MYCOBIOLOGY
2019, VOL. 47, NO. 2, 256–260
https://doi.org/10.1080/12298093.2019.1616377

RESEARCH NOTE

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Neuraminidase Inhibitors from the Fruiting Body of *Glaziella splendens*

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ABSTRACT

Neuraminidase (NA) cleaves the glycosidic bond linkages of sialic acids to release the mature virions from infected cells and has been an attractive therapeutic target for anti-influenza agents. In our ongoing investigation of NA inhibitors in mushroom extracts, we found that the extract the fruiting body of *Glaziella splendens* potently inhibited neuraminidase. The fruiting bodies of *G. splendens* were extracted and partitioned successively with hexane, ethyl acetate, and butanol. The ethyl acetate soluble-layer was subjected to silica gel and Sephadex LH-20 column chromatographies, and MPLC to obtain five compounds (**1**–**5**). Their structures were determined by spectroscopic methods. NA inhibitory activity of these compounds was evaluated using NAs from recombinant rvH1N1, H3N2, and H5N1 influenza A viruses. One compound (**1**) was elucidated as a new azaphilone derivative, and four compounds (**2**–**5**) were identified as entonaemin A, comazaphilone D, rubiginosin A, and entonaemin B, respectively. Compounds **3** and **4** showed considerable inhibitory activity against three types of neuraminidases with the IC_{50} values of 30.9, 41.8, and 35.7 μM for **3** and 46.5, 50.4, and 29.9 μM for **4**, respectively. This study reveals that the fruiting bodies of *G. splendens* possess azaphilone derivatives with the NA inhibitory activity. This is the first report on the isolation of neuraminidase inhibitors from the fruiting bodies of *G. splendens*.

ARTICLE HISTORY
Received 6 March 2019
Revised 30 April 2019
Accepted 5 May 2019

KEYWORDS
Azaphilone; *Glaziella splendens*; Neuraminidase inhibitor

Influenza is a highly contagious respiratory disease resulting from infection with the influenza virus. It usually occurs annually during summer and winter seasons as epidemics, but the virus has also caused several pandemics due to outbreaks of novel viral mutants and cross-species transmission to humans, which have resulted in considerable mortality and morbidity [1]. The influenza viruses are RNA viruses belonging to the family Orthomyxoviridae, which is divided into three types, types A, B, and C on the basis of their nucleoproteins and matrix proteins [2]. Each of these types can be further categorized into diverse serotypes based on two main proteins of glycoproteins, hemagglutinin and neuraminidase (NA). Currently, two types of anti-influenza drugs are used to target NA and the M2 ion channel. However, M2 ion channel blockers cause severe side effects and have been associated with high levels of drug resistance [3]. NA, also called as sialidase, plays an important role in the release of mature virions from infected cells [4]. For these reasons, it has been selected as an attractive therapeutic target. Two NA inhibitors, oseltamivir and zanamivir, have been used to treat influenza viral infections [5]. However, the long-term use of these two influenza drugs can cause a high emergence of drug resistance and numerous side effects [6].

As a part of an ongoing effort for NA inhibitory compounds in mushrooms, we found that a chloroform/methanol (1:1, v/v) extract of the fruiting bodies of *G. splendens* exhibited significant H5N1 NA inhibitory activity. The mushroom, *G. splendens* belongs to the family Glaziellaceae, and is characterized by hollow, gelatinous stromata that accumulate liquid [7]. Azaphilone derivatives we isolated from *G. splendens* have been reported in the literature to display anti-microbial activity [8]. In this study, we report the isolation, structure elucidation, and NA inhibitory activity of compounds **1**–**5** (Figure 1).

The fruiting bodies of *G. splendens* were collected from Jeju Island, Korea, 2015. The fruiting bodies were ground and extracted twice with chloroform/methanol (1:1, v/v) at room temperature. The extract was evaporated to remove the solvents. The crude extract (33 g) was partitioned successively with hexane, ethyl acetate, and butanol. The ethyl acetate-soluble layer (1.88 g) was subjected to silica gel column chromatography, and eluted with a gradient of $CHCl_3$:MeOH (100:1 → 0:100, v/v) to yield

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Supplemental data for this article can be accessed here.

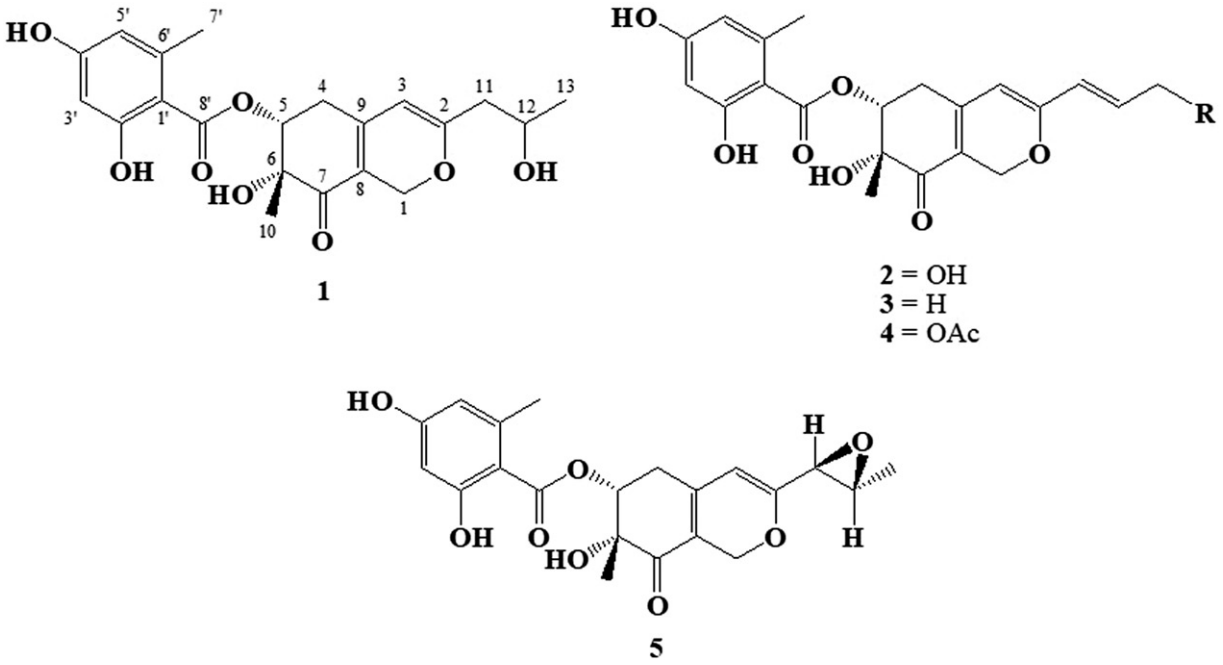

Figure 1. Structures of compounds **1**–**5**.

two fractions. One fraction (320.3 mg) was further separated using Sephadex LH-20 column chromatography eluted with MeOH to give compounds **1** (6.0 mg) and **2** (6.2 mg). The other fraction (244.0 mg) was subjected to medium pressure column chromatography equipped with RediSep Rf C_{18} reversed-phase column (43 g) and eluted with a gradient of 40–100% aqueous MeOH to yield compounds **3** (5.6 mg, t_R: 145 min), **4** (6.1 mg, t_R: 176 min), and **5** (6.3 mg, t_R: 236 min).

Compound **1** was obtained as an amorphous yellowish powder with the specific rotation value ($[\alpha]_D^{25}$) of +52.6 ($c = 0.52$, MeOH), and showed UV maxima (log ϵ) at 215 (4.00), 264 (4.01), 304 (4.23), and 356 (4.54) nm. The molecular formula was determined to be $C_{21}H_{24}O_8$ by high-resolution ESI-mass data (*m/z* 427.1364 $[M+Na]^+$, Δ − 0.5 mmu). The ^{1}H NMR spectrum of compound **1** showed signals due to two *meta*-coupled aromatic methines at δ 6.14 and 6.13, one olefinic methane at δ 5.38, two oxygenated methines at δ 5.57 and 4.00, three non-equivalent methylenes at δ 4.97/4.79, 3.04/2.75, and 2.36/2.30, and three methyls at δ 2.23, 1.41, and 1.19. The ^{13}C NMR spectrum displayed the presence of two carbonyl carbons at δ 197.3 and 172.2, seven sp^2 quaternary carbons at δ 167.4, 166.4, 164.1, 149.3, 145.0, 113.9, and 105.8, three sp^2 methine carbons at δ 112.7, 104.2, 101.8, two oxygenated methine carbons at δ 78.4 and 66.7, one oxygenated quaternary carbon at δ 75.6, one oxygenated methylene carbon at δ 65.1, two methylene carbons at δ 44.7 and 33.0, and three methyl carbons at δ 24.6, 23.7, and 23.5 (Table 1). All proton-bearing carbons were established by the HMQC spectrum and two partial structures, –CH_2–CH(–O) – and CH_3–CH(–O)–CH_2–, were determined by the ^{1}H–^{1}H COSY spectrum. The chemical structure was determined by the HMBC spectrum, which showed long-range correlations from the methylene protons at δ 4.97/4.79 (H-1) to the carbons at δ 197.3 (C-7), 167.4 (C-2), 149.3 (C-9), and 113.9 (C-8), and from the olefinic methine proton at δ 5.38 (H-3) to the carbons at δ 167.4 (C-2), 113.9 (C-8), and 33.0 (C-4), from the methylene protons at δ 3.04/2.75 (H-4) to the carbons at δ 113.9 (C-8), 104.2 (C-3), and 75.6 (C-6), and from the methine proton at δ 5.57 (C-5) to the

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **1** in CD_3OD.

	1	
No.	δ_C	δ_H (*J* in Hz)
1	65.1	4.97 (d, 12.3 Hz)[a] 4.79 (d, 12.3 Hz)
2	167.4	
3	104.2	5.38 (s)
4	33.0	3.04 (br dd, 19.5, 2.1 Hz) 2.75 (br dd, 19.5, 2.1 Hz)
5	78.4	5.57 (dd, 2.1 Hz)
6	75.6	
7	197.3	
8	113.9	
9	149.3	
10	23.7	1.41 (s)
11	44.7	2.36 (dd, 14.2, 7.6 Hz) 2.30 (dd, 14.2, 5.2 Hz)
12	66.7	4.00 (m)
13	23.5	1.19 (d, 6.1 Hz)
1′	105.8	
2′	166.4	
3′	101.8	6.13 (d, 2.4 Hz)
4′	164.1	
5′	112.7	6.14 (d, 2.4 Hz)
6′	145.0	
7′	24.6	2.23 (s)
8′	172.2	

[a]Proton multiplicity and coupling constants in parenthesis.

carbons at δ 197.3 (C-7) and 149.3 (C-9), establishing the presence of azaphilone moiety in **1**. The long-range correlations from the methyl protons at δ 2.23 (H-7′) to the carbons at δ 145.0 (C-6′), 112.7 (C-5′), and 105.8 (C-1′), from the methine proton at δ 6.13 (H-3′) to the carbons at δ 112.7 (C-5′) and 105.8 (C-1′), and from the methine proton at δ 6.14 (H-5′) to the carbons at δ 105.8 (C-1′) and 101.8 (C-3′) revealed the presence of an orsellinic acid moiety. The long-range correlation from the methine proton at δ 5.57 (H-5) to the carbonyl carbon at δ 172.2 (C-8′) connected the orsellinic acid to the azaphilone moiety via ester linkage. Furthermore, the long-range correlations from H-3 to C-11, from H-11 to C-2, C-3, C-12, and C-13, and from H-10 to C-5, C-6, and C-7 unambiguously established the structure of **1**, as shown in Figure 2. The NOESY correlation between H-5 and H-10 revealed the *cis*-configuration of C-5 and C-6. To determine the absolute stereochemistry of C-12 via application of the Mosher's methods, compound **1** (1.0 mg) was treated with (*R*)-(-)-α-methoxy–α-(trifluoromethyl)phenylacetic acid chloride (5 µL) and 4-(dimethylamino)pyridine (1 crystal) in pyridine (300 µL) at room temperature for 24 h. However, MTPA esterification of **1** was not successful. The stereochemistry of C-12 still remains unknown. Accordingly, compound **1** was determined to be a new azaphilone derivative and was named glaziellin A.

The known compounds were identified as entonaemin A (**2**), comazaphilone D (**3**), rubiginosin A (**4**), and entonaemin B (**5**), by comparing spectroscopic data with previously reported literatures [9–12].

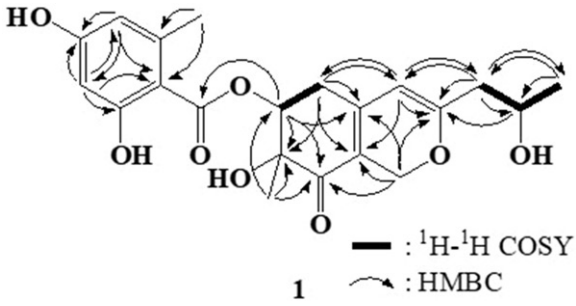

Figure 2. ^{1}H–^{1}H COSY and HMBC correlations of compound **1**.

All compounds (**1**–**5**) were evaluated using neuraminidases from recombinant rvH1N1, H3N2, and H5N1 influenza A viruses, and zanamivir was used as a positive control. Compounds **3** and **4** exhibited considerable NA inhibitory activity against the three influenza A types with IC_{50} values of 30.9, 41.8, and 35.7 µM for **3** and 46.5, 50.4, and 29.9 µM for **4**, respectively. However, compounds **1**, **2**, and **5** showed weak NA inhibitory activity with IC_{50} values of 235.8, 230.6, and 165.4 µM for **1**, 243.8, 260.9, and 233.3 µM for **2**, and 177.4, 185.6, and 164.2 µM for **5**, respectively. The positive control zanamivir exhibited with IC_{50} values of 12.2, 9.2, and 2.9 nM, respectively (Table 2). Interestingly, compounds **3** and **4** exhibited higher activity than compounds **1** and **5**, suggesting that the double bond at C-11 contributed to enhance NA inhibitory activity. However, the hydroxylation of C-13 in compound **2** considerably decreased the NA inhibitory activity. These results imply that the alkyl chain of C-2 plays an important role for NA inhibitory activity. We also investigated the inhibition type of compounds **3** and **4** using enzyme-inhibitor kinetic studies at different concentrations. The kinetic parameters were calculated using SigmaPlot Enzyme Kinetics Module (Systat, San Jose, CA). To study the inhibition type of compounds **3** and **4**, double reciprocal Lineweaver–Burk plots were used. The inhibition type of compounds **3** and **4** was non-competitive (Figure 3). The inhibition constants (K_i) were determined by Dixon plots. The K_i values of compounds **3** and **4** were 35.3 ± 0.6 and 42.0 ± 1 µM, respectively.

In this study, five azaphilone derivatives including one new compound were isolated from the fruiting body of *G. splendens* through silica gel and Sephadex LH-20 column chromatographies, and MPLC. Chemical structures were elucidated by spectroscopic methods, mainly NMR and mass analyses. Compounds **3** and **4** exhibited significant inhibition activity against neuraminidases from recombinant rvH1N1, H3N2, and H5N1 influenza A viruses with IC_{50} values of 30.9, 41.8, and 35.7 µM for **3** and 46.5, 50.4, and 29.9 µM for **4**, respectively. The inhibition type of these compounds was non-competitive. This is the first report on the isolation of

Table 2. Neuraminidase inhibitory activity of compounds **1**–**5**.

Compounds	IC_{50} (µM)[a]			Inhibition type (H3N2, K_i, µM)
	H1N1	H3N2	H5N1	
1	230.6 ± 9.7	235.8 ± 2.8	165.4 ± 3.6	N.T[b]
2	260.9 ± 3.7	243.8 ± 3.8	233.3 ± 5.7	N.T.
3	41.8 ± 0.6	30.9 ± 0.1	35.7 ± 0.2	Non-competitive (35.3)
4	50.4 ± 1.1	46.5 ± 2.5	29.9 ± 0.6	Non-competitive (42.0)
5	185.6 ± 7.5	177.4 ± 1.3	164.2 ± 4.6	N.T.
Zanamivir (nM)	9.2 ± 0.1	12.2 ± 0.2	2.9 ± 0.1	Competitive

[a]Results were obtained from three independent experiments.
[b]N.T.: not tested.

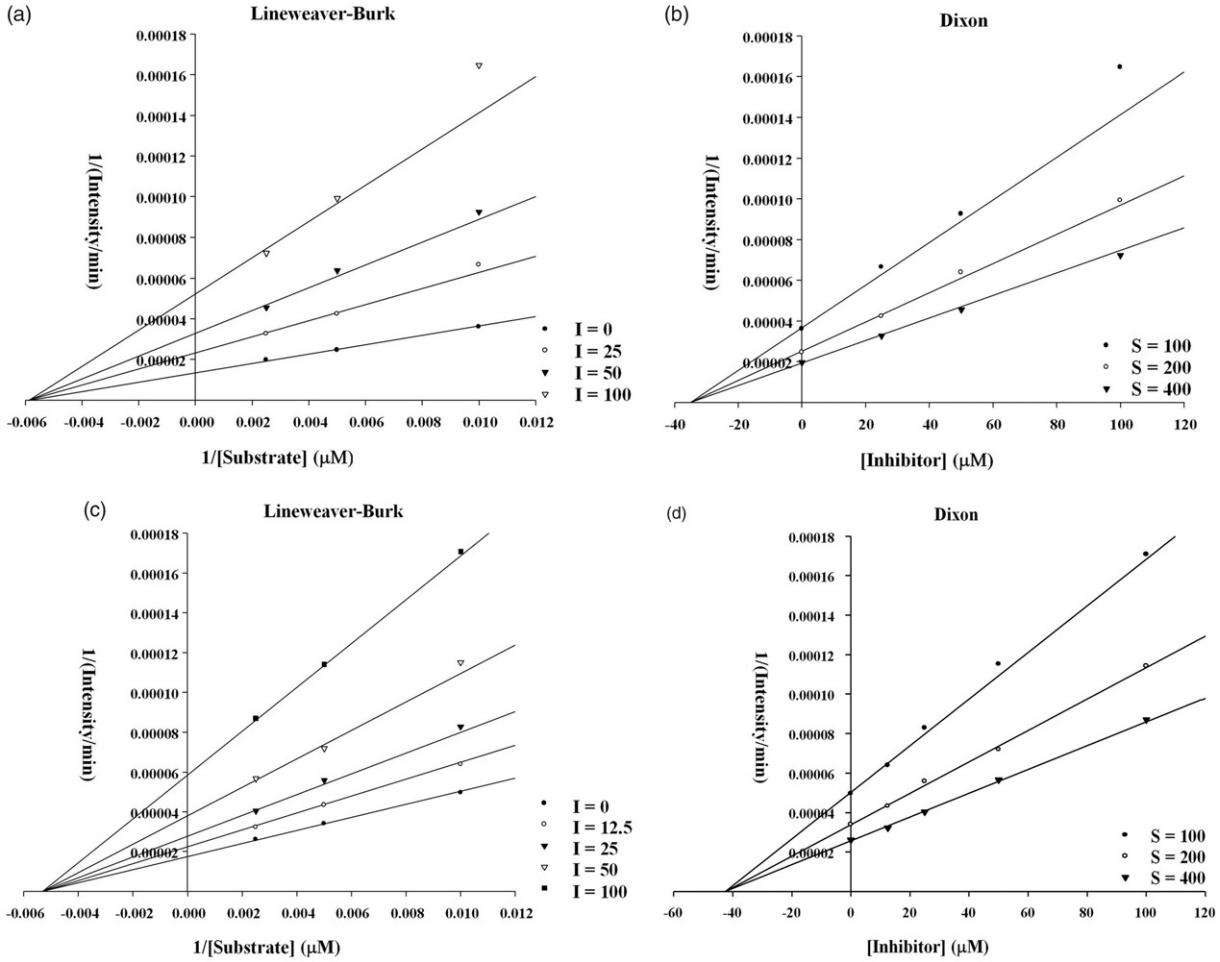

Figure 3. Graphical determination of inhibition type for compounds **3** and **4**. (A and C) Lineweaver–Burk plots for NA inhibition by compounds **3** and **4**; (B and D) Dixon plots for NA inhibition by compounds **3** and **4**.

neuraminidase inhibitors from the fruiting bodies of *G. splendens*.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF-2016R1A2B2014430). Authors thank Ms Ji-Young Oh, Center for University Research Facility (CURF) at Chonbuk National University, for NMR measurement.

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