Chemical Constituents of *Gymnopilus spectabilis* and Their Antioxidant Activity

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Gymnopilus spectabilis, a hallucinogenic mushroom belonging to the family Cortinariaceae, is found growing in dense clusters on stumps and logs of hardwoods and conifers. It contains the hallucinogenic alkaloid psilocybin and its strongly bitter taste makes it undesirable as an edible. In an effort to identify chemical constituents of Korean native wild mushrooms, 4,6-decadiyne-1,3,8-triol (1), ergosta-4,6,8(14), 22-tetraen-3-one (2), bisnoryangonin (3), and hispidin (4) were isolated from the methanolic extract of the fruiting bodies of *G. spectabilis*. Their structures were assigned on the basis of various spectroscopic studies. Compounds 3 and 4 displayed significant scavenging activity against the ABTS radical cation, DPPH radical, and superoxide radical anion, while 1 and 2 exhibited no antioxidant activity.

KEYWORDS : Antioxidant, Bisnoryangonin, 4,6-Decadiyne-1,3,8-triol, Ergosta-4,6,8,22-tetraen-3-one, *Gymnopilus spectabilis*, Hispidin

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial medicines. They produce diverse classes of secondary metabolites with interesting biological activities and, thus, have the potential as valuable chemical resources (Zjawiony, 2004; Berger and Guss, 2005). We investigate on chemical constituents of Korean native wild mushrooms to make a chemical library. In addition, we search for antioxidative metabolites in view of the fact that free radical is implicated in the pathogenesis of various diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation, and aging processes. *Gymnopilus spectabilis*, a poisonous mushroom belonging to the family Cortinariaceae, is found growing in dense clusters on stumps and logs of hardwoods and conifers. It contains the hallucinogenic alkaloid psilocybin, and its strongly bitter taste makes it undesirable as an edible.

Gymnoprenols, major substances possessed the structures of a novel type of polyisoprenepolyols with 45 to 60 carbon atoms, were previously isolated from this mushroom (Nozoe *et al.*, 1983), and the bitter principle was known as gymnoprenol F renamed as gymnopilin (Nozoe *et al.*, 1983). Also cerevisterol and acetylenic compounds were isolated and characterized from *G. spectabilis* (Kusano *et al.*, 1986). As part of our ongoing efforts to characterize antioxidants and chemical components from the fruiting body of *G. spectabilis*, 4,6-decadiyne-1,3,8-triol (1), ergosta-4,6,8(14), 22-tetraen-3-one (2), bisnoryangonin (3), and hispidin (4) were isolated (Fig. 1). In this paper, we describe the isolation, structure determination, and free

radical scavenging activity of compounds 1~4 and their proposed biogenesis.

Materials and Methods

General methods. ESI-MS was taken on a Navigator mass spectrometer in positive and negative modes. NMR spectra were obtained on a Varian UNITY Inova NMR spectrometer with ¹H NMR at 400 MHz and ¹³C NMR at 100 MHz in CD₃OD, CDCl₃ or a mixture of CD₃OD and CDCl₃. Chemical shifts were given in ppm (δ) using tetramethylsilane (TMS) as internal standard.

Fungal materials. The specimen of the fungus *G. spectabilis* was collected at Gyeonggi province in Korea, and identified. A voucher specimen (No. 311) was deposited in the herbarium of Bioactive Metabolites Research Center, Korea Research Institute of Bioscience and Biotechnology.

Extraction and isolation. Compounds 1~4 were isolated as shown in Fig. 1. The dried fruiting bodies of *G. spectabilis* (615 g) were extracted twice with methanol at room temperature for 2 days. After the removal of the methanol under reduced pressure, the resulting solution was partitioned between ethyl acetate and H₂O. The half of ethyl acetate-soluble fraction was chromatographed on a column of silica gel eluted with a gradient with increasing amount of methanol in chloroform to give two fractions. The first fraction was further purified by consecutive Sephadex LH-20 column chromatographies eluted with chloroform-methanol (1:1, v/v) and methanol, respectively. Finally, preparative silica gel TLC developed with

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Fig. 1. Structures of compounds 1–4.

chloroform-methanol (20 : 1, v/v) provided compound **2** (4 mg). The other fraction was purified by Sephadex LH-20 column chromatography eluted with methanol, followed by preparative reversed-phase TLC developed with 50% aqueous acetonitrile to afford compound **4** (2 mg). The other half of ethyl acetate-soluble fraction was subjected to a column of Sephadex LH-20 eluted with methanol to afford compound **1** (6 mg) and compound **3** (4 mg).

Superoxide radical anion scavenging activity. Superoxide anion scavenging activity was evaluated by the xanthine/xanthine oxidase method with minor modifications (Beauchamp and Fridovich, 1971). In brief, each well of a 96-well plate containing the 100 μ l 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 samples. Plates were 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. Trolox, BHA, and caffeic acid were used as standards.

ABTS radical cation decolorization assay. Evaluation of free radical scavenging activity was carried out by using ABTS radical cation decolorization assay (Re *et al.*,

1999). The activity is based on the ability of the antioxidant to scavenge the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS^{•+}) with spectrophotometric analysis. According to Re *et al.* (1999), ABTS was dissolved in H₂O to a concentration of 7 mM. The ABTS 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 the addition of 0.1 ml of the ABTS radical cation solution (A734 nm = 0.700) to the ethanol solution of the compound (5 μ l) and mixing for 6 min, the absorbance was measured by microplate reader using VERSAmax (Molecular Devices Co., USA). For standards, trolox, BHA, and caffeic acid were used.

DPPH radical scavenging activity. Sample dissolved in 5 μ l of DMSO was added to 95 μ l of 150 μ M DPPH ethanol solution (Blois, 1958). After vortex mixing, the mixture was incubated for 20 min at room temperature, and the absorbance was measured at 517 nm using a microplate reader (Molecular Devices Co., USA). The differences in absorbance between the test sample and control (DMSO) were measured. Trolox, butylated hydroxyanisole (BHA), and caffeic acid were used as standards.

Results and Discussion

Structures of compounds 1–4. The dried fruiting bodies of the fungus *G. spectabilis* were extracted twice with

Table 1. ^1H and ^{13}C NMR spectral data of compounds **1**, **3**, and **4**^a

No.	Decadiyne (1)		Bisnoryangonin (3)		Hispidin (4)	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	59.0	3.75 (2H, m) ^b				
2	41.3	1.87 (2H, m)	167.8		167.8	
3	60.2	4.53 (1H, t, $J = 7.5$)		quenched		quenched
4	81.2		173.7		173.7	
5	68.9		102.2	5.94 (1H, s)	102.0	6.06 (1H, s)
6	69.1		161.6		161.9	
7	81.6		116.0	6.53 (1H, d, $J = 16.5$)	116.9	6.53 (1H, d, $J = 16.0$)
8	64.3	4.29 (1H, d, $J = 7.5$)	136.6	7.25 (1H, d, $J = 16.5$)	137.2	7.26 (1H, d, $J = 16.0$)
9	31.7	1.67 (2H, qd, $J = 7.5, 2.4$)	128.0		128.8	
10	9.8	0.98 (3H, t, $J = 7.5$)	130.1	7.35 (1H, d, $J = 8.4$)	114.8	7.00 (1H, d, $J = 1.8$)
11			116.6	6.71 (1H, d, $J = 8.4$)	148.6	
12			159.9		146.7	
13			116.6	6.71 (1H, d, $J = 8.4$)	116.5	6.76 (1H, d, $J = 8.4$)
14			130.1	7.35 (1H, d, $J = 8.4$)	121.9	6.90 (1H, dd, $J = 1.8, 8.4$)

^aNMR data were measured at 400 MHz for proton and at 100 MHz for carbon.^bProton resonance integral, multiplicity, and coupling constant ($J = \text{Hz}$) are in parentheses.

methanol. The methanolic extract was partitioned between ethyl acetate and H_2O . Repeated chromatographic separations of the ethyl acetate-soluble fraction led to the purification of compounds **1**–**4**.

The molecular weight of compound **1** was determined to be 182 by the ESI-mass measurements, which exhibit quasi-molecular ion peaks at m/z 205 $[\text{M}+\text{Na}]^+$ and m/z 387 $[2\text{M}+\text{H}]^+$ in the positive mode, and m/z 181 $[\text{M}-\text{H}]^-$ and m/z 363 $[2\text{M}-\text{H}]^-$ in the negative mode. The ^1H NMR spectrum of **1** in mixture of CD_3OD and CDCl_3 exhibited signals due to a methyl protons at δ 0.98, two methylene protons at δ 1.67 and 1.87, an oxygenated methylene protons at δ 3.75, and two methine protons at δ 4.29 and 4.53 (Table 1). In the ^{13}C NMR spectrum, one methyl car-

bon at δ 9.8, three methylene carbons at δ 31.7, 41.3, and 59.0, two oxygenated methine carbons at δ 60.2 and 64.3, and four quaternary carbons at δ 68.9, 69.1, 81.2, and 81.6 were evident. Unique chemical shift values of four quaternary carbons at δ 68.9, 69.1, 81.2, and 81.6 suggested the presence of diyne moiety in **1** (Table 1). The structure of **1** was determined on the basis of HMBC correlations, as summarized in Fig. 2. The HMBC correlations of H-1 to C-2 and C-3, H-2 to C-1, C-3, and C-4, H-3 to C-1, C-2, C-4, and C-5, H-8 to C-6, C-7, C-9, and C-10, H-9 to C-7, C-8, and C-10, and H-10 to C-8 and C-9 were observed. Therefore, the structure of **1** was unambiguously determined to be 4,6-decadiyne-1,3,8-triol. This compound was previously isolated from this mushroom

Fig. 2. Isolation procedures of compounds **1**–**4**.

by Kusano *et al.* (1986).

The molecular weight of **2** was established by the ESI-mass measurements, which provided a quasi-molecular ion peak at m/z 391 [M-H] in negative mode, suggesting the molecular weight to be 392. The ^1H NMR spectrum of **2** in mixture of CD_3OD and CDCl_3 exhibited signals due to six methyl protons at δ 0.8–1.2, methylene and methine protons at δ 1.3–2.7, and five methine protons at δ 5.2, 5.3, 5.7, 6.1 and 6.7, suggesting that this compound was a member of triterpenoids. The structure of **2** was determined on the basis of HMBC and ^1H - ^1H COSY experiments, as summarized in Fig. 2. HMBC correlations from H-28 to C-24, C-26, and C-27, from H-27 to C-24 and C-26, from H-25 to C-23 and C-24, from H-21 to C-17, C-20, and C-22, from H-18 to C-12, C-13, C-14, and C-17, from H-19 to C-1, C-5, C-9 and C-10, from H-4 to C-2, C-5, and C-6, from H-6 to C-4, C-5, C-8 and C-10, and from H-7 to C-5 and C-9 were observed. Based on these HMBC correlations and ^1H - ^1H COSY data, the structure of **2** was determined as ergosta-4,6,8(14), 22-tetraen-3-one, which was previously isolated from the fungi *Alternaria alternata* (Seitz and Paukstelis, 1977), *Acremonium coenohialum* (Davis *et al.*, 1986), and *Scleroderma polyrhizum* (Gonzalez *et al.*, 1983). However, it was found for the first time in *Gymnopilus spectabilis*.

Compound **3** was obtained as yellow powder, and its ESI-mass in negative mode provided a quasi-molecular ion peak at m/z 229 [M-H], suggesting the molecular weight to be 230. The ^1H NMR spectrum of **3** in CD_3OD exhibited signals due to singlet methine proton at δ 5.94, two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond at δ 6.53 and 7.25, and 1,4-disubstituted benzene peaks at δ 6.71 and 7.35 (Table 1). In the ^{13}C NMR spectrum, seven sp^2 methine carbons, one ester carbonyl, and four sp^2 quaternary carbons including three oxygenated carbons were evident (Table 1). The structure of **3** was established by the HMBC spectrum, which showed long-range correlations from singlet methine proton at δ 5.94 to C-3, C-4, C-6, and C-7, from olefinic methine protons at δ 6.53 and 7.25 to C-5, C-6, C-9, and C-10, from methine protons at δ 6.71 to C-9, C-11, and C-12, and from methine protons at δ 7.35 to C-8, C-10, and C-12, as summarized in Fig. 2. Therefore, the structure of **3** was identified as bisnoryangonin, which was found in *Pholiota* sp. (Brady and Benedict, 1972), *Polyporus* sp. (Nambudiri, 1974), and *Gymnopilus* sp. (Hatfield and Brady, 1969).

Compound **4** was obtained as yellow powder, and its molecular weight was established by the ESI-mass measurements, which provided a quasi-molecular ion at m/z 245 [M-H] in negative mode, suggesting the molecular weight to be 246. The ^1H NMR spectrum of **4** was very similar to that of **3**, except for signals attributed to a 1,2,4-trisubstituted benzene at δ 6.76, 6.90, and 7.00 instead of

Fig. 3. HMBC correlations of compounds 1–3.

1, 4-disubstituted benzene at δ 6.71 and 7.35 showed in compound **3** (Table 1). From these results, compound **4** was identified as hispidin that was ubiquitous in mushroom metabolite. Hispidin was reported as a selective PKC- β inhibitor from *Phellinus pomaceus* (Klaar and Steglich, 1977; Gonindard *et al.*, 1997).

Antioxidant activity. A main property of antioxidant is the ability to scavenge free radicals. Therefore, we evaluated the free radical scavenging efficacy of compounds 1–4 by using superoxide radical anion, ABTS radical cation, and DPPH radical scavenging assay methods. For DPPH and ABTS radical scavenging activity, results were expressed in terms of trolox equivalent antioxidant capacity (TEAC, IC_{50} of μM compound/ IC_{50} of μM trolox).

Results from radical scavenging assay are presented in Table 2. Bisnoryangonin and hispidin were capable of scavenging DPPH, ABTS, and superoxide radicals in a concentration-dependent manner. Bisnoryangonin exhibited approximately 3–5 times higher activity than trolox and was comparable to BHA and caffeic acid, which were used as controls. Hispidin also showed potent scavenging activity but was less active than bisnoryangonin. Up to

Table 2. Free radical scavenging activity

Compounds	TEAC ^{a,b}		Superoxide ^c IC ₅₀ (μM) ^b
	DPPH ^c	ABTS ^d	
Decadiyne (1)	— ^f	—	—
Ergosta-tetraen-3-one (2)	—	—	—
Bisnoryangonin (3)	0.21 ± 0.22	0.23 ± 0.52	8.05 ± 0.61
Hispidin (4)	0.31 ± 0.81	2.27 ± 0.71	34.9 ± 4.0
Caffeic acid	0.11 ± 0.21	0.17 ± 0.26	16.6 ± 2.5
BHA	0.35 ± 0.20	0.11 ± 0.20	—

^aExpressed as IC₅₀ of μM compound/IC₅₀ of μM trolox.^bResults presented as the mean (n = 3) ± SD.^cα,α-diphenyl-β-picrylhydrazyl.^d2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).^eXanthine/xanthine oxidase.^fNo activity up to 200 μM.

date, bisnoryangonin has been reported as an antimicrobial substance. In this study, we found for the first time that bisnoryangonin had potent antioxidant activity. However, 4,6-decadiyne-1,3,8-triol and ergosta-4,6,8(14),22-tetraen-3-one exhibited no free radical scavenging activity.

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