

RESEARCH NOTE

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Neuraminidase Inhibitors from the Culture Broth of *Phellinus linteus*

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During the search for neuraminidase inhibitors from medicinal fungi, we found that the culture broth of *Phellinus linteus* exhibited potent inhibitory activity. Solvent partition, Sephadex LH-20 column chromatography, and high-performance liquid chromatography (HPLC) were performed for purification of two active substances from the culture broth. According to ¹H NMR measurements and comparison of HPLC retention times with those of authentic compounds, their chemical structures were identified as hispidin and hypholomine B. Compounds (hispidin) 1 and 2 (hypholomine B) inhibited neuraminidase, with IC₅₀ values of 13.1 and 0.03 μM, respectively.

KEYWORDS : Hispidin, Hypholomine B, Neuraminidase inhibitor, *Phellinus linteus*

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial medicines. They produce a large variety of secondary metabolites with unique chemical structures and interesting biological activities. *Phellinus linteus* is cultivated as a folk medicine in Korea and is known to exhibit various biological activities, such as anticancer, antioxidative, anti-angiogenic, anti-inflammatory and anti-viral effects [1-5]. However, except for polysaccharides, a limited number of bioactive metabolites have been reported from *P. linteus*.

Neuraminidases, called sialidases, catalyze hydrolysis of terminal neuraminic-acid residues from newly formed virions and from host cell receptors [6], and are therefore involved in release of progeny virus from infected cells. Specifically, cleavage of the α-ketosidic bond linking a terminal neuraminic-acid residue to an adjacent oligosaccharide moiety by neuraminidase, which is also important for trafficking of virus in the mucus layer of the respiratory tract, allowing the virus access to underlying epithelial cells, has been reported [7]. Therefore, neuraminidase that plays an important role in viral proliferation is a drug target for the prevention of the spread of influenza infection. Neuraminidase inhibitors, such as zanamivir administered by inhalation and oseltamivir administered orally, have been used for combating influenza infection [8]. However, the long-term efficacy of these drugs is often limited by toxicity and the almost inevitable selection of drug-resistant viral mutants [9]. In our ongoing effort to identify

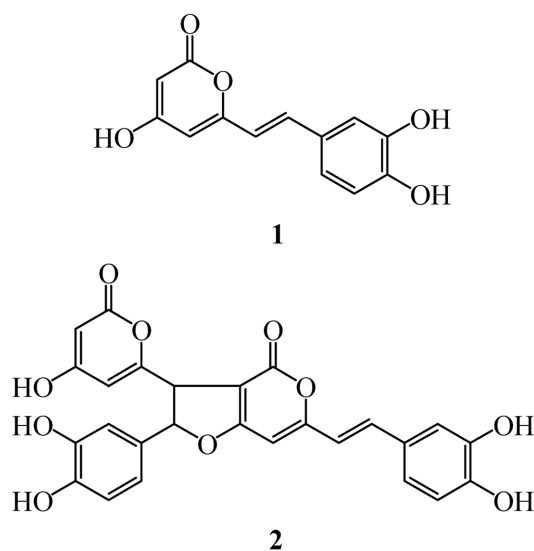


Fig. 1. Structures of compounds 1 (hispidin) and 2 (hypholomine B).

bioactive substances from fungi, primarily medicinal fungi [10, 11], two neuraminidase inhibitors have been isolated from the culture broth of *P. linteus* (Fig. 1). This paper describes the isolation, structure determination, and neuraminidase inhibitory activity of these compounds.

A strain of *P. linteus*, which was obtained from the Rural Development Administration (RDA) in Korea, was grown in potato dextrose broth medium at 28°C for 10

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days. Extraction of three liters of the culture broth was performed using ethyl acetate, with vigorous shaking. Neuraminidase inhibitory activity was observed in the ethyl acetate-soluble portion. Following concentration of the ethyl acetate-soluble portion under reduced pressure, the concentrate was subjected to a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column eluted with methanol to give two active fractions. A Sephadex LH-20 column with 70% aqueous methanol was used for chromatography of one fraction, followed by purification with preparative reversed-phase high-performance liquid chromatography (HPLC) with 60% aqueous methanol with 0.04% trifluoroacetic acid to give compound **1**. Sephadex LH-20 column chromatography, with 70% aqueous methanol, was performed for purification of the other fraction, followed by preparative reversed-phase HPLC using the same solvent used for compound **1** to afford compound **2**.

The chemical structure of compound **1** was determined according to ^1H NMR spectrum and by comparison of HPLC retention time with that of the authentic compound. The ^1H NMR spectrum of compound **1** in CD_3OD exhibited signals due to a 1,2,4-trisubstituted benzene at δ 7.01 (d, $J = 1.5$ Hz), 6.93 (dd, $J = 8.3, 1.5$ Hz) and 6.76 (d, $J = 8.3$ Hz), two olefinic methines attributable to a *trans*-1,2-disubstituted double bond unit at δ 7.29 (d, $J = 16.0$ Hz) and 6.58 (d, $J = 16.0$ Hz), and a sp^2 singlet methine at δ 6.09. These spectroscopic data were well matched with those of hispidin, which was previously isolated by our group as an antioxidant [12]. Thus, analytical HPLC equipped with a reversed-phase C_{18} column (150 \times 4.6 mm i.d.) was performed for comparison of retention time of compound **1** with that of authentic hispidin. A linear gradient using water acidified with 0.04% trifluoroacetic acid (v/v) and methanol, at a flow rate of 1 mL/min, was used, starting at 10% aqueous methanol and reaching 100% methanol within a period of 30 min. Consequently, the retention time of compound **1** was the same as that of hispidin. Thus, compound **1** was identified as hispidin, a ubiquitous compound in genera *Phellinus* and *Inonotus* [12].

The structure of compound **2** was assigned using the same methods employed for compound **1**. The ^1H NMR spectrum of compound **2** in CD_3OD exhibited signals due to two 1,2,4-trisubstituted benzenes at δ 7.03 (d, $J = 2.0$ Hz), 6.93 (dd, $J = 8.3, 2.0$ Hz), 6.72 (d, $J = 8.3$ Hz) and 6.79 (d, $J = 2.0$ Hz), 6.78 (d, $J = 8.3$ Hz), 6.70 (dd, $J = 8.3, 2.0$ Hz), two olefinic methines attributable to a *trans*-1,2-disubstituted double bond unit at δ 7.35 (d, $J = 16.1$ Hz) and 6.63 (d, $J = 16.1$ Hz), two sp^3 methines at δ 5.76 (d, $J = 6.4$ Hz) and 4.30 (d, $J = 6.4$ Hz), and two sp^2 singlet methines at δ 6.36 and 6.08. Based on findings from an extensive literature survey, we observed that ^1H NMR spectrum was in good agreement with that of hypholomine B [12]. Thus, analytical HPLC equipped

with a reversed-phase C_{18} column (150 \times 4.6 mm i.d.), using the same solvent used for compound **1**, was performed for comparison of the retention time of compound **2** with that of authentic hypholomine B. Consequently, the retention time for compound **2** was the same as that of hypholomine B, revealing that compound **2** was identical to hypholomine B.

A previously reported method, with minor modifications, was used in performance of the neuraminidase inhibition assay [13]. Briefly, 50 μL neuraminidase (N2876; Sigma, St. Louis, MO, USA) solution (0.05 U/mL) was pre-mixed with 10 μL of sample solution at different concentrations in 90 μL of 50 mM sodium acetate buffer (pH 5.0) in a cuvette. Then, 4-methylumbelliferyl- α -D-*N*-acetylneuraminic acid sodium salt hydrate (M8639; Sigma) 0.05 mM in buffer (pH 5.0) was added (50 μL) to the mixture as a substrate in order to start the reaction at 37°C. Quantification of 4-methylumbelliferone was performed immediately by fluorometric determination using a POLARstar OPTIMA

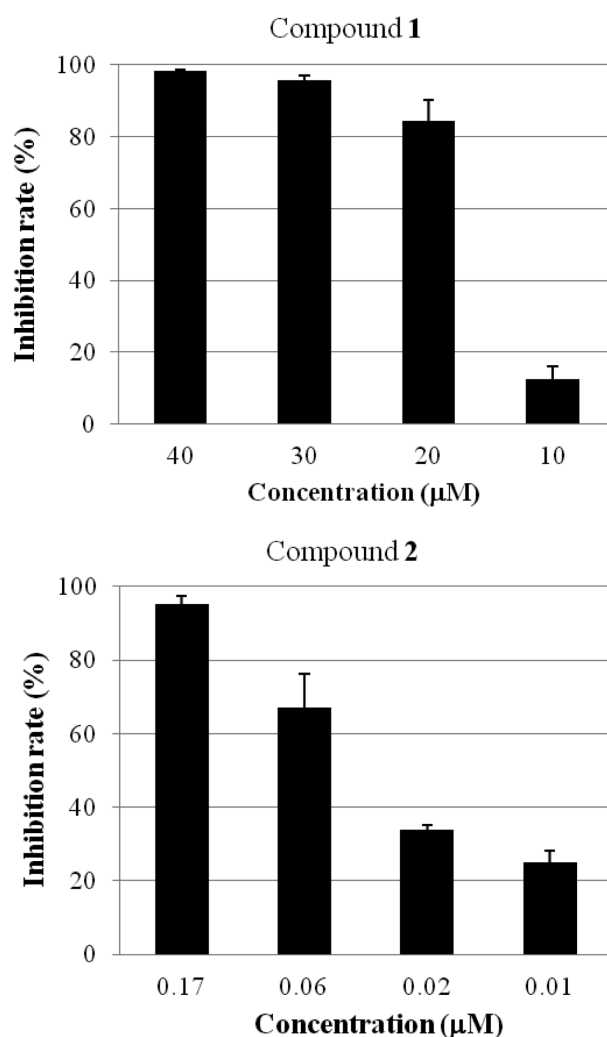


Fig. 2. Neuraminidase inhibitory activity of compounds **1** (hispidin) and **2** (hypholomine B).

multi-detection microplate reader (BMG Labtech, Offenburg, Germany). The excitation and emission wavelengths were 355 nm and 460 nm, respectively. As a result, compounds **1** and **2** exhibited neuraminidase inhibitory activity with IC₅₀ values of 13.1 and 0.03 μM, respectively, in a dose-dependent manner (Fig. 2). Compound **2** induced potent inhibition of neuraminidase and exhibited approximately 430-times higher activity than compound **1**. To the best of our knowledge, this is the first study to report on hispidin and hypholomine B as neuraminidase inhibitors. Details regarding the mode of action of hispidin and hypholomine B for inhibition of neuraminidase are under investigation.

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References

- Zhu T, Kim SH, Chen CY. A medicinal mushroom: *Phellinus linteus*. *Curr Med Chem* 2008;15:1330-5.
- Lee IK, Yun BS. Styrylpyrone-class compounds from medicinal fungi *Phellinus* and *Inonotus* spp., and their medicinal importance. *J Antibiot (Tokyo)* 2011;64:349-59.
- Lee YS, Kim YH, Shin EK, Kim DH, Lim SS, Lee JY, Kim JK. Anti-angiogenic activity of methanol extract of *Phellinus linteus* and its fractions. *J Ethnopharmacol* 2010;131:56-62.
- Kim HG, Yoon DH, Lee WH, Han SK, Shrestha B, Kim CH, Lim MH, Chang W, Lim S, Choi S, et al. *Phellinus linteus* inhibits inflammatory mediators by suppressing redox-based NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. *J Ethnopharmacol* 2007;114:307-15.
- Ichinohe T, Ainai A, Nakamura T, Akiyama Y, Maeyama J, Odagiri T, Tashiro M, Takahashi H, Sawa H, Tamura S, et al. Induction of cross-protective immunity against influenza A virus H5N1 by an intranasal vaccine with extracts of mushroom mycelia. *J Med Virol* 2010;82:128-37.
- von Itzstein M. The war against influenza: discovery and development of sialidase inhibitors. *Nat Rev Drug Discov* 2007;6:967-74.
- Klenk HD, Rott R. The molecular biology of influenza virus pathogenicity. *Adv Virus Res* 1988;34:247-81.
- Zhang J, Yu K, Zhu W, Jiang H. Neuraminidase pharmacophore model derived from diverse classes of inhibitors. *Bioorg Med Chem Lett* 2006;16:3009-14.
- Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet* 2003;362:1733-45.
- Jang YW, Lee IK, Kim YS, Seok SJ, Yu SH, Yun BS. Chemical constituents of the fruiting body of *Xylaria polymorpha*. *Mycobiology* 2009;37:207-10.
- Lee IK, Jung JY, Yeom JH, Ki DW, Lee MS, Yeo WH, Yun BS. Fomitoside K, a new lanostane triterpene glycoside from the fruiting body of *Fomitopsis nigra*. *Mycobiology* 2012;40:76-8.
- Jung JY, Lee IK, Seok SJ, Lee HJ, Kim YH, Yun BS. Antioxidant polyphenols from the mycelial culture of the medicinal fungi *Inonotus xeranticus* and *Phellinus linteus*. *J Appl Microbiol* 2008;104:1824-32.
- Kim JY, Jeong HJ, Park JY, Kim YM, Park SJ, Cho JK, Park KH, Ryu YB, Lee WS. Selective and slow-binding inhibition of shikonin derivatives isolated from *Lithospermum erythrorhizon* on glycosyl hydrolase 33 and 34 sialidases. *Bioorg Med Chem* 2012;20:1740-8.