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Research Note

Neuraminidase Inhibitors from the Fruiting Body of *Phellinus igniarius*

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Abstract During our ongoing investigation of neuraminidase inhibitors from medicinal fungi, we found that the fruiting bodies of *Phellinus igniarius* exhibited significant inhibitory activity against neuraminidase from recombinant H3N2 influenza viruses. Two active compounds were isolated from the methanolic extract of *P. igniarius* through solvent partitioning and Sephadex LH-20 column chromatography. The active compounds were identified as phelligridins E and G on proton nuclear magnetic resonance (^{1}H NMR) and electrospray ionization mass measurements. These compounds inhibited neuraminidases from recombinant rvH1N1, H3N2, and H5N1 influenza viruses, with IC_{50} values in the range of 0.7~8.1 μM.

Keywords Neuraminidase inhibitor, Phelligridin E, Phelligridin G, *Phellinus igniarius*

Neuraminidase, also known as sialidase, is an important glycoprotein in influenza viruses that cleaves sialic acid from the infected cell surface and releases virus progeny [1]. It plays a major role in viral proliferation and infecting other cells [2]. Thus, neuraminidase is an attractive therapeutic drug target for the treatment of influenza. Currently, neuraminidase inhibitors such as zanamivir (Relenza; Glaxo Wellcome Inc., Research Triangle Park, NC, USA) and oseltamivir (Tamiflu; Roche, Nutley, NJ, USA) are used for the treatment of influenza. However, several problems are associated with these drugs, including a high level of drug resistance and diverse side effects such as vomiting and nausea [3, 4]. Therefore, next-generation neuraminidase inhibitors are urgently needed.

Mushrooms are both a nutritional food source and a traditional medicine. They produce various classes of bioactive secondary metabolites with unique chemical structures [5, 6]. In previous studies, we reported the isolation of neuraminidase inhibitors from *Phellinus baumii* and *P. linteus* and their biological properties [7-9]. As part of an ongoing effort to identify neuraminidase inhibitors from medicinal fungi, we found that the methanolic extract of fruiting bodies of *P. igniarius* exhibited significant H3N2 neuraminidase inhibitory activity. *Phellinus igniarius*, belonging to the family Hymenochaetaceae, has been used to treat fester, abdominalgia, and bloody gonorrhea in traditional medicine in Korea, Japan, and China and has been reported to possess anti-oxidative, anti-proliferative, anti-metastatic, and anti-influenza activities [10-12]. In this study, we describe the isolation, structure determination, and biological properties of neuraminidase inhibitors from the methanolic extract of *P. igniarius*.

Isolation of neuraminidase inhibitors. In order to isolate neuraminidase inhibitors, the fruiting bodies of *P. igniarius* were extracted using methanol for 2 days at room temperature. The methanolic extract was concentrated under reduced pressure, and the resultant residue was partitioned twice with ethyl acetate. The ethyl acetate-soluble layer was subjected to Sephadex LH-20 column chromatography (Pharmacia, Uppsala, Sweden) eluted with methanol to yield two active fractions. One active fraction was further purified by Sephadex LH-20 column chromatography eluted with 70% aqueous methanol to provide compound **1**. The other fraction was subjected to Sephadex LH-20 column chromatography with 70% aqueous methanol as the eluting

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solvent to yield compound **2**.

Structure determination of neuraminidase inhibitors. The chemical structures of compounds **1** and **2** were determined via electrospray ionization (ESI)-mass and proton nuclear magnetic resonance (^{1}H NMR) spectrum measurements. The molecular weight of compound **1** was 474 on ESI-mass measurement, where it exhibited a quasi-molecular ion peak at *m/z* 475 $[M+H]^+$. The ^{1}H NMR spectrum of **1** in DMSO-d_6 demonstrated seven methine

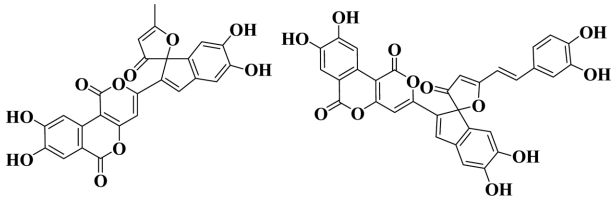

Fig. 1. Chemical structure of compounds **1** (phelligridin E) and **2** (phelligridin G).

Table 1. Neuraminidase inhibitory activity of compounds **1** and **2**

Compound	Neuraminidase inhibition (IC_{50}, μM)			Kinetic mode (K_i, μM)
	H1N1	H3N2	H5N1	
Phelligridin E (**1**)	8.1 ± 0.1	6.6 ± 0.4	1.0 ± 0.3	Noncompetitive (7.1)
Phelligridin G (**2**)	8.0 ± 1.0	5.2 ± 0.6	0.7 ± 0.1	Noncompetitive (6.9)
Zanamivir (nM)	1.3 ± 0.1	8.0 ± 1.5	3.1 ± 0.1	NT

NT, not tested.

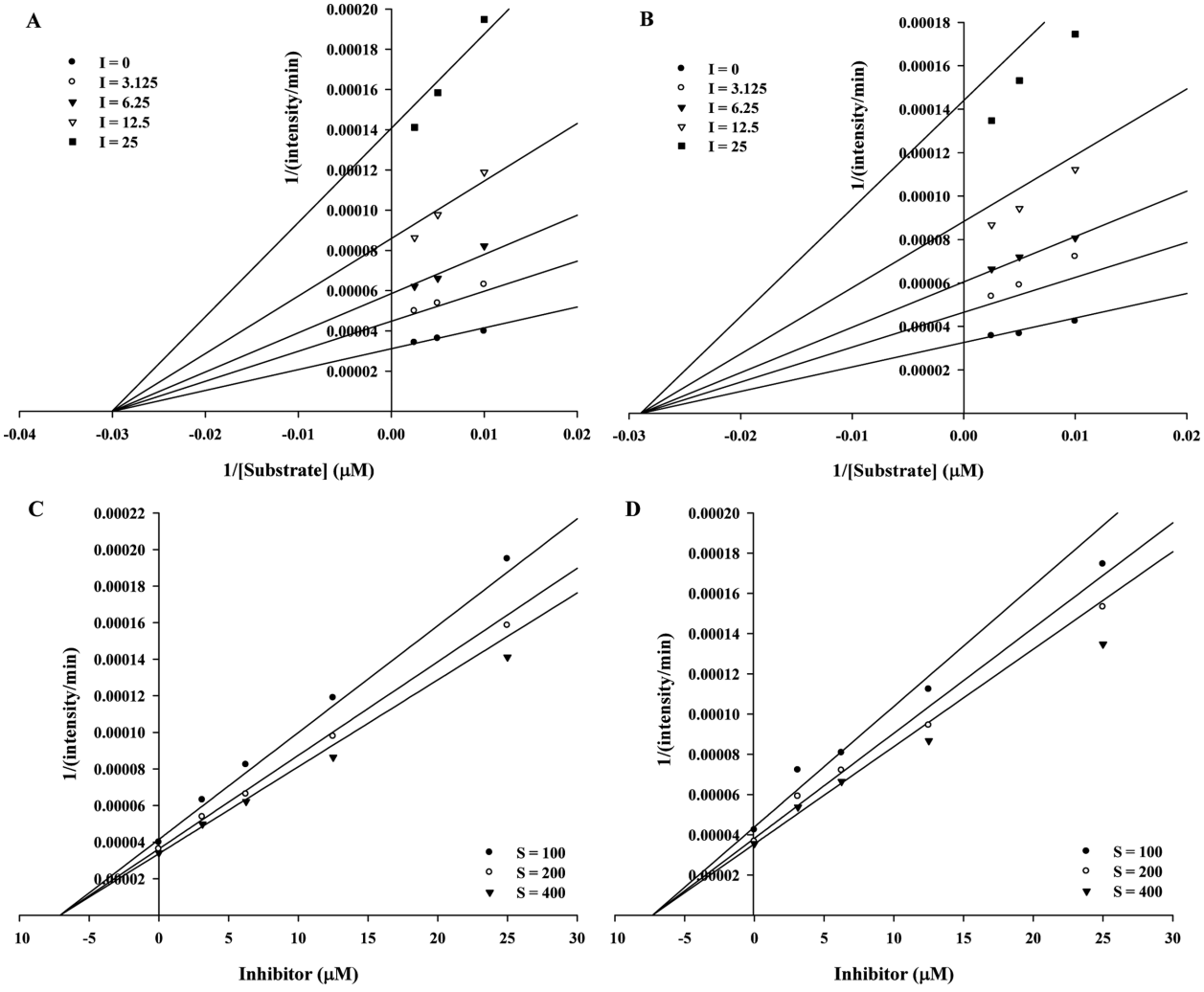

Fig. 2. Graphical representation of the neuraminidase inhibition of isolated compounds. A, B, Lineweaver-Burk plots of the neuraminidase inhibition of compounds **1** and **2**; C, D, Dixon plots of the neuraminidase inhibition of compounds **1** and **2**.

protons at δ 8.24, 7.85, 7.47, 6.97, 6.61, 6.15, and 6.09 and one methyl signal at δ 2.50. Based on literature review, compound **1** was identified as phelligridin E; the molecular weight and ^{1}H chemical shift values were in good agreement with those previously reported for phelligridin E [13].

The chemical structure of compound **2** was also determined via ESI-mass and ^{1}H NMR spectrum measurements. The molecular weight of compound **2** was 594 on ESI-mass measurement, and it demonstrated a quasi-molecular ion peak at *m/z* 595 $[M+H]^+$. The ^{1}H NMR spectrum of compound **2** in DMSO-d_6 showed three aromatic methine signals assignable to a 1,2,4-trisubstituted benzene moiety at δ 7.17 (d, J = 2.1 Hz), 7.10 (dd, J = 8.2, 2.1 Hz), and 6.79 (d, J = 8.2 Hz), two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond at δ 7.54 (d, J = 16.2 Hz) and 7.10 (d, J = 16.2 Hz), and seven methine singlets at δ 8.28, 7.86, 7.48, 7.01, 6.64, 6.25, and 6.02. These spectral data were consistent with those of phelligridin G previously reported in the literature [14]. Therefore, compounds **1** and **2** were identified as phelligridin E and phelligridin G, respectively, as shown in Fig. 1.

Neuraminidase inhibition activity. The inhibitory activity of compounds **1** and **2** against neuraminidases from recombinant rvH1N1, H3N2, and H5N1 influenza viruses was evaluated. Neuraminidase inhibition assay was performed in 96-well plates as previously described [15], with some modifications. Briefly, the substrate, 50 μL of 0.2 mM MUNANA (2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt; Sigma, St. Louis, MO, USA), was mixed with 90 μL of 50 mM Tris buffer (containing 200 mM NaCl, 5 mM $CaCl_2$, pH 7.5) at room temperature. Ten microliters of the sample and 50 μL of H1N1 neuraminidase (50 ng/mL) were added to each well. The mixture was then recorded at the excitation and emission wavelengths of 365 nm and 445 nm, respectively, using a POLAR OPTIMA (BMG LABTECH, Ortenberg, Germany). In the case of H3N2 and H5N1 neuraminidases, 25 mM MES buffer (containing 500 mM NaCl, 5 mM $CaCl_2$, pH 6.5) and 50 mM MES buffer (containing 500 mM NaCl, 5 mM $CaCl_2$, pH 6.5) were used, respectively, and H5N1 required an activation period of 24 hr at 37°C before assay. The other methods were the same as used for H1N1 neuraminidase. A neuraminidase inhibitor, zanamivir (Relenza) was used as a positive control. Compounds **1** and **2** showed H3N2 neuraminidase inhibition activity with an IC_{50} value of 6.7 μM, respectively, in a dose-dependent manner. Inhibitory activity of compounds **1** and **2** against H1N1 and H5N1 neuramanidases were also evaluated. These compounds significantly inhibited the H1N1 and H5N1 neuramanidases with IC_{50} values in the range of 8.0~1.0 μM in a dose-dependent manner (Table 1).

We investigated the inhibition type of compounds **1** and **2** using kinetic parameters. To study the mode of inhibition, Dixon plots were used to distinguish the mechanism of neuraminidase enzyme action and to confirm the inhibition constant (K_i). The data were measured using Sigmaplot ver. 1.3 (Systat, Chicago, IL, USA). Compounds **1** and **2** displayed noncompetitive inhibitory activity, as indicated by a decrease in Vmax while Km remained stable at increasing inhibitor concentrations. The inhibition constant (K_i) values of compounds **1** and **2** were 7.1 ± 0.3 and 6.9 ± 0.3, respectively (Fig. 2).

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