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New antioxidant polyphenols from the medicinal mushroom *Inonotus obliquus*

In-Kyoung Lee, Young-Sook Kim, Yoon-Woo Jang, Jin-Young Jung and Bong-Sik Yun*

Functional Metabolites Research Center, KRIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea

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Abstract—The fruiting body of *Inonotus obliquus*, a medicinal mushroom called chaga, has been used as a traditional medicine for cancer treatment. Although this mushroom has been known to exhibit potent antioxidant activity, the mechanisms responsible for this activity remain unknown. In our investigation for free radical scavengers from the methanolic extract of this mushroom, inonoblins A (**1**), B (**2**), and C (**3**) were isolated along with the known compounds, phelligridins D (**4**), E (**5**), and G (**6**). Their structures were established by extensive spectroscopic analyses. These compounds exhibited significant scavenging activity against the ABTS radical cation and DPPH radical, and showed moderate activity against the superoxide radical anion.

Free radicals have been implicated in the pathogenesis of various diseases, including myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, and cancer-initiation, as well as in the aging process.[1,2] There is considerable evidence that antioxidants could help to prevent these diseases because they have the capacity to quench free radicals.[3] Although some synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), exhibit potent free radical scavenging effects, they have been demonstrated to exert toxicological effects as compared with natural antioxidants.[4,5] Thus, the demand for alternative and safe antioxidants from natural sources has gradually grown.

In contrast, medicinal mushrooms produce various classes of secondary metabolites with potent antioxidant activity. As part of our ongoing efforts to search for free radical scavengers from medicinal mushrooms, new antioxidant polyphenols, inonoblins A (**1**), B (**2**), and C (**3**), have been isolated together with the known compounds phelligridins D (**4**), E (**5**), and G (**6**) from the methanolic extract of the fruiting body of *Inonotus obliquus* (Fig. 1). *I. obliquus* (Pers.: Fr.) Pil. [syn; *Fuscoporia obliqua* (Pers.: Fr.) Aoshima], also called chaga mushroom, is a white-rot fungus that belongs to family Hymenochaetaceae,[6] and is a medicinal mushroom that is widely distributed in Europe, Asia, and North America. This mushroom, which preferably lives on tree trunks, usually of *Betula* (birch) and rarely on *Ulmus*, *Alnus*, and *Fraxinus*, has been used as a folk medicine for cancer treatment in Russia and western Siberia, and has also been used to prevent and treat heart, liver, and stomach diseases and tuberculosis.[7,8] In a previous investigation on the chemical constituents of this mushroom, inotodiol,[9] trametenolic acid,[10] inonotsuoxides,[11] and other triterpenes[12–14] with antitumor and antifungal activity were reported. Although the extract of this mushroom is known to exhibit potent antioxidant activity,[15] the mechanisms responsible for this activity remain unknown. In this paper, we describe the isolation and structural determination of antioxidant polyphenols isolated from the fruiting body of *I. obliquus*, as well as their free radical scavenging activity.

The ground fruiting body of the fungus *I. obliquus* (3 kg) was extracted twice with MeOH at room temperature. After the removal of MeOH under reduced pressure, the aqueous solution was partitioned between *n*-hexane, chloroform, ethyl acetate, and *n*-butanol and water, in that order. Compounds **1**, **2**, and **4**–**6** were purified from the ethyl acetate-soluble portion by the bioassay-guided fractionation using an ABTS (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) radical scavenging assay. The ethyl acetate extract was chromatographed on a Sephadex LH-20 column eluted with MeOH to

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* Corresponding author. Tel.: +82 42 860 4339; fax: +82 42 860 4595; e-mail: ybs@kribb.re.kr

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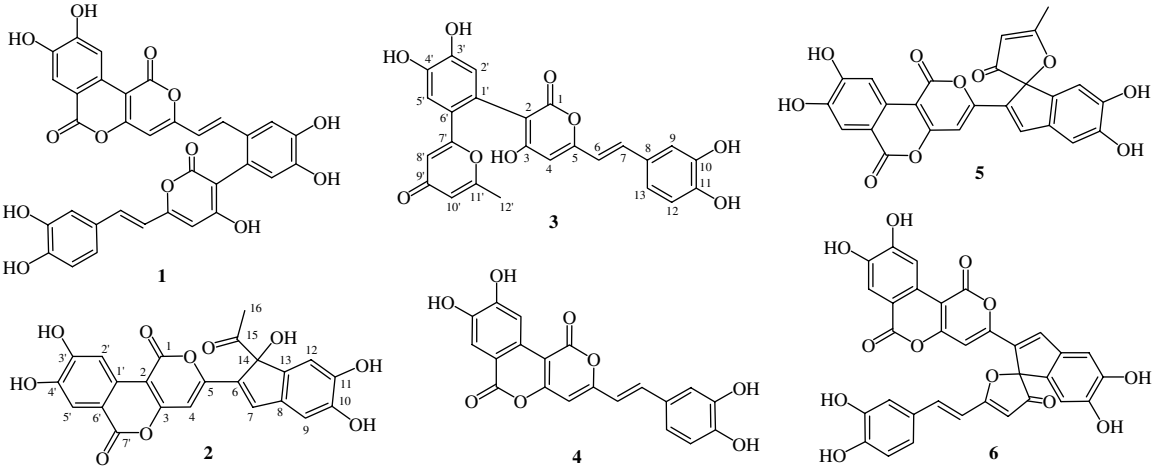

Figure 1. Structures of compounds **1**–**6**.

yield five yellow fractions (fractions 1–5). Fraction 1 was rechromatographed on a Sephadex LH-20 column eluted with 70% aqueous MeOH, followed by preparative reversed-phase TLC developed with 60% aqueous MeOH, to afford compound **2** (4 mg). Fraction 2 was subjected to a Sephadex LH-20 column eluted with 70% aqueous MeOH to yield compound **5** (7 mg). Fraction 3 was rechromatographed on a Sephadex LH-20 column eluted with 70% aqueous MeOH, followed by preparative reversed-phase TLC developed with 70% aqueous MeOH, to yield compound **4** (10 mg). Fraction 4 was chromatographed on a Sephadex LH-20 column eluted with 70% aqueous MeOH to yield compound **1** (7 mg). Compound **6** (3 mg) was purified by chromatography with a Sephadex LH-20 column eluted with 70% aqueous MeOH, followed by preparative reversed-phase HPLC on a column eluted with 52% aqueous MeOH containing 0.04% trifluoroacetic acid. Compound **3** was purified from the *n*-butanol-soluble portion. After concentration of the butanol extract under reduced pressure, the aqueous solution was subjected to a Diaion HP-20 column. The column was washed with 30% aqueous MeOH, and the active substance was then eluted with 50% aqueous MeOH. The active eluate was concentrated and chromatographed on a reversed-phase Sep-pak column eluted with a gradient of increasing MeOH (20–90%) in water. The active fraction was further purified by chromatography with a Sephadex LH-20 column eluted with 70% aqueous MeOH, followed by preparative reversed-phase TLC developed with 50% aqueous MeOH, to yield compound **3** (5 mg).

Compounds **4**–**6** were identified as phelligridins D, E, and G, respectively, on the basis of their physicochemical properties and NMR spectral data, which were in good agreement with those published previously. Compound **1**, designated as inonoblin A, was isolated for the first time in this study, and its physicochemical properties and structural determination were reported previously.[16] Recently, this compound was re-isolated from another medicinal fungus, *Phellinus igniarius*, which is used for the treatment of wounds, abdominalgia, and bloody gonorrhea in traditional Chinese medicine.[17]

Compound **2** was obtained as a yellow powder, and its positive electron spray ionization (ESI) mass provided a quasi-molecular ion peak at *m*/*z* 473 $[M+Na]^+$, while the negative ESI-mass yielded a quasi-molecular ion peak at *m*/*z* 449 $[M-H]^-$. The molecular formula was found to be $C_{23}H_{14}O_{10}$ according to its high resolution mass spectrometry result (*m*/*z* 449.0455 $[M-H]^-$, −5.4 mmu) in combination with ^1H and ^{13}C NMR spectral data. The UV absorption maxima at 253 and 389 nm, similar to compound **1**, suggested that compound **2** had the same chromophore as did compound **1**. In the ^1H NMR spectrum in CD_3OD, six sp^2 methine singlets at δ 8.29, 7.66, 7.52, 6.99, 6.78, and 6.39, and a methyl singlet at δ 1.81 were evident (Table 1). The ^{13}C NMR spectrum revealed the presence of 23 carbons, including one overlapping carbon at δ 111.9. According to the HMQC spectrum, these carbon peaks were established as one methyl, six sp^2 methines, and 16 quaternary carbons, including one ketone and two ester carbonyls and six oxygenated sp^2 carbons. The structure of compound **2** was determined by the HMBC spectrum, as shown in Figure 2. A structural moiety of 8,9-dihydroxypyrano[4,3-*c*]isochromene-1,6-dione was assigned by the long-range correlations from H-4 to C-2, C-3, and C-5, H-2′ to C-2, C-4′, and C-6′, and H-5′ to C-1′, C-3′, C-4′, and C-7′, and these proton and carbon chemical shifts were in good agreement with the corresponding protons and carbons of compound **1**.[17] Additional long-range correlations from H-7 to C-8, C-13, and C-14, H-9 to C-11 and C-13, and H-12 to C-8 and C-10 revealed the presence of an indene-5,6-diol moiety. Finally an acetyl group was connected to C-14 by the HMBC correlations from H-16 to C-14 and C-15, and an indene moiety was conjugated to C-5 by the long-range correlations from H-4 to C-6 and H-7 to C-5. Compound **2** was optically inactive, which suggests that the biogenetic formation of the chiral center (C-14) is non-stereoselective. From the above results, the structure of compound **2** was established as a new polyphenol antioxidant as shown in Figure 1.

Compound **3** was obtained as a yellow powder, and its molecular weight was established to be 462 Da by the

Table 1. ^{1}H and ^{13}C NMR data of compounds **2** and **3** in CD_3OD[a]

No.	2		3	
	δ_C	δ_H	δ_C	δ_H
1	161.7		166.4	
2	101.2		104.6	
3	162.0		168.0	
4	98.8	6.39 (1H, s)[b]	101.3	6.19 (1H, s)
5	155.4		160.5	
6	137.7		116.6	6.59 (1H, d, J = 16.0)
7	140.1	7.66 (1H, s)	137.4	7.30 (1H, d, J = 16.0)
8	134.8		128.8	
9	111.9	6.99 (1H, s)	114.9	7.02 (1H, d, J = 2.0)
10	148.3		146.8	
11	148.7		148.7	
12	111.3	6.78 (1H, s)	116.6	6.77 (1H, d, J = 8.4)
13	139.8		122.0	6.94 (1H, dd, J = 8.4, 2.0)
14	90.4			
15	206.5			
16	22.6	1.81 (3H, s)		
1′	128.7		124.2	
2′	111.9	8.29 (1H, s)	120.6	6.81 (1H, s)
3′	154.9		149.3	
4′	148.4		146.6	
5′	115.5	7.52 (1H, s)	116.7	7.10 (1H, s)
6′	113.4		125.0	
7′	160.9		169.0	
8′			112.4	6.31 (1H, d, J = 2.0)
9′			172.7	
10′			113.8	6.16 (1H, br d, J = 2.0)
11′			169.0	
12′			19.6	2.21 (3H, s)

[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.

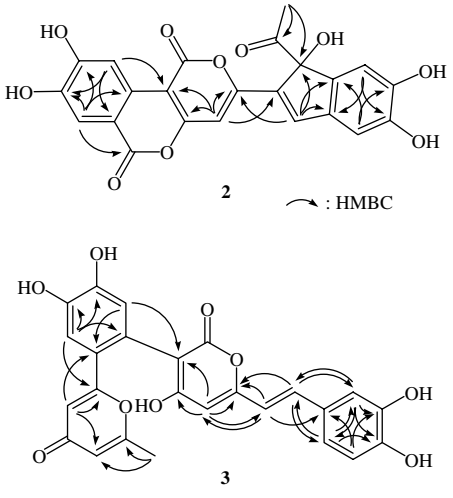

Figure 2. HMBC correlations for compounds **2** and **3**.

electron spray ionization (ESI) mass measurements providing quasi-molecular ion peaks at m/z 463 $[M+H]^+$ in the positive ion mode and at m/z 461 $[M-H]^-$ in the negative ion mode. The molecular formula was established to be $C_{25}H_{18}O_9$ according to its high resolution mass spectrometry data (m/z 461.0903 $[M-H]^-$, +3.0 mmu) in combination with ^{1}H and ^{13}C NMR spectral data. The UV absorption maxima at 248, 323, and 385 nm suggested that compound **3** had a hispidin moiety. The ^{1}H NMR spectrum in CD_3OD with a trace of trifluoroacetic acid-*d* showed signals resulting from a 1,2,4-trisubstituted benzene at δ 7.02 (1H, d, J = 2.0 Hz), 6.94 (1H, dd, J = 8.4, 2.0 Hz), and 6.77 (1H, d, J = 8.4 Hz), three sp^2 methine singlets at δ 7.10, 6.81, and 6.19, two olefinic methine signals attributable to a *trans*-1,2-disubstituted double bond at δ 7.30 (1H, d, J = 16.0 Hz) and 6.59 (1H, d, J = 16.0 Hz), two *meta*-coupled sp^2 methine doublets at δ 6.31 (1H, d, J = 2.0 Hz) and 6.16 (1H, d, J = 2.0 Hz), and a methyl singlet at δ 2.21 (Table 1). The ^{13}C NMR spectrum revealed the presence of 25 carbons, including the overlapping carbons at δ 116.6 and 169.0, and the HMQC spectrum established 11 proton-bearing carbons. The structure of compound **3** was determined according to 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, and C-6, H-6 to C-4, C-5, and C-8, H-7 to C-5, 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 the hispidin moiety. Long-range correlations from H-2′ to C-4′ and C-6′ and H-5′ to C-1′, C-3′, and C-4′ revealed the presence of a 4,5-disubstituted catechol moiety that was, in turn, connected to C-2 by the long-range correlation from H-2′ to C-2. A 2-methyl-4*H*-pyran-4-one moiety was also suggested by the HMBC correlations from H-8′ to C-7′ and C-10′, and from the methyl protons of H-12′ to C-10′ and C-11′. Finally, a 4*H*-pyran-4-one group was conjugated to a catechol moiety by the correlations from H-5′ to C-7′ and H-8′ to C-6′. Therefore, compound **3** was established as a new hispidin-class polyphenol antioxidant. Based on its structural features, this compound seems to be biogenerated by the oxidative coupling of precursor hispidin and hispolon, which might be catalyzed by mushroom peroxidase.[17]

The antioxidant activities of compounds **1**–**6** were evaluated by measuring free radical scavenging activity using three different assays, the ABTS radical scavenging activity assay, the DPPH radical scavenging activity assay, and the superoxide radical anion scavenging activity assay (Table 2). ABTS radical scavenging activity was carried out by using an ABTS radical cation decolorization assay with minor modifications.[18] All of the compounds isolated exhibited higher ABTS cation radical scavenging activity than trolox and caffeic acid, well-known antioxidants that were used as controls. In addition, compounds **4** and **5** showed higher activities than a synthetic antioxidant, BHA, while compounds **1** and **6** were comparable to BHA. Although less active than BHA, compounds **2** and **3** exhibited significant $ABTS^{\cdot+}$ cation radical scavenging activities. To investigate the scavenging effect to the DPPH radicals, each concentration of compounds **1**–**6** 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

Table 2. Free radical scavenging activities of compounds **1**–**6**

Compounds	TEAC[a,b]		Superoxide[e] IC_{50}[b] (μM)
	ABTS[c]	DPPH[d]	
Inonoblin A (**1**)	0.43	1.45	93.6 ± 4.4
Inonoblin B (**2**)	0.58	1.42	90.8 ± 5.3
Inonoblin C (**3**)	0.65	0.82	97.6 ± 5.5
Phelligridin D (**4**)	0.33	1.51	85.5 ± 3.5
Phelligridin E (**5**)	0.40	1.57	120.2 ± 7.6
Phelligridin G (**6**)	0.43	1.48	92.3 ± 4.7
Caffeic acid	0.66	0.41	10.2 ± 2.3
BHA	0.43	1.34	34.3 ± 1.6

[a] Expressed as IC_{50} of compound (μM)/IC_{50} of trolox (μM).
[b] Results presented as the mean ($n = 3$) ± SD.
[c] 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid).
[d] α,α-Diphenyl-β-picrylhydrazyl.
[e] Xanthine/xanthine oxidase.

reader. Compound **3** showed potent DPPH radical scavenging effect and higher activity than trolox or BHA. However, the other compounds were less active than caffeic acid, BHA, and trolox, which were used as controls. Superoxide radical scavenging activity was evaluated by the xanthine/xanthine oxidase method with minor modifications.[19] Results indicated that compounds **1**–**4** and **6** exhibited moderate superoxide radical anion scavenging activity with IC_{50} values in the range of 85–98 μM, approximately nine times and three times less active than caffeic acid and BHA, respectively, as described in Table 2.

The medicinal fungus, *I. obliquus*, of the Hymenochaetaceae family has been used as a traditional medicine for the treatment of cancer. Although the antioxidant activity of *I. obliquus* is well characterized,[15] the compounds responsible for this antioxidant activity have yet to be determined. In this study, we focused on the secondary metabolites with free radical scavenging activities in the fruiting body of *I. obliquus*, and found six major polyphenol antioxidants, inonoblins A–C and phelligridins D, E, and G. These compounds might be responsible for the antioxidant activity of the medicinal mushroom, *I. obliquus*.

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18. 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.
19. Each well of a 96-well plate containing the 100 μL of 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.