	
1′	130.7	
2′, 6′	129.6	7.89 d (7.8)
3′, 5′	128.3	7.33 d (7.8)
4′	132.7	7.43 t (7.8)

a) Assignments were performed on the basis of the heteronuclear single quantum correlation (HSQC) spectrum of **7**.

activities. Therefore, **6** was subjected to the modified Mosher's ester procedure to determine the absolute configuration of the hydroxyl group at C-3.[18] Negative $\Delta\delta_{S-R}$ value for (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetic acid [(*S*)-MTPA] ester (**6b**) and (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid [(*R*)-MTPA] ester (**6a**) of **6** was obtained for H_3-20 on the left side of the MTPA plane, and positive values were obtained for H_3-19 and H-6 on the right side (Fig. 2), indicating that the absolute configuration at C-3 is *S*. Stereochemical assignments at other chiral centers of **6** were further correlated from axial H-3 [δ_H 3.11 (dd, J=8.9, 6.2 Hz)] on the basis of the NOESY spectrum. Essentially similar NOEs of **6** to those reported in ref. 5, namely from H-3 to H_3-18 (δ_H 1.03) and H-5 (δ_H 1.12), from H-5 to H-6 (δ_H 5.66), from H-2′ (δ_H 7.96) to H-19 (δ_H 0.90) and H-20 (δ_H 1.44), and from H-20 to H-8 (δ_H 2.39) confirmed the absolute structure of **6** as shown in Fig. 1. On the basis of the co-occurrence with **6**, compounds **4**, **5**, and **7** were concluded to be of the same absolute configuration.

Compounds **4**—**7** showed potent IC_{50} of 2.5, 2, 4, and 50 μg/ml, respectively, in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) cytotoxic assay against KB cells. Next, we assayed compounds **4**—**7** for their inhibition of NO production in the LPS/IFNγ-induced murine macrophages. The inorganic free radical NO is produced by the oxidation of L-arginine by NO synthase and its

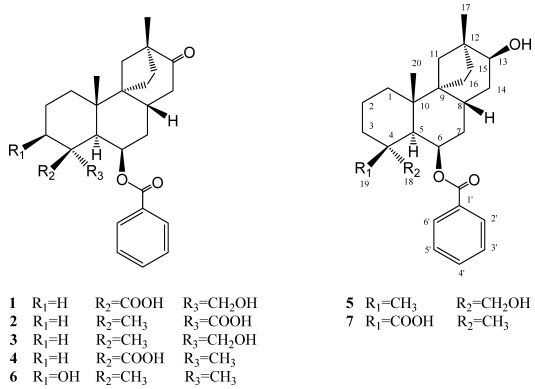

Fig. 1. Chemical Structures of **1**—**7**

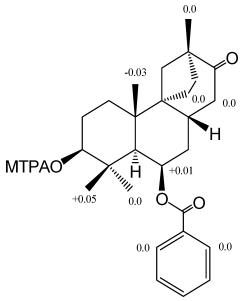

Fig. 2. $\Delta\delta_{S-R}$ Values (in ppm) Obtained for (*S*)- and (*R*)-MTPA Esters (**6b**, **a**) of **6**

overproduction can stimulate tumor growth and metastasis by promoting the migratory, invasive, and angiogenic potentials of tumor cells.[6] However, weak activities were observed for **4**—**7** with IC_{50} of 150, 50, 100, and 900 μg/ml, respectively. The multidrug resistance (MDR) was also evaluated by calcein efflux studies. The membrane transport proteins belonging to the ATP-binding cassette (ABC) family such as P-glycoprotein (Pgp), which is encoded by the *mdr1* gene, and members of the multidrug resistance associated protein family, especially MRP1, MRP2, and MRP3, are directly involved in this process.[7] The fluorescent organic anion calcein has been used as a probe of MRP activity in intact cells. In cellular studies, the acetoxymethyl ester of calcein (calcein-AM) given to cells diffused into the cells, where it was hydrolyzed to fluorescent calcein. In the present study, the diterpenoids **4**—**7** were able to moderately inhibit MRP at IC_{50} of 15, 30, 10, and 20 μg/ml, respectively. The MIC_{50} values of **4**—**7** against *Escherichia coli*, *Bacillus subtillis*, and *Candida albicans*, and **5**—**7** against *Staphylococcus aureus* and MRSA could not be determined because complete inhibition was not observed up to the concentration of 1 mg/ml. Only selective inhibition of **4** against *S. aureus* (MIC_{50} 500 μg/ml), MRSA B26 strain (MIC_{50} 125 μg/ml), and the clinical isolate MRSA K1 strain (MIC_{50} 125 μg/ml) was observed.

Experimental

General Procedure Optical rotations were measured on a Union Giken PM-101 digital polarimeter. FT-IR spectra were recorded on a Horiba FT-710 spectrophotometer. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a JEOL JNM α-400 NMR spectrometer with tetramethyl silane as the internal standard. Negative-ion HR-FAB-MS were measured on a JEOL SX-102 mass spectrometer with PEG-400 as the calibration matrix. HPLC was carried out with a JASCO PU-1580 pump and UV-2075 Plus detector (set at 254 nm) on YMC ODS columns (150×4.6 mm i.d. in analytical

and 150×20 mm i.d. in preparative scales) at the corresponding flow rates of 0.5 and 5 ml/min. Silica gel 60 (0.063—0.200 mm, Merck) and YMC ODS gel were used for open column chromatography (CC). TLC was carried out on Merck TLC glass plates (silica gel 60 F_{254}), and detected by spraying with 10% H_2SO_4 in 50% EtOH, followed by heating on a hot plate at 200 °C. (*R*)-(+)- and (*S*)-(−)-MTPAs were purchased from Nacalai Tesque (Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Cica (Japan), and 4-dimethylaminopyridine (4-DMAP) from Wako Pure Chemical Industries (Japan).

Microorganisms The microorganisms used in this study *Escherichia coli*, *Bacillus subtillus*, *Staphylococcus aureus*, *Candida albicans*, and methicillin-resistant *Staphylococcus aureus* (MRSA) B26, and the clinical isolate strain MRSA K1 from patients of Hiroshima University Hospital (Hiroshima, Japan) were from the Collection of Professor M. Sugiyama of the Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima (Japan).

Plant Material The dried aerial parts of *S. dulcis* were collected in the suburbs of Hanoi, Northern Vietnam, and identified by Dr. Tran Ngoc Ninh (Institute of Ecology and Biological Resources, Vietnam Academy of Natural Science and Technology, Hanoi, Vietnam) in January 2005. A voucher specimen (No. TNST 2005-1) is deposited in the Herbarium of the Institute of Ecology and Biological Resources.

Extraction and Isolation The dried and powdered aerial parts of *S. dulcis* (1.0 kg) were extracted with MeOH and the MeOH extract (73.6 g) obtained was successively fractionated with *n*-hexane and CH_2Cl_2. On the basis of their similar TLC separation, the *n*-hexane- (16.8 g) and CH_2Cl_2-soluble fractions (8.2 g) were combined and fractionated by silica gel CC using *n*-hexane and *n*-hexane–EtOAc (6 : 1, 3 : 1, 1 : 1). Pooled fraction eluted with *n*-hexane–EtOAc (3 : 1) (3.8 g) was subjected to successive separations on silica gel CC (*n*-hexane–EtOAc, 2 : 1) and ODS gel CC (MeOH–H_2O, 7 : 3, 4 : 1), and preparative ODS HPLC (MeOH–H_2O, 7 : 3) to give adrenalin (5.0 mg), 6-methoxybenzoxazolinone (180.5 mg), 4-*epi*-scopadulcic acid (**4**) (12.1 mg), a mixture of lupeol and betulinic acid (5.0 mg), nirtetralin (6.3 mg), niranthin (3.5 mg), and a mixture of dulcidiol (**5**) and scopadulcic acid C (**7**) (10.9 mg). The mixture of **5** and **7** was further separated by semi-preparative HPLC on a polyamine II column (YMC, 250×4.6 mm i.d.) using MeCN–H_2O (9 : 1) to afford **5** (7.0 mg) and **7** (2.3 mg). The same treatment of pooled fraction eluted with *n*-hexane–EtOAc (1 : 1) (3.8 g) gave 4′,5-dihydroxy-3,7-dimethoxyflavone (2.0 mg), acacetin (3.0 mg), 3′-hydroxy-4′,5,7-trimethoxyflavone (1.0 mg), and *iso*-dulcinol (**6**) (9.1 mg).

Iso-dulcinol (**6**): Amorphous powder. ^{1}H-NMR ($CDCl_3$)[19] δ: 0.90 (3H, s, H_3-19), 1.02 (1H, s, H_3-17), 1.03 (3H, s, H_3-18), 1.12 (1H, d, J=2.0 Hz, H-5), 1.44 (3H, s, H_3-20), 1.48 (1H, d, J=12.0 Hz, H-11a), 1.55 (1H, dt, J=12.7, 3.4 Hz, H-1a), 1.59 (1H, m, H-2a), 1.67 (1H, m, H-1b), 1.68 (3H, m, 2H-7, H-15a), 1.75 (1H, d, J=12.0 Hz, H-11b), 1.77 (1H, m, H-16a), 1.78 (1H, m, H-2b), 1.90 (1H, dd, J=16.4, 12.0 Hz, H-14a), 2.01 (1H, m, H-16b), 2.19 (1H, dd, J=16.4, 6.1 Hz, H-14b), 2.39 (1H, m, H-8), 3.11 (1H, dd, J=8.3, 6.8 Hz, H-3), 5.66 (1H, br s, H-6), 7.39 (2H, t, J=7.3 Hz, H-3′, H-5′), 7.50 (1H, t, J=7.3 Hz, H-4′), 7.96 (2H, d, J=7.3 Hz, H-2′, H-6′). ^{13}C-NMR data ($CDCl_3$) were superimposable with literature data.[5] Negative-ion HR-FAB-MS: *m*/*z* 423.2574 $[M-H]^-$ (Calcd for $C_{27}H_{35}O_4$: 423.2594).

Scopadulcic acid C (**7**): Amorphous powder. $[\alpha]_D^{25}$ −21.7° (c=0.23, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 3592—2651, 3430, 3064, 2929, 2865, 1706, 1456, 1384, 1280, 1108, 1025, 756. ^{1}H- and ^{13}C-NMR: see Table 1. Negative-ion HR-FAB-MS: *m*/*z* 439.2461 $[M-H]^-$ (Calcd for $C_{27}H_{35}O_5$: 439.2484).

Preparation of (*R*)- and (*S*)-MTPA Esters (6a, b) from *Iso*-dulcinol (6) A solution of **6** (4.1 mg) in 1 ml of dehydrated CH_2Cl_2 was reacted with (*R*)-MTPA (50 mg) in the presence of EDC (36 mg) and 4-DMAP (40 mg) and the resulting mixture was occasionally stirred at 25 °C for 24 h. After the addition of 1 ml of H_2O and 1 ml of CH_2Cl_2, the solution was washed successively with 5% HCl, $NaHCO_3$-saturated H_2O, and brine. The organic layer was dried over Na_2SO_4 and then evaporated under reduced pressure. The residue was purified by silica gel CC (*n*-hexane–EtOAc, 4 : 1) to give the ester, **6a** (2.3 mg). Using a similar procedure, **6b** (5.0 mg) was prepared from **6** (3.5 mg) with (*S*)-MTPA (50 mg), EDC (36 mg) and 4-DMAP (40 mg).

Iso-dulcinol (*R*)-MTPA Ester (**6a**): Amorphous powder. ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, s, H_3-19), 0.92 (3H, s, H_3-17), 1.03 (3H, s, H_3-18), 1.47 (3H, s, H_3-20), 1.92 (1H, dd, J=16.1, 12.2 Hz, H-14a), 2.03 (1H, br d, J=13.9 Hz, H-16a), 2.20 (1H, dd, J=16.1, 6.4 Hz, H-14b), 3.48 (3H, s, –OCH_3), 4.59 (1H, br s, H-3), 5.64 (1H, br s, H-6), 7.19—7.50 (8H, m, aromatic protons), 7.93 (2H, d, J=7.2 Hz, H-2′, H-6′).

Iso-dulcinol (*S*)-MTPA Ester (**6b**): Amorphous powder. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, s, H_3-19), 0.92 (3H, s, H_3-17), 1.03 (3H, s, H_3-18), 1.44 (3H, s, H_3-20), 1.92 (1H, dd, J=16.1, 11.7 Hz, H-14a), 2.03 (1H, br d, J=13.4 Hz, H-16a), 2.21 (1H, dd, J=16.1, 5.9 Hz, H-14b), 3.48 (3H, s, –OCH_3), 4.67 (1H, br s, H-3), 5.65 (1H, br s, H-6), 7.19—7.50 (8H, m, aromatic protons), 7.93 (2H, d, J=7.2 Hz, H-2′, H-6′).

Antibacterial Susceptibility Assay Susceptibility tests were performed using a broth microdilution assay according to National Committee for Clinical Laboratory Standards (NCCLS) reference methods.[20] Assays were performed using Mueller-Hinton broth (Difco). The bacterial inocula were adjusted to yield a density of 5×10^5 colony forming units (CFU)/ml. Samples were diluted directly in 96-well microtiter plates by serial 2-fold dilution using a multichannel pipette. Microtiter plates were incubated during 24 h at 35 °C and were read using a microtiterplate reader (Molecular Devices) at 620 nm as well as by visual observation. The MIC_{50} was determined as a 50% decrease in the optical density. Amphotericin B, penicillin G, and streptomycin were used as positive controls.

Antifungal Succeptibility Assay against *Candida albicans* Broth microdilution testing was performed according to the guidelines in NCCLS document M27-A2[21] by using the spectrophotometric method of inoculum preparation, an inoculum concentration of 1.5×10^3 cells/ml, and RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer. Samples were diluted directly in 96 well microtiter plates by serial 2-fold dilution using a multichannel pipette. Microtiter plates were incubated for 24 h at 35 °C and were read using a microtiterplate reader (Molecular Devices) at 620 nm. Amphotericin B, penicillin G, and streptomycin were used as positive controls.

MTT Cytotoxicity Assay This assay was performed using human epidermoid carcinoma KB cells and the viability was estimated by the colorimetric MTT assay.[22] Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/ml of kanamycin and 0.5 μg/ml of amphotericin B were used as the cell culture medium. The test compounds were dissolved in DMSO and added to the each well of the 96-well micro-titration plates at 1% as the final concentration. KB cells (5×10^3 cells/well) were cultured in a CO_2 incubator at 37 °C for 72 h and then MTT solution was added to each well and the plates incubated for a further 4 h. The viability was compared to that of control cells incubated in the same medium without the test compounds. The cytotoxic activity was evaluated as the IC_{50} (the concentration of the compound required for 50% inhibition of cell growth). Mitomycin C was used as a positive control, which showed 50% inhibition at 10 ng/ml.

Measurements of NO Production in LPS/IFNγ-Induced Murine Macrophage Cells Murine macrophage cell line RAW 264.7 was obtained from American Type Cell Culture Collection. RAW 264.7 cells were cultured in plastic dishes containing DMEM supplemented with 10% FBS in a humidified incubator containing 5% CO_2. The cells were cultured in 96-well plates (5×10^5) containing DMEM supplemented with 10% FBS for 24 h to become nearly confluent. The cells were then cultured with DMSO as a vehicle or samples in the presence of stimuli of LPS (10 μg/ml)/IFNγ (20 ng/ml) for 24 h. Nitrite production, an indicator of NO synthesis, was measured in the culture supernatant of macrophages, as described previously[23] using Griess reagent. Optical density at 550 nm was measured using a microplate reader (Molecular Devices).

Calcein Efflux Assay Calcein efflux assay was performed using a previously described method[24] with minor modifications to exclude possible side effects of test compounds on the uptake of calcein. A human erythroleukemia cell line, K562, was used for this assay. The cells were labeled with calcein-AM (Invitrogen) according to the instructions of the manufacturer. Then 2×10^4 labeled cells were seeded in each well of 96-well microtiter plates with or without the test compounds. After a 4 h culture, the supernatant was harvested and the released fluorescence with excitation at 485 nm and emission at 530 nm was measured. Verapamil (10 μM) and probenecid (2.5 mM) were used as the reference compounds and showed 71.7% and 37.0% inhibition, respectively.

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