Antioxidative Constituents from the Aerial Part of *Piper elongatum* VAHL.

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Received January 20, 1997; Accepted May 12, 1997

Six aromatic compounds, asebogenin (1), 2',6'-dihydroxy-4'-methoxydihydrochalcone (2), 3-geranyl-4-methoxybenzoic acid (3), 3-geranyl-4-hydroxybenzoic acid (4), nervogenic acid (5) and 2,2-dimethyl-6-carboxyl-8-prenylchromene (6) were isolated from the methanol extract of the aerial part of *Piper elongatum* VAHL., whose leaves are used as a folk medicine in South America. The structures of 1-6 were elucidated by MS, 1H-NMR and 13C-NMR spectroscopies, and chemical evidence. Among these compounds, 1 showed stronger antioxidative activity than that of α-tocopherol, and 4 and 5 exhibited higher activity than that of tert-butyl-4-hydroxyanisole (BHA) using the ferric thiocyanate method.

Keywords: *Piper elongatum* VAHL., antioxidative effect, dihydrochalcone, benzoic acid, dimethylchromene, thiocyanate method, *Piper aduncum* L.

*Piper elongatum* VAHL. (syn. *Piper aduncum* L.) is a small tree commonly found in the lowlands of the Amazon, and its leaves are used as a folk medicine for the treatment of dermatosis in South America (Fournet et al., 1994). In earlier investigations of *P. elongatum* VAHL., phenylpropene, benzoic acid and flavonoid derivatives from the fruits, and prenylated benzoic acid derivatives, chromenes and dihydrochalcones from its leaves were reported (Burke & Nair, 1986; Orjala et al., 1993a, b, 1994). In the course of our studies on natural antioxidants (Ono et al., 1995, 1997), the methanol extract of this aerial part revealed stronger antioxidative activity than BHA which is a synthetic antioxidant, using linoleic acid as the substrate for the ferric thiocyanate method. In this paper, we report the isolation and structure elucidation of six aromatic compounds from the methanol extract of the aerial part of *P. elongatum* VAHL., and their antioxidative effects evaluated using the ferric thiocyanate method.

Materials and Methods

The plant material of *P. elongatum* VAHL. was purchased from Fundacion para la Investigacion Tecnologica del Recurso Agrobiologico Andino in Peru. Melting points (mp) were determined on a Yanagimoto apparatus and are uncorrected. The infra-red (IR) spectrum was taken on a Hitachi IR spectrometer model 270-30. The proton (1H) and the carbon-13 (13C) nuclear magnetic resonance (NMR) spectra were recorded on JEOL-GX-400 spectrometers; chemical shifts were recorded on a δ (ppm) scale with tetramethylsilane (TMS) as the internal standard. The ion fast atom bombardment mass spectrum (FAB-MS) and the electron impact mass spectrum (EI-MS) were obtained on a JEOL JMS-DX-303 HF (FAB-MS; matrix, glycerol/MeOH. EI-MS; ionization voltage, 70 eV). The visible absorptions were measured with a Shimadzu UV-140-02 spectrometer (Shimadzu, Kyoto). Column chromatography was carried out with Sephadex LH 20 (Pharmacia Fine Chemicals, Uppsala, Sweden), Diaion HP 20 (Mitsubishi Chemical Industries Co., Ltd., Tokyo) and Cosmolsil 75C18 ODS (Nacalai Tesque, Inc., Kyoto). Thin layer chromatography (TLC) was done with silica gel 60 F$_{254}$ (Merck Art. 5554; Merck, Darmstadt, Germany) and RP-18 F$_{254}$ (Merck Art. 13724) and detection was achieved by spraying the plates with 10% H$_2$SO$_4$-MeOH reagent, followed by heating or by spraying with FeCl$_3$ reagent. High performance liquid chromatographic (HPLC) separation was run on a Micro Pump Shimadzu LC-6A with a UV-Detector Shimadzu SPD-6A or with a RI-Detector Shimadzu RID-6A. For HPLC column chromatography, Inertsil ODS-2 (GL Sciences, Tokyo, 20 mm i.d. ×250 mm) with the MeOH-H$_2$O system as the developing solvent was used.

Extraction and isolation The air-dried and powdered aerial part of *P. elongatum* VAHL. (5.00 kg) was extracted with MeOH (13 l) under reflux, and the solvent was removed under reduced pressure to afford a brown extract (288 g). This extract was defatted by treatment of hexane (300 ml ×2) to give a hexane-soluble fraction (7.66 g) and the residue (275 g). This residue was successively chromatographed over Diaion HP 20 with 40%, 60%, 80%, MeOH, MeOH and acetone to afford fractions 1 (62.6 g), 2 (35.7 g), 3 (37.4 g), 4 (93.1 g) and 5 (39.1 g). Fraction 3 (0.83 g) was chromatographed over Sephadex LH 20 with MeOH to give fractions 6 (478 mg) and 7 (192 mg). Fraction 7 was crystallized from MeOH to give 1 (125 mg). The chromatography of fraction 6 over Sephadex LH 20 with MeOH afforded fraction 8 (414 mg). Fraction 8 was subjected to Sephadex LH 20 with 85% MeOH to give fractions 9 (92 mg), 10 (36 mg) and 11 (203 mg). Fraction 10 was chromatographed over Sephadex LH 20 with MeOH to afford 5 (15 mg). Fraction 4 (0.98 g) was subjected to
Sephadex LH 20 with MeOH to afford 2 (31 mg).

Negative FAB-MS m/z: 286 [M-H]-. 1H-NMR (in CDCl3, 400 MHz) δ: 1.58 (6H, s, 2'-CH3, 6'-CH3), 3.30 (4H, d, J = 7.3 Hz, 1'-CH2, 5'-CH2), 5.28 (2H, br t, J = 7.3 Hz, 2'-H, 2''-H), 7.51 (2H, s, 6-H, 8-H).

1H-NMR (in CDCl3, 400 MHz) δ: 1.76 (12H, s, 4'-CH3), 4'-CH3), 5'-CH3), 5'-CH3), 3.34 (4H, d, J = 7.3 Hz, 1'-H, 1''-H), 5.28 (2H, br t, J = 6.1 Hz, 2'-H, 2''-H), 7.27 (2H, s, 2-H, 6-H).

13C-NMR (in DMSO-d6, 100 MHz) δ: 167.4 (COOH), 156.4 (4-C), 132.0 (3'-C, 5'-C), 128.4 (2-C, 6-C), 127.9 (3-C, 5-C), 122.1 (2'-C, 2''-C), 121.5 (1-C), 28.1 (1'C, 1''-C), 25.5 (4''-C, 4'-C), 17.6 (5-C, 5''-C).

6: A white powder. Positive FAB-MS m/z: 273 [M+H]+. EI-MS m/z: 272 ([M]+). 1H-NMR (in DMSO-d6, 400 MHz) δ: 1.39 (6H, s, 2-CH3), 1.70 (3H, d, J = 7.3 Hz, 7-H), 1.69 (3H, s, 5'-CH3), 3.23 (1H, d, J = 7.3 Hz, 1-H', 1''-H'), 5.22 (1H, t, J = 7.3 Hz, 2'-H'), 5.80 (1H, d, J = 9.5 Hz, 3-H'), 6.48 (1H, d, J = 9.5 Hz, 4-H'), 7.53 (1H, d, J = 1.8 Hz, 5-H'), 7.58 (1H, s, 7-H). 13C-NMR (in DMSO-d6, 100 MHz) δ: 167.1 (COOH), 153.8 (9-C), 131.7 (3-C'), 131.2 (3-C'), 130.6 (7-C'), 128.4 (8-C'), 125.8 (5-C), 122.7 (10-C'), 121.2 (2'-C'), 121.6 (4-C'), 120.3 (6-C'), 77.1 (2-C'), 27.8 (CH3-x2), 27.7 (1'-C'), 25.5 (4''-C'), 17.7 (5''-C'). 13C-NMR (in CD3OD, 100 MHz) δ: 169.0 (COOH), 154.7 (9-C'), 134.2 (3-C'), 131.9 (5-C or 7-C'), 131.2 (5-C or 7-C'), 129.4 (8-C'), 126.9 (4-C'), 124.0 (10-C'), 122.9 (3-C or 2-C'), 122.5 (3-C or 2-C'), 121.0 (6-C'), 77.5 (2-C'), 28.7 (1'C'), 28.1 (CH3-x2), 25.8 (4''-C'), 17.9 (5''-C').

Acetylation of 1 Compound 1 (5 mg in Ac2O-pyridine (1: 1, 1 ml) was allowed to stand at room temperature overnight. After removal of the reagent under a stream of N2, the residue was suspended in H2O (1 ml) and then extracted with ether (1 ml). The ether layer was concentrated to afford 1a (6 mg).

1a: 1H-NMR (in CDCl3, 400 MHz) δ: 2.16 (6H, s, 2'COCH3, 6'COCH3), 2.28 (3H, s, 4-COCH3), 2.96 (2H, m, 3'H, 5'H), 3.04 (2H, m, 2'H, 4'H), 3.79 (3H, s, 4'-OCH3), 6.56 (2H, d, J = 6.7 Hz, 1'-H2, 5'-H2), 7.16 (1H, br t-like, J = 2.2 Hz, 4-H).

2: An amorphous powder. Negative FAB-MS m/z: 271 [M-H]-. 1H-NMR (in CDCl3, 400 MHz) δ: 2.94 (2H, m, 3'-CH2), 3.33 (2H, m, 2''-CH2), 3.75 (3H, t, J = 13.2 Hz, 4-OCH3), 5.92 (2H, t, J = 13.2 Hz, 3'-H, 5'-H), 7.16 (1H, br t-like, J = 2.2 Hz, 4-H), ca. 7.23 (4H, 2-H, 3-H, 5-H, 6-H).

3: An amorphous powder. Negative FAB-MS m/z: 271 [M-H]-. 1H-NMR (in CDCl3, 400 MHz) δ: 3.00 (2H, m, 3'-CH2), 3.40 (2H, m, 2''-CH2), 3.79 (3H, s, 4'-OCH3), 6.00 (2H, s, 3'-H, 5'-H), 7.18 (1H, br t-like, J = 2.2 Hz, 4-H), ca. 7.27 (4H, 2-H, 3-H, 5-H, 6-H).

3a: (6 mg) from 3, 3a (3 mg) from 4 and 6a (3 mg) from 6.

4: A white powder. 1H-NMR (in DMSO-d6, 400 MHz) δ: 1.55 (3H, s, 10'-CH3), 1.61 (3H, s, 8'-CH3), 1.67 (3H, s, 9'-CH3), ca. 2.01 (2H, 4'-H'), ca. 2.05 (2H, 5'-H'), 3.28 (2H, d, J = 7.3 Hz, 1'-H2), 3.86 (3H, s, 4'-OCH3), 5.07 (1H, t, J = 6.8 Hz, 6'-H), 5.26 (1H, d, J = 6.7 Hz, 2'-H), 7.03 (1H, d, J = 8.8 Hz, 5-H), 7.71 (1H, d, J = 2.2 Hz, 2-H), 7.81 (1H, d, J = 2.2, 8.8 Hz, 6-H).

Preparation of the ferric thiocyanate method (Ono et al., 1995). A mixture of 2.5% ethanol solution (1.3 ml) of each sample in a vial with a cap
and placed in the dark at 40°C to accelerate the oxidation. At intervals during incubation, this assay solution (0.10 ml) was diluted with 75% ethanol (9.70 ml), which was followed by adding 30% ammonium thiocyanate (0.10 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.10 ml) to the reaction mixture, the absorbance due to the developed red color was measured at 500 nm.

Results and Discussion
The aerial part of *P. elongatum* VAHL was extracted with methanol under reflux. This extract, which showed a stronger antioxidative effect than BHA (Fig. 1), was defatted with hexane. The residue was successively subjected to Diaion HP 20 column chromatography, Sephadex LH 20 column chromatography and HPLC on octadecyl silica (ODS) to give six compounds (1-6).

**Structural elucidation of 1-6**

Compound 1 was obtained as colorless needles, mp 172–173°C and it showed an \([M-H]^-\) ion peak at \(m/z\) 287 in the negative FAB-MS. The \(^1H\)-NMR spectrum of 1 gave the signals due to four aromatic protons for an \(A_2B_2\) pattern \(\delta 7.04 (2H, dd, J=2.0, 6.4 \text{ Hz})\), \(6.70 (2H, dd, J=2.0, 6.4 \text{ Hz})\), two equivalent aromatic protons \(\delta 5.92 (2H, s)\) and four methylene protons \(\delta 3.27 (2H, m), 2.85 (2H, m)\). The \(^13C\)-NMR spectrum of 1 indicated signals due to one carbonyl carbon \(\delta 207.5\), eight aromatic carbons \(\delta 168.2, 166.3, 157.2, 134.7, 131.1, 116.4, 106.8, 95.1\), one methoxyl carbon \(\delta 56.6\) and two methylene carbons \(\delta 48.2, 32.1\). Furthermore, the acetate of 1 (1a) showed signals of three acetyl groups in the \(^1H\)-NMR spectrum. These data indicated 1 to be a dihydrochalcone derivative composed of three hydroxy groups and one methoxyl group. The assignments of the connecting location for these substituents were based on the chemical shifts and splitting patterns of the aromatic proton signals in the \(^1H\)-NMR spectrum and the nuclear Overhauser effect (NOE) difference spectrum. In the NOE spectrum of 1, irradiation of the signal of the methoxyl protons at \(\delta 3.75\) gave the NOE enhancement of the signal at \(\delta 5.92 (2H, s)\). From this evidence, 1 was found to be identical with assebogenin, which is the aglycone of assebotin (Terai et al. 1973) (Fig. 2).

Compound 2 was obtained as an amorphous powder, and the negative FAB-MS afforded an \([M-H]^-\) ion peak at \(m/z\)
m/z 273. In the 1H-NMR spectrum, 6 showed signals due to two meta-coupled aromatic protons [δ 7.51 (2H, s), 7.53 (2H, s)], four olefinic protons [δ 5.22 (1H, t, J = 7.3 Hz), 5.23 (2H, d, J = 7.3 Hz), 5.80 (1H, d, J = 9.5 Hz)], one methyl group [δ 2.31 (3H, s)], and two quaternary methyl groups [δ 1.39 (6H, s)]. The 13C-NMR spectrum of 6 gave the signals due to one carboxyl carbon (δ 167.1), one oxygenated aromatic carbon (δ 153.8), four olefinic carbons (δ 131.2, 130.6, 125.8, 121.6), five carbons for a prenyl group (δ 131.7, 122.1, 27.7, 25.5, 17.7), one quaternary carbon (δ 77.1) and one methyl carbon (δ 17.7). The location of these groups were decided on the basis of the 13C-NMR data and the NOE difference spectrum of 6. The NOE correlations are illustrated in Fig. 3. Furthermore, 6 was treated with diazomethane to afford 6a, which showed the signal of only one methoxyl group [(δ 3.86 and δ 55.7) in 4, observed in the NMR data of 3. Furthermore, methylation of 4 by the diazomethane-ether gave 3a. From the above evidence, 4 was found to be identical with 3-geranyl-4-hydroxybenzoic acid, which was isolated from Lindera umbellata (Tanaka et al., 1984) and Piper murrayananum (Seeram et al., 1996) (Fig. 2).

Compound 5 was obtained as an amorphous powder, and it revealed the signals of two aromatic protons [δ 7.51 (2H, s)], two olefin protons [δ 5.28 (2H, br t, J = 7.3 Hz)], four methylene protons [δ 3.30 (4H, d, J = 7.3 Hz)] and four methyl groups [(δ 1.72 (6H, s), 1.68 (6H, s))] in the 1H-NMR spectrum of 5. The 13C-NMR spectrum of 5 showed the signals of one carbonyl carbon (δ 167.4), four aromatic carbons (δ 156.4, 128.4, 127.9, 121.5) and five carbons for a prenyl group (δ 132.0, 122.1, 28.1, 25.5, 17.6). From these data, 5 was suggested to be nervogenic acid (Orjala et al., 1993a), which was confirmed by the 1H- and 13C-NMR data when compared to those of an authentic sample (Abe & Yamauchi, 1985) (Fig. 2).

Compound 6 was obtained as a white powder, and it exhibited an [M+H]+ ion peak in the positive FAB-MS at m/z 273. In the 1H-NMR spectrum, 6 showed signals due to two meta-coupled aromatic protons [δ 7.51 (1H, s), 7.53 (1H, d, J = 1.8 Hz)], two cis-coupled olefin protons [δ 6.48 (1H, d, J = 9.5 Hz), 5.80 (1H, d, J = 9.5 Hz)], one prenyl group [δ 5.22 (1H, t, J = 7.3 Hz), 3.23 (2H, d, J = 7.3 Hz), 1.70 (3H, s), 1.69 (3H, s)] and two quaternary methyl groups [δ 1.39 (6H, s)]. The 13C-NMR spectrum of 6 gave the signals due to one carboxyl carbon (δ 167.1), one oxygenated aromatic carbon (δ 153.8), four olefinic carbons (δ 131.2, 130.6, 125.8, 121.6), five carbons for a prenyl group (δ 131.7, 122.1, 27.7, 25.5, 17.7), one quaternary carbon (δ 77.1) and one methyl carbon (δ 17.7). The location of these groups were decided on the basis of the 13C-NMR data and the NOE difference spectrum of 6. The NOE correlations are illustrated in Fig. 3. Furthermore, 6 was treated with diazomethane to afford 6a, which showed the signal of only one methoxyl group [(δ 3.86 (3H, s))] in the 1H-NMR spectrum. These data indicated 6 to be a dimethylchromene derivative, composed of one carboxyl group and one prenyl group. Accordingly, the structure of 6 was determined to be 2,2-dimethyl-6-carboxyl-8-prenyl-chromene (Orjala et al., 1993b), which was the aglycone of Anodendrosin H and Anodendrosin I (Abe & Yamauchi, 1985).
Antioxidants from Piper elongatum VAHL.

1985) (Fig. 2).

As far as we know, compounds 5 and 6 have previously been isolated from the dried leaves of Piper aduncum L. (Piper elongatum VAHL.) (Orjala et al., 1993a, b), but this report is the first example of the isolation of 1, 2, 3 and 4 from this plant, and further, 3 is regarded as a new compound.

Antioxidative effect of compounds 1-6 The antioxidative activities of 1-6 were evaluated using the ferric thiocyanate method (Ono et al., 1995). Compounds 1, 4 and 5 showed stronger antioxidative activity than that of α-tocopherol. Furthermore, 4 and 5 exhibited higher activity than that of BHA at the final concentration of 0.02% (Fig. 4). These data indicated that the antioxidative activities of 1, 4 and 5 depended upon the occurrence of the hydroxyl group on the benzene ring. However, the activity of the methanol extract was higher than those of 1, 4 and 5. Therefore, it was suggested that more antioxidative active constituents than these compounds would be present in the methanol extract and/or a synergistic effect might be recognized in combination with some compounds.

Recently, J. Orjala et al. (1993a, b, 1994) reported that 1, 2, 5 and 6 showed antibacterial activities, but the antioxidative activities of 1-6 are described for the first time.

Further investigations of the constituents of this plant are in progress.

Acknowledgments We express our appreciation to Mr. K. Takeda and Mr. T. Iriguchi of Kumamoto University for their measurement of the NMR spectra and MS. This study was supported in part by a Grant-in-Aid for Encouragement of Young Scientists (No. 08772048) from the Ministry of Education, Science, Sports and Culture of Japan and by the General Research Organization of Tokai University.

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