Acylated Kaempferol Glycosides from Laurus nobilis Leaves and Their Inhibitory Effects on Na\(^+/K^+\)-Adenosine Triphosphatase

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Na\(^+/K^+\)-adenosine triphosphatase (ATPase) inhibitors have considerable therapeutic potential against some heart diseases like congestive heart failure and cardiac arrhythmias. Through bioassay-guided separation of the leaf extract of Laurus nobilis, six acylated kaempferol glycosides (compounds 1—6) were isolated. Their structures were determined on the basis of spectroscopic analysis and comparison with reported data. All the isolates were subjected to in vitro bioassays to evaluate their inhibitory activities against Na\(^+/K^+\)-ATPase from porcine cerebral cortex and bacterial growth. These studies led to the identification of compounds 1—6 as potent Na\(^+/K^+\)-ATPase inhibitors, with IC\(_{50}\) values in the range of 4.0±0.1—10.4±0.6 μM. These compounds also exhibited a broad spectrum of antibacterial activity. In particular, compounds 4 and 6 showed potent inhibitory activities against several bacterial strains, except Escherichia coli, with minimum inhibitory concentration (MIC) values in the range of 0.65—2.08 μg/mL. Thus, L. nobilis-derived acylated kaempferol glycosides may have a potential to be leads for the development of Na\(^+/K^+\) ATPase inhibitors (1—6) and antibacterial agents (4, 6).

Key words Laurus nobilis; acylated kaempferol glycoside; Na\(^+/K^+\)-adenosine triphosphatase; antibacterial activity

Na\(^+/K^+\)-adenosine triphosphatase (ATPase) is an ubiquitous sodium pump in the membrane of most eukaryotic cells, which is essential to establish and maintain high K\(^+\) and low Na\(^+\) concentration in the cytoplasm. The establishment of an electrochemical gradient for Na\(^+\) across the plasma membrane is vital for cell functions as diverse as the propagation of nerve signals, volume regulation, nutrient absorption, and pH regulation. Since this pump is the only known receptor for toxic cardiac glycosides such as digoxin and ouabain, which are used to treat some heart diseases like congestive heart failure and cardiac arrhythmias, a new type of less toxic natural regulator of this pump might be useful for clinical purposes.

Laurus nobilis L. (fam. Lauraceae), known commonly as Bay, Sweet Bay, True Laurel or Roman Laurel, is natively distributed in the Mediterranean Basin and has been used as a fixture in European and North American cuisines and also as a flavor in many classic French dishes. Previous phytochemical investigations on L. nobilis leaves and fruits have resulted in the isolation of sesquiterpene lactones, alkaloids, glycosylated flavonoids, and monoterpenes. Roots and leaves are also a source of sesquiterpene lactones, and two distinct chemical races were found containing, respectively, laurenobiolide and costunolide as major compounds. It has been reported that L. nobilis has analgesic, anti-inflammatory, anti-convulsant and antioxidant activities. From the essential oil, various volatile components were identified with antimicrobial activities against bacteria, yeast, and some molds.

Our group has been interested in the search for biologically active secondary metabolites from natural resources. During our continuing program with this aim, we encountered the L. nobilis leaves whose crude extract exhibited significant inhibitory activity toward Na\(^+/K^+\)-ATPase. In this paper, we report the inhibition of Na\(^+/K^+\)-ATPase from porcine cerebral cortex by the total extract of L. nobilis, along with the isolation of the active compounds involved and their structure–activity relationships. In addition, we investigated the inhibitory effects of these compounds against several representative bacteria.

MATERIALS AND METHODS

Chemicals The ¹H- and ¹³C-NMR spectra were recorded on Bruker AMX-500 and Varian Gemini 2000 spectrometers in MeOD solutions. Mass spectrometric data were obtained from the Korea Basic Science Institute (Daegu) on a Jeol JMS 700 mass spectrometer. Optical rotations were measured on a JASCO P-1020 polarimeter using a 1 cm cell. UV spectra were recorded on a Hitachi U-3010 spectrophotometer, and IR spectra were recorded on a JASCO 300E FT-IR spectrometer. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All solvents used were spectral grade or distilled from glass prior to use.

Plant Materials The plant material of Laurus nobilis was acquired as leaves of the trees purchased from Turkey (Orege Forest Agricultural and Food Products Foreign Trade Ltd.) in August 2007, and verified by Dr. H.-J. Lee, Korea Forest Research Institute, Seoul, Korea. A voucher specimen (No. NPRI-Q003) has been deposited at the herbarium in the Natural Products Research Institute, Seoul National University, Seoul, Korea.

Isolation and Identification The dried leaves of Laurus nobilis (1.5 kg) were extracted repeatedly with CH\(_2\)Cl\(_2\) (2 L×2) and MeOH (2 L×2). The combined crude extract (270 g) was partitioned between 10% aq MeOH (196.3 g) and n-hexane (57.8 g) and then former was re-partitioned between H\(_2\)O (116.8 g) and CHCl\(_3\) (67.8 g). The CHCl\(_3\) layer was subjected to silica normal-phase vacuum flash chromatography using sequential mixtures of n-hexane and EtOAc as eluents. The fraction eluted with 70% EtOAc in n-hexane from flash chroma-
tography (481 mg) was separated by semi-preparative HPLC (YMC ODS-A column, 1 cm×25 cm, 30% aqueous MeOH) to yield in order of elution, compounds 1—6 as amorphous solid. Final purification of individual compound was then accomplished by HPLC (YMC CN column, 1 cm×25 cm, 50% aqueous MeOH) to afford 9.4, 4.6, 36.4, 41.3, 157.2, and 52.3 mg of 1—6, respectively.

**Alkaline Hydrolysis of Compound 5** Nonacylated afzelin (kaempferol-3-O-rhamnopyranoside) was prepared from compound 5 by alkaline hydrolysis method.\(^1\) A sample of compound 5 (7.0 mg) in 10% methanolic KOH (2 mL) and MeOH (5 mL) was refluxed for 5 h at 60°C. After neutralization with 1 n HCl (5 mL), the solvent was evaporated to dryness and the residue extracted with MeOH. The extract was purified by HPLC (YMC CN column, 0.46 cm×25 cm, 60% aqueous MeOH) to afford 2 mg of afzelin (kaempferol-3-O-rhamnopyranoside). \(^1\)H-NMR data of Afzelin (acetone-\(_6\)) δ: 12.71 (1H, s), 7.85 (2H, d, \(J=8.7 \text{ Hz}\)), 7.01 (2H, d, \(J=8.7 \text{ Hz}\)), 6.47 (1H, \(d, J=2.3 \text{ Hz}\)), 6.26 (1H, d, \(J=2.3 \text{ Hz}\)), 5.53 (1H, d, \(J=1.5 \text{ Hz}\)), 4.20 (1H, dd, \(J=3.3, 1.5 \text{ Hz}\)), 3.67 (1H, dd, \(J=8.7, 3.6 \text{ Hz}\)), 3.30 (2H, m), 0.88 (3H, d, \(J=5.7 \text{ Hz}\)).

**Assay for Na\(^+\)/K\(^+\)-ATPase Inhibitory Activity** The inhibitory activity of compounds 1—6 against Na\(^+\)/K\(^+\)-ATPase from porcine cerebral cortex (SigmaChemical Co.) was measured by a fluorometric method.\(^1\) This method is based on the online determination of change in fluorescence due to the formation of the fluorescent compound 3-O-methylfluorescein from the parent compound 3-O-methylfluorescein phosphate. The reaction mixture containing 50 μM 3-O-methylfluorescein phosphate, 50 mM creatinine phosphate, 4 mM MglCl\(_2\), 0.5 mM ethylene glycol bis(aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), and 80 mM Tris–HCl (pH 7.2) was prewarmed at 37°C. Subsequently, 0.005 unit of Na\(^+\)/K\(^+\)-ATPase from porcine cerebral cortex was added. To activate the Na\(^+\)/K\(^+\)-ATPase, 10 μL of 0.01 M KCl was added, giving a final concentration of 10 mM KCl, and incubated for 30 min. Fluorescence was assayed in SpectraMAX Genini XS fluorometer (Molecular Devices Co.) with the reaction mixture in a thermostatically controlled 96-well plate. The excitation wavelength was 470 nm, and the emission wavelength was 510 nm. IC\(_{50}\) values were expressed as the drug concentrations inhibiting enzyme activity by 50% by setting the fluorescence in the absence of an inhibitor as 100%. Ouabain (Sigma Chemical Co.) was used as a positive control; all experiments were carried out in triplicate.

**Assay for Antibacterial Activity** The isolated compounds were evaluated against a panel of bacterial strains *Staphylococcus aureus* (ATCC 6538p), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (IFO 12708), *Proteus vulgaris* (ATCC 3851), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* (ATCC 35270). Bacteria were grown overnight in Standard methods (SM) broth (Difco Lab.) at 37°C, harvested by centrifugation, and then washed twice with sterile distilled water. Each test compound was dissolved with SM broth in 96-well plates to prepare serial twofold dilutions in the range of 100—0.05 μg/mL. Ten microliters of the broth containing approximately 10\(^5\) cfu/mL of test bacteria was added to each well of 96-well plate. Culture plates were incubated for 24 h at 37°C. Optical density (OD) was tested at 600 nm to measure bacterial growth as negative control showing 100% OD. The minimum inhibitory concentration (MIC) values were calculated as the lowest concentration showing 100% growth inhibition of the strains OD values. Ampicillin and ciprofloxacin were used as positive controls; all experiments were carried out in triplicate.

**RESULTS AND DISCUSSION**

**Purification and Identification of Effective Compounds** The combined crude extract (CH\(_2\)Cl\(_2\) and MeOH) of the leaves of *L. nobilis* was divided into fractions soluble in 10% acq MeOH and \(n\)-hexane and then former was re-partitioned between \(H_2O\) and CHCl\(_3\). The CHCl\(_3\) fraction was active against a (porcine cerebral cortex) Na\(^+\)/K\(^+\)-ATPase from showing 97% inhibition at 100 μg/mL. This fraction subjected to silica normal-phase vacuum flash chromatography. The fraction eluted with 70% EtOAc in \(n\)-hexane was separated by semi-preparative HPLC to yield six active compounds. The molecular structures of all isolates were determined using spectroscopic evidence and comparison with literature data. The metabolites were identified as kaempferol-3-O-α-L-(3′,4′-di-E-di-p-coumaroyl)-rhamnopyranoside (1), kaempferol-3-O-α-L-(3′,4′-di-Z-p-coumaroyl)-rhamnopyranoside (2), kaempferol-3-O-α-L-(3′,4′-di-E-p-coumaroyl)-rhamnopyranoside (3), kaempferol-3-O-α-L-(2′,4′-di-Z-p-coumaroyl)-rhamnopyranoside (5), and kaempferol-3-O-α-L-(2′,4′-di-E-p-coumaroyl)-rhamnopyranoside (6). The spectroscopic data (\(^1\)H-NMR, MS) of these compounds were in good agreement with those reported previously.\(^5\) The chemical structures of compounds 1—6 isolated from *L. nobilis* leaves are shown in Fig. 1.

**Na\(^+\)/K\(^+\)-ATPase Inhibitory Activities of Compounds 1—6** The *in vitro* inhibitory activities, expressed as IC\(_{50}\) values, of the compounds 1—6 against Na\(^+\)/K\(^+\)-ATPase are shown in Table 1 and are compared to that of a known Na\(^+\)/K\(^+\)-ATPase inhibitor, ouabain (IC\(_{50}\) 4.6±0.1 μM).\(^1\) As shown in Table 1, compounds 1—6 exhibited potent Na\(^+\)/K\(^+\)-ATPase inhibitory activities, with IC\(_{50}\) values in the range of 4.0±0.1—10.4±0.6 μM. In particular, the inhibitory activity of the compound 4 (IC\(_{50}\) 4.0±0.1 μM) was similar to that of ouabain. The compounds 1—6 consist of kaempferol, rhamnose, and coumaroyl moieties. To understand more about the activity of these compounds, nonacylated afzelin (kaempferol-3-O-rhamnopyranoside) was separated from the crude extract by semi-preparative HPLC, and the IC\(_{50}\) values were determined. As shown in Table 1, the nonacylated afzelin (IC\(_{50}\) 4.0±0.1 μM) was less active than the acylated one (IC\(_{50}\) 4.6±0.1 μM). This result suggests that the acylation of the rhamnopyranoside moiety plays an important role in the inhibition of the Na\(^+\)/K\(^+\)-ATPase.
nopyranoside)\textsuperscript{11} was prepared from compound 5 (kaempferol-3-O-\textalpha-L-(3',4'-di-E-p-coumaroyl)-rhamnopyranoside) by alkaline hydrolysis method.\textsuperscript{12} The inhibitory activities of \( p \)-coumaric acid and two nonacylated parent compounds (kaempferol and afzelin) were investigated. \( p \)-Coumaric acid and nonacylated compounds, however, did not show Na\(^+\)/K\(^+\)-ATPase inhibitory activity (Table 1). These results suggest that each one of these compounds alone is not enough for the inhibition of Na\(^+\)/K\(^+\)-ATPase. Thus, it seems that the intact structures of compounds 1—6 are important for exerting Na\(^+\)/K\(^+\)-ATPase inhibitory activity. Comparison of the structure–activity relationship for the acylated kaempferol glycosides indicated that compound 3, which has \( E \)-\( p \)-coumaroyl group at C-3\(^{\prime\prime}\) in the rhamnopyranoside ring, was more active than compounds 1 and 2, which contain \( Z \)-\( p \)-coumaroyl groups at C-3\(^{\prime\prime}\) position, respectively. The compounds 5 and 6 showed almost the same IC\(_{50}\) values against Na\(^+\)/K\(^+\)-ATPase. As in compound 4, which was the most potent inhibitor of the compounds tested, the importance of \( E \)-\( p \)-coumaroyl group at C-2\(^{\prime\prime}\) and \( Z \)-\( p \)-coumaroyl group at C-4\(^{\prime\prime}\) in the rhamnopyranoside ring for high inhibitory capacity of acylated kaempferol glycosides was found in this study.

**Antibacterial Activities of Compounds 1—6** The antibacterial activity of the compounds 1—6 were also evaluated against several representative Gram-positive and Gram-negative bacteria using micro-broth dilution method. As shown in Table 2, compounds 1—6 showed potent inhibitory activity against Gram-positive and Gram-negative bacteria, except *Escherichia coli*. In particular, compounds 4 and 6 exhibited strong antibacterial activities with MIC values in the range of 0.65—2.08 \( \mu \)g/mL. The activity of 4 and 6 was a little weaker than those of ampicillin. In antibacterial activity
inhibitory activity in the test concentration range. These results also indicate that the intact structures of compounds 1—6 are required for exerting antibacterial activity. By comparing chemical structures of these compounds, it was found that compounds 4 and 6, which have p-coumaroyl groups at C-2' and C-4' in the rhamnopyranoside ring, were more active than compounds 1—3, which contain p-coumaroyl groups at C-3' and C-4' positions (Fig. 1). In addition, compound 6 (MICs = 0.65—1.56 μg/mL), which has Z-p-coumaroyl group at C-2', was more active than compound 5 (MICs = 6.25—25 μg/mL), which contain E-p-coumaroyl group at C-2' position. Taken together, our data indicated that the Z-p-coumaroyl moiety at C-2' and E-p-coumaroyl moiety at C-4' of acylated kaempferol glycosides might be important for potent antibacterial activity.

In previous studies, compounds 5 and 6 isolated from L. nobilis were found to exhibit strong antibacterial activity against methicillin-resistant (MRSA, MICs = 1—2 μg/mL) and methicillin-sensitive Staphylococcus aureus (MSSA, MIC = 0.5 μg/mL),17 and the effects of these compounds with fluoroquinolones were found to be synergistic.18 The MIC of compound 6 (0.65 μg/mL) in this study (Table 2) was similar to that of MSSA in a previous study, even though our study was performed using different bacterial strains. The MIC of compound 5 (25 μg/mL) for S. aureus, however, was significantly higher compared to the reported value.17 Although the effects of compound 5 on S. aureus strains are not completely understood, the difference of MIC values may be partially due to the effects of culture media and/or bacterial strain. It has been well known that fluoroquinolones including ciprofloxacin exert their antibacterial activities on cells by inhibiting with the DNA gyrase and topoisomerase IV.19 Glycosylated flavonoids and flavonoids including kaempferol have been reported to show effects as inhibitors of bacterial type II topoisomerases, such as DNA gyrase and topoisomerase IV.19,20 These previous studies indicated a possible interaction of acylated kaempferol glycosides or their cleavage product with the DNA gyrase and topoisomerase IV. In our study, all bacterial strains tested were sensitive to ciprofloxacin (Table 2). In contrast, compounds 1—6, nonacylated compounds and p-coumaric acid did not exhibit inhibitory activities against E. coli. Currently, we cannot fully understand this result, and are not in a position to elucidate the action mechanism of these compounds. Therefore, a more advanced chemical and biological study for target molecular will be needed to identify the action mechanism of acylated kaempferol glycosides against this bacteria.

In conclusion, we discovered that acylated kaempferol glycosides 1—6 isolated from L. nobilis leaves inhibit Na+/K+ -ATPase. We also found that the intact structures of these compounds are important for exerting Na+/K+ -ATPase inhibitory activity. The isolated compounds 4 and 6 also exhibited strong and broad spectrum of antibacterial activities. These results indicated that acylated kaempferol glycosides might serve as leads to develop potent Na+/K+ ATPase inhibitors and antibacterial agents.

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