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Hispidin analogs from the mushroom *Inonotus xeranticus* and their free radical scavenging activity

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Abstract—Three new free radical scavengers were isolated from the methanolic extract of the fruiting bodies of *Inonotus xeranticus* (Hymenochaetaceae), along with the known compound davallialactone. Their structures were established as hispidin analogs by extensive NMR spectral data. Compounds **3** and **4** displayed significant scavenging activity against the superoxide radical anion, ABTS radical cation, and DPPH radical, while**1** and **2** exhibited potent antioxidative activity only against ABTS radical cation.

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 processes.[1–3] Therefore, there is growing interest in free radical scavengers having the potential as protective agents against these diseases.

Mushrooms are ubiquitous in nature and produce various classes of primary and secondary metabolites, many that exhibit significant antimicrobial, antitumor, and antiviral activities. Some of them are nutritionally functional foods and important sources of physiologically beneficial medicines in the Orient. In spite of their potential for drug development, few bioactive metabolites have been reported from mushrooms as compared with the higher plants and microbes. *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] In a previous investigation, we isolated several hispidin derivatives from a yellow antioxidant extract of the fruiting bodies of *I. xeranticus*.[5] As part of our ongoing efforts to characterize antioxidant constituents from the fruiting body of *I. xeranticus*, three new hispidin derivatives named inoscavin C (**1**), methylinoscavin C (**2**), and methyldavallialactone (**4**) have been isolated together with the known compound davallialactone (**3**).[6,7] We herein report the isolation, structure determination, and free radical scavenging activity of compounds **1**–**4**.

The ground fruiting bodies of *I. xeranticus* (3 kg) 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 between ethyl acetate and H_2O. Compounds **1**–**4** were purified from the ethyl acetate-soluble portion through the bioassay-guided isolation using ABTS radical scavenging assay. The ethyl acetate extract was chromatographed twice on a column of Sephadex LH-20 with different elution solvents, $CHCl_3$/MeOH (1:1, v/v) and MeOH, respectively. A yellow antioxidant fraction active against ABTS radical was concentrated and then subjected to a column of ODS eluting with a gradient of increasing methanol (40–100%) in water 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 reverse-phase TLC with 70% aqueous MeOH to give **1** (inoscavin C, 3 mg) and **2** (methylinoscavin C, 2 mg). The other was purified by Sephadex LH-20 column chromatography with 70% aqueous MeOH, followed by preparative reversed-phase TLC with 50% aqueous MeOH to give **3** (davallialactone, 20 mg) and **4** (methyldavallialactone, 1 mg) (Fig. 1).

The molecular formula of compound **1** was determined to be $C_{23}H_{16}O_8$ by high-resolution ESI-MS (*m*/*z*

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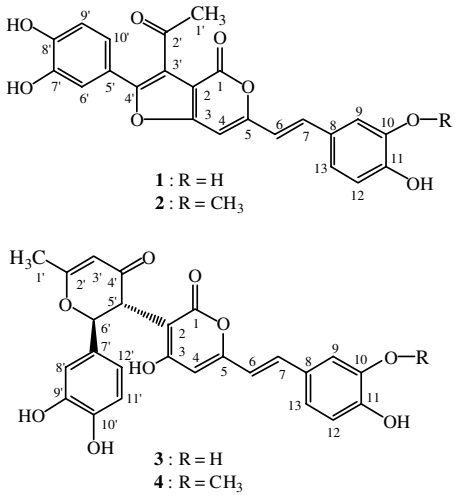

Figure 1. Structures of compounds **1**–**4**.

421.0952 $[M+H]^+$, +3.4 mmu) in combination with ^1H and ^{13}C NMR data. Its IR spectrum showed absorption bands for hydroxyl (3446 cm^{-1}), conjugated carbonyl (1700 cm^{-1}), and aromatic ring (1603 and 1558 cm^{-1}). The UV maxima at 260 and 400 nm suggested that **1** was a hispidin derivative. The ^1H NMR spectrum in CD_3OD showed six aromatic methine signals assignable to two 1,2,4-trisubstituted benzene moieties at δ 7.05 (1 H, d, J = 2.0 Hz), 6.95 (1H, dd, J = 8.0, 2.0 Hz), and 6.77 (1H, d, J = 8.0 Hz), and δ 7.23 (1H, d, J = 2.0 Hz), 7.16 (1H, dd, J = 8.4, 2.0 Hz), and 6.83 (1H, d, J = 8.4 Hz), a methine singlet at δ 6.80, two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond at δ 7.33 (1H, d, J = 16.0 Hz) and 6.68 (1H, d, J = 16.0 Hz), and a methyl singlet at δ 2.65 (Table 1). The ^{13}C NMR spectrum revealed the presence of 23 carbons comprised of one methyl, 9 sp^2 methines, and 13 quaternary carbons including an ester carbonyl, an α,β-unsaturated ketone carbonyl, and seven oxygenated sp^2 carbons. Proton-bearing carbons were assigned by the aid of an HMQC spectrum (Table 1). HMBC data revealed the three partial structures, hispidin moiety, 3,4-dihyroxyphenyl group, and acetyl group, 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, 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-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 of hispidin moie-

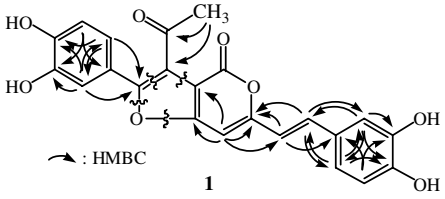

Figure 2. HMBC correlations for **1**.

Table 1. ^1H and ^{13}C NMR spectral data of compounds **1**–**4** in CD_3OD[a]

No.	1		2	3		4
	δ_C	δ_H	δ_H	δ_C	δ_H	δ_H
1	158.7			168.8		
2	108.0			97.3		
3	161.0			165.5		
4	94.7	6.80 (1H, s)[b]	6.84 (1H, s)	100.0	5.95 (1H, s)	5.94 (1H, s)
5	158.7			159.1		
6	115.8	6.68 (1H, d, J = 16.0)	6.81 (1H, d, J = 16.0)	115.7	6.45 (1H, d, J = 16.0)	6.56 (1H, d, J = 16.0)
7	135.3	7.33 (1H, d, J = 16.0)	7.40 (1H, d, J = 16.0)	136.0	7.20 (1H, d, J = 16.0)	7.25 (1H, d, J = 16.0)
8	127.9			127.8		
9	113.6	7.05 (1H, d, J = 2.0)	7.20 (1H, d, J = 2.0)	113.8	6.98 (1H, d, J = 2.0)	7.14 (1H, d, J = 2.0)
10	145.6			145.4		
10-OMe			3.92 (3H, s)			3.88 (3H, s)
11	147.4			147.2		
12	115.4	6.77 (1H, d, J = 8.0)	6.81 (1H, d, J = 8.0)	115.5	6.75 (1H, d, J = 8.0)	6.77 (1H, d, J = 8.0)
13	120.7	6.95 (1H, dd, J = 8.0, 2.0)	7.08 (1H, dd, J = 8.0, 2.0)	120.8	6.89 (1H, dd, J = 8.0, 2.0)	7.00 (1H, dd, J = 8.0, 2.0)
1′	22.0	2.65 (3H, s)	2.66 (3H, s)	20.8	2.09 (3H, s)	2.09 (3H, s)
2′	197.5			175.9		
3′	119.9			104.1	5.49 (1H, s)	5.48 (1H, s)
4′	155.3			194.7		
5′	119.9			46.8	4.28 (1H, d, J = 13.2)	4.28 (1H, d, J = 13.4)
6′	114.5	7.23 (1H, d, J = 2.0)	7.24 (1H, d, J = 2.0)	83.2	5.76 (1H, d, J = 13.2)	5.78 (1H, d, J = 13.4)
7′	145.3			128.9		
8′	148.0			114.7	6.86 (1H, d, J = 1.6)	6.86 (1H, d, J = 2.0)
9′	115.2	6.83 (1H, d, J = 8.4)	6.83 (1H, d, J = 8.4)	144.7		
10′	120.1	7.16 (1H, dd, J = 8.4, 2.0)	7.17 (1H, dd, J = 8.4, 2.0)	145.7		
11′				114.9	6.67 (1H, d, J = 8.0)	6.68 (1H, d, J = 8.0)
12′				119.7	6.70 (1H, dd, J = 8.0, 1.6)	6.70 (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.

ty.[5,8] Long-range correlations from H-6′ to C-4′, C-7′, C-8′, and C-10′, H-9′ to C-7′ and C-11′, and H-10′ to C-4′, C-6′, and C-8′ revealed the presence of a 3,4-dihyroxyphenyl moiety, and the correlations from H-1′ to C-2′ and C-3′ established the presence of acetyl group. By the process of elimination, C-3′ (δ 119.9) should be connected to C-4′ (δ 155.3) to make a double bond, and to satisfy the molecular formula $C_{23}H_{16}O_8$ and 16° of unsaturation, two sp^2 oxygenated carbons of C-3 (δ 161.0) and C-4′ (δ 155.3) should be connected to each other by an ethereal oxygen bridge. Therefore, the structure of **1** was unambiguously determined to be a new hispidin analog.

Compound **2** was obtained as a yellow powder and its molecular formula was established as $C_{24}H_{18}O_8$ by high-resolution ESI-MS (*m*/*z* 435.1113 $[M+H]^+$, +3.9 mmu). The ^1H NMR spectrum of **2** was very similar to that of **1**, except for an additional methoxyl signal at δ 3.92. The structure of **2** was assigned by interpretation of HMBC spectrum, which exhibited a long-range correlation from aromatic methoxyl protons at δ 3.92 to an oxygenated carbon at δ 145.6 corresponding to C-10. Other HMBC correlations were consistent with those of inoscavin C. Therefore, the structure of **2** was determined to be methylinoscavin C.

Compound **3** was isolated as a major component of yellow antioxidant pigment, and its ESI-MS gave a quasi-molecular ion at *m*/*z* 487 $[M+Na]^+$ in the positive mode and *m*/*z* 463 $[M-H]^-$ in the negative mode, implying its molecular weight to be 464. The ^1H NMR spectrum of **3** revealed the signals due to a methyl at δ 2.09 and three methines at δ 4.28, 5.49, and 5.76, and a 1,2,4-trisubstituted benzene moiety, in addition to hispidin signals. The above spectroscopic data were well matched with those of davallialactone, which was previously reported from the rhizoma of a fern *Davallia mariesii*.[7] Compound **3** was ascertained to be identical to davallialactone on the basis of 2D NMR analysis including HMQC and HMBC, and a specific rotation value of +221°. Although compound **3** was previously reported from the plant as a cytotoxic substance against BALB/3T3 cells transformed by H-ras oncogene,[6] this is the first report from microbial origin as a potent free radical scavenger.

Compound **4** was isolated as a small amount, and its molecular formula was determined to be $C_{22}H_{18}O_8$ by high-resolution ESI-MS (*m*/*z* 501.1202 $[M+Na]^+$, +4.7 mmu). The ^1H NMR spectrum of **4** closely resembled that of **3**, except for an additional methoxyl signal at δ 3.88. The structure of **4** was determined by a detailed comparison of ^1H NMR spectrum with that of **3**. The proton chemical shifts of **3** and **4** were almost same except for the signals derived from the trisubstituted benzene in hispidin moiety, and the difference of chemical shift values between **3** and **4** was very similar to that between **1** and **2**, suggesting that **3** and **4** were in the structural relationship of **1** and **2**. Consequently, **4** was proposed to be methyldavallialactone.

The antioxidant activity of compounds **1**–**4** was evaluated by measuring free radical scavenging effects using three different assays, the superoxide radical anion scavenging activity assay, ABTS radical cation decolorization assay, and DPPH radical scavenging activity assay (Table 2). Superoxide anion scavenging activity was evaluated by the xanthine/xanthine oxidase method.[9] 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. As a result, **1** and **2** exhibited no activity up to 100 μM, while **3** and **4** exhibited potent superoxide radical scavenging activity with IC_{50} values of 2.3 and 5.4 μM, which were comparable to BHA and caffeic acid, well-known antioxidants used as a control.

Table 2. Free radical scavenging activities of compounds **1**–**4** (IC_{50}, μM)

Compound	Superoxide radical	ABTS radical	DPPH radical
1	>100	7.8	>100
2	>100	12.3	>100
3	2.3	0.8	3.4
4	5.4	1.5	18.7
Vitamin E	>100	5.7	12.3
Caffeic acid	2.9	2.8	31.7
BHA	9.5	0.8	22.0

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. Compounds **1**–**4** potently scavenged the ABTS radical cation, as described in Table 2. Compound **3** of the compounds tested showed activity higher than those of vitamin E and caffeic acid, and comparable activity to a synthetic antioxidant BHA against the ABTS radical cation.

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.[11] Compounds **3** and **4** exhibited significant DPPH radical scavenging effect with IC_{50} values of 3.4 and 18.7 μM, respectively, while **1** and **2** showed no activity against the DPPH radical.

From the above results, compounds **3** and **4** displayed the scavenging activity against all of free radical species used in this experiment, while **1** and **2** showed the activ-

ity only against the ABTS radical cation. Compound **3** was the most potent scavenger among all the compounds tested against a broad spectrum of radical species and showed activity higher than those of the known antioxidants, vitamin E, caffeic acid, and BHA.

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