J. Nat. Prod. 2007, 70, 1043−1045 1043

Cyathusals A, B, and C, Antioxidants from the Fermented Mushroom *Cyathus stercoreus*

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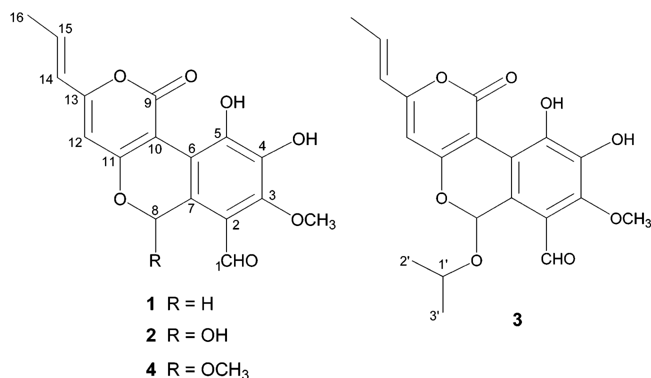
Received December 27, 2006

Three new polyketide-type antioxidative compounds, cyathusals A (**1**), B (**2**), and C (**3**), and the known pulvinatal (**4**) were obtained from the fermented mushroom *Cyathus stercoreus*. The structures of the compounds were characterized on the basis of NMR and mass spectroscopic data. Cyathusals A, B, and C and pulvinatal showed free radical scavenging activities on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with EC_{50} values of 41.6, 46.0, 26.6, and 28.6 μM, respectively, and on the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) cation radical with EC_{50} values of 7.9, 11.1, 9.1, and 8.4 μM, respectively.

Reactive oxygen species and free radicals can react with biomolecules including lipids, proteins, and DNA and may cause disease, such as brain disorders, atherosclerosis, and cancer.[1,2] Antioxidants are good in protection and as therapeutic means against diseases related to oxidative damage.[3,4] In past decades, much attention has been paid to the natural antioxidant defense network. As a result, many natural antioxidants have been found from various natural sources and have been utilized in foods, medicines, and cosmetics.[5,6] In the course of our efforts to find natural antioxidants from Korean mushrooms, we have isolated several novel antioxidants, such as inoscavin A,[7] sterin C,[8] and hirsutenols D−F.[9] In our continued screening program for natural antioxidants from basidiomycetes, *Cyathus stercoreus* was chosen for chemical investigation.

C. stercoreus is a basidiomycete belonging to the bird's nest fungi due to its appearance of the fruiting bodies. Mushrooms of the genus *Cyathus* have been known to produce some interesting antibiotic compounds, such as cyathins and striatins.[10,11] Here, we describe the isolation, structural determination, and antioxidant activity of three new metabolites, cyathusals A (**1**), B (**2**), and C (**3**), together with the known compound pulvinatal (**4**) from the fermented *C. stercoreus*. Cyathusal A (**1**) was isolated as yellow, amorphous powder. Its molecular formula was deduced as $C_{17}H_{14}O_7$ from HREIMS analysis (*m/z* 330.0740 $[M]^+$) in combination with ^{13}C NMR data. The ^1H NMR spectrum showed resonances for one aldehyde (δ_H 10.36), three olefinic (δ_H 6.86, 6.12 and 6.10), one *O*-methyl (δ_H 4.07), one methylene (δ_H 5.61), one methyl (δ_H 1.98), and two hydroxy (δ_H 12.24 and 6.07) protons. The ^{13}C NMR and HMQC spectra depicted 17 carbons attributable to one aldehyde (δ_C 191.2), one ester (δ_C 165.6), 12 olefinic (δ_C 170.1, 159.4, 150.8, 147.7, 138.8, 138.0, 123.4, 122.0, 118.6, 108.8, 101.7, and 100.5), one methylene (δ_C 67.1), one *O*-methyl (δ_C 62.0), and one methyl (δ_C 18.9) carbon. The $^1H-^1H$ COSY correlations of H-14/H-15/H-16 indicated the presence of a propenyl group linked to an olefinic carbon. The HMBC correlations of the aldehydic proton (H-1) with C-3 and C-7, C4-OH with C-3 and C-5, and C5-OH with C-4 and C-6 suggested the presence of a substituted aromatic ring with an aldehyde, an *O*-methyl, and two hydroxy groups. Also, the long-range correlations of H-12 with C-14 and C-10, and H-15 with C-13, indicated a propenyl-substituted unsaturated δ-lactone moiety. Finally, the HMBC correlation of H-8 with C-11 and C-6 showed the connection of the lactone ring with an aromatic ring via the $-O-C_8-$ bridge. The key HMBC correlations of **1** are shown in Figure 1. From the above spectrometric analysis, the



structure of **1** was similar to that of pulvinatal (**4**) with C-8 *O*-methyl being replaced by hydrogen.[12]

Cyathusal B (**2**) was obtained as light yellow, amorphous powder, $[\alpha]_D$ 0. The molecular formula was determined as $C_{17}H_{14}O_8$ on the basis of HREIMS (*m/z* 346.0688 $[M]^+$) and ^{13}C NMR analysis. ^1H and ^{13}C NMR data of **2** were similar to those of **1**; however **2** showed deshielded H-8 (δ_H 7.23) and C-8 (δ_C 96.7) resonances. The structural confirmation of **2** was accomplished by analysis of the HMBC spectrum, which showed correlations of H-8 with C-11 and C-16. These spectroscopic data indicated that **2** is the C-8-oxygenated analogue of compound **1**.

Cyathusal C (**3**) was obtained as yellow crystals, $[\alpha]_D$ 0. The molecular formula was determined as $C_{20}H_{20}O_8$ on the basis of HREIMS (*m/z* 388.1159 $[M]^+$) and ^{13}C NMR analysis. Its ^1H and ^{13}C NMR spectra were similar to those of **1**. However, the ^1H and ^{13}C NMR spectra of **3** showed a methine [δ_H 4.30 (H-1′); δ_C 73.7-(C-1′)] and two methyl [δ_H 1.26 (H-3′), 1.18 (H-2′); δ_C 23.8(C-3′), 22.5(C-2′)] resonances and deshielded H-8 (δ_H 7.24) and C-8 (δ_C 95.4) resonances due to the addition of one more oxygen at C-8. The COSY spectrum suggested the presence of an isopropanoxy group by the correlations of H-1′ with H-2′ and H-3′. In the HMBC spectrum, the correlation of H-1′ with C-8 indicated the attachment of the isopropanoxy group at C-8. Therefore, **3** was defined as the C-8 isopropanoxy analogue of compound **1**.

Compound **4** was identified as pulvinatal by comparison with published data. Pulvinatal was initially isolated from *Nidularia pulvinata* as an inducer of the differentiation of HL-60 promyelocytic leukemia cells.[12]

In this study, the preliminary isolation experiment of **4** suggested that if methanol is used in the isolation process, the aldehyde group of this compound can react with two methanol molecules to form acetal (**5**) (Figure 2). This was proven by ^1H NMR analysis. The aldehydic proton resonance of **4** at δ_H 10.43 disappeared and the acetal proton resonance was observed at δ_H 5.67. Also, two

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10.1021/np060637h CCC: $37.00 © 2007 American Chemical Society and American Society of Pharmacognosy
Published on Web 05/19/2007

Table 1. NMR Spectroscopic Data for Cyathusals A (**1**), B (**2**), and C (**3**)

	cyathusal A (**1**)		cyathusal B (**2**)		cyathusal C (**3**)	
position	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	191.2	10.36, s	190.6	10.41, s	190.7	10.42, s
2	118.6		117.6		117.4	
3	150.8		151.4		151.6	
4	138.8		139.5		139.6	
5	147.7		147.7		148.0	
6	108.8		108.2		108.7	
7	122.0		121.2		122.0	
8	67.1	5.61, s	96.7	7.23, d (5.2)	95.4	7.24, s
9	165.6		165.6		165.9	
10	101.7		101.1		102.1	
11	170.1		165.8		166.1	
12	100.5	6.10, s	101.1	6.22, s	101.3	6.17, s
13	159.4		159.0		159.1	
14	123.4	6.12, dd (16.0, 1.6)	121.7	6.11, dd (15.6, 1.6)	122.0	6.12, dd (15.6, 1.6)
15	138.0	6.86, dq (16.0, 7.0)	137.7	6.87, dq (15.6, 6.8)	137.7	6.87, dq (15.6, 6.8)
16	18.9	1.98, dd (7.0, 1.6)	18.9	1.99, dd (6.8, 1.6)	18.9	1.98, dd (6.8, 1.6)
1′					73.7	4.30, m (6.0)
2′					22.5	1.18, d (6.0)
3′					23.8	1.26, d (6.0)
3-OCH_3	62.0	4.07, s	62.0	4.10, s	62.0	4.07, s
4-OH		6.07, s		6.18, s		6.16, s
5-OH		12.24, s		12.90, s		12.76, s
8-OH				3.94, d (5.2)		

[a] ^1H and ^{13}C NMR were measured at 400 and 100 MHz, respectively, in $CDCl_3$, and solvent resonances were used as reference.

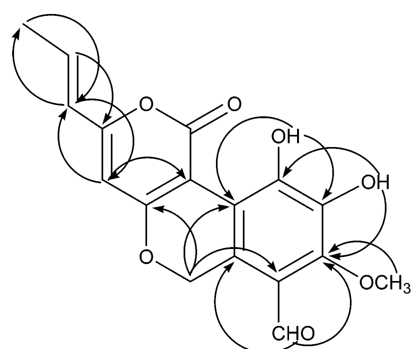

Figure 1. HMBC correlations for cyathusal A (**1**).

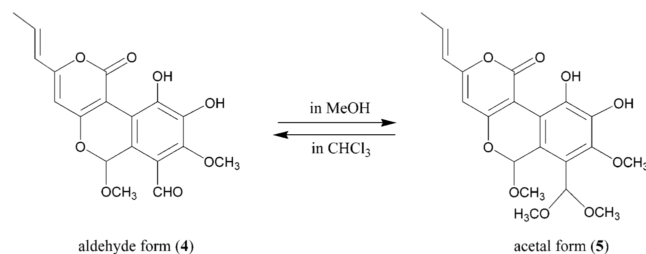

Figure 2. Acetal formation of pulvinatal (**4**) in MeOH.

O-methyl resonances at δ_H 3.47 and 3.50 emerged. This interesting chemical property of **4** provides some advantages in the isolation procedure with the high product yield and also with the protection of the aldehyde group. We obtained **4** simply by the addition of methanol to the ethyl acetate extract. In this process, the acetal formation occurred in methanol solution and the crystallized acetal **5** was obtained. Then, the acetal group of **5** was converted into the aldehyde group by hydrolysis in chloroform solution. In the preliminary isolation, we also isolated the acetal forms of **1**, **2**, and **3**. Therefore, to clarify that the acetal forms are the artifacts formed in methanol, we employed the isolation process without the use of alcoholic solvents, and this yielded the aldehyde compounds **1**, **2**, and **3**.

Chemical instability of the hemiacetal center (C-8) of **2** may cause many artifacts by replacement of the hydroxy group with other nucleophiles. Thus, compound **3** is considered to be an artifact derived from **2** because the isopropanoxy group is biosynthetically unusual. However, the isolation experiments employed only non-nucleophilic solvents.

Table 2. DPPH and ABTS Radical Scavenging Activity of **1**, **2**, **3**, and **4**

	EC_{50} value (μM) ± SD	
sample	DPPH	$ABTS^{•+}$
1	41.59 ± 2.384	7.86 ± 0.138
2	46.01 ± 0.417	11.06 ± 0.440
3	26.64 ± 0.840	9.10 ± 0.092
4	28.59 ± 0.948	8.36 ± 0.154
BHA	97.09 ± 5.522	11.70 ± 0.261
Trolox	37.61 ± 1.687	14.54 ± 0.083

The antioxidant activity of **1**−**4** was evaluated by DPPH and ABTS radical scavenging activity assays and compared with that of reference antioxidants, BHA and Trolox (Table 2). The antioxidant activity was defined as an amount of antioxidant necessary to decrease the initial DPPH and ABTS radical concentration by 50% [EC_{50} (μM)]. DPPH radical scavenging activities of **3** and **4** were higher than those of Trolox and BHA, while **1** and **2** showed almost the same activity as those of the reference antioxidants. In the ABTS radical scavenging assay, **1**−**4** showed higher activity than those of Trolox and BHA.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. UV and FT-IR spectra were recorded on a Pharmacia Biotech Ultrospec 3000 UV/visible spectrometer and a Shimazu 8400S FT-IR spectrometer, respectively. ^1H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded on a Varian INOVA 400 MHz NMR spectrometer. $CDCl_3$ was used as a solvent for the NMR experiments, and the solvent resonances were used as an internal reference. For the 2D heteronuclear correlation spectroscopy, the refocusing delays were optimized for $^1J_{CH} = 145$ Hz and $^nJ_{CH} = 10$ Hz. ESI and HREI mass spectra were acquired using a Finnigan Navigator 30086 and a JMS-700 Mstation high-resolution mass spectrometer system, respectively. HPLC was carried out using a Waters HPLC system equipped with Waters 996 photodiode array detector and Millenium32 software using a C_{18} column (J'sphere ODS-H80, 150 × 20 mm, 4 μm, YMC Co.). Trolox and BHA were purchased from Aldrich Co. DPPH and ABTS were purchased from Sigma Co.

Mushroom Strain. The mushroom strain, *C. stercoreus* (IUM00802), was kindly donated by the culture collection of wild mushroom species of the University of Incheon, Korea.

Fermentation and Isolation. The *C. stercoreus* strain was maintained in solid potato dextrose agar medium. For submerged seed culture, the strain was grown in YPS medium (20 g of glucose, 2 g of yeast extract, 5 g of bactopeptone, 0.5 g of $MgSO_4$, and 1 g of KH_2PO_4 per liter of dH_2O) under shaking at 26 °C for 1 week. For fermentation, the seed cultures were inoculated to 5 L fermentors containing 3 L of the same medium. Fermentation was carried out at 28 °C with aeration and agitation. After 2 weeks of fermentation, a total of 30 L of culture was harvested, and the culture broth was separated from the mycelium by filtration. The mycelium was extracted with 80% aqueous acetone. The culture broth and the evaporated mycelium extract were combined and extracted with EtOAc. The EtOAc extract (12 g) was divided into the $CHCl_3$-soluble fraction and the residue. The $CHCl_3$-soluble fraction was separated by reversed-phase C_{18} open column chromatography using a solvent gradient from 30% to 100% MeCN. Further purification by reversed-phase HPLC with 50% MeCN afforded **1** (34.6 mg), **2** (6.0 mg), and **3** (12.6 mg). From the residue fraction, **5** was obtained by crystallization from MeOH. Then, **5** was converted into **4** (400 mg) by hydrolysis in $CHCl_3$ solution.

Cyathusal A (1): yellow, amorphous powder; UV (MeCN) λ_{max} (log ϵ) 237 (5.42), 256 (5.32), 302 (5.09), 372 (5.16) nm; IR (KBr) ν_{max} 3475, 2954, 2921, 2853, 1733, 1679, 1659, 1622, 1577, 1531, 1432, 1406, 1352, 1313, 1294, 1242, 1219 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode) m/z 329 $[M - H]^-$; HREIMS m/z 330.0740 $[M]^+$ (calcd for $C_{17}H_{14}O_7$, 330.0739).

Cyathusal B (2): light yellow, amorphous powder; $[\alpha]^{20}_D$ 0 (*c* 0.04, MeCN); UV (MeCN) λ_{max} (log ϵ) 194 (5.36), 231 (5.23), 303 (4.86), 361 (4.96) nm; IR (KBr) ν_{max} 3412, 2923, 2854, 1666, 1632, 1585, 1530, 1416, 1348, 1313, 1215 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode) m/z 345 $[M - H]^-$; HREIMS m/z 346.0688 $[M]^+$ (calcd for $C_{17}H_{14}O_8$, 346.0689).

Cyathusal C (3): yellow crystals (MeCN); mp 184−185 °C; $[\alpha]^{20}_D$ 0 (*c* 0.24, MeCN); UV (MeCN) λ_{max} (log ϵ) 228 (5.33), 258 (5.20), 303 (4.98), 362 (5.15) nm; IR (KBr) ν_{max} 3224, 2968, 2921, 1664, 1629, 1578, 1528, 1414, 1352, 1319, 1242, 1215 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS (negative ion mode), m/z 387 $[M - H]^-$; HREIMS m/z 388.1159 $[M]^+$ (calcd for $C_{20}H_{20}O_8$, 388.1158).

Pulvinatal (4): yellow crystals (MeCN); mp 189−190 °C; $[\alpha]^{20}_D$ 0 (*c* 0.72, MeCN); UV (MeCN) λ_{max} (log ϵ) 227 (5.38), 257 (5.25), 303 (5.05), 363 (5.17) nm; IR (KBr) ν_{max} 3427, 2940, 2845, 1673, 1662, 1633, 1581, 1531, 1418, 1343, 1312, 1238, 1207 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.99 (3H, dd, $J = 6.8$, 1.6 Hz, H-16), 3.65 (3H, s, R-OMe), 4.08 (3H, s, 3-OMe), 6.12 (1H, dd, $J = 15.6$, 1.6 Hz, H-14), 6.16 (1H, s, 4-OH), 6.21 (1H, s, H-12), 6.87 (1H, dq, $J = 15.6$, 7.0 Hz, H-15), 6.96 (1H, s, H-8), 10.43 (1H, s, H-1), 12.77 (1H,s 5-OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.9 (C-16), 59.0 (8-OMe), 62.0 (3-OMe), 97.0 (C-8), 101.3 (C-12), 101.9 (C-10), 108.4 (C-6), 117.8 (C-2), 121.4 (C-7), 122.0 (C-14), 137.9 (C-15), 139.7 (C-4), 147.9 (C-5), 151.6 (C-3), 159.2 (C-13), 165.8 (C-9), 165.9 (C-11), 190.8 (C-1); ESIMS (negative ion mode) m/z 359 $[M - H]^-$; HREIMS m/z 360.0842 $[M]^+$ (calcd for $C_{18}H_{16}O_8$, 360.0845).

Pulvinatal-acetal (5): light brown crystals (MeOH); mp 193−194 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.95 (3H, dd, $J = 6.8$, 1.6 Hz, H-16), 3.38 (3H, s, R-OMe), 3.47 (3H, s, 1-OMe), 3.50 (3H, s, 1-OMe), 3.93 (3H, s, 3-OMe), 5.67 (1H, s, H-1), 6.09 (1H, dd, $J = 15.6$, 1.6 Hz, H-14), 6.17 (1H, s, H-12), 6.73 (1H, s, H-8), 6.82 (1H, dq, $J = 15.6$, 7.0 Hz, H-15), 11.74 (1H, s, 5-OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.8 (C-16), 55.2 (8-OMe), 56.2 (1-OMe), 56.4 (1-OMe), 61.3 (3-OMe), 98.5 (C-8), 101.4 (C-12), 101.6 (C-10), 103.1 (C-1), 108.7 (C-6), 118.8 (C-2), 120.0 (C-7), 122.2 (C-14), 136.9 (C-15), 139.8 (C-4), 142.5 (C-3), 145.9 (C-5), 158.7 (C-13), 165.1 (C-9), 165.5 (C-11); EIMS m/z 406 $[M]^+$.

DPPH Radical Scavenging Activity. DPPH radical scavenging activity was assessed according to the method of Lee et al.[13] with minor modifications. Ten microliters of diluted samples, standard antioxidant solutions, or DMSO (control) was added to 190 μL of a 1.5×10^{-4} M EtOH solution of DPPH radical in a well of a 96-well plate. The absorbance of the reaction mixture at 517 nm was measured at steady state after 30 min of incubation at room temperature (25 °C) using a microplate reader. The concentration-dependent scavenged DPPH radical was calculated from absorptions at steady state by

$$\text{scavenged DPPH (\%)} = (1 - A_{test}/A_{control}) \times 100$$

where A_{test} is the absorbance of a sample at a given concentration after 30 min reaction time and $A_{control}$ is the absorbance recorded for 10 μL of DMSO. The EC_{50} value is defined as the concentration of sample that scavenges 50% of the DPPH radical.

ABTS Radical Scavenging Activity. Spectrophotometric analysis of ABTS cation radical scavenging activity was conducted according to the method of Re et al. (1999).[14] ABTS radical was produced by the reaction between 7.0 mM ABTS/H_2O and 2.45 mM potassium persulfate for 12 h in the dark at room temperature. The ABTS radical solution was diluted with PBS until $A_{734} = 0.7$. The reaction was initiated by adding 190 μL of ABTS radical solution to 10 μL of sample solution disolved in DMSO at 25 °C. After 7 min, the absorbance at 734 nm was recorded and the reduction of the absorbance was plotted as a function of the sample's concentration. The EC_{50} values of the test compounds, which lead to 50% loss of the ABTS radical, were calculated in the same way as described in the DPPH assay.

Acknowledgment. This research was supported by a grant from the KRIBB−JEJU joint research program funded by Jeju Special Self-Governing Province, South Korea.

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NP060637H