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Free radical scavengers from the medicinal mushroom *Inonotus xeranticus* and their proposed biogenesis

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Abstract—New free radical scavengers, inoscavin D (**1**) and methylinoscavin D (**2**), were isolated from the methanolic extract of the fruiting bodies of *Inonotus xeranticus* (Hymenochaetaceae), along with the known compounds phelligridin D (**3**), 3,4-dihydroxybenzaldehyde (**4**), and 3,4-dihydroxybenzoic acid (**5**). Their structures were established by various spectroscopic analyses. Compounds **1** and **3** were proposed to be biosynthesized from the oxidative coupling of the precursor hispidin with 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid, respectively. These compounds exhibited significant scavenging activity against the ABTS radical cation, and compounds **2** and **4** displayed moderate superoxide radical scavenging activity.

Mushrooms are ubiquitous in nature, and some of them are important sources of physiologically beneficial medicines. They produce various classes of secondary metabolites with interesting biological activity and thus have the potential to be used as valuable chemical resources for drug discovery. We have screened free radical scavengers from medicinal mushrooms in view of the fact that free radicals are implicated in the pathogenesis of various human diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation, and aging process.[1–3] *Inonotus xeranticus* (Berk.) Imaz. Et Aoshi. (Hymenochaetaceae), a medicinal mushroom widely distributed in East Asia including Korea, Japan, and China, is a saprophytic fungus preferably living on deciduous trees.[4] In a previous investigation, we isolated several hispidin derivatives from a yellow antioxidant fraction of the fruiting body of *I. xeranticus*.[5] As part of our ongoing efforts to characterize antioxidant constituents from the fruiting body of *I. xeranticus*, new hispidin derivatives named inoscavin D (**1**) and methylinoscavin D (**2**) have been isolated together with the known compound phelligridin D (**3**), 3,4-dihydroxybenzaldehyde (**4**), and 3,4-dihydroxybenzoic acid (**5**) (Fig. 1). In this paper, we report the isolation, structure determination, and free radical scavenging activity of compounds **1**–**5** and their proposed biogenesis.

The ground fruiting body of the fungus *I. xeranticus* (3 kg) was extracted twice with MeOH at room temperature for 2 days. After removal of MeOH under reduced pressure, the resultant was partitioned between *n*-hexane, ethyl acetate, and *n*-butanol and water, consecutively. Compounds **1**–**3** were purified from the ethyl acetate-soluble portion by the bioassay-guided fractionation using ABTS (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) radical scavenging assay. The ethyl acetate extract was chromatographed on a column of Sephadex LH-20 with MeOH to afford two yellow antioxidant fractions. One was subjected to a column of ODS (reversed-phase resin) eluting with a gradient of increasing methanol (40–100%) in water and rechromatographed on a column of Sephadex LH-20 with 70% aqueous MeOH to give two antioxidant fractions. Each fraction was finally purified by preparative reversed-phase TLC with 70% aqueous MeOH to afford **1** (3 mg) and **2** (2 mg). The other was purified on a column of ODS eluting with a gradient of increasing methanol (50–100%) in water, followed by preparative reversed-phase TLC with 70% aqueous MeOH to provide **3** (4 mg). The BuOH-soluble fraction was evaporated under reduced pressure and the residue was dissolved

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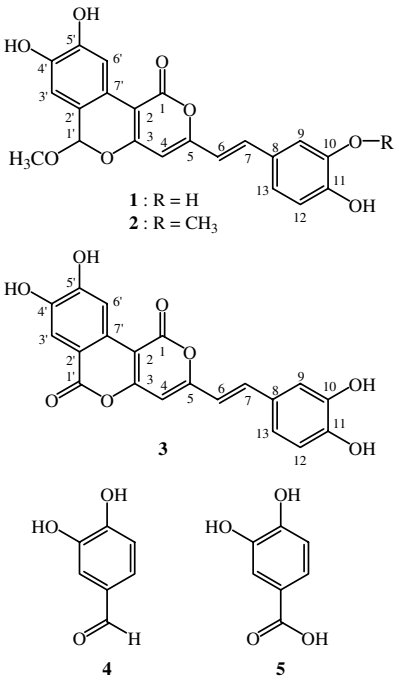


Figure 1. Structures of compounds **1**–**5**.

in H_2O and subjected to a column of Diaion HP-20. The column was washed by 30% aqueous MeOH, and compounds **4** and **5** were eluted by 50% aqueous MeOH. Finally, **4** (4 mg) and **5** (3 mg) were purified by Sephadex LH-20 column chromatography with 70% aqueous MeOH, followed by preparative reversed-phase TLC developed with 30% aqueous MeOH.

Compound **1** was isolated as a yellow amorphous powder, and its positive electron spray ionization (ESI) mass provided a quasi-molecular ion peak at *m*/*z* 419 $[M+Na]^+$, while the negative ESI-mass gave a quasi-molecular ion peak at *m*/*z* 395 $[M-H]^-$. The molecular formula of **1** was established as $C_{21}H_{16}O_8$ by high resolution ESI mass data (*m*/*z* 397.0954 $[M+H]^+$, +3.7 mmu) in combination with ^1H and ^{13}C NMR data. The UV absorption maxima at 257 and 414 nm suggested that **1** had a hispidin moiety, which is ubiquitous in the fungi belonging to the hymenochaetaceae family.[6,7] The ^1H NMR spectrum in CD_3OD showed three aromatic methine signals assignable to a 1,2,4-trisubstituted benzene moiety at δ 7.05 (1H, d, J = 2.0 Hz), 6.96 (1H, dd, J = 8.0, 2.0 Hz), and 6.78 (1H, d, J = 8.0 Hz), three methine singlets at δ 8.08, 6.74, and 6.36 in aromatic region, two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond at δ 7.33 (1H, d, J = 16.0 Hz) and 6.63 (1H, d, J = 16.0 Hz), a methine singlet at δ 6.06, and a methyl singlet at δ 3.53 (Table 1). The ^{13}C NMR spectrum revealed the presence of 21 carbons, which were established as one methoxyl methyl, nine methines, and 11 quaternary carbons including an ester carbonyl and six oxygenated sp^2 carbons by the aid of the HMQC spectrum. The structure of **1** was determined by the HMBC spectrum, as shown in Figure 2. The hispidin moiety was assigned by the long-range correlations from H-4 to C-2, C-3, C-5,

Table 1. ^1H and ^{13}C NMR data of compounds **1** and **2** in CD_3OD[a]

No.	1		2
	^{13}C	^1H	^1H
1	163.3		
2	101.4		
3	163.0		
4	101.8	6.36 (1H, s)[b]	6.38 (1H, s)
5	159.9		
6	116.7	6.63 (lH, d, J = 16.0)	6.74 (1H, d, J = 16.0)
7	136.8	7.33 (lH, d, J = 16.0)	7.39 (1H, d, J = 16.0)
8	129.0		
9	114.7	7.05 (lH, d, J = 2.0)	7.20 (1H, d, J = 2.0)
10	146.8		
10-OMe			3.91 (3H, s)
11	148.7		
12	116.6	6.78 (lH, d, J = 8.0)	6.81 (1H, d, J = 8.0)
13	122.0	6.96 (lH, dd, J = 8.0, 2.0)	7.08 (lH, dd, J = 8.0, 2.0)
1′	101.7	6.06 (1H, s)	6.07 (1H, s)
1′-OMe	55.9	3.53 (3H, s)	3.54 (3H, s)
2′	114.0		
3′	113.9	6.74 (1H, s)	6.75 (1H, s)
4′	146.8		
5′	147.7		
6′	112.2	8.08 (1H, s)	8.08 (1H, s)
7′	118.9		

[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.

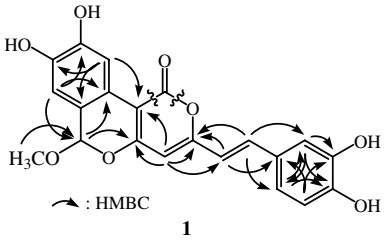


Figure 2. HMBC correlations for **1**.

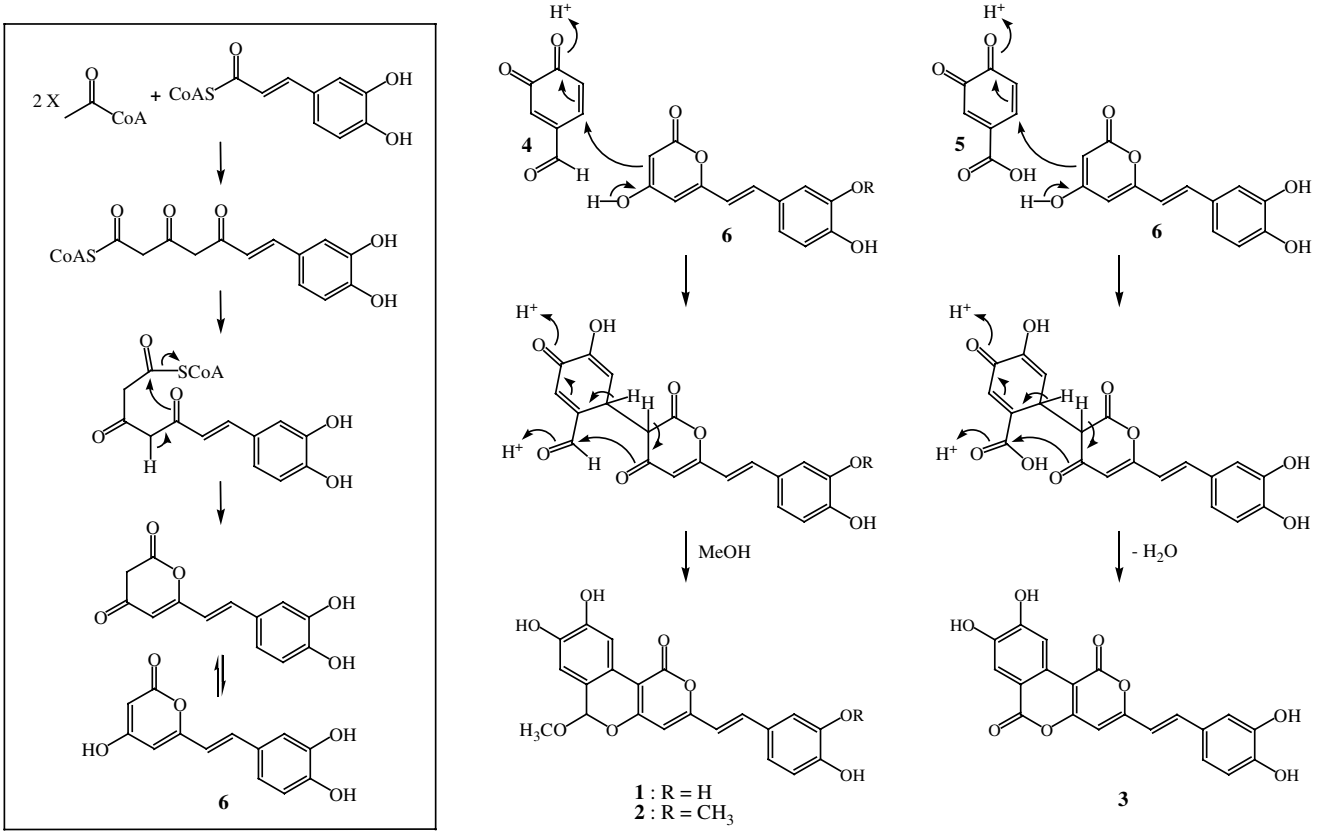
and C-6, H-6 to C-5 and C-8, H-7 to C-9 and C-13, H-9 to C-7, C-10, C-11, and C-13, H-12 to C-8 and C-10, and H-13 to C-7, C-9, and C-11, and these chemical shift values were consistent with the corresponding protons and carbons of hispidin moiety.[5,7] Long-range correlations from H-3′ to C-5′ and C-7′, and H-6′ to C-2′ and C-4′ revealed the presence of a 1,2,4,5-tetrasubstituted benzene moiety. In addition, the three bonded correlations from H-3′ to C-1′, H-1′ to C-3, C-3′, and C-7′ and H-6′ to C-2 were evident. The chemical shift of C-1′ at δ 101.7 suggested the possibility for C-1′ to be an sp^2 carbon, but the long-range correlation from the methyl protons at δ 3.53 to C-1′ implied that C-1′ was an sp^3 acetal. By the process of elimination, a remaining ester carbonyl carbon at δ 163.3 should be positioned to C-1. Compound **1** was optically inactive, suggesting that the biogenetic formation of the chiral center (C-1′) is nonstereoselective. From the above results, the structure of **1** was unambiguously determined to be a new antioxidant with hispidin moiety.

Compound **2** was obtained as a yellow powder and its molecular formula was established as $C_{22}H_{18}O_8$ by high resolution ESI-MS (*m*/*z* 411.1046 $[M+H]^+$, −2.8 mmu). The ^1H NMR spectrum of **2** was very similar to that of **1**, except for an additional methoxyl methyl signal at δ 3.91. The structure of **2** was assigned by interpretation of the HMBC spectrum, which exhibited a long-range correlation from the methyl protons at δ 3.91 to an oxygenated quaternary carbon corresponding to C-10. Therefore, the structure of **2** was determined as a methylated **1**.

The molecular weight of compound **3** was established as 380 Da by the ESI-MS providing a quasi-molecular ion at *m*/*z* 379.4 $[M-H]^-$ in the negative mode. The ^1H NMR spectrum of **3** measured in CD_3OD revealed the signals due to a 1,2,4-trisubstituted benzene at δ 7.05 (1H, br s), 6.96 (1H, br d, $J = 8.0$ Hz), and 6.77 (1H, d, $J = 8.0$ Hz), three sp^2 methine singlets at δ 8.38, 7.57, and 6.48, and two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond at δ 7.37 (1H, d, $J = 15.6$ Hz) and 6.66 (1H, d, $J = 15.6$ Hz). The above spectroscopic data were in good agreement with those of phelligridin D, which was previously isolated from the fungus *Phellinus igniarius*.[7] Compound **3** was ascertained to be identical to phelligridin D on the basis of the HMBC experiment. Although compound **3** was previously reported as a potent cytotoxic substance against several human cancer cell lines, this is the first report from the fungus *I. xeranticus* as an antioxidant. Compounds **4** and **5** were isolated from the BuOH-soluble portion, and their structures were determined to be 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid, respectively, on the basis of mass and NMR spectroscopic analyses.

The mushrooms belonging to *Phellinus* and *Inonotus* spp. produce a wide variety of hispidin derivatives possessing unprecedented carbon skeleton, and these compounds were proposed to be biogenerated by the oxidative coupling of precursor hispidin with phenolic compounds or additional hispidin which might be catalyzed by mushroom peroxidase.[5,7] We proposed the biogenesis of compounds **1**–**3** as shown in Scheme 1 on the basis of their chemical structures and the co-isolation of compounds **4** and **5** from same fungal source. Hispidin is known to be synthesized by two different mechanisms; one is from phenylalanine via a cinnamyl derivative that is combined with either acetate or malonate through the polyketide pathway,[8,9] and the other is by the condensation of 4-hydroxy-6-methyl-2-pyrone, which is formed by the reaction of three molecules of acetyl-SCoA and one molecule of 3,4-dihydroxybenzoyl-SCoA (or 3,4-dihydroxybenzaldehyde).[7] Compounds **1** and **3** would be biosynthesized by the oxidative coupling of hispidin with 3,4-dihydroxybenzaldehyde (**4**) and 3,4-dihydroxybenzoic acid (**5**), respectively, and the condensation process was proposed as shown in Scheme 1.

The antioxidant activity of compounds **1**–**5** was evaluated by measuring free radical scavenging activity using three different assays, the superoxide radical anion scav-



Scheme 1. Proposed biosynthetic mechanism of compounds **1**–**3**.

Table 2. Free radical scavenging activities of compounds **1**–**5** (IC_{50}, μM)

Compound	ABTS radical	Super oxide radical	DPPH radical
1	12.5	>100	>100
2	8.7	40.7	>100
3	13.7	>100	>100
4	5.0	25.3	29.4
5	15.7	>100	27.0
Vitamin E	8.8	>100	11.5
Caffeic acid	2.8	2.9	30.0
BHA	1.0	10.3	22.4

enging activity assay, ABTS radical cation decolorization assay, and DPPH radical scavenging activity assay (Table 2). ABTS radical scavenging activity was carried out by using ABTS radical cation decolorization assay with minor modifications.[10] ABTS was dissolved in water to a concentration of 7 mM. The $ABTS^{\cdot+}$ cation radical was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and by allowing the mixture to stand in the dark for 12 h. After adding 0.1 mL of the ABTS radical cation solution to 5 μL of antioxidant compounds in ethanol, the absorbance was measured by ELISA reader at 734 nm after mixing up to 6 min. Although less activity than caffeic acid and a synthetic antioxidant BHA, all of compounds tested exhibited significant $ABTS^{\cdot+}$ cation radical scavenging activity. Compounds **2** and **4** among them were comparable to vitamin E, as described in Table 2.

Superoxide anion scavenging activity was evaluated by the xanthine/xanthine oxidase method.[11] In brief, each well of a 96-well plate containing the 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 samples 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. In result, **1**, **3**, and **5** exhibited no activity up to 100 μM, while **2** and **4** exhibited moderate superoxide radical scavenging activity with IC_{50} values of 40.7 and 25.3 μM, respectively.

To investigate the scavenging effect to the DPPH radicals, each concentration of **1**–**4** was added to 95 μL of 150 μM DPPH ethanol solution, the mixture was incubated for 20 min at room temperature, and the absorbance was measured at 517 nm using an ELISA reader. Compounds **4** and **5** showed potent DPPH radical scavenging effects with IC_{50} values of 29.4 and 27.0 μM, respectively, while compounds **1**–**3** showed no activity up to 100 μM.

Acknowledgments

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