A New Monoterpenoid Glycoside from *Myrica esculenta* and the Inhibition of Angiotensin I-Converting Enzyme

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Received July 4, 2010; accepted July 23, 2010; published online July 26, 2010

One new monoterpenoid glycoside, myresculoside (1), and eleven known compounds, were isolated from methanol extract of *Myrica esculenta* leaves by repeated column chromatography. The effects of these compounds on angiotensin I-converting enzyme (ACE) inhibition were investigated. Compounds 3 and 4 showed the most potent ACE inhibition with rates of 29.97% and 25.63% at concentration of 100 μM, respectively. Compounds 5, 6, and 11 showed weak activity with inhibitory rates of 0.07—1.41% at concentration of 100 μM.

Key words *Myrica esculenta*; myresculoside; angiotensin I-converting enzyme inhibitor; antihypertensive

The renin angiotensin system (RAS) is a hormone system that regulates blood pressure and water balance. Excessive activation of this system is considered to be the main cause of hypertension.[1)] Angiotensin I-converting enzyme (ACE) is the most important regulatory site of RAS. ACE catalyses the conversion of angiotensin I, an inactive decapeptide, into angiotensin II, an octapeptide with potent vasoconstrictive activity. In fact, a number of synthetic ACE inhibitors have been widely used in patients with cardiac failure and renal disease. Moreover, ACE inhibitors have been identified and isolated from a wide range of plant and animal sources.[2—4)]

Myrica esculenta Buch-Ham. ex. D. Don. (Myricaceae) is abundant found in India, South China, Japan, Malaysia, and Vietnam. In Vietnam, the plant is widely distributed in mountainous areas at altitudes ranging from 1000 to 2200 m above sea level. The barks are used in Vietnamese folk medicine to treat catarrhal fever, cough, sore throat, and skin disease.[5)] Several studies on the chemical constituents of *M. esculenta* have reported the presence of triterpenoids[6)] and tannins.[7)] We have been studying the effects of components of Myrica species on cardiovascular diseases. Here we report the isolation of a new monoterpenoid glycoside and eleven known compounds from *M. esculenta*, and discuss their ACE inhibitory activity.

Results and Discussion

Dried leaves of *M. esculenta* were extracted with MeOH and fractionated with chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water. From these extracts and subsequently separation, one new and eleven known compounds were isolated and their structures were elucidated using physicochemical and spectroscopic methods. (Fig. 1)

Compound **1** was obtained as an amorphous powder and its molecular formula was determined to be $C_{21}H_{36}O_{11}$ by electrospray ionization mass spectrometry (EI-MS) at *m*/*z* 487 $[M+Na]^+$ (positive) and high-resolution electrospray ionization mass spectrometry (HR-EI-MS) at *m*/*z* 465.2360 $[M+H]^+$ (Calcd $C_{21}H_{37}O_{11}$ for 465.2336). The ^{1}H-NMR spectrum of **1** (methanol-d_4) showed signals for three tertiary methyl groups at δ 0.94 (3H, s) and 1.19 (6H, s), and two anomeric protons at δ 4.36 (1H, d, J=8.4 Hz) and 4.90 (1H, d, J=2.4 Hz) suggesting the presence of two sugar units, as listed in Table 1. The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra revealed 21 carbon signals, 10 of which were assigned to a monoterpenoid moiety, with the remaining 11 assigned to the two monosaccharide moieties. The aglycone of **1** was concluded to be 4-hydroxy-1,8-cineole.[8)] Moreover, acid hydrolysis of **1**

Table 1. The NMR Spectral Data of Compound **1** in CD_3OD

Pos.	**1**	
	δ_C [a)]	δ_H [b)] mult. (J in Hz)
Aglycone		
1	71.3	—
2	34.5	1.71 (m)
3	29.0	1.83 (m)
		2.08 (m)
4	77.6	—
5	26.5	1.91 (m)
6	34.3	1.77 (m)
7	26.9	0.94 (s)
8	78.2	—
9	25.7	1.19 (s)
10	25.6	1.19 (s)
4-Glc		
1′	98.4	4.36 (d, 8.4)
2′	75.2	3.03 (dd, 8.4, 9.0)
3′	78.5	3.21 (t, 9.0)
4′	71.9	3.11 (t, 9.0)
5′	76.4	3.27 (m)
6′	68.8	3.45 (dd, 6.6, 11.4)
		3.85 (dd, 1.8, 11.4)
(1″→6″) Api		
1″	110.9	4.90 (d, 2.4)
2″	78.0	3.78 (d, 2.4)
3″	80.6	—
4″	74.9	3.66 (d, 9.6)
		3.86 (d, 9.6)
5″	65.6	3.49 (s)

a) 150 MHz, *b*) 600 MHz. Assignments were done by HMQC, HMBC, COSY and ROESY experiments; glc, β-D-glucopyranosyl; api, β-D-apiofuranosyl.

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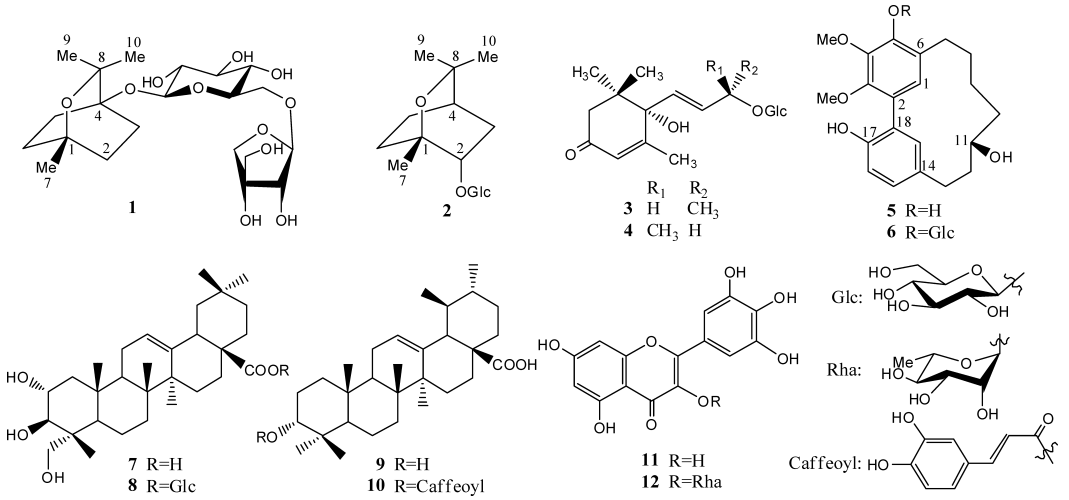

Fig. 1. Structures of **1**—**12**

Table 2. Angiotensin I-Converting Enzyme Inhibitory Activities of Compounds **1**—**12**

Compounds	ACE inhibition rate (%)
1	0
2	0
3	29.97±4.77
4	25.63±1.35
5	1.61±1.35
6	0.07±1.47
7	0
8	0
9	0
10	0
11	1.41±1.71
12	0
Captopril[a)]	88.64±2.57

a) Captopril (4.6 μM) was used as positive control. Percentage of enzyme inhibition of compounds at the concentration of 100 μM. Data presented is the mean±S.D. of samples run in triplicate.

revealed D-apiose and D-glucose as sugar components (identified as trimethylsilyl (TMS) derivatives by a gas chromatography (GC) method).

The ^{13}C-NMR spectral data of **1** showed the presence of one β-apiofuranosyl moiety[9)] and one β-glucopyranosyl moiety. The heteronuclear multiple bond correlations (HMBC) between the glc H-1′ (δ 4.36) and C-4 of the aglycone (δ 77.6), between api H-1″ (δ 4.90) and glc C-6′ (δ 68.8), between glc H-6′ (δ 3.45, 3.85) and api C-1″ (δ 110.9) were observed (see Fig. 2). These observations suggested the sequence of sugar linkages of **1** and sugar moiety located at C-4 of the aglycone. The carbon signals of the sugar moiety were superimposable on those of characteristic monoterpenoid glycosides isolated from *Glehnia littoralis*.[9)] Consequently, the structure of **1** was determined to be 4-hydroxy-1,8-cineole 4-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, a new compound named myresculoside.

The other compounds were characterized as (1*S*,2*S*,4*R*)-2-hydroxy-1,8-cineole β-D-glucopyranoside (**2**),[8)] corchoionoside C (**3**),[10)] (6*S*,9*R*)-roseoside (**4**),[10)] myricanol (**5**),[11)] 5-*O*-β-D-glucopyranosyl myricanol (**6**),[12)] arjunolic acid (**7**),[13)] arjunglucoside (**8**),[13)] 3-*epi*-ursonic acid (**9**),[14)] 3-*O*-(*E*)-caffeoylursonic acid (**10**),[15)] myricetin (**11**),[16)] and myricitrin

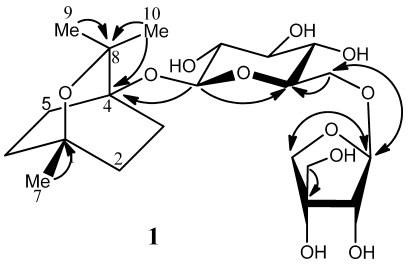

Fig. 2. Selected HMBC Spectrum of Compound **1**

(**12**)[17)] (see Fig. 1). Their structures were established on the basis of spectral and chemical evidence, which were in good agreement with those reported in the literature. Compounds **2**—**4**, **8**, and **10** were the first time isolated from this genus.

The rabbit lung ACE inhibitory activities of these twelve compounds were compared (see Table 2). All samples were prepared on a constant volume basis (100 μM). Among the 12 compounds, two megastigmanes (**3**, **4**) showed high ACE inhibitory activity with rates of 29.97% and 25.63%, respectively, at concentration of 100 μM, two phenylheptanoids (**5**, **6**) and one flavonoid (**11**) showed weak activity. Other compounds, including triterpenoids and monoterpenoids and their glycosides showed no activity. Control of hypertension by inhibition of ACE with antihypertensive agent such as Captopril® may be an important strategy to manage hypertension, which is a cardiovascular disease factor and often results from long-term diabetes mellitus. Therefore, the above results provide a strong biochemical rationale for clinical studies focused on megastigmanes (**3**, **4**) of *M. esculenta* for the management of hypertension.

Experimental

General Experimental Procedures Optical rotations were determined on a Jasco DIP-370 automatic polarimeter (Jasco, Tokyo, Japan). Preparative HPLC was conducted using a Waters HPLC system (600 pump, 600 controller, and 996 photodiode array detector; Waters Corporation, Milford, MA, U.S.A.). The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz; JEOL, Tokyo, Japan), and FAB-MS was performed using a JEOL JMS-HX/HX110A tandem mass spectrometer. EI-MS was performed on an AGILENT 1200 Series LC-MSD Trap spectrometer (Agilent Technologies, Palo Alto, CA, U.S.A.). GC spectra were recorded on a Shidmazu-2010 spectrometer (Shimadzu, Kyoto, Japan). Column chromatography was performed using a silica-gel (Kieselgel 60, 70—

230 mesh and 230—400 mesh, Merck, Whitehouse Station, NJ, U.S.A.) or YMC RP-18 resins (30—50 μm, Fujisilisa Chemical Ltd., Kasugai, Aichi, Japan), and thin layer chromatography (TLC) using pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

Plant Material The leaves of *M. esculenta* were collected in Hoabinh Province, Vietnam in June, 2006, and identified by Dr. Ninh Khac Ban (Institute of Ecology and Biological Resources, VAST). A voucher specimen (ME0606) was deposited at the Herbarium of Institute of Marine Biochemistry, VAST, Vietnam.

Extraction and Isolation The dried leaves of *M. esculenta* (2.0 kg) were extracted with MeOH three times under reflux for 15 h to yield 240 g of a dark solid extract, which was then suspended in water and successively partitioned with chloroform ($CHCl_3$) and ethyl acetate (EtOAc) to obtain $CHCl_3$ (ME1, 79.4 g), EtOAc (ME2, 60.3 g), and water (ME3, 72.5 g) extracts after removing solvent *in vacuo*. ME1 was chromatographed on a silica gel column and eluted with *n*-hexane-acetone gradient (100 : 1-1 : 1, v/v) to obtain four subfractions, ME1A (30.5 g), ME1B (16.4 g), ME1C (18.0 g), and ME1D (10.1 g). The ME1B fraction was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (30 : 1, v/v) to obtain **9** (8.0 mg) and **10** (6.4 mg). The ME1C fraction was further chromatographed on a silica gel column eluting with CH_2Cl_2-MeOH (20 : 1, v/v) to give three smaller fractions, ME1C1—ME1C3. The ME1C2 fraction was chromatographed on an YMC RP-18 column eluting with acetone–water (5 : 1, v/v) to yield **5** (8.6 mg) and **7** (6.0 mg). ME2 was chromatographed on a silica gel column and eluted with $CHCl_3$-MeOH gradient (50 : 1-1 : 1, v/v) to obtain four subfractions, ME2A—ME2D. The ME2B fraction was chromatographed on a silica gel column eluting with acetone–MeOH (8 : 1, v/v) to yield **11** (8.0 mg). The ME2C fraction was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (6 : 1, v/v) to give three smaller fractions, ME2C1—ME2C3. Fraction ME2C1 was chromatographed on an YMC RP-18 column eluting with MeOH-water (3 : 1, v/v) to yield **6** (7.0 mg) and **12** (8.5 mg). The water soluble fraction ME3 was chromatographed on a Diaion HP-20P column (Mitsubishi Chem. Ind. Co., Tokyo, Japan) eluting with water containing increasing concentrations of MeOH (100% H_2O, 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH) to obtain four fractions, ME3A—ME3D. Fraction ME3B was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH-H_2O (5 : 1 : 0.1, v/v/v) to give three fractions, ME3B1—ME3B3. The ME3B2 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2-MeOH-H_2O (5 : 1 : 0.1, v/v/v) to obtain compound **8** (14.0 mg). The ME3B3 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2-acetone-H_2O (1 : 2.5 : 0.1, v/v/v) to obtain the new compound **1** (50.0 mg) and compound **2** (18.5 mg). Fraction ME3D was chromatographed on a silica gel column using CH_2Cl_2-MeOH-H_2O (6 : 1 : 0.05, v/v/v) to give four fractions ME3D1—ME3D4. The ME3D4 fraction was further separated on a YMC RP-18 column eluting with acetone-MeOH-H_2O (1 : 2 : 2, v/v/v) to obtain compounds **3** (5.0 mg) and **4** (8.0 mg).

Myresculoside (**1**): An amorphous white powder, $[\alpha]_D^{25}$ −14.3° (c=0.5, MeOH); positive EI-MS *m/z*: 503 $[M+Na]^+$, HR-EI-MS Found *m/z*: 465.2360 $[M+H]^+$ (Calcd $C_{21}H_{37}O_{11}$ for 465.2336); and ^{1}H- and ^{13}C-NMR: see Table 1.

Acid Hydrolysis of 1 Compound **1** (2.0 mg) was dissolved in 1.0 N HCl (dioxane–H_2O, 1 : 1, v/v, 1.0 ml) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 ml of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 ml) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 ml of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 ml, each), and the organic layer was analyzed by GC: Column: column of SPB-1 (0.25 mm×30 m); detector FID, column temp 210 °C, injector temp. 270 °C, detector temp. 300 °C, carrier gas He (2.0 ml/min). The retention times of persilylated glucose and apiose were founded to be 14.11 and 6.70 min, respectively, when compared with the standard solutions prepared by the same reaction from the standard monosaccharides. The retention times of persilylated D-glucose, L-glucose, D-apiose, and L-apiose rhamnose were 14.11, 14.26, 6.70, and 6.95 min, respectively.

Angiotensin I-Converting Enzyme (ACE) Inhibition Assay ACE inhibition was assayed by a modification of the method of Kwon *et al.*[18] The substrate, hippuryl-histidyl-leucine (HHL) and ACE from rabbit lung (EC 3.4.15.1) were purchased from Sigma (St. Louis, MO, U.S.A.). Aliqouts (50 μl) of each sample were incubated with 100 μl of 1.0 M NaCl-borate buffer (pH 8.3) containing 2.0 mU ACE solution at 37 °C for 10 min. Then 100 μl of 5.0 mM HHL was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μl of 0.5 N HCl. The hippuric acid formed was detected and quantified by HPLC. A volume of 5 μl of each sample was injected using Agilent an ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies) equipped with DAD 1100 diode array detector. The solvents used for gradient were 10 mM phosphoric acid (pH 2.5) and 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for the next 5 min, then decreased to 0% for last 5 min (total run time: 18 min). The analytical column used was a Nucleosil 100-5C18, 250×4.6 mm i.d., using with packing material with a particle size of 5 μm at a flow rate 1 ml/min at ambient temperature. During each run the chromatogram was recorded at 228 nm and integrated using Agilent Chemstation enhanced integrator to detect liberated hippuric acid. Pure hippuric acid (purchased from Sigma Chemical Co.) was used to calibrate the standard curve and retention time. The percent inhibition was calculated as follows:

$$\%\ \text{inhibition} = \left(\left[\frac{E^{\text{Control}} - E^{\text{Sample}}}{E^{\text{Control}} - E^{\text{Blank}}} \right] \right) \times 100$$

Acknowledgments This study was supported by the Priority Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815), Republic of Korea. The authors would like to thank the Korean Basic Science Institute (KBSI) for performing the NMR experiments.

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