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Cyathuscavins A, B, and C, new free radical scavengers with DNA protection activity from the Basidiomycete *Cyathus stercoreus*

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ABSTRACT

Three new polyketides, cyathuscavins A (**1**), B (**2**), and C (**3**) were isolated from the mycelium culture of *Cyathus stercoreus*. The structures of the compounds were elucidated on the basis of NMR and mass spectroscopic data. Antioxidant activities of the compounds were evaluated by the scavenging ability against $ABTS^{+}$, DPPH, and superoxide anion radicals. Cyathuscavins A–C showed significant antioxidant activity comparable to those of reference antioxidants, BHA and Trolox. Cyathuscavins A–C protected supercoiled plasmid DNA from Fe^{2+}/H_2O_2-induced breakage.

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Reactive oxygen species (ROS) including hydroxyl radical, superoxide anion, and hydrogen peroxide are generated in the process of cellular oxygen metabolism and are important in the cell signaling.[1,2] However, these species can be increased by imbalance between oxidation and antioxidant defense system. Excess ROS are reactive and toxic enough to cause cell injury by destruction of pivotal bio-molecules.[3] Pathogenesis of several diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, and inflammations was associated with ROS.[4,5] Especially oxidative DNA damage by ROS has been believed to be a risk factor for carcinogenesis and related to cancer, neurodegenerative disease, and aging.[6] Hydroxyl radical (.OH), a highly reactive oxygen species, is generated from hydrogen peroxide (H_2O_2) in the presence of transition metal ions, especially iron which is known as Fenton reaction. It rapidly attacks almost all cellular bio-molecules including nucleotides, proteins, sugars, and lipids.[7] For preventing radical-induced damage and disease, much attention has been paid to natural antioxidants as a defense.

Mushrooms are abundant natural resources that have structurally unique compounds with diverse bioactivities.[8] We reported polyketide antioxidative compounds, cyathusals A, B, and C from the fermented metabolite of *Cyathus stercoreus*.[9] Further investigation of the metabolite to find new antioxidants possessing free radical scavenging and DNA protection activities led three new polyketides designated cyathuscavins A (**1**), B (**2**), and C (**3**), together with the known 4-hydroxy-6-propenyl-pyran-2-one (**4**) (Fig. 1).

Mycelium culture of *C. stercoreus* was conducted as described previously.[9] Thirty liters of the culture broth were filtered to separate broth from mycelium. The mycelial cake was extracted with 80% aqueous acetone and concentrated under vacuum. The filtered broth and the mycelium extract were combined and extracted with EtOAc. The EtOAc extract was purified by SPE C_{18} cartridge (Altech, 10 g/75 mL) eluting with 30–80% aqueous MeOH, sequentially. The fraction, eluted with 30% aqueous MeOH, was further purified by Sephadex LH-20 column chromatography (80% aq MeOH), and reverse-phase HPLC (20% aqueous MeOH containing 0.04% triflouroacetic acid) to afford **1** (12.0 mg), **2** (212.3 mg), and **3** (113.2 mg).

Cyathuscavin A (**1**)[10] was obtained as white powder which is optically inactive. The molecular formula of **1** was determined as $C_{17}H_{14}O_8$ on the basis of HREIMS analysis ($[M]^+$ $m/z = 346.0688$) and ^{13}C NMR data (Table 1). The IR spectrum of **1** indicated the presence of hydroxyl (3402 cm^{-1}) and carbonyl groups (1732 cm^{-1}). In the ^1H and ^{13}C NMR spectra measured in DMSO-d_6, the resonances of one carboxyl (δ_H 9.71), two hydroxy (δ_H 8.90, 11.06), one *O*-methyl (δ_H 3.86), and one methylene (δ_H 5.31) protons suggested *O*-methyl and hydroxy orsellinic acid moieties in combination with all substituted aromatic carbon resonances at δ_C 147.0, 143.7, 141.1, 130.4, 114.6, and 114.0. In addition, three olefinic (δ_H 6.52, 6.26, and 6.10) and one methyl (δ_H 1.90) proton resonances, and one ester (δ_C 162.9), six olefinic

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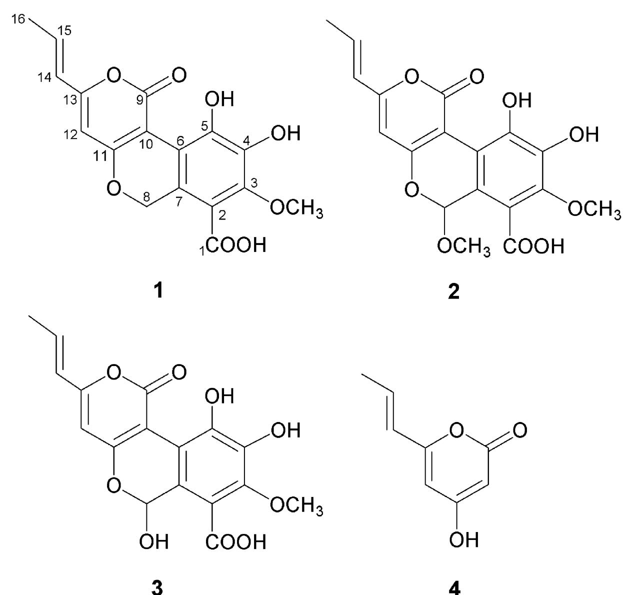

Figure 1. Structures of cyathuscavins A (**1**), B (**2**), C (**3**), and 4-hydroxyl-6-propenyl-pyran-2-one (**4**).

(δ_C 170.1, 157.6, 133.5, 124.2, 101.0, and 97.0), and one methyl (δ_C 18.8) carbon resonances depicted 4-hydroxyl-6-propenyl-pyran-2-one moiety, which was isolated as a separated compound (**4**), by the aid of COSY and HMBC analyses. The HMBC correlation of H-8 (δ_H 5.31) with C-11 (δ_C 170.1) and C-6 (δ_C 114.0) allowed the connected structure of two tetraketide moieties.[9] The structure of **1** is closely related with that of cyathusal A, but the aldehyde group of cyathusal A was replaced into carboxyl group in **1**.

Cyathuscavin B (**2**)[11] was obtained as white powder, $[\alpha]_D$ 0. The HREIMS data ($[M]^+$ m/z = 376.0792) and ^{13}C NMR data for compound **2** suggested the molecular formula as $C_{18}H_{16}O_9$. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** and the subtle difference is the additional proton resonance at δ_H 3.47 and carbon resonance at δ_C 56.2 (Table 1). The HMBC correlation of *O*-methy proton (δ_H 3.47) with 8-C (δ_C 100.7) completed the structure of **2** as C-8 *O*-methyl analog of **1**.

Table 2
DPPH, $ABTS^+$, and O_2^- scavenging activity of **1**, **2**, **3** and **4**

Sample	EC_{50} value (μM) ± SD		
	DPPH	$ABTS^+$	O_2^-
1	19.31 ± 1.26	14.85 ± 0.58	21.90 ± 3.73
2	13.92 ± 2.59	9.84 ± 0.36	25.12 ± 2.51
3	19.63 ± 2.18	7.08 ± 0.40	NA[a]
4	>100	>50	NA
BHA	99.22 ± 1.27	9.929 ± 0.52	82.56 ± 2.64
Trolox	22.10 ± 1.34	11.78 ± 0.31	NA

[a] NA = not active.

Cyathuscavin C (**3**)[12] was obtained as white powder, $[\alpha]_D$ 0. The molecular formula of **3** was determined as $C_{17}H_{14}O_9$ by HREIMS analysis ($[M]^+$ m/z = 362.0636). The NMR and MS spectroscopic data of **3** was very similar to those of **1**. Due to the additional hydroxyl group (δ_H 7.86) substituted at C-8, **3** showed the downfield-shifted resonances of H-8 at δ_H 6.58 and C-8 at δ_c 97.0 instead of the resonances at δ_H 5.31 and δ_C 66.7 in **1**. Therefore, the structure of **3** was determined as a hydroxylated compound of **1** at C-8. In the isolation procedure using methanol, the conversion of cyathuscavin C (**3**) into cyathuscavin B (**2**) was observed by HPLC analysis. This was considered to be caused by the presence of unstable hemiacetal center of C-8 in cyathuscavin C (**3**) as it had been observed in cyathusal B.[9] The highly reactive hemiacetal center of cyathuscavin C (**3**) can provide the simple method to synthesize multipotent antioxidants also with the construction of combinatorial library.

The antioxidant activity of **1**–**4** was evaluated by their free radical scavenging activity against $ABTS^+$, DPPH, and O_2^- radicals as described previously with minor modification[13–19] and compared with that of the reference antioxidants, BHA (butylated hydroxyanisole), and Trolox (Table 2). Compounds **1**–**3** showed higher scavenging activity than reference antioxidants on DPPH radical scavenging activity assay. Compound **3** exhibited the highest activity on the $ABTS^+$ radical scavenging activity assay and others showed similar activity with the reference antioxidants. On the superoxide radical scavenging assay, **1** and **2** showed higher activities than BHA, while **3** showed no activity as Trolox did. In all assays, compound **4** did not show any activity.

Table 1
NMR spectroscopic data for cyathuscavins A (**1**), B (**2**), and C (**3**)

Position	Cyathuscavin A (**1**)		Cyathuscavin B (**2**)		Cyathuscavin C (**3**)	
	δ_C[a]	δ_H (*J* in Hz)[a]	δ_C[a]	δ_H (*J* in Hz)[a]	δ_C[a]	δ_H (*J* in Hz)[a]
1	167.5		166.9		166.8	
2	114.6		116.2		116.5	
3	141.1		142.9		142.9	
4	143.7		143.9		143.9	
5	147.0		148.3		147.8	
6	114.0		113.8		113.6	
7	130.4		127.7		130.7	
8	66.7	5.31, s	100.7	6.49, s	97.0	6.58, s
9	162.9		162.7		162.7	
10	97.0		96.7		95.4	
11	170.1		167.7		168.0	
12	101.0	6.10, s	100.5	6.11, s	100.4	6.11, s
13	157.6		157.9		157.8	
14	124.2	6.26, dd (15.6, 1.6)	124.1	6.26, dd (15.6, 1.6)	124.1	6.26, dd (16.0, 1.6)
15	133.5	6.52, dq (15.6, 6.8)	133.9	6.50, dq (15.6, 6.8)	133.8	6.52, dq (16.0, 6.8)
16	18.8	1.90, d (6.8)	18.8	1.90, d (6.8)	18.8	1.90, d (6.8)
3-OCH_3	60.0	3.86, s	60.4	3.89, s	60.6	3.90, s
8-OCH_3			56.2	3.47, s		
1-OH		9.71, s		9.81, s		9.69, s
8-OH						7.86, s
4-OH		8.90, s		9.12, s		8.97, s
5-OH		11.06, s		11.15, s		11.14, s

[a] ^1H and ^{13}C NMR were measured at 400 and 100 MHz, respectively, in DMSO-d_6 and solvent resonances were used as reference.

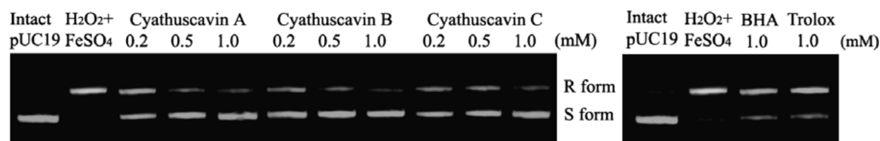

Figure 2. Protection activity of cyathuscavins A–C (left) and reference BHA and Trolox (right) against supercoiled plasmid DNA breakage. Relaxed DNA is indicated as R form and supercoiled DNA is indicated as S form.

Compounds **1**, **2**, and **3** protected supercoiled DNA from the Fenton reaction-mediated DNA breakage. The intact supercoiled DNA (S form) was broken into relaxed (R form) by Fe^{2+}/H_2O_2-induced hydroxyl radical.[20,21] Compound **2** showed 90% of protective activity at 1 mM, and compounds **1** and **3** possessed over 70% of protective activity at the same concentration. However, BHA and Trolox showed lower supercoiled DNA protection effect than compounds **1**–**3** (Fig. 2). These results are corresponded with radical scavenging activity of compounds **1**–**3**.

These results suggest that the activities of the compounds **1**–**3** would be due to their abilities to scavenge free radicals derived from the phenolic moiety and to chelate metal ions by means of the *o*-dihydroxy group of the structures. It has been an well-known fact that phenolic hydroxyl groups are important for radical scavenging activity. The strong free radical scavenging activity of compounds **1**–**3** may be due to the presence of catechol moiety whereas **4** has no phenolic group.[22] The absence of superoxide activity of compound **3** may be due to its chemical instability of the hemiacetal center (C-8) that could lead to unexpected side reactions. The metal ion chelating ability of the catechol moiety in compounds **1**–**3** might collaborate with free radical scavenging ability to the DNA protection from the Fenton-mediated breakage.[23]

Up to date, isolation of bioactive compounds from mushrooms has been done mainly on the products of fruiting bodies. However, collection of fruiting bodies is an onerous work and is limited by collecting season and area. Also the small amounts of the collected samples are limited to find metabolites from mushrooms. Thus, mycelial culture of basidiomycetes originated from wild mushrooms would be suggested as an alterative, consistent, and controllable method that can obtain various bioactive metabolites from mushrooms.

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10. *Cyathuscavin A* (**1**)*:* White powder; $[\alpha]_D^{20}$ 0 (*c* 0.24, MeOH); UV (MeCN) λ_{max} (log ε) 220.0 (4.55), 265.0 (3.83), and 330.5 (3.95) nm; IR (KBr) ν_{max} 3402, 2957, 2710, 2642, 1732, 1668, and 1560 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode) *m/z* 345 [M−H]$^-$; ESIMS (positive ion mode) *m/z* 369 [M+Na]$^+$; HREIMS *m/z* 346.0688 [M]$^+$ (calcd 346.0689 for $C_{17}H_{14}O_8$).
11. *Cyathuscavin B* (**2**)*:* White powder; $[\alpha]_D^{20}$ 0 (*c* 0.91, MeOH); UV (MeCN) λ_{max} (log ε) 221.5 (4.66), 273.5 (3.93), and 332.0 (4.09) nm; IR (KBr) ν_{max} 3394, 3097, 2945, 2847, 1755, 1670, 1626, and 1564 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode) *m/z* 375 [M−H]$^-$; HREIMS *m/z* 376.0792 [M]$^+$ (calcd 376.0794 for $C_{18}H_{16}O_9$).
12. *Cyathuscavin C* (**3**)*:* White powder; $[\alpha]_D^{20}$ 0 (*c* 0.47, MeOH); UV (MeCN) λ_{max} (log ε) 221.0 (4.56), 271.5 (3.85), and 331.5 (3.97) nm; IR (KBr) ν_{max} 3354, 3180, 3005, 2966, 2853, 1726, 1668, and 1560 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode), *m/z* 361 [M−H]$^-$; HREIMS *m/z* 362.0636 [M]$^+$ (calcd 362.0638 for $C_{17}H_{14}O_9$).
13. *ABTS cation radical scavenging activity:* The total antioxidant activity of the compounds was determined using the $ABTS^+$ scavenging assay of Re et al.[14] The ABTS cation radical was produced by the reaction between 7.0 mM ABTS and 2.45 mM potassium persulfate in water for 12 h in the dark at room temperature. The $ABTS^+$ solution was diluted with PBS until A_{734} = 0.7. The reaction was initiated by adding 190 µl of $ABTS^+$ to 10 µl sample solution at 25 °C. The percentage of reduction of A_{734} was recorded and was plotted as a function of the sample's concentration. The EC_{50} values of test compounds, which lead to 50% loss of the $ABTS^+$ radical, were calculated by the same equation as described in the DPPH assay.
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15. DPPH radical scavenging activity was determined by using the method of Blois[16] with minor modifications. The solution of the sample (10 µL) in ethanol was added to 90 µL of a 0.15-mM DPPH radical in ethanol in a 96-well plate. The sample solution refers to the tested compounds and the reference antioxidants at various concentrations, as well as ethanol as a control. The solutions of the tested compounds had concentrations ranging from 3 to 1000 µg/ml, whereas the concentrations of the solutions of the reference compounds varied from 0.1 to 1000 µg/ml. The reaction leading to the scavenging of DPPH radical was complete within 10 min at 25 °C. The absorbance of the mixture was then measured at 517 nm using a microplate reader. The reduction of DPPH radical was expressed as percentage:Scavenged DPPH (%) = $(1 - A_{test}/A_{control}) \times 100$, where A_{test} is the absorbance of a sample at a given concentration after 10 min. reaction time, and $A_{control}$ is the absorbance recorded for 10 µL ethanol. The EC_{50} value is defined as the concentration of sample that causes 50% loss of the DPPH radical.
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17. Superoxide radical scavenging activity was measured by employing a modified method of Beauchamp and Fridovich[18] as described by Kim et al.[19] The mixture consisted of 140 µL of 0.030 mM riboflavin, 1 mM EDTA, 0.60 mM methionine and 0.030 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 µL of a sample solution, which includes the test compounds and the reference compound at various concentrations in DMSO, as well as DMSO as a control. The photoinduced reactions to generate superoxide anion were carried out in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated at 25 °C for 8 min. The photochemically reduced riboflavin generated superoxide anion, which reduced NBT to form the blue formazan. The un-illuminated reaction mixture was used as a blank. Reduction of NBT was measured by the absorbance change at 560 nm before and after irradiation using a microplate. Scavenging activity was calculated from the absorbance changes of control and test samples: Scavenging activity (%) = $(1 - \Delta A_{sample}/\Delta A_{control}) \times 100$, where ΔA_{sample} is the change of the absorbance in the wells containing the tested compounds, and $\Delta A_{control}$ is the change of the absorbance in the wells containing DMSO instead of the tested compounds. The EC_{50} value is defined as the concentration of substrate that causes 50% loss of the reduced NBT. The assays were performed in triplicate and the absorbance changes were averaged before calculation.
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20. DNA was prepared by described in Ref. 21 *Escherichia coli* DH5α cells were transformed with pUC19 plasmid DNA and were grown in LB medium containing ampicillin (50 µg/mL) at 37 °C for overnight. Plasmid DNA was purified using the QIAprep-spin Plasmid Kit (Quiagen Inc., Chatsworth, USA). Evaluation of DNA protection effect from Fenton-reaction-mediated DNA breakage was conducted by using supercoiled plasmid DNA.[21] Five microliters of Tris–HCl buffer (100 mM, pH 7.5), 10 µL of plasmid DNA (0.2 µg/µL), 1 µL of different concentration of samples, and 2 µL of hydrogen peroxide (50 mM) were mixed. The reaction was initiated by adding 2 µL of ferrous sulfate (5 mM) into the mixture. After 10 min, the reaction was

stopped by addition of 10 μL of stop solution (8 M urea, 50% sucrose, 50 mM EDTA, 0.1% bromophenol blue). The reactants were electrophoresed in 1% of agarose gel. After running, agarose gel was stained with ethium bromide and then analyzed with image analyzer.

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