Antioxidant *Ortho*-Benzoyloxyphenyl Acetic Acid Ester, Vaccihein A, from the Fruit of Rabbiteye Blueberry (*Vaccinium ashei*)

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A new *ortho*-benzoyloxyphenyl acetic acid ester, called vaccihein A (1), was isolated from the fruit of rabbiteye blueberry (*Vaccinium ashei*). The chemical structure was determined on the basis of spectroscopic data. Compound 1 had antioxidative activity using the ferric thiocyanate method. In addition, 1 showed a scavenging effect on the stable free radical 1,1-diphenyl-2-picrylhydrazyl.

Key words *Vaccinium ashei*; blueberry; Ericaceae; benzoyloxyphenyl acetic acid ester; vaccihein A; antioxidative activity

Blueberries (Ericaceae) are cultivated worldwide, and the fruit is used not only as foodstuff but also for the treatment of eyestrain. Representative species of blueberry include highbush blueberry (*Vaccinium corymbosum* L.), lowbush blueberry (*Vaccinium angustifolium* AIT.), rabbiteye blueberry (*Vaccinium ashei* READE), and bilberry blueberry (*Vaccinium myrtillus* L.),[1] all of which contain large amounts of phenolics, especially anthocyanins.[2] The present paper describes the isolation and structure elucidation of a new *ortho*-benzoyloxyphenyl acetic acid ester from the MeOH extract of *V. ashei* and the antioxidant evaluation of **1**.

The MeOH extract was successively subjected to Diaion HP 20, MCI gel CHP 20P, and silica gel column chromatography as well as HPLC on ODS to afford **1**. Compound **1**, trivially named vaccihein A, was obtained as a white powder. In the electron impact (EI)-MS, **1** showed an $[M]^+$ ion peak at *m/z* 378 along with a base ion peak at *m/z* 181 $[C_9H_9O_4]^+$. The molecular formula of **1** was determined to be $C_{18}H_{18}O_9$ by high-resolution (HR) positive FAB-MS. The IR spectrum of **1** revealed the absorption of hydroxyl groups at 3390 cm^{-1} and ester carbonyl groups at 1730 cm^{-1}. The ^{1}H-NMR spectrum of **1** showed signals due to four aromatic protons (δ 7.30, 2H, s; δ 6.27, 1H, d, J=1.0 Hz; δ 6.12, 1H, d, J=1.0 Hz), three methoxyl groups (δ 3.84, 6H; δ 3.48, 3H), and two equivalent methylene protons (δ 3.40, 2H, s). The ^{13}C-NMR spectrum of **1** gave signals assignable to two ester carbonyl carbons (δ 171.3, 163.6), 12 aromatic carbons (δ 157.0, 156.7, 150.7, 147.7 (×2), 141.5, 118.2, 107.3 (×2), 105.0, 100.8, 99.9), three methoxyl carbons (δ 56.1 (×2), 51.4), and one methylene carbon (δ 28.7). From these data, it was presumed that **1** has a 3,5-dimethoxy-4-hydroxy benzoyl group, a 2,4,6-trisubstituted benzyl group, and one ester group. Furthermore, the heteronuclear mutiple bond correlation (HMBC) spectrum of **1** indicated correlations between the respective protons and carbons, as illustrated in Fig. 1. Consequently, **1** was elucidated to be methyl 2-(3,5-dimethoxy-4-hydroxybenzoyloxy)-4,6-dihydroxyphenyl acetate, which corresponds to an oxide that has undergone carbon–carbon cleavage between the 2- and 3-positions of 3-*O*-methyl malvidin (**2**) (Fig. 2).

The antioxidative activity of **1** was evaluated using linoleic acid as the substrate in the ferric thiocyanate method.[3] Compound **1** showed moderate activity in comparison with α-tocopherol and BHA (Fig. 3). Furthermore, the scavenging effect of **1** on the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was examined.[4] Compound **1** exhibited more potent activity than L-cysteine at a concentration of 0.20 mM (Fig. 4).

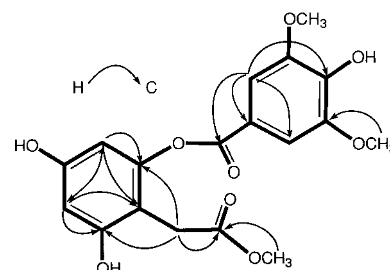

Fig. 1. ^{1}H–^{13}C Long-Range Correlations Observed for **1** in the HMBC Spectrum

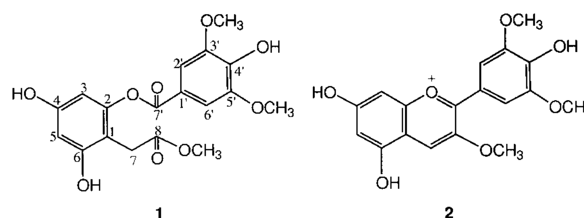

Fig. 2. Structures of **1** and **2**

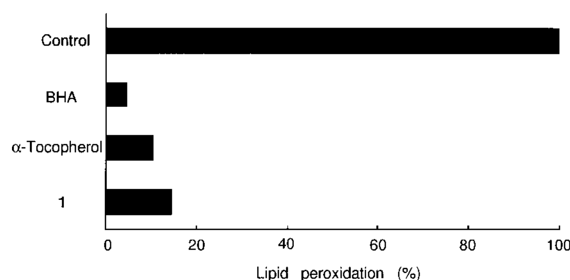

Fig. 3. Antioxidative Activities of **1**, α-Tocopherol, and BHA 5 d after Lipid Peroxidation

The final concentration of each sample tested was 0.5 mM. A control containing no added samples or standards in its values represents 100% lipid peroxidation.

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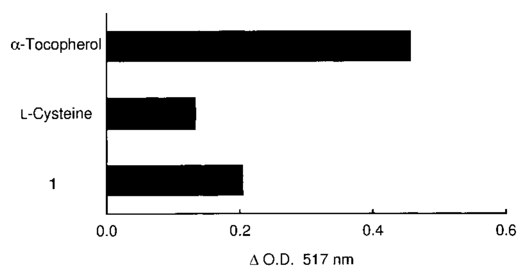

Fig. 4. DPPH Radical Scavenging Effects of **1**, L-Cysteine, and α-Tocopherol

The final concentration of each sample tested was 0.02 mM. ΔO.D.=O.D. of control at 517 nm (1.122)−O.D. of sample. DPPH; 0.1 mM.

Experimental

All the instruments and the materials used were the same as cited in the previous reports[5,6] unless otherwise specified.

Plant Material Fruit of *V. ashei* was collected in June 2000 at the orchard of Kyushu Tokai University, Kumamoto prefecture, Japan, and identified by Professor Haruki Komatsu, Kyushu Tokai University School of Agriculture.

Extraction and Isolation Crushed fresh fruit of *V. ashei* READE (1192 g) was soaked in MeOH (1192 ml) for 24 h at room temperature (this procedure was repeated three times), and the solvent was removed under reduced pressure to give a dark purple syrup (155.6 g). The MeOH extract (145.2 g) was subjected to Diaion HP 20 (H_2O, 70% MeOH, MeOH, acetone) to give fraction (fr.) 1 (129.7 g), fr. 2 (6.3 g), fr. 3 (0.7 g), and fr. 4 (0.8 g). Chromatography of fr. 2 (5912 mg) over MCI gel CHP 20P (H_2O–MeOH, gradient) afforded frs. 5—17. Fr. 15 (1260 mg) was subjected to silica gel [$CHCl_3$–MeOH–H_2O (14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5)] to give frs. 18—24. Fr. 18 (37 mg) was subjected to HPLC (COSMOSIL 5C18-AR-II, Nacalai Tesque Inc., 250 mm×20 mm i.d., 55% MeOH) to give **1** (9 mg).

Vaccihein A (**1**): White powder. IR (KBr) cm^{-1}: 3390 (OH), 1730 (COOR). EI-MS *m/z* (rel. int.): 378 $[M]^+$ (1), 181 (100). Positive FAB-MS *m/z*: 401 $[M+Na]^+$, 379 $[M+H]^+$. HR positive FAB-MS *m/z*: 401.0840 $[M+Na]^+$ (Calcd. for $C_{18}H_{18}O_9Na$, 401.0849). ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 7.30 (2H, s, H-2′, H-6′), 6.27 (1H, d, J=1.0 Hz, H-3), 6.12 (1H, d, J=1.0 Hz, H-5), 3.84 (6H, s, OCH_3-3′, OCH_3-5′), 3.48 (3H, s, OCH_3-8), 3.40 (2H, s, H_2-7). ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 171.3 (C-8), 163.6 (C-7′), 157.0 (C-4), 156.7 (C-2), 150.7 (C-6), 147.7 (C-3′, C-5′), 141.5 (C-4′), 118.2 (C-1′), 107.3 (C-2′, C-6′), 105.0 (C-1), 100.8 (C-5), 99.9 (C-3), 56.1 (OCH_3-3′, OCH_3-5′), 51.4 (OCH_3-8), 28.7 (C-7).

Assay of Antioxidative Activity Antioxidative activity of the test sample was assayed using the ferric thiocyanate method.[3] A mixture of 2.51% linoleic acid–EtOH solution (0.80 ml), 0.05 M phosphate buffer (pH 7.0, 1.60 ml), EtOH (0.60 ml), and H_2O (0.80 ml) was added to 10 mM EtOH solution (0.20 ml) of each sample in a vial with a cap and placed in darkness at 40 °C to accelerate oxidation. After the fifth day of incubation, this assay solution (0.05 ml) was diluted with 75% EtOH (4.85 ml), followed by the addition of 30% ammonium thiocyanate (0.05 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.05 ml) to the reaction mixture, the absorbance of the red color developed was measured at 500 nm. The control sample was prepared in the same manner by mixing all the same chemicals and ingredients except for the test compounds. α-Tocopherol and BHA were used as standard samples.

Assay of Scavenging Effect on DPPH The method of Uchiyama *et al.*[4] was applied in a slightly modified manner. The EtOH solution (1.00 ml) of each test sample was added to a mixture of 0.1 M acetic acid buffer (pH 5.5, 1.00 ml) and 0.5 mM DPPH EtOH solution (0.50 ml) in a test tube and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. α-Tocopherol and L-cysteine were used as standard samples.

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References

1) Sato M., *Shokuhin Kogyo*, **43**, 34—39 (2000).
2) Prior L. P., Cao G., Martin A., Sofic E., McEwen J., O'Brien C., Lischer N., Ehlenfeldt M., Kalt W., Krewer G., Mainland C. M., *J. Agric. Food Chem.*, **46**, 2686—2693 (1998).
3) Kikuzaki H., Nakatani N., *J. Food Sci.*, **58**, 1407—1410 (1993).
4) Uchiyama M., Suzuki Y., Fukuzawa K., *Yakugaku Zasshi*, **88**, 678—683 (1968).
5) Ono M., Ito Y., Kinjo J., Yahara S., Nohara T., Niiho Y., *Chem. Pharm. Bull.*, **43**, 868—871 (1995).
6) Ono M., Yamamoto M., Yanaka T., Ito Y., Nohara T., *Chem. Pharm. Bull.*, **49**, 82—86 (2001).