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A New Antioxidant from the Marine Sponge-derived Fungus *Aspergillus versicolor*

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Abstract – A chemical investigation of the marine-derived fungi *Aspergillus versicolor* led to the isolation of a new aromatic polyketide (**1**), The structure was elucidated by spectroscopic analysis, and its radical-scavenging activity, reducing power, and inhibitory activity to lipid oxidation were investigated. Those activities of compound **1** were compared with standard antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiarybutylhydroquinone (TBHQ), and ascorbic acid (V_C). Compound **1** showed antioxidant activity comparable to that of BHA, and siginificantly higher than that of BHT.
Keywords – aromatic polyketide, marine fungus, *Aspergillus versicolor*, antioxidant

Introduction

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of free radicals. The interaction of these reactive species with molecules of a lipid nature produces new radicals: hydroperoxides and various peroxides (Torel *et al.*, 1986). This group of radicals may interact with biological systems in a clearly cytotoxic manner. In this respect, phenols have been shown to possess an impotant antioxidant activity toward these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationship between different parts of their chemical structure (Visioli *et al.*, 1998). Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as BHA and BHT, which widely used in food industry, were deemed to have a carcinogenic potential (Branen 1975; Imadia *et al.*, 1983). Thus, a need for alternative natural and safe sources of food antioxidants has been created, and the search for natural antioxidants, has notably increased (Mojca *et al.*, 2005).

The fungal genus *Aspergillus* has been reported to produce a considerable number of bioactive compounds (Belofsky *et al.*, 1998; Fremlin *et al.*, 2009). In our previous study on bioactive compounds from the sponge-derived fungus *Aspergillus versicolor*, three polyketides, a new peptide, and a series of anthraquinone and xanthone derivatives (Lee *et al.*, 2007, 2010) were isolated by bioactivity-guided fractionations. In a continuing study on the same fungus, a new aromatic polyketide (**1**) was isolated. This paper describes the isolation, structure elucidation, and antioxidant evaluation of the isolated compound.

Material and methods

General experimental procedures – The IR spectrum was recorded using a JASCO FT/IR-410 spectrometer. The UV spectrum was recorded using a GE Ultrospec 2100 pro UV/visible spectrophotometer. 1D and 2D NMR spectra were recorded on Varian UNITY 400 and Varian INOVA 500 spectrometers. Chemical shifts are reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD). FABMS data were obtained on a JEOL JMS SX-102A. HRFABMS data were obtained on a JEOL JMS SX-101A. HPLC was performed on a Gilson 370 pump with an YMC packed J'sphere ODS-H80 column (250 × 10 mm, 4 μm, 80 Å) using a Shodex RI-71 detector.

Standard BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tertiarybutylhydroquinone),

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V_C (ascorbic acid), DPPH (1,1-diphenyl-2-picrylhydrazyl), and TCA (trichloroacetic acid) were purchased from Sigma (St. Louis, MO, USA). All the other chemicals were analytical grade supplied by Fluka or Sigma Co.

Animal material – The sponge was collected by hand using SCUBA (20 m in depth) in 2004 off the coast of Jeju Island, Korea. This specimen was identified as *Petrosia* sp. and the morphology of the sponge specimen was described elsewhere (Lim *et al.*, 2001)

Fungal strain – The fungal strain was isolated from a marine sponge *Petrosia* sp. Following a rinse with sterile sea water, small pieces of the surface and inner tissue of the sponge were homogenized and then inoculated on malt extract agar (MEA) petri dishes. The sterilized MEA medium (prepared with 75% sea water) contained glucose (20 g/L), malt extract (20 g/L), agar (20 g/L), peptone (1 g/L), and antibiotics (10,000 Units/mL penicillin and 10 mg/mL streptomycin, 5 mL/L). Emerging fungal colonies were transferred to the same media in a petri dish and incubated at 25 °C for 10 - 14 days to allow colony development. The pure fungal strain, designated as PF10M, was identified as *Aspergillus versicolor* by a morphological and biochemical analysis.

Extraction and isolation – Fermentation was performed in 100 mL malt media in 300 mL Erlenmeyer flasks for subculture. For the massive culture, 100 mL of subculture was transferred into a 2 L Erlenmeyer flask each containing 20 g/L malt media, and fermentation was carried out on a rotary shaker (37 °C, 150 rpm, 21 days). The cultured fungus (20 L) was extracted with 10 L of EtOAc, to afford the EtOAc extract, which was partitioned between n-hexane and 90% MeOH. The 90% MeOH layer was subjected to a stepped-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) eluting with 40% to 100% MeOH to afford 14 fractions. Fraction 2 was subjected to Silica gel 60 (15 - 40 μm) column chromatography eluting with a gradient solvent system of 100% CH_2Cl_2 to 100% MeOH, to afford 6 subfractions. Subfraction 2 (30 mg) was subjected to RP-HPLC (YMC packed J'sphere ODS-H80 column, 250 × 10 mm, 4 μm, 80 Å), eluted with 15% aqueous MeOH to afford compound **1** (5.0 mg).

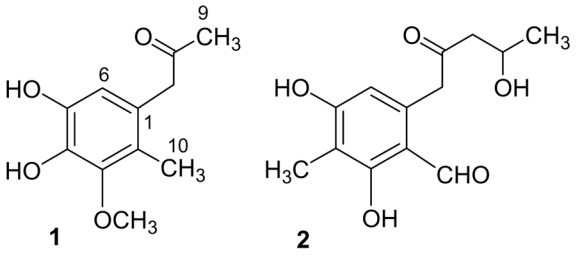
Aspergillin A (**1**) : brown oil; UV (MeOH) λ_{max} (log ε) 298 (2.39) nm; IR ν_{max} 3425, 2925, 2854, 1718, 1635, 1459, 1384, 1095, 1026, 668 cm^{-1}; ^1H NMR data (CD_3OD 500 MHz) δ_H 6.41 (1H, s, H-6), 3.71 (3H, s, -OCH_3), 3.59 (2H, s, H-7), 2.09 (3H, s, H-9), 2.01 (3H, s, H-10); ^{13}C NMR data (CD_3OD 100 MHz) δ_C 208.8 (C-8), 146.9 (C-5), 143.8 (C-3), 137.2 (C-4), 124.2 (C-1), 121.0 (C-2), 113.2 (C-6), 59.4 (-OCH_3), 49.0 (C-7), 27.7 (C-9), 10.6 (C-10); (–)-FABMS *m/z* 209 $[M - H]^-$; (–)-HRFABMS *m/z* 209.0806 $[M - H]^-$ (calcd for $C_{11}H_{13}O_4$, 209.0814).

Radical-scavenging activity (RSA) assay – The antioxidant activity of compound **1** was measured in terms of hydrogen donating or radical scavenging ability using modified DPPH method (Hatano *et al.*, 1988). A methanolic solution (0.1 mL) of the sample at various concentration were added to 0.1 mL of DPPH (150 μM) solution. Then the mixture was shaken vigorously and left to stand for 30 min in the dark (until stable absorption values were obtained) and the resulting color was measured spectrophotometrically at 520 nm using a Bio-Rad 680 microplate reader. An increasing intensity of yellow color was related to higher radical-scavenging power. The radical-scavenging activity (RSA) was calculated according to the following equation:

% RSA = (1 – absorbance of sample/absorbance of blank) × 100. In this study, antioxidants BHA, BHT, and V_C at 5, 10, 20, 40, and 100 μg/mL were used as a positive control.

Reducing power – Reducing power of compound **1** was determined according to the method of Oyaizu (Oyaizu, 1986). Various concentrations of sample solutions (0.5 mL) were placed into tubes. To these tubes 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide were added and mixed gently. The mixture was incubated at 50 °C in a water bath for 20 min. A 0.5 mL of 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 3,000 rpm for 5 min. The top layer of the supernatant (0.5 mL) was transferred into tubes containing 0.5 mL of distilled water and 0.1 mL of 1% ferric chloride ($FeCl_3 \cdot 6H_2O$). After shaking, the mixture was left at room temperature for 5 min and then absorbance was recorded at 700 nm using a UV-VIS spectrophotometer (Shimadzu UV-2401 PC). Here, BHA, BHT, and V_C were employed as positive controls. The higher the absorbance, the stronger the reducing power of the sample was recognized.

Antioxidant activity in linoleic acid emulsion system – The antioxidant activity of compound **1** was determined by the thiocyanate method (Duh *et al.*, 1999). Different concentrations of the sample in methanol were mixed with linoleic acid emulsion in potassium phosphate buffer (0.02 M, pH 7.0). Fifty milliliter of linoleic acid emulsion was prepared by mixing and homogenising 280.4 mg linoleic acid, 280.4 mg of Tween-20 as emulsifier, and 50 mL of phosphate buffer (0.2 M, pH 7.0). The reaction mixture was incubated at 37 ± 0.5 °C. Aliquots of 0.1 mL were taken at various intervals during incubation. The degree of oxidation was measured by sequentially adding ethanol (5 mL, 75% v/v), ammonium thiocyanate (0.1 mL,



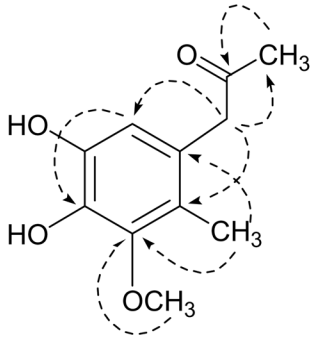


Fig. 1. Key HMBC correlations of compound **1**.

30% w/v), and ferric chloride (0.1 mL, 0.02 M in 3.5% HCl w/v) to sample solution (0.1 mL), and then reading the absorbance at 500 nm. Solutions devoid of compound **1** were used as blank control. The degree of oxidation was measured every 24 h and the data were average of triplicate analyses.

The inhibition of lipid peroxidation in percent was calculated by the following equation:

LPI (%) = (1 – absorbance of sample/absorbance of blank) × 100. Antioxidants BHA, BHT, and TBHQ at 100 μg/mL were used as positive controls.

Statistical analysis – Experimental results were mean ± S.D. of three parallel measurements. P values ± 0.05 were regarded as significant.

Results and discussion

Structure elucidation – Compound **1** was obtained as a brown oil that showed a pseudomolecular ion at *m/z* 209 $[M-H]^-$ in the negative ion (–)-FABMS spectrum, the exact mass was measured to be 209.0806 by (–)-HRFABMS, which corresponded to the molecular formula $C_{11}H_{13}O_4$ (Δ –0.8 mmu), implying five degrees of unsaturation.

Due to the molecular formula and 12 protons evident from ^{1}H NMR, two protons had to be present as hydroxyl group. The ^{13}C NMR spectrum contained 11 resonances of one methoxyl group, two methyl, one methylene, and

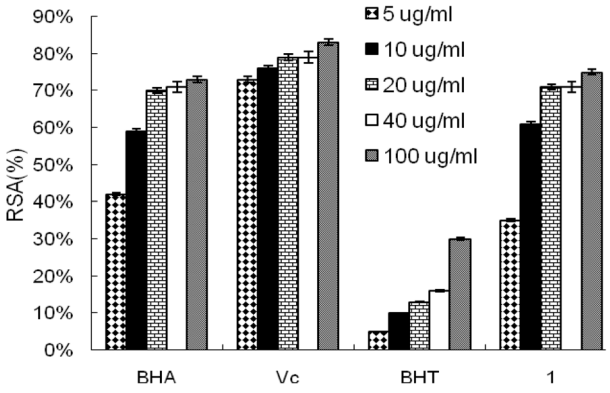


Fig. 2. Radical scavenging activity (RSA) of compound **1** and standard antioxidants BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and V_C (ascorbic acid) on DPPH radicals. Values were expressed as mean ± SD (n = 3).

six quaternary carbons, including one carbonyl carbon (δ_C 208.8). One aromatic methine and five quaternary, sp^2-hybridized carbons suggested a pentasubstituted aromatic system. From ^{1}H and ^{13}C NMR spectra, an acetonyl chain (CH_2-7 $\delta_H/_C$ 3.59/49.0, C-8 δ_C 208.8, CH_3-9 $\delta_H/_C$ 2.09/27.7) was deduced and confirmed by ^{1}H-^{13}C HMBC correlations (Fig. 1). The methylene proton (δ_H 3.59) showed cross-peaks with carbon signals at δ_C 121.0 (C-2) and 113.2 (C-6) suggesting that the acetonyl moiety was located at C-1. The ^{1}H NMR spectrum dispalyed one aromatic proton (CH-6 δ_H 6.41), one methyl (CH_3-10 δ_H 2.01), and one methoxyl group (-OCH_3 δ_H 3.71). The HMBC measurements revealed the substitution pattern. The chemical shift of the methyl proton (δ_H 2.01) was significantly shifted downfield, and the HMBC correlation to δ_C 143.8 (C-3) and δ_C 124.2 (C-1) were observed. The position of methoxyl group was confirmed by the correlation of -OCH_3 (δ_H 3.71) to C-3 in the HMBC spectrum. Based on the molecular formula, the remaining substituents at C-4 and C-5 should be hydroxyl groups. And the chemical shift of C-3 (143.8), C-4 (137.2), and C-5 (146.9) further confirmed this 4,5-dihydroxy-3-methoxyl subsititution. On the basis of the aforementioned observations, compound **1** was characterized as 1-(4,5-dihydroxy-3-methoxy-2-methylphenyl)-propan-2-one, and we propose a trivial name aspergillin A. It shares similar structural motif with the previously isolated aromatic polyketide 2,4-dihydroxy-6-(4-hydroxy-2-oxopentyl)-3-methylbenzaldehyde (**2**) from the same strain (Lee *et al.*, 2010).

Radical-scavenging activity (RSA) – The free radical DPPH possesses a characteristic absorption at 520 nm (purple in color), which decreases significantly on exposure to radical-scavengers (by providing hydrogen

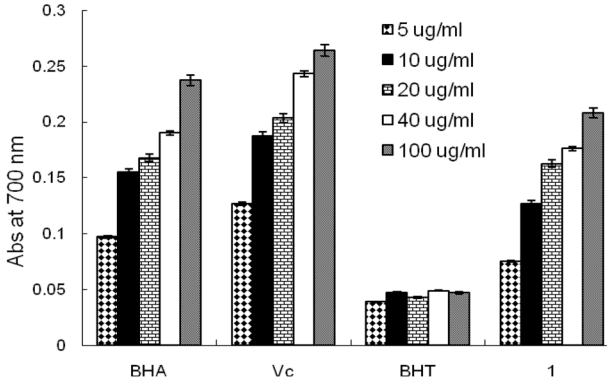


Fig. 3. Reducing power of compound **1** and standard antioxidants BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and V_C (ascorbic acid). Values were expressed as mean ± SD (n = 3).

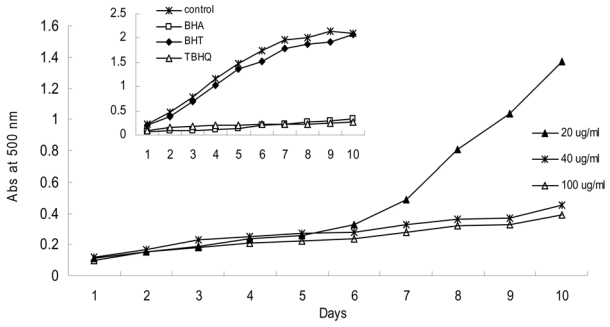


Fig. 4. Antioxidant properties of compound **1** and standard antioxidants determined with the FTC methods. Data were mean ± SD values (n = 3). Inset graph shows activities of blank control, BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and TBHQ (tertiarybutylhydroquinone) at the concentration of 100 μg/mL.

atoms or by electron donation). Free radical-scavenging activity is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening compounds with RSA (Amarowicz *et al.*, 2004).

The results for RSA of compound **1**, BHA, BHT and Vc are presented in Fig. 2. The scavenging effect of compound **1** on DPPH radicals was siginificant and concentration-dependent. The activity of compound **1** was comparable to those of BHA and Vc, and much higher than that of BHT.

Reducing power – Fig. 3 shows the reducing power of compound **1**. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each test sample. The presence of a reductant cause the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's prussian blue at 700 nm, the Fe^{2+} concentration can be monitored (Kumaran, 2006), a higher absorbance at 700 nm indicates a higher reducing power. The reducing power of compound **1** was significant and concentration-dependent. It showed activity comparable to that of BHA and Vc, and higher than that of BHT.

Total antioxidant activity in a linoleic acid emulsion system – The total antioxidant activity of compound **1** in a linoleic acid emulsion system was determined by ferric thiocyanate (FTC) assay. The FTC method is used to measure the peroxide level during the initial stage of lipid oxidation. During linoleic acid oxidation, peroxides are formed and oxidize Fe^{2+} to Fe^{3+}, resulting in a maximum absorbance at 500 nm. Low absorbance value indicates high level of antioxidative activity (Duh *et al.*, 1999).

Fig. 4 shows the effect of compound **1** on lipid peroxidation compared to TBHQ, BHA, and BHT. Compound **1** exhibited significant antioxidant activity at the concentrations higher than 40 μg/mL. At the concentration of 100 μg/mL, compound **1** showed the highest inhibition activity (87%) after 144 hours, which was comparable to those of BHA (93%) and TBHQ (91%), and much higher than that of BHT (15%).

These results suggest that compound **1** might serve as a possible supplement for food and cosmetics, and as a potential antioxidant for biological systems susceptible to free radical-mediated reactions. Therefore, further study regarding its *in vivo* activity and safety would be rewarding.

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