Hispidin Derivatives from the Mushroom *Inonotus xeranticus* and Their Antioxidant Activity

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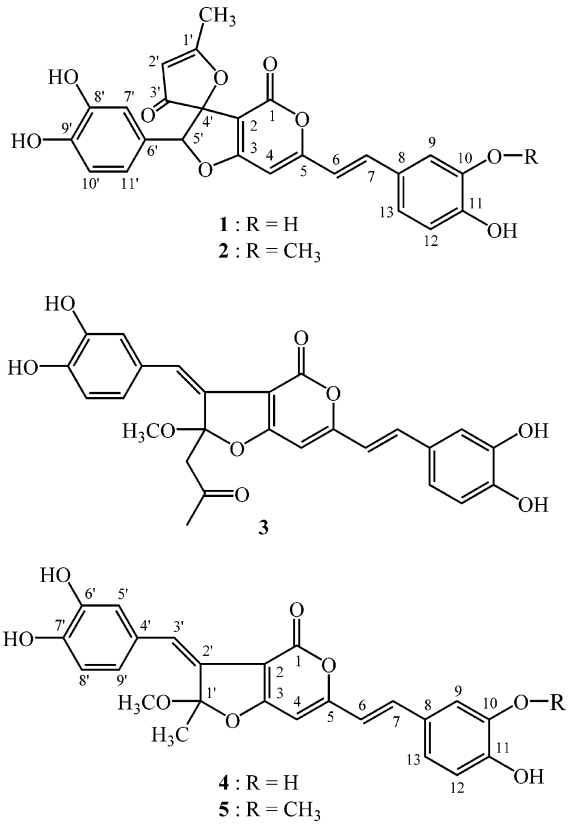
In an effort to identify antioxidants from edible and medicinal mushrooms, three new hispidin derivatives, methylinoscavin A (**2**), inoscavin B (**4**), and methylinoscavin B (**5**), together with the known compounds inoscavin A and phelligridin F, were isolated from the methanolic extract of the fruiting bodies of *Inonotus xeranticus*. Their structures were determined on the basis of spectroscopic analyses.

Mushrooms are ubiquitous in nature and produce structurally unique and biologically active secondary metabolites. Some of them are nutritionally functional foods, and some are important sources of physiologically beneficial medicines in Asia. We have screened free radical scavengers from basidiomycetes in view of the fact that free radicals are implicated in the pathogenesis of various diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation, and aging processes.[1−3] *Inonotus xeranticus* (Berk.) Imaz. Et Aoshi. (Hymenochaetaceae), widely distributed in East Asia including Korea, Japan, and China, is a saprophytic fungus preferably living on deciduous trees such as *Quercus* species.[4] Although *Inonotus* species have been used for the treatment of gastrointestinal cancer, cardiovascular disease, tuberculosis, liver or heart diseases, stomach ailments, and diabetes as a traditional medicine,[5] *I. xeranticus* is not used. In a previous investigation we found that *I. xeranticus* produced a considerable quantity of yellow antioxidant pigment and reported the isolation of inoscavin A (**1**) as a major component of the pigment.[6] As part of our ongoing efforts to characterize antioxidant constituents from the fruiting body of *I. xeranticus*, three new hispidin derivatives named methylinoscavin A (**2**), inoscavin B (**4**), and methylinoscavin B (**5**) have been isolated together with the known compound phelligridin F (**3**).[7] The isolation, structure determinations, and antioxidant activities of these compounds are described in this paper.

Fresh fruiting bodies of *I. xeranticus* were extracted twice with MeOH. The methanolic extract was partitioned between *n*-hexane and H_2O and then between ethyl acetate and H_2O. Repeated chromatographic separations of the ethyl acetate-soluble fraction led to the purification of five antioxidant substances, **1**−**5**.

Compound **2** was obtained as a yellow powder, and its molecular formula was established as $C_{26}H_{20}O_9$ by HRESIMS. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1**,[6] except for signals of an additional methoxyl group at δ 3.92 in the ^{1}H NMR spectrum and δ 56.5 in the ^{13}C NMR spectrum. The structure of **2** was assigned by interpretation of the HMBC spectrum, which exhibited a long-range correlation from aromatic methoxyl protons at δ 3.92 to C-10 at δ 149.5. Other HMBC correlations were consistent with those of **1**. Therefore, the structure of **2** was determined to be methylinoscavin A, as shown.

The molecular weight of compound **3** was established to be 478 by ESIMS, providing quasi-molecular ions at *m*/*z* 477 $[M - H]^-$ in the negative mode and *m*/*z* 501 $[M + Na]^+$ in the positive mode. Its ^{1}H and ^{13}C NMR data were in good agreement with those of phelligridin F, a hispidin analogue isolated from the mushroom *Phellinus igniarius*.[7] Compound **3** was confirmed to be identical to phelligridin F by interpretation of 2D NMR spectra including HMQC, HMBC, and NOESY.[7]



1 : R = H
2 : R = CH_3

3

4 : R = H
5 : R = CH_3

The molecular formula of compound **4** was determined to be $C_{24}H_{20}O_8$ by HRESIMS in combination with ^{1}H and ^{13}C NMR data. Its IR spectrum showed absorption bands for hydroxyl (3450 cm^{-1}), conjugated carbonyl (1656 cm^{-1}), and aromatic rings (1605 and 1543 cm^{-1}). The ^{1}H NMR spectrum in CD_3OD showed six aromatic methine signals assignable to two 1,2,4-trisubstituted benzene moieties at δ 7.06 (1H, d, J = 2.0 Hz), 6.98 (1H, dd, J = 8.0, 2.0 Hz), and 6.79 (1H, d, J = 8.0 Hz) and δ 7.24 (1H, d, J = 2.0 Hz), 7.02 (1H, dd, J = 8.4, 2.0 Hz), and 6.74 (1H, d, J = 8.4 Hz), two methine singlets at δ 7.37 and 6.37, two olefinic methine peaks assigned to a *trans*-1,2-disubstituted double bond unit at δ 7.39 (1H, d, J = 16.0 Hz) and 6.67 (1H, d, J = 16.0 Hz), and singlets at δ 3.37 and 1.75 attributable to methoxyl and methyl groups, respectively. The ^{13}C NMR spectrum revealed the presence of 24 carbons comprised of one methyl, one methoxyl, 10 sp^2 methines, and 12 quaternary carbons including an ester carbonyl and six oxygenated sp^2 carbons. Twelve proton-bearing carbons were

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Table 1. ^{1}H and ^{13}C NMR Spectral Data for Compounds **2**, **4**, and **5** in CD_3OD[a]

	2		**4**		**5**	
no.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	160.6		160.6		160.9	
2	99.6		104.1		104.3	
3	176.8		171.0		171.2	
4	95.7	6.51 (1H, s)[b]	95.8	6.37 (1H, s)	96.0	6.39 (1H, s)
5	167.0		163.5		163.8	
6	117.0	6.83 (1H, d, $J = 16.0$)	116.9	6.67 (1H, d, $J = 16.0$)	117.3	6.78 (1H, d, $J = 16.0$)
7	139.7	7.51 (1H, d, $J = 16.0$)	138.0	7.39 (1H, d, $J = 16.0$)	137.9	7.46 (1H, d, $J = 16.0$)
8	128.5		128.7		128.8	
9	111.5	7.24 (1H, d, $J = 2.0$)	114.8	7.06 (1H, d, $J = 2.0$)	111.3	7.22 (1H, d, $J = 2.0$)
10	149.5		146.7		149.5	
10-OCH_3	56.5	3.92 (3H, s)			56.5	3.92 (3H, s)
11	150.4		148.8		150.2	
12	116.6	6.83 (1H, d, $J = 8.4$)	116.5	6.79 (1H, d, $J = 8.0$)	116.6	6.82 (1H, d, $J = 8.4$)
13	123.9	7.12 (1H, dd, $J = 8.4, 2.0$)	122.2	6.98 (1H, dd, $J = 8.0, 2.0$)	123.6	7.10 (1H, dd, $J = 8.4, 2.0$)
1′	192.9		118.2		118.2	
1′-CH_3	16.6	1.98 (3H, s)	23.3	1.75 (3H, s)	23.2	1.76 (3H, s)
1′-OCH_3			51.6	3.37 (3H, s)	49.9	3.37 (3H, s)
2′	105.1	5.58 (1H, s)	129.2		129.3	
3′	203.1		126.2	7.37 (1H, s)	126.4	7.37 (1H, s)
4′	94.5		128.7		128.8	
5′	95.9	5.66 (1H, s)	117.4	7.24 (1H, d, $J = 2.0$)	117.4	7.24 (1H, d, $J = 2.0$)
6′	123.2		146.2		146.4	
7′	115.5	6.71 (1H, d, $J = 2.0$)	146.8		147.0	
8′	146.3		116.3	6.74 (1H, d, $J = 8.4$)	116.4	6.74 (1H, d, $J = 8.4$)
9′	147.8		123.5	7.02 (1H, dd, $J = 8.4, 2.0$)	123.6	7.02 (1H, dd, $J = 8.4, 2.0$)
10′	115.9	6.75 (1H, d, $J = 8.0$)				
11′	120.3	6.59 (1H, dd, $J = 8.0, 2.0$)				

[a] NMR data were measured at 400 MHz for proton and at 100 MHz for carbon. [b] Proton resonance integral, multiplicity, and coupling constant (J = Hz) are in parentheses.

assigned by the aid of an HMQC spectrum, as shown in Table 1. NMR analysis implied that the structure of **4** was very similar to that of **3**, except for the disappearance of a ketone and a sp^3 methylene signal in **3**. The structure of **4** was unambiguously established by the HMBC spectrum. The hispidin moiety was revealed by the long-range correlations from H-4 to C-2, C-5, and C-6, H-6 to C-5 and C-8, H-7 to C-9 and C-13, H-9 to C-11 and C-13, H-12 to C-8 and C-10, and H-13 to C-7, C-9, and C-11, and these chemical shift values were in good agreement with the corresponding protons and carbons in **3**. Long-range correlations from H-3′ at δ 7.37 to C-5′ and C-9′, H-5′ to C-7′ and C-9′, H-8′ to C-4′ and C-6′, and H-9′ to C-5′ and C-7′ suggested the presence of a 3,4-dihydroxybenzylidene moiety. Additional HMBC cross-peaks from H-3′ to C-2 at δ 104.1, from the methyl protons at δ 1.75 to C-1′ and C-2′, and from the methoxyl protons at δ 3.37 to C-1′ at δ 118.2 were evident. The proton and carbon chemical shifts of the methoxyl methyl at δ 3.37 and 51.6, respectively, were relatively shifted upfield, suggesting that the carbon at δ 118.2 that was long-range coupled with the methoxyl protons at δ 3.37 should be an sp^3 acetal carbon. To satisfy the molecular formula $C_{24}H_{20}O_8$, 15 degrees of unsaturation, and the HMBC cross-peaks from H-3′ to C-2 at δ 104.1 and the methyl protons at δ 1.75 to C-1′ and C-2′, C-3 should be connected to C-1′ by an ethereal oxygen bridge. Consequently, the structure of **4** was determined to be a new hispidin analogue as shown. The stereochemistry at C-1′ remains to be determined.

Compound **5** was obtained as a yellow powder, and its molecular formula $C_{25}H_{22}O_8$ was established by HRESIMS. The ^{1}H and ^{13}C NMR spectra of **5** were very similar to those of **4**. However, signals for an additional methoxyl group were observed in **5**. HMBC showed a long-range correlation from the methoxyl protons at δ 3.92 to C-10 at δ 149.5, and other correlations were consistent with those of inoscavin B. Therefore, **5** was determined to be methyl-inoscavin B.

The antioxidant activity of compounds **1**−**5** was evaluated by measuring free radical scavenging effects using three different assays: the superoxide anion scavenging activity assay, ABTS radical cation decolorization assay, and DPPH radical scavenging activity assay. Compounds **1**−**5** exhibited moderate superoxide radical scavenging activity with IC_{50} values of 20−90 μM (BHA, 10 μM). Compounds **2** (IC_{50} = 20 μM) and **5** (IC_{50} = 38 μM), with the hispidin moiety masked by a methyl group, showed about 4 and 2 times higher activity than **1** (IC_{50} = 78 μM) and **4** (IC_{50} = 95 μM), without the methyl group, respectively. The IC_{50} value of compound **3** was 40 μM. Compounds **2**, **3**, and **5** displayed significant ABTS radical scavenging activity, with IC_{50} values of 10.7, 11.4, and 13.1 μM (vitamin E, 6 μM), respectively, while all of the compounds tested exhibited no DPPH radical scavenging activity.

Experimental Section

General Experimental Procedures. Specific rotation was determined using a JASCO P-1020 polarimeter. ESIMS was taken on a Navigator mass spectrometer in positive and negative modes, and HRESIMS was obtained on an ABI Mariner mass spectrometer with poly(ethylene glycol) as internal standard. UV and IR spectra were recorded on a Shimadzu UV-300 and a FT-IR Equinox 55 spectrometer, respectively. NMR spectra were obtained on a Varian UNITY Inova NMR spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz in CD_3OD. Chemical shifts are given in ppm (δ) using TMS as internal standard.

Mushroom Material. The fresh mushroom *I. xeranticus* (3 kg) was collected at chonan-si, Korea, in April 2005, and identified by staff at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The voucher specimen (No. Y011) is deposited in the herbarium of Antioxidants Research Laboratory, Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation. The ground fruiting bodies of *I. xeranticus* were extracted twice with MeOH at room temperature for 2 days. After removal of MeOH under reduced pressure, the resulting solution was partitioned between *n*-hexane and H_2O and then ethyl acetate and H_2O. The ethyl acetate-soluble fraction was subjected to a column of Sephadex LH-20 eluted with $CHCl_3$/MeOH (1:1, v/v). A yellow antioxidant fraction active against ABTS radical was concentrated in vacuo and then chromatographed on a column of ODS eluting with a gradient of increasing MeOH (40−100%) in H_2O to give two antioxidant fractions. One was rechromatographed on a column of

Sephadex LH-20 with 70% aqueous MeOH and, then, finally purified by preparative reversed-phase TLC with 70% aqueous MeOH to provide **1** (inoscavin A, R_f 0.32, 10 mg) and **2** (methylinoscavin A, R_f 0.27, 2 mg). The other was purified by Sephadex LH-20 column chromatography with 70% aqueous MeOH, followed by preparative reversed-phase TLC with 70% aqueous MeOH, to give **4** (inoscavin B, R_f 0.18, 4 mg), **5** (methylinoscavin B, R_f 0.14, 6 mg), and **3** (phelligridin F, R_f 0.32, 7 mg).

Methylinoscavin A (2): yellow powder; $[\alpha]_D$ −3.5 (*c* 0.04, MeOH); UV λ_{max} (MeOH) (log ϵ) 214 (3.89), 221 (3.85), 260 (sh, 3.67), 389 (3.70) nm; IR ν_{max} 3430, 2923, 2852, 1708, 1631, 1597, 1555, 1364, 1286, 1122 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS (positive mode) *m*/*z* 499.5 $[M + Na]^+$, (negative mode) *m*/*z* 475.5 $[M - H]^-$; HRESIMS *m*/*z* 499.0984 $[M + Na]^+$ (calcd for $C_{26}H_{20}O_9Na$, 499.1005).

Inoscavin B (4): orange powder; $[\alpha]_D$ −5.7 (*c* 0.07, MeOH); UV λ_{max} (MeOH) (log ϵ) 213 (3.56), 220 (3.50), 264 (sh, 3.20), 419 (3.30) nm; IR ν_{max} 3450, 2923, 2852, 1656, 1632, 1605, 1543, 1460, 1287, 1117 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS (positive mode) *m*/*z* 437.4 $[M + H]^+$, (negative mode) *m*/*z* 435.5 $[M - H]^-$; HRESIMS *m*/*z* 437.1230 $[M + H]^+$ (calcd for $C_{24}H_{21}O_8$, 437.1231).

Methylinoscavin B (5): yellow powder; $[\alpha]_D$ −15.5 (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ϵ) 214 (3.80), 219 (3.70), 257 (sh, 3.56), 410 (3.60) nm; IR ν_{max} 3447, 2924, 2852, 1647, 1632, 1508, 1385, 1287, 1123 cm^{-1}; ^1H NMR and ^{13}C NMR in CD_3OD, see Table 1; ESIMS (positive mode) *m*/*z* 473.6 $[M + Na]^+$, (negative mode) *m*/*z* 449.6 $[M - H]^-$; HRESIMS *m*/*z* 473.1217 $[M + Na]^+$ (calcd for $C_{25}H_{22}O_8Na$, 473.1206).

Superoxide Anion Scavenging Activity. Superoxide anion scavenging activity was evaluated by the xanthine/xanthine oxidase method with minor modifications.[8] In brief, each well of a 96-well plate containing 100 μL of the following reagents—50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.04 mM NBT (nitroblue tetrazolium), 0.18 mM xanthine, 250 mU/mL xanthine oxidase—and each concentration of each sample was incubated for 30 min at 37 °C in the dark. The xanthine oxidase catalyzes the oxidation of xanthine to uric acid and superoxide, and the superoxide reduces NBT to blue formazan. The reduction of NBT to blue formazan was measured at 560 nm in a microplate reader. For each point, background was corrected by subtracting the values derived from the no-xanthine oxidase control.

ABTS Radical Cation Decolorization Assay. Evaluation of free radical scavenging activity was carried out by using the ABTS radical cation decolorization assay.[9] The activity is based on the ability of the antioxidant to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) with spectrophotometric analysis, according to Re et al. ABTS was dissolved in H_2O to a concentration of 7 mM. The $ABTS^{\bullet+}$ cation radical was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 h. After adding 0.1 mL of the ABTS radical cation solution (A_{734nm} = 0.700) to 5 μL of antioxidant compounds in EtOH, the absorbance was measured by ELISA reader using VERSAmax (Molecular Devices Co.) after mixing up to 6 min.

DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity. Each concentration of test samples dissolved in 5 μL of DMSO was added to 95 μL of 150 μM DPPH ethanol solution. After vortex mixing, the mixture was incubated for 20 min at room temperature, and the absorbance was measured at 517 nm using an ELISA reader (Molecular Devices Co.).[10] Differences in absorbance between test samples and control (DMSO) were measured, and the activity was also compared with those of vitamin E and caffeic acid.

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