

Antioxidant Hispidin Derivatives from Medicinal Mushroom *Inonotus hispidus*

Li-feng ZAN,^{a,b,#} Jian-chun QIN,^{b,#} Ya-mei ZHANG,^b Yan-hua YAO,^b Hai-ying BAO,^{*,a} and Xiang LI^{*,b,c}

^aEngineering Research Centre of Edible and Medicinal Fungi, Ministry of Education, Jilin Agricultural University; Changchun, Jilin 130118, China; ^bCollege of Plant Sciences, Jilin University; Changchun, Jilin 130062, China; and ^cSaskatoon Research Centre, Agriculture and Agri-food Canada; Saskatoon, S7N 0X2, Saskatchewan, Canada.

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Two natural antioxidants, named inonosin A (**1**) and B (**2**), were isolated from the methanolic extract of the fruit bodies of *Inonotus hispidus*, together with (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**), hispidin (**4**) and 3,4-dihydroxybenzaldehyde (**5**). Their structures were identified by means of extensive NMR and MS data analysis. Compounds **1**, **2** and **4** exhibited significant scavenging activity against the 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation. Compound **1** also showed moderate cytotoxicity against a human breast carcinoma cell line (MCF-7) with IC₅₀ values of 19.6 μM.

Key words *Inonotus hispidus*; antioxidant; cytotoxicity; hispidin derivative

The fungal kingdom includes many species produce variety of structurally unusual and bioactive secondary metabolites.^{1–3)} *Inonotus hispidus* (basidiomycetes) is a parasitic fungus preferably living on deciduous trees such as fraxinus, quercus, sorbus and malus.⁴⁾ *I. hispidus* has been used as traditional medicines for the treatment of dyspepsia, cancer, diabetes and other stomach problems in the northeast region and Xinjiang province of China.⁵⁾ Previous chemical investigations indicated that *I. hispidus* produced a large variety of yellow-brown polyphenol pigments with a styrylpyrone skeleton, which had been reported to exhibit antimicrobial, antioxidant, antiviral and anti-inflammatory activities.^{6–11)} Within the context of our searching for biologically active and new structurally metabolites with potential application in human health from Chinese fungi species,^{12,13)} we have examined the chemical constituents of a sample of *I. hispidus*, which was collected from Inner Mongolia of China. These efforts resulted in the identification of two novel metabolites: inonosin A (**1**) and B (**2**), together with (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**), hispidin (**4**) and 3,4-dihydroxybenzaldehyde (**5**) (Fig. 1). This report describes the isolation and structural determination of the compounds **1** and **2**, as well as their antioxidant and cytotoxic activities.

Inonosin A (**1**) was obtained as a yellow amorphous powder with molecular formula of C₁₅H₁₄O₆ established base on positive high resolution-electrospray ionization-mass spectra (HR-ESI-MS) at *m/z* 291.0867 [M+H]⁺ (Calcd 291.0869). The ¹H-NMR spectrum of **1** showed signals attributable to the ABX system at δ 6.74 (d, *J*=7.8 Hz, H-13), 6.90 (dd, *J*=7.8, 1.5 Hz, H-14) and 7.02 (d, 1.5 Hz, H-10), an AM spin system originating from a *trans*-1,2-disubstituted double bond unit at δ 6.49 (d, *J*=15.7 Hz, H-8) and 7.21 (d, *J*=15.7 Hz, H-7), which suggested a dihydroxystyryl in the structure, a singlet at δ 5.33 (1H), a methyl group at δ 1.50 and a methylene moiety at δ 2.43, 2.84 (d, *J*=16.0 Hz, H-3). The ¹³C-NMR spectrum of **1** (Table 1) showed signals for 15 carbons, with the multiplicities of the carbon signals determined from the APT spectrum, as follows: seven quaternary, six methine, one methylene and one methyl carbon atoms. Comprehensive analysis of the 2D-NMR data, including the results of ¹H–¹H correlation spectroscopy (COSY), ¹H-de-

tected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC), experiments, were used to elucidate the planar structure of **1**. The ¹H–¹H COSY spectrum (Fig. 2) showed the following correlations: H₁₃–H₁₄ and H₇–H₈. In the HMBC spectrum, the correlations from H-10 to C-12, C-14 and C-8, H-13 to C-11, C-9, H-14 to C-8, C-10 and C-12, both H-7 and H-8 to C-9, C-10 and C-14 confirmed the presence of the *para*-hydroxystyryl moiety (Fig. 2). The long-range correlations from H-3 to C-1, C-2, C-4 and C-5, H-15 to C-1, C-2 and C-3, H-5 to C-6, C-3 and C-7, both H-7 and H-8 to C-5 and C-6, revealed two possible partial structures which linked to the styryl moiety (Fig. 3). To confirm whether there is a free carboxylic acid exists, **1** was methylated by diazomethane method. ¹H-NMR spectrum of the products showed no methoxyl group. Consequently, the structure of **1** was unambiguously established as 6-(11,12-dihydroxystyryl)-2-hydroxy-

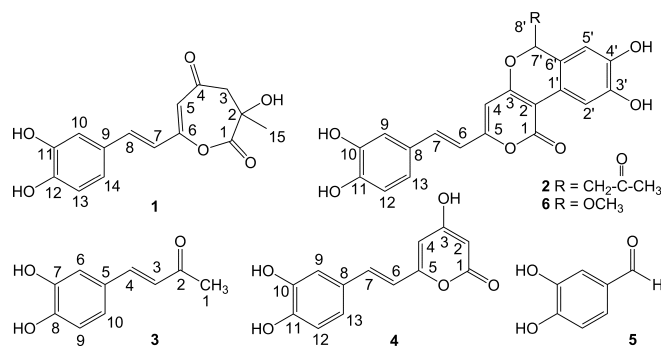


Fig. 1. Chemical Structure of Compounds **1**–**6**

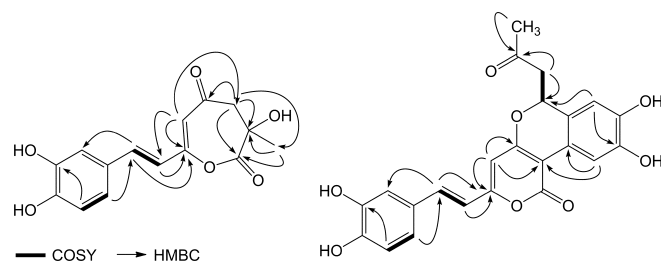


Fig. 2. Key HMBC and COSY Correlations of Compounds **1** and **2**

* To whom correspondence should be addressed. e-mail: li_xiang@jlu.edu.cn; baohaiying2008@126.com

These authors contributed equally to this work.

2-methyl-5,6-dihydro-oxepine-1,4-dione and named inonotusin A. **1** was found optically inactive which showed no cotton effect in circular dichroism (CD) spectrum, indicated that either chiral center (C-2) is non-stereoselective or **1** was isolated as racemic mixture.

Inonotusin B (**2**) was obtained as a yellow amorphous powder and gave a $[M+Na]^+$ ion at m/z 445.0907 in the HR-ESI-MS, which corresponds to a molecular formula of $C_{23}H_{18}O_8$ (Calcd 445.0899). The IR spectrum (KBr) implies the presence of hydroxyls (3337 cm^{-1}), carbonyl group (1679 cm^{-1}) and aromatic rings ($1597, 1530, 1435\text{ cm}^{-1}$). The ^1H - and ^{13}C -NMR spectroscopic data (Table 1) were similar to those of inoscavin D (**6**) except that the 7'-methoxyl moiety of inoscavin D was replaced by a 2-oxopropyl unit.¹¹⁾ This was confirmed by long-range correlations from H-5' to C-7', H-10' to C-8' and C-9' and H-8' to C-6', C-7', C-9' and C-10' (Fig. 2). Therefore, the structure of **2** was assigned as 5-(10,11-dihydroxystyryl)-3',4'-dihydroxy-7'-(2-oxopropyl)pyrano-isochromen-1-one and designated inonotusin B. **2** was also found optically inactive which same as **6** suggested that the biogenetic formation of the chiral center (C-1') is nonstereoselective.¹¹⁾ Based on the structural features, **2** was proposed to be biosynthesized by oxidative conjugating of (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) and hispidin (**4**).^{14,15)}

Compounds **1**, **2** and **4** potently scavenged the ABTS radi-

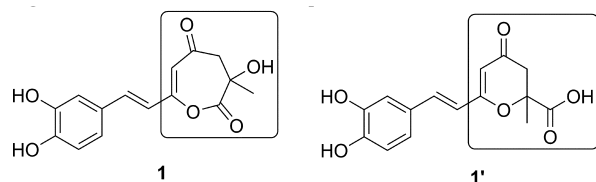


Fig. 3. Possible Structures of Compound **1**

Table 1. NMR Spectra Data for **1**–**4**^{a)}

C No.	1		2		C No.	2		3	4
	δ_{H}	δ_{C}	δ_{H}	δ_{C}		δ_{H}	δ_{C}		
1	—	173.8	—	160.3 ^{b)}	1'	—	119.0 ^{b)}	27.3	169.2
2	—	84.2	—	100.2	2'	7.90 (s)	111.8	202.1	94.5
3	2.43, 2.84 (d, 16.0)	45.8	—	162.6 ^{b)}	3'	—	145.8	128.5	161.8
4	—	192.3	6.34 (s)	104.6	4'	—	146.1	138.3	101.6
5	5.33 (s)	104.8	—	158.5 ^{b)}	5'	6.59 (s)	112.3	127.9	158.3
6	—	168.7	7.21 (d, 15.7)	116.3	6'	—	117.0	114.6	116.8
7	7.21 (d, 15.7)	119.6	6.72 (d, 15.7)	135.0	7'	5.76 (dd, 8.8, 4.1)	74.7	145.8	134.2
8	6.49 (d, 15.7)	136.9	—	127.3	8'a	2.88 (dd, 4.1, 16.7)	48.8	146.1	128.0
9	—	127.3	7.05 (d, 1.8)	114.4	8'b	3.08 (dd, 8.8, 16.7)	—	115.8	115.3
10	7.02 (d, 1.5)	114.6	—	145.5	9'	—	205.6 ^{b)}	121.5	145.8
11	—	146.1	—	145.8	10'	2.16 (s)	30.7	—	145.9
12	—	148.0	6.78 (d, 8.5)	116.3	—	—	—	—	116.0
13	6.74 (d, 7.8)	116.3	6.97 (dd, 8.5, 1.8)	120.6	—	—	—	—	121.3
14	6.90 (dd, 7.8, 1.5)	120.8	—	—	—	—	—	—	—
15	1.50 (s)	25.4	—	—	—	—	—	—	—

a) **1**–**4** were measured in $\text{DMSO}-d_6$ at 500 MHz for ^1H - and 125 MHz for ^{13}C -NMR. b) Signals were extracted from HMBC spectrum.

Table 2. Antioxidant Capacity of Compounds **1**–**5** ($\mu\text{mol Trolox}/\mu\text{mol Compound}$)

Compounds	1	2	3	4	5	Quercetin
TEAC	12.71 \pm 3.57	59.53 \pm 9.70	23.88 \pm 0.59	14.47 \pm 0.7	5.90 \pm 0.87	4.78 \pm 0.58

cal cation in which compound **2** showed the highest activity at the level of $59.53\pm 9.70\ \mu\text{mol Trolox}/\mu\text{mol compound}$ (Table 2). Compounds **1**–**5** were also tested for cytotoxic activity against two cell lines, human breast carcinoma (MCF-7) and human hepatoblastoma (HepG-2). Compounds **2**–**5** were noncytotoxic to the two cell lines. Compound **1** showed moderate cytotoxicity against MCF-7 with IC_{50} values of $19.6\ \mu\text{M}$.

Experimental

Fungi Material The sporocarps of *I. hispidus* growing on the *Fraxinus mandshurica* were collected from the Daqinggou National Nature Reserve, Inner Mongolia, China and identified by Prof. Dr. Tolgor, Mycological Institute of Jilin Agricultural University, China. The voucher specimen was deposited in the Herbarium of Mycology in Jilin Agricultural University (HMJAU4208), China.

Extraction and Isolation The ground fruiting body (2 kg) was extracted with MeOH at room temperature for $3\times 24\text{ h}$. After the solvent was removed under reduced pressure at 35°C , a dark brown residue (26 g) was obtained. The residue was suspended in water and then partitioned with petroleum ether, EtOAc, and *n*-BuOH successively. The *n*-BuOH fraction (8 g) was separated by column chromatography on silica gel eluting with a gradient increasing MeOH (5–30%) in CH_2Cl_2 to give 7 fractions (Fr. 1–7). Fraction Fr. 3 was chromatographed over Sephadex LH-20 eluted with MeOH, and further purified by preparative HPLC to afford compounds **1** (8 mg) and **3** (10 mg). Fraction Fr. 4 was chromatographed on silica gel eluting with CH_2Cl_2 :MeOH (9:1), and further purified by preparative HPLC to afford compound **2** (2 mg). Fraction Fr. 6 was directly subjected to preparative HPLC to afford compounds **4** (12 mg) and **5** (17 mg).

Inonotusin A (1): $C_{15}H_{14}O_6$, yellow amorphous powder. UV λ_{max} (MeOH) 247, 363 nm. IR ν_{max} (cm^{-1}): 3157, 1670, 1557, 1526, 1415. Positive-ion ESI-MS m/z : 291 $[M+H]^+$, HR-ESI-MS at m/z 291.0867 $[M+H]^+$, Calcd: 291.0869. For the ^1H - and ^{13}C -NMR spectral data (see Table 1).

Inonotusin B (2): $C_{23}H_{18}O_8$, yellow amorphous powder. UV λ_{max} (MeOH) 267, 375 nm. IR ν_{max} (cm^{-1}): 3337, 1679, 1597, 1530, 1435. Positive-ion ESI-MS m/z : 423 $[M+H]^+$, HR-ESI-MS $[M+Na]^+$ m/z : 445.0907, Calcd: 445.0899. For the ^1H - and ^{13}C -NMR spectral data (see Table 1).

Diazomethane Derivative of Compound 1 Freshly prepared diazomethane reagent (1 ml in MeOH) was added to a 2 ml toluene solution containing 5 mg of **1** at 4°C with stirring. This solution was gradually

warmed to room temperature. After 2 h, the solvent was removed under vacuum to yield the crude products.

Antioxidant Assay The antioxidant activities of compounds **1**–**5** were measured by the Trolox equivalent antioxidant capacity (TEAC) assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, U.S.A.). The TEAC value was calculated on the basis of the ability of the compounds to scavenge the blue-green ABTS^{•+} radical cation [2,20-azino-bis(3-ethylbenzthiazoline-6-sulfonate)] relative to the scavenging ability of the water-soluble vitamin E analogue, Trolox.

Cytotoxicity Five-day *in vitro* Sulforhodamine B (SRB) cytotoxicity tests against human tumors cell lines were carried out using modified protocols for MCF-7 (breast cancer), HepG-2 (human hepatoblastoma) and the normal cells were used as control. Generally, 5×10^3 /ml cells were placed in a 24-well plate and treated with compounds **1**–**5**. The plate was incubated at 37 °C for 5 d. Then the medium was removed from the 24-well plate, and 10% ice-cold trichloroacetic acid (TCA) (1 ml) was added. The plate was kept at 4 °C for 2 h after which was washed four times with cold water, then stained with SRB (Sigma St. Louis, MO, U.S.A.). After washing with 1% acetic acid, the bound dye was solubilized with Tris base A (Sigma-Aldrich) and 100 μ l of each sample were transferred into a 96-well plate, and then read at 492 nm.

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