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NA^+-K^+-ATPASE INHIBITORS FROM *LYSIMACHIA JAPONICA*

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In continuation of our research for bioactive compounds in plants, we have found that the methanolic extract of *Lysimachia japonica* Thumb (Primulaceae) markedly inhibited the Na^+-K^+-ATPase activity. The whole plants of the species have been used medicinally as an antitussive (1). Previously, salicylic acid and some common flavonoids have been isolated (2). The present note deals with the isolation and characterization of the Na^+-K^+-ATPase inhibitors, 6-tridecylresorcylic acid and grevillol, from *Lysimachia japonica*.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were obtained on a Yanagimoto micro melting point apparatus and are uncorrected. Ir spectra were obtained on a Shimadzu IR-27G photometer. Uv spectra were recorded on a Hitachi 200-20 spectrophotometer. Pmr spectra were recorded on a Hitachi R-22 spectrometer.

PLANT MATERIAL.—The plant material examined in the present study was collected in Tokushima, Japan, and a voucher specimen is on deposit in our institute.

NA^+-K^+-ATPASE ASSAY.—Na^+-K^+-ATPase (0.36 unit/mg protein) isolated from porcine cerebal cortex was purchased from Sigma Chemical Company. The enzyme reaction was carried out at 37° in 0.5 ml reaction medium containing (mM): NaCl, 100; KCl, 20; $MgCl_2$, 5; Tris HCl, 50; pH 7.4. ATPase activity was measured from the amount of inorganic phosphate released from ATP during a 10-min incubation. Enzyme (65.0 μg/ml) was incubated with various concentrations of samples and ouabain (10^{-5} M) in the presence or absence of 100 mM NaCl and 15 mM KCl. After a 5-min preincubation, reactions were initiated by the addition of 5 mM ATP and, after 10 min, terminated by the addition of 0.5 ml ice-cold 10% (v/v) trichloroacetic acid to the medium. Inorganic phosphate was determined by the method of Martin and Doty (3). Na^+-K^+-ATPase activity was calculated from the difference between the quantity of inorganic phosphate liberated in the presence and absence of NaCl and KCl.

ISOLATION OF THE NA^+-K^+-ATPASE INHIBITORS.—Dried whole plants (4.28 kg) were extracted three times with boiling MeOH, and the combined methanolic extract was evaporated to dryness to yield a residue (916 g). The residue was partitioned between equal volumes of EtOAc and H_2O. The aqueous layer was separated and reextracted twice with EtOAc. The combined EtOAc extract was concentrated to dryness to afford a residue (396 g). A 100-g sample of the residue was chromatographed on a silica gel 60 (Merck 70-230 mesh, 1500 g) column, which was eluted with 5% MeOH in $CHCl_3$. Fractions of 500-ml volume were collected and combined using tlc. Rechromatography of the residue (18 g) from the bioactive fractions of the first column was performed on a column packed with silica gel 60 (Merck 230-400 mesh, 500 g) and eluted with 5% MeOH in $CHCl_3$ to afford two active compounds (**1** and **2**). Compound **2** (1.8 g) was eluted first, followed by **1** (4.0 g). Further purification of the compound was conducted using a Sephadex LH-20 (100 g) column with a solution of 50% MeOH in EtOAc: compound **1**, $C_{20}H_{32}O_4$, colorless needles, mp 127-128° (EtOAc); ir, ν max (KBr) 3370, 1620, 1245 cm^{-1}; uv, λ max (EtOH) 260 (log ε 3.90), 300 (3.55) nm, ms, (rel. int.) *m/z* 336 (M^+, 2.6%), 124 (100); pmr, (CD_3OD) δ 0.88 (3H, bt, J=6Hz, $-CH_3$), 1.27 (22H, bs, $-(CH_2)_{11}-$), 2.88 (2H, bt, J=7.5Hz, $-CH_2-$), 6.16 (2H, m, aromatic protons). *Anal.* calcd for $C_{20}H_{32}O_4$: C, 71.39; H, 9.59. Found: C, 71.41; H, 10.05. Compound **2**, $C_{19}H_{32}O_2$, colorless needles, mp 82-84° (C_6H_6); ir, ν max (KBr) 3350, 1600, 1160 cm^{-1}; uv, λ max (EtOH) 276 (log ε 3.34), 282 (3.33) nm; ms, (rel. int.) *m/z* 292 (M^+, 15.4%), 124 (100). 123 (24.3); pmr, (CD_3OD) δ 0.87 (3H, bt, J=6Hz, $-CH_3$), 1.26 (22H, bs, $-(CH_2)_{11}-$), 2.43 (2H, bt, J=7.5Hz, $-CH_2-$), 6.09 (3H, m, aromatic protons).

METHYLATION OF **1**.—To an ethereal solution of **1** (316 mg) was added Et_2O containing CH_2N_2 until a faint yellow color persisted in the solution. After evaporation of the Et_2O, the re-

sidue was chromatographed on a silica gel 60 (Merck 230-400 mesh, 9 g) column and eluted with 5% Me_2CO in *n*-hexane to yield a dimethylated derivative (**3**, 168 mg), followed by a methyl ester (**4**, 152 mg). Derivative **3**, $C_{22}H_{36}O_4$, colorless needles, mp 56-57° (*n*-hexane); ir, ν max (KBr) 1640, 1610, 1320, 1250, 1150 cm^{-1}; ms, (rel. int.) *m/z* 364 (M^+, 19.5%), 196(100), 177(26.4), 164(24.1); pmr, ($CDCl_3$) δ 0.86 (3H, bt, J=6Hz, $-CH_3$), 1.25 (22H, bs, $-(CH_2)_{11}-$), 2.82 (2H, bt, J=7.5Hz, $-CH_2-$), 3.75 (3H, s, $C_{(4)}-OCH_3$), 3.87 (3H, s, $-COOCH_3$), 6.24 (2H, m, aromatic protons). Derivative **4**, $C_{21}H_{34}O_4$, colorless needles, mp 91-92° ($CHCl_3$); ir, ν max (KBr) 3350, 1660, 1320, 1260, 1170 cm^{-1}; ms, (rel. int.) *m/z* 350 (M^+, 20.9%), 182(100), 163(30.1), 150(25.1); pmr, ($CDCl_3$) δ 0.88 (3H, bt, J=6Hz, $-CH_3$), 1.25 (22H, bs, $-(CH_2)_{11}-$), 2.83 (2H, bt, J=7.5Hz, $-CH_2-$) 3.90 (3H, s, $-COOCH_3$), 6.22 (2H, m, aromatic protons).

ACETYLATION OF **2**.—Compound **2** (682 mg) was dissolved in pyridine (1 ml) and Ac_2O (1 ml) and left to stand overnight. The reaction mixture was worked up as usual to afford a diacetylated derivative (**5**, 860 mg). Derivative **5**, $C_{23}H_{36}O_4$, colorless needles, mp 47-48° (EtOH); ir, ν max (KBr) 1770, 1210, 1190 cm^{-1}; ms, (rel. int.) *m/z* 376 (M^+, 1.6%), 334 (19.1), 293 (21.3), 292 (90.7), 124 (24.5), 123 (28.2); pmr, ($CDCl_3$) δ 0.88 (3H, bt, J=6Hz, $-CH_3$), 1.24 (22H, bs, $-(CH_2)_{11}-$), 2.25 (6H, s, $-OCOCH_3\times2$), 2.60 (2H, bt, J=7.5Hz, $-CH_2-$), 6.74 (3H, m, aromatic protons).

RESULTS AND DISCUSSION

From the whole plants of *L. japonica*, two Na^+-K^+-ATPase inhibitors were isolated in crystalline form by chromatographic procedures. The molecular formula $C_{20}H_{32}O_4$ of **1** was determined by elemental analysis and mass spectrum. Its uv, ir and pmr spectra suggest the presence of an aromatic ring. In the pmr spectrum, a broad triplet methyl (δ 0.88), broad singlet-like methylene (1.27), and a broad triplet methylene (2.88) are assigned to a C_{13} straight chain attached to the aromatic ring. The four oxygen functions are assigned to two phenolic groups and one carboxylic group by the formation of a methyl ester (**4**). Its 1,2,3,5,-tetrasubstitution is determined by the *meta* coupling pattern of the aromatic protons in the pmr spectrum, uv comparison with orsellinic acid and inference from anacardic acids and cardols (4). Thus, **1** is 6-tridecylresorcylic acid, which was detected in immature seeds of *Ginkgo biloba* L. by gc/ms (4). Compound **2** is shown to have a molecular formula, $C_{19}H_{32}O_2$, which differs from that of **1** by CO_2, suggesting that **2** should be a decarboxylated derivative of **1**, that is grevillol (5). Compound **2** is identified as grevillol by comparison of physico-chemical data of **2** and its diacetate **5** with those in reference (5).

As shown in Figure 1, **1** and **2** inhi-

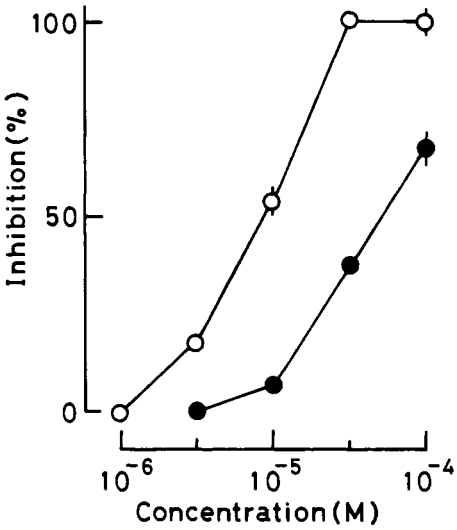
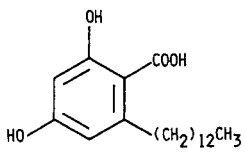


FIGURE 1. Inhibitory effects of compounds **1** (○) and **2** (●) on the Na^+-K^+-ATPase activities of porcine cerebral cortex. The enzyme reaction was carried out at 37° for 10 min. Symbols and vertical bars indicated means of S.E. of mean (n=4).



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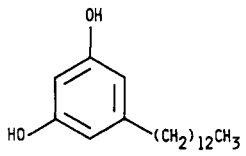

2

bited Na^+-K^+-ATPase activity in a concentration-dependent manner. The 50% inhibitory concentrations (IC_{50}) of **1** and **2** were 8×10^{-6} M and 5×10^{-5} M, respectively. On the basis of IC_{50}, the potency of **1** was approximately six times greater than that of **2**, a decarboxylated derivative, indicating that the carboxyl function in **1** is important for biological activity. In addition, IC_{50} of ouabain was approximately 6×10^{-7} M. Considering the IC_{50} values, **1** was about one-thirteenth as potent as ouabain in the inhibitory activity.

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