Mycobiology

Research Note

Neuraminidase Inhibitors from the Fermentation Broth of *Phellinus linteus*

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Abstract During a search for neuraminidase inhibitors derived from medicinal fungi, we found that the fermentation broth of *Phellinus linteus* exhibited potent neuraminidase inhibitory activity. Through bioassay-guided fractionation, two active compounds were purified from the ethyl acetate-soluble portion of the fermentation broth of *P. linteus*. These structures were identified as inotilone (**1**) and 4-(3,4-dihydroxyphenyl)-3-buten-2-one (**2**) by spectroscopic methods. Compounds **1** and **2** inhibited H1N1 neuraminidase activity with IC_{50} values of 29.1 and 125.6 μM, respectively, in a dose-dependent manner. They also exhibited an antiviral effect in a viral cytopathic effect reduction assay using MDCK cells. These results suggest that compounds **1** and **2** from the culture broth of *P. linteus* would be good candidates for the prevention and therapeutic strategies towards viral infections.

Keywords 4-(3,4-Dihydroxyphenyl)-3-buten-2-one, Anti-influenza agent, Inotilone, Neuraminidase inhibitor, *Phellinus linteus*

Influenza viruses are enveloped RNA viruses that belong to the family Orthomyxoviridae, and cause significant morbidity and mortality in humans through epidemics or pandemics [1]. Influenza viruses are classified into various serotypes on the basis of two surface glycoproteins: hemagglutinin and neuraminidase. Neuraminidase (EC 3.2.1.18) plays an important role in viral proliferation and is therefore a drug target for prevention of the spread of influenza [2]. Currently, the preferred treatment for influenza virus infection is the use of neuraminidase inhibitors such as oseltamivir (Tamiflu) and zanamivir (Relenza) [3]. However, toxicity due to long-term exposure to these drugs and the appearance of viral strains that are resistant to these antiviral drugs highlight the urgent need for next-generation neuraminidase inhibitors [4].

Mycobiology 2014 June, **42**(2): 189-192
http://dx.doi.org/10.5941/MYCO.2014.42.2.189
pISSN 1229-8093 • eISSN 2092-9323

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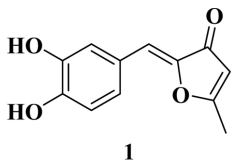
Received April 2, 2014
Revised May 13, 2014
Accepted May 15, 2014

Phellinus linteus is a species of mushroom belonging to the Hymenochaetaceae family, which is indigenous mainly to tropical regions of America, Africa and East Asia [5]. It is one of many medicinal mushrooms that have been widely used in East Asia, especially in Korea, China, and Japan, as health booster and ancient herbal medicine [6]. *P. linteus* is known as Sangwhang in Korea [7] and produces abundant bioactive compounds such as protocatechuic acid, caffeic acid, hispidin, davallialactone, hypholomine B, interfungins A, and inoscavin A [8-11]. The extract and compounds of *P. linteus* exhibit various biological activities including anti-cancer, anti-oxidative, anti-angiogenic, anti-inflammatory and anti-viral effects [6, 12-17]. During the search for neuraminidase inhibitors from medicinal fungi, two neuraminidase inhibitors were isolated from the fermentation broth of *P. linteus* (Fig. 1). This paper describes the isolation,



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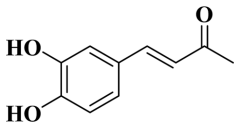

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Fig. 1. Structures of compounds **1** (inotilone) and **2** (4-(3,4-dihydroxyphenyl)-3-buten-2-one).

structure determination, and neuraminidase inhibitory activity of these compounds.

P. linteus was obtained from the Korea National College of Agriculture and Fisheries, Korea. The strain was fermented on potato dextrose broth (26 L) at 27°C for 30 days. The fermentation broth was partitioned with ethyl acetate by vigorous shaking, and the ethyl acetate-soluble portion exhibited potent neuraminidase inhibitory activity at the concentration of 50 μg/mL. Following the concentration of the ethyl acetate-soluble portion under reduced pressure, the concentrate was subjected to a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column and eluted with methanol resulting in 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/0.04% trifluoroacetic acid, which resulted in compound **1** (6.8 mg). The other fraction was purified by Sephadex LH-20 column chromatography eluted with 70% aqueous methanol, followed by preparative reversed-phase HPLC using the same solvent used for compound **1,** to afford compound **2** (6.3 mg).

The structure of compound **1** was determined by the mass as well as the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) measurements. The molecular weight of compound **1** was established by the electrospray ionization (ESI)-mass measurement, which provided a quasi-molecular ion peak at *m*/*z* 219.0 $[M + H]^{+}$, suggesting a molecular weight of 218. The ^{1}H NMR spectrum of compound **1** in CD_3OD exhibited signals due to δ 7.34 (1H, d, *J* = 2.0 Hz, ArH), 7.16 (1H, dd, *J* = 8.4, 2.0 Hz, ArH), 6.80 (1H, d, *J* = 8.4 Hz, ArH), 6.49 (1H, s, CH), 5.80 (1H, s, CH), and 2.55 (3H, s, CH_3). In the ^{13}C NMR spectrum, twelve carbons were evident including a carbonyl carbon at δ 187.0, four oxygenated sp^2 carbons at δ 180.9, 148.4, 145.7, and 144.6, five sp^2 methine carbons at δ 123.1, 118.2, 116.2, 112.3, 105.7, one sp^2 quaternary carbon at δ 125.0, and one methyl carbon at δ 15.9. Consequently, compound **1** was identified as inotilone by comparing measured ^{1}H and ^{13}C NMR spectra with those reported in the literature [18].

The structure of compound **2** was determined by mass and ^{1}H NMR measurements. The molecular weight of compound **2** was established by the ESI-mass, which provided a quasi-molecular ion peak at *m*/*z* 177.0 $[M-H]^{-}$, suggesting a molecular weight of 178. The ^{1}H NMR spectrum of compound **2** in CD_3OD exhibited signals due to δ 7.51 (1H, d, *J* = 16.4 Hz), 7.07 (1H, d, *J* = 2.4 Hz), 6.98 (1H, dd, *J* = 2.4, 8.4 Hz), 6.78 (1H, d, *J* = 8.4 Hz), 6.54 (1H, d, *J* = 16.4 Hz), and 2.32 (3H, s, CH_3). These spectroscopic data were well matched with those of 4-(3,4-dihydroxyphenyl)-3-buten-2-one.

We then investigated the inhibitory effects of compounds **1** and **2** against neuraminidase from recombinant influenza A virus H1N1 (rvH1N1). A previously reported method was used for the neuraminidase inhibition assay, with minor modifications [19]. In brief, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt (MUNANA, Cat. No M8639; Sigma, St. Louis, MO, USA), at the final concentration of 0.2 mM, was mixed with 90 μL of 50 mM Tris buffer (pH 7.5) at room temperature. Ten microliters of sample solution and 50 μL of rvH1N1 (50 ng/mL) were added to a well in a plate. The mixture was recorded at excitation and emission wavelengths of 365 nm and 445 nm, respectively, with a POLAR OPTIMA (BMG LABTECH, Ortenberg, Germany). Zanamivir (Relenza) and quercetin, which were used as positive controls, inhibited neuraminidase with IC_{50} values of 0.0015 and 37.2 μM, respectively, in this assay system. As a result, compounds **1** and **2** exhibited neuraminidase inhibitory activity with IC_{50} values of 29.1 and 125.6 μM, respectively, in a concentration-dependent manner (Table 1).

Table 1. H1N1 neuraminidase inhibitory activity of compounds **1** and **2**

Compounds	IC_{50} (μM)[a]
Inotilone (**1**)	29.1 ± 2.8
4-(3,4-Dihydroxyphenyl)-3-buten-2-one (**2**)	125.6 ± 0.6
Quercetin	37.1 ± 0.7
Zanamivir (nM)	1.5 ± 0.2

[a]Results are presented as mean IC_{50} values obtained from three independent experiments carried out in triplicate ± SD.

Antiviral effect and cytotoxicity were evaluated by the SRB method using the cytopathic effect (CPE) reduction method [20]. In brief, MDCK cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. Then, 0.09 mL of diluted virus suspension and 0.01 mL of medium supplemented with trypsin-EDTA and containing 10 μg/mL of compounds **1** and **2** was added to each well. After incubation at 37°C in 5% CO_2 for 2 days, the morphology of cells was observed under a microscope at a magnification of 32 × 10 (AXIOVERT10; Zeiss, Jena, Germany), and images were recorded. After MDCK cells had undergone 2-day infection with the influenza A/WS/33 virus, mock cells or cells treated with compounds **1**, **2** or oseltamivir showed typical spread-out shapes and normal morphology. At this concentration, no signs of cytotoxicity were observed. Infection with influenza A/WS/33 virus in the absence of compounds resulted in a severe CPE (Fig. 2). Addition of compounds **1** and **2** to influenza A/WS/33 virus-infected MDCK cells inhibited the formation of a visible CPE with IC_{50} values of 61.5 and 52.3 μM, respectively, while oseltamivir prevented CPE formation with an IC_{50} value of 64.7 μM. These results revealed that compounds **1** and **2** were more effective than the positive control oseltamivir against influenza virus H1N1 (Table 2).

In conclusion, inotilone and 4-(3,4-dihydroxyphenyl)-3-buten-2-one isolated from the fermentation broth of *P. linetus* were shown to be effective against H1N1 neuraminidase and the influenza A/WS/33 virus. Therefore, the potential of these compounds for use in the treatment of viral influenza infections merits additional attention.

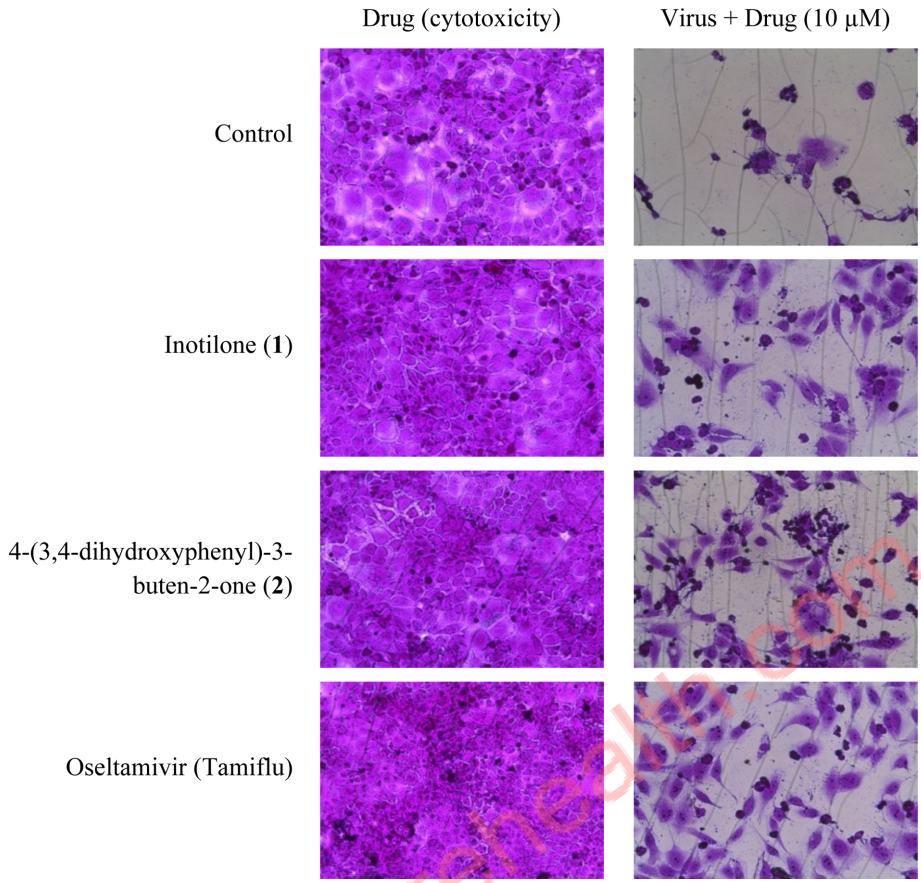

Fig. 2. Effects of compounds **1** and **2** on influenza A/WS/33 virus-induced cytopathic effect.

Table 2. Antiviral activity of compounds **1** and **2** against influenza A virus in MDCK cells[a]

Compounds (μM)	CC_{50}[b]	IC_{50}[c]	TI[d]
Inotilone (**1**)	> 100	61.5 ± 6.4	> 1.6
4-(3,4-Dihydroxyphenyl)-3-buten-2-one (**2**)	> 100	52.3 ± 5.4	> 1.9
Oseltamivir (Tamiflu)	176.4	64.7	1.6

[a]Results are presented as mean IC_{50} values obtained from three independent experiments carried out in triplicate ± SD.
[b]Concentration required to reduce cell growth by 50%.
[c]Concentration required to inhibit virus-induced cytopathic effect by 50%.
[d]Therapeutic index = CC_{50}/IC_{50}.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Technology Development Program for Bio-industry, Ministry for Food, Agriculture, Forestry and Fisheries as well as support from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009796012014), Rural Development Administration, Republic of Korea.

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