NOTE

Cytotoxic and anti-inflammatory disulfide compounds from the fruiting bodies of *Boletus pseudocalopus*

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Boletus pseudocalopus (Boletaceae) is an inedible mushroom that mainly causes gastrointestinal irritation, and is widely distributed in Korea and Japan.¹ It is recognized by its yellow fruiting bodies and the blue staining when the fruit bodies are cut or brushed.^{1,2} A previous phytochemical study of this mushroom reported the isolation of cytotoxic grifolin derivatives.² During our screening for new cytotoxic metabolites from Korean wild mushrooms, it was found that the MeOH extract of *B. pseudocalopus* showed significant cytotoxicity against three human tumor cell lines (A549, Hs746T and H3122).³ Three new cytotoxic fatty acid esters, calopusins A–C, were isolated from its CHCl₃-soluble fraction by the bioassay-guided isolation, and were recently reported.³ As a part of our continuing efforts to study the bioactive constituents of the MeOH extract of *B. pseudocalopus*, we have investigated its *n*-BuOH-soluble fraction in the search for structurally interesting and bioactive metabolites. Repeated column chromatographic separation of the *n*-BuOH-soluble fraction of the MeOH extract resulted in the isolation of two disulfide compounds including a new compound, 3-methylbutyl hydrodisulfide (**1**) and phenylethyl hydrodisulfide (**2**), which was previously reported but only as a synthetic product,⁴ together with three known polar compounds including erythritol (**3**),⁵ arabinitol (**4**)⁵ and uracil (**5**)⁶ (Figure 1). The structures of **1** and **2** were elucidated on the basis of 1D and 2D NMR spectroscopic data analysis, and the known compounds **3**–**5** were identified by comparison of the physical and spectroscopic data with the values in the literature. Here, we describe the isolation and structural elucidation of **1** and **2**, as well as the biological activities of the isolates **1**–**5**.

The fresh fruiting bodies of *B. pseudocalopus* were collected at Mt. Gaya, Hapcheon-Gun of Gyeongsangnam-do, Korea, in August 2006. The mushroom was authenticated by one of the authors (K.R.L.). The air-dried and powdered fruiting bodies of *B. pseudocalopus* (139 g) were extracted twice with 80% aqueous MeOH (each 1.5 l × 2 days) at room temperature and filtered. The filtrate was concentrated under vacuum to give a crude MeOH extract (10 g), which was partitioned with *n*-hexane, CHCl₃ and *n*-BuOH by using H₂O, yielding 600, 700 and 2100 mg of residues, respectively.

The CHCl₃-soluble fraction was investigated in a previous study³ and the main fraction, the *n*-BuOH-soluble fraction, was selected for the current phytochemical investigation. The *n*-BuOH-soluble fraction (2 g) was separated over a silica gel (50 g, 230–400 mesh, Merck, Darmstadt, Germany) open column chromatography with CHCl₃-MeOH-H₂O (16:7:1) to yield eight fractions (B1–B8). Fraction B2 (150 mg) was purified by RP-C₁₈ semi-preparative HPLC (Econosil RP-18 column, Alltech, Nicholasville, KY, USA, 10 μm, 250 mm × 10 mm i.d.) using a solvent of MeOH-H₂O (85:15) to yield compound **5** (4 mg). Fraction B4 (240 mg) was purified by normal-phase semi-preparative HPLC (Apollo Silica column, Alltech, 5 μm, 250 mm × 10 mm i.d.) using a solvent system of CHCl₃-MeOH (4:1) to afford compounds **2** (8 mg) and **3** (6 mg). Compound **4** (30 mg) was isolated from fraction B6 (410 mg) by the purification of RP-C₁₈ semi-preparative HPLC (Econosil RP-18 column) with MeOH-H₂O (85:15). Finally, fraction B7 (270 mg) was subjected to passage over a Waters Sep-Pak Vac 6 cc (Milford, MA, USA) with a solvent of CHCl₃-MeOH-H₂O (9:5:1) to obtain the new compound **1** (24 mg).

The physico-chemical properties of compound **1** are summarized in Table 1. The molecular formula of **1** was determined to be C₅H₁₂S₂ from the [M+Na]⁺ peak at *m/z* 159.0273 (calcd. for C₅H₁₂S₂Na, 159.0278) in the positive-ion high resolution (HR)-ESIMS spectrum. The structure of compound **1** was mainly elucidated by analysis of NMR spectra including 2D NMR. The ¹H NMR spectrum (Table 2) of **1** showed characteristic signals for two methylene protons at δ_H 2.93 (2H, t, *J* = 7.0 Hz) and 1.54 (2H, q, *J* = 7.0 Hz), a methine proton at δ_H 1.67 (1H, m) and two methyl groups at δ_H 0.96 (6H, d, *J* = 7.0 Hz). The ¹³C NMR (Table 2) and DEPT spectra displayed two methylene carbons at δ_C 37.9 and 36.2, a methine carbon at δ_C 25.6 and two overlap methyl carbons at δ_C 21.3. Interpretation of the HMQC data allowed all single bond proton and carbon correlations to be assigned (Table 1). An analysis of the ¹H-¹H COSY and HMBC spectra for **1** led to be assigned the gross structure seen in Figure 2. The cross-peaks between H-1 (δ_H 2.93) and H-2 (δ_H 1.54), H-2 (δ_H 1.54) and H-3 (δ_H 1.67), H-3 (δ_H 1.67) and H-4 (δ_H 0.96), and H-3 (δ_H 1.67) and

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H-5 (δ_{H} 0.96) were observed in the ^1H - ^1H COSY spectrum (Figure 2). It allowed us to establish the isopentane unit, which was further supported by key HMBC correlations from H-1 to C-3 (δ_{C} 25.6) and H-4 and H-5 to C-2 (δ_{C} 36.2). The NMR spectra of **1** were generally similar to those of 3-methyl-1-butanol,⁷ but the apparent differences were the signals attributable to C-1 and C-2. In the case of 3-methyl-1-butanol,⁷ the substituent of the hydroxyl group (OH) at C-1 in the isopentane unit led to downfield shift of the signals [δ_{H} 3.58 (H-1), δ_{C} 61.2 (C-1); δ_{H} 1.42 (H-2), δ_{C} 42.6 (C-2)] for C-1 and C-2, compared with those [δ_{H} 2.93 (H-1), δ_{C} 37.9 (C-1); δ_{H} 1.54 (H-2), δ_{C} 36.2 (C-2)] of **1**. The characteristic signals for C-1 and C-2 allowed us to expect the substituent at C-1 to be an amine (NH_2) or thiol group (SH). However, a survey of the literature revealed that compound **1** does not have the expected substituents at C-1 when compared with

the NMR data of the related compounds.^{8,9} The NMR data of 3-methylbutylamine displayed the resonances of C-1 (δ_{C} 40.6) and C-2 (δ_{C} 43.7) in the ^{13}C NMR spectrum,⁸ whereas the NMR data of butane-1-thiol showed a similar chemical shift of C-2 (δ_{C} 37.1) to

Figure 2 Key COSY (bold) and HMBC (arrow) correlations of **1** and **2**.

Table 3 Antiproliferative activities of compounds **1**–**2** against four cultured human cancer cell lines

Compound	IC_{50} (μM) ^a			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	18.75	>30.0	9.91	>30.0
2	>30.0	>30.0	13.55	>30.0
Doxorubicin ^b	0.012	0.008	0.017	0.857

^a IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

^bDoxorubicin was used as the positive control.

Table 4 Inhibitory effect of compounds **1**–**5** on nitric oxide (NO) production in LPS-activated BV-2 cells

Compound	IC_{50} (μM) ^a	Cell viability (%) ^b
1	40.98	98.1 ± 3.8*
2	60.94	101.8 ± 2.3
3	73.60	101.9 ± 3.1
4	71.87	102.3 ± 2.9
5	34.55	101.1 ± 2.9
NMMA ^c	18.29	101.3 ± 4.5

Abbreviation: LPS, lipopolysaccharide.

Results are averages of three independent experiments, and data are expressed as mean ± s.d. (* P -value < 0.05).

^a IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.

^bCell viability after treatment with 20 μM of each compound and LPS is expressed as a percentage (%) of the group treated with LPS only.

^cNMMA was the positive control.

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

Table 1 Physico-chemical properties of compounds **1** and **2**

	Compound 1	Compound 2
Appearance	Colorless gum	Colorless gum
Molecular formula	$\text{C}_5\text{H}_{12}\text{S}_2$	$\text{C}_8\text{H}_{10}\text{S}_2$
Molecular weight	136	170
HR-ESIMS m/z	$[\text{M}+\text{Na}]^+$	$[\text{M}+\text{Na}]^+$
Calcd	159.0278 ($\text{C}_5\text{H}_{12}\text{S}_2\text{Na}$)	193.0122 ($\text{C}_8\text{H}_{10}\text{S}_2\text{Na}$)
Found	159.0273	193.0125
UV (MeOH) λ_{max} (log ϵ)		263 (2.4), 217 (3.9)
IR (KBr) ν_{max} (cm^{-1})	2947, 2935, 2494, 1451	2949, 2937, 2502, 1661, 1456

Table 2 NMR spectroscopic data for compounds **1** and **2**^a

Position	1					2		
	δ_{H} (CD_3OD)	δ_{C} (CD_3OD)	δ_{H} (CDCl_3)	δ_{C} (CDCl_3)	HMBC	δ_{H} (CD_3OD)	δ_{C} (CD_3OD)	HMBC
1	2.93 t (7.0)	37.9 t	3.00 t (7.0)	38.4 t	C-2, 3		136.6 s	
2	1.54 q (7.0)	36.2 t	1.67 q (7.0)	36.3 t	C-1, 3, 4, 5	7.28 m	128.5 d	C-4, 6, 7
3	1.67 m	25.6 d	1.70 m	25.7 d	C-1, 2, 4, 5	7.34 td (8.0, 1.5)	128.8 d	C-1, 5
4	0.96 d (7.0)	21.3 q	0.92 d (7.0)	22.1 q	C-2, 3, 5	7.25 m	127.1 d	C-2, 6
5	0.96 d (7.0)	21.3 q	0.92 d (7.0)	22.1 q	C-2, 3, 4	7.34 td (8.0, 1.5)	128.8 d	C-1, 3
6						7.28 m	128.5 d	C-2, 4, 7
7						2.95 t (7.5)	33.3 t	C-1, 2, 6, 8
8						3.17 t (7.5)	40.7 t	C-1, 7
SSH			3.24 s		C-1			

^a ^1H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

the corresponding one of **1**, but upfield shifted resonance of C-1 (δ_C 24.6).⁹ On the other hand, the ^1H NMR spectrum of **1** was found to be very similar to that of pentyl hydrodisulfide,¹⁰ although the acidic proton of SSH was not detected owing to usage of the NMR solvent of CD_3OD . The existence of the hydrodisulfide group (SSH) could be suggested by the IR spectrum of **1** exhibiting IR absorption at 2494 cm^{-1} attributed to SSH. Furthermore, the presence of the hydrodisulfide group was confirmed on the basis of the molecular formula, $\text{C}_5\text{H}_{12}\text{S}_2$ established by the HR-ESIMS data. To identify the acidic proton of SSH in compound **1**, ^1H NMR spectrum (Table 2) of **1** was additionally measured in CDCl_3 where the acidic proton of SSH was observed at δ_{H} 3.24 (1H, s). The proton signal of SSH showed HMBc correlation with C-1 (δ_C 38.4). Thus, the structure of **1** was elucidated as 3-methylbutyl hydrodisulfide, and is shown in Figure 1.

Compound **2** was obtained as a colorless gum, and the molecular formula $\text{C}_8\text{H}_{10}\text{S}_2$ was established by HR-ESIMS. The physico-chemical properties of **2** are summarized in Table 1. The structure of compound **2** was determined by analysis of NMR spectra including 2D NMR (Figure 2), where characteristic signals for the phenylethyl unit were clearly assigned. The presence of a hydrodisulfide group was also confirmed by the downfield shifted signals of C-7 and C-8 and the IR absorption at 2502 cm^{-1} , together with the molecular formula, $\text{C}_8\text{H}_{10}\text{S}_2$ established by the HR-ESIMS data. Thus, the structure of **2** was determined as phenylethyl hydrodisulfide (**2**), which was previously reported but only as a synthetic product.⁴ Full NMR data assignments of **2** were reported in this study for the first time (Table 2).

The antiproliferative activities of compounds **1**–**5** were evaluated by determining their inhibitory effects on four human tumor cell lines, namely A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma) and HCT-15 (colon adenocarcinoma) using the SRB bioassay.¹¹ After continuous exposure to the compounds for 48 h, compound **1** showed antiproliferative activity against A549 and SK-MEL-2 cells with IC_{50} values of 18.75 and $9.91\text{ }\mu\text{M}$, respectively, and compound **2** exhibited the activity against the SK-MEL-2 cell line with an IC_{50} value of $13.55\text{ }\mu\text{M}$, but the other compounds (**3**–**5**) were inactive ($\text{IC}_{50} > 30.0\text{ }\mu\text{M}$) (Table 3).

In addition, the anti-inflammatory activities of compounds **1**–**5** were evaluated by measuring the produced nitric oxide (NO) levels in lipopolysaccharide-activated microglia BV-2 cells.¹² After 24 h incubation, compounds **1** and **2** displayed an anti-inflammatory effect by inhibiting NO levels with IC_{50} values of 40.98 and $60.94\text{ }\mu\text{M}$, respectively, in lipopolysaccharide-stimulated BV-2 cells without cytotoxicity, while compound **5** significantly exhibited the inhibitory activity with an IC_{50} of $34.55\text{ }\mu\text{M}$ (Table 4).

Disulfide compounds and other structurally related compounds have been reported mainly as volatile components from *Allium* vegetables (including garlic, onions, leeks, chives and scallions) used throughout the world for their sensory characteristics as well as their

apparent health benefits.¹³ The sulfur compounds have been reported to have a variety of pharmacological properties.^{14–18} For example, diallyldisulfide and diallyltrisulfide showed antiproliferative and proapoptotic effects in human epithelial cancer and neuronal cell lines,^{14,15} apoptosis in the leukemic HL-60 cell line through activation of caspase-3¹⁶ and inhibition of NO synthesis in lipopolysaccharide-activated macrophages.¹⁷

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