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Chalcones as novel influenza A (H1N1) neuraminidase inhibitors from *Glycyrrhiza inflata*

Trong Tuan Dao [a], Phi Hung Nguyen [a], Hong Sik Lee [b], Eunhee Kim [b], Junsoo Park [c], Seong Il Lim [d], Won Keun Oh [a,*]

[a] *BK21 Project Team, College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, Republic of Korea*
[b] *Choong Ang Vaccine Laboratory, 59-3 Hwaam-dong, Yuseong-gu, Daejeon 305-348, Republic of Korea*
[c] *Division of Biological Science and Technology, Yonsei University, Wonju 220-100, Republic of Korea*
[d] *Traditional Food Research Group, Korea Food Research Institute, Sungnam 463-746, Republic of Korea*

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ABSTRACT

The emergence of highly pathogenic influenza A virus strains, such as the new H1N1 swine influenza (novel influenza), represents a serious threat to global human health. During our course of an anti-influenza screening program on natural products, one new licochalcone G (**1**) and seven known (**2**–**8**) chalcones were isolated as active principles from the acetone extract of *Glycyrrhiza inflata*. Compounds **3** and **6** without prenyl group showed strong inhibitory effects on various neuraminidases from influenza viral strains, H1N1, H9N2, novel H1N1 (WT), and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells. In addition, the efficacy of oseltamivir with the presence of compound **3** (5 μM) was increased against H274Y neuraminidase. This evidence of synergistic effect makes this inhibitor to have a potential possibility for control of pandemic infection by oseltamivir-resistant influenza virus.

As influenza viruses belong to the *Orthomyxoviridae* family, and type A is clinically the most important in three types of viruses A, B, and C.[1] The genome of influenza A viruses encodes 11 proteins, including two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which 16 HA (H1–H16) and 9 NA (NA1–NA9) subtypes have been identified.[2] NA (also called sialidase) cleaves sialic acid from cell surface and releases progeny virions from infected cells.[3a,b] NA may promote viral movement through respiratory tract mucus, thus enhancing viral infectivity. When the influenza virus is deficient in NA activity, virus progeny aggregates at the surface of the infected cell, severely impairing further spread of viruses to other cells.[3]

Oseltamivir, zanamivir, and peramivir which impair the efficient release of viruses from the infected host cell, were used for the NA inhibitors.[4a] However, there are reported that adults receiving peramivir and oseltamivir (Tamiflu) are the cases of nausea and vomiting.[4b] Furthermore, the high-level's drug resistance is caused by the substitutions of single amino acid in the NA proteins.[4b] For these reasons, researches for new antiviral compounds from natural products are needed for the development of new therapeutic agents in the battle of influenza virus.[4c]

During the course of an anti-influenza screening program on natural products, the acetone extract of *Glycyrrhiza inflata* was found to possess the potential NA inhibitor. The genus *Glycyrrhiza* (Leguminosae) is comprised of approximately 30 species, of which some have been used worldwide since ancient times as a medicine and a sweetening agent (licorice).[5a] Saponins, flavonoids, coumarins, and stilbenoids have been reported as constituents of this genus, which have been found to relate to a wide range of biological activities.[5] In order to identify the compounds with inhibitory activity against influenza NA by bioassay-guided fractionation, the acetone extract of *G. inflata* was subjected to a succession of chromatographic procedures.[6] Herein, we describe the isolation of eight chalcone derivatives (**1**–**8**) as the active principles, including licochalcone G (**1**),[7] licochalcone A (**2**),[8] echinantin (**3**),[8b] 5-prenylbutein (**4**),[9] licochalcone D (**5**),[8b] isoliquiritigenin (**6**),[10] licoagrochalcone A (**7**),[11] and kanzonol C (**8**).[12] The structure of the new compound (**1**) and the known compounds (**2**–**8**) was identified by 1D and 2D NMR analyses and confirmed by a comparison of the physicochemical and spectroscopic data with those published in the literature (Fig. 1).

Compound **1** was obtained as a yellow amorphous powder. Its UV spectrum exhibited absorption maxima at 258 and 380 nm, indicating the presence of aromatic rings. The IR spectrum showed absorption bands at 3420 cm^{-1} for one or more hydroxy groups and 1699 cm^{-1} for conjugated carbonyl functionality. The ^{1}H

* Corresponding author. Tel./fax: +82 62 230 6370.
E-mail address: wkoh@chosun.ac.kr (W.K. Oh).

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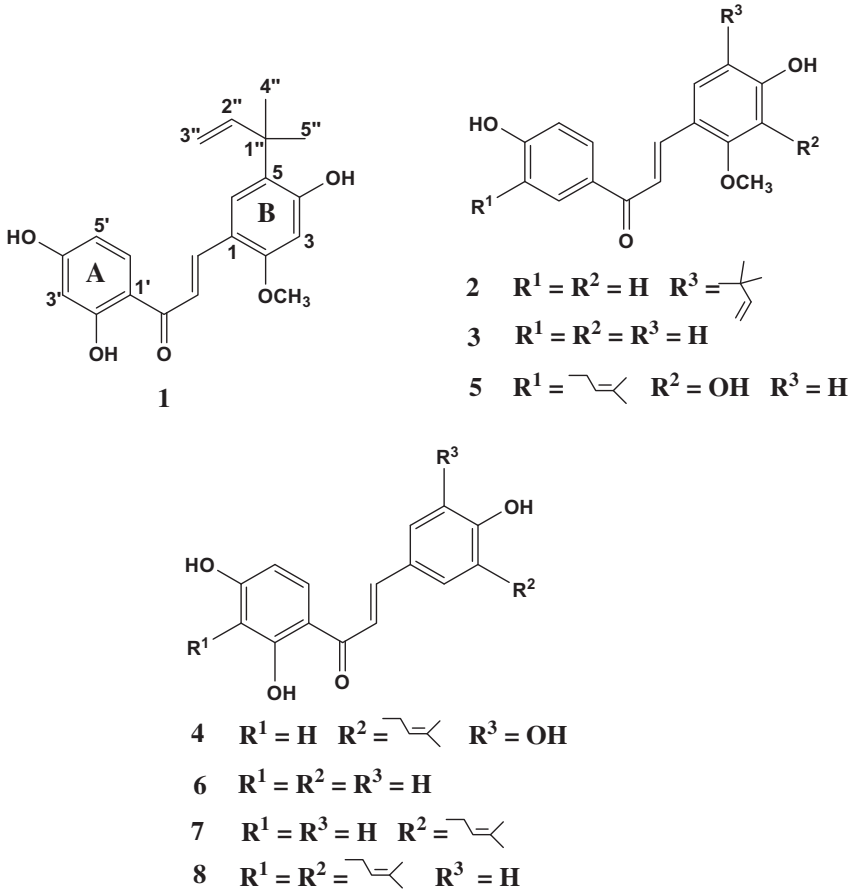

Figure 1. Chemical structure of compounds **1**–**8** isolated from *Glycyrrhiza inflata*.

NMR spectrum (Table 1) showed signals for a methoxy group δ_H 3.87 (3H, s, 2-OCH_3), two *trans*-olefinic protons [δ_H 7.64 (1H, d, J = 15.5 Hz, H-α) and 7.98 (1H, d, J = 15.5 Hz, H-β)], 1,1-dimethylallyl group [δ_H 1.51 (6H, s, 4″-CH_3 and 5″-CH_3), 4.99 (1H, dd, J = 11.0, 1.5 Hz, H-3″), 5.04 (1H, dd, J = 17.0, 1.5 Hz, H-3″) and 6.30 (1H, dd, J = 17.0, 11.0 Hz, H-2″)], two aromatic protons [δ_H 6.58 (1H, s, H-3) and 7.58 (1H, s, H-6)], and ABX-type aromatic protons [δ_H 6.94 (1H, d, J = 2.0 Hz, H-3′), 7.56 (1H, dd, J = 8.0, 2.0 Hz, H-5′) and 7.60 (1H, d, J = 8.0 Hz, H-6′)]. Consistent with the above ^{1}H NMR analysis, the ^{13}C NMR spectrum of this compound displayed signals corresponding to the methoxy group δ_C 56.0 (2-OCH_3), two olefinic carbons [δ_C 119.7 (C-α) and 139.8 (C-β)], 1,1-dimethylallyl group [δ_C 27.6 (C-4″ and C-5″), 110.9 (C-3″), 148.7 (C-2″) and 40.8 (C-1″)], a conjugated ketone δ_C 188.5, and twelve carbons of the two aromatic rings. 1D NMR spectroscopic data of **1** were similar to those of licochalcone A, with an exception of an additional hydroxy group attached to

Table 1
NMR spectroscopic data for compound **1**[a]

Position	δ_H (J in Hz)	δ_C	HMBC (J_{H-C})
α	7.64 d (15.5)	119.7 d	C-β, C=O
β	7.98 d (15.5)	139.8 d	C-α, C-2, C-6, C=O
1		122.7 s	
2		159.8 s	
3	6.58 s	101.1 d	
4		150.5 s	
5		127.6 s	
6	7.58 s	129.4 d	C-1, C-4, C-1″
1′		132.5 s	
2′		159.9 s	
3′	6.94 d (2.0)	115.6 d	C-1′, C-4′
4′		145.8 s	
5′	7.56 dd (8.0, 2.0)	116.1 d	C-4′, C-6′
6′	7.60 d (8.0)	129.4 d	C-5′, C-2′, C=O
1″		40.8 s	
2″	6.30 dd (17.0, 11.0)	148.7 d	C-5, C-1″, C-4″, C-5″
3″	5.04 dd (17.0, 1.5)	110.9 t	C-1″, C-2″
	4.99 dd (11.0, 1.5)		C-1″, C-2″
4″-CH_3	1.51 s	27.6 q	C-5, C-1″, C-2″, C-5″
5″-CH_3	1.51 s	27.6 q	C-5, C-1″, C-2″, C-4″
2-OCH_3	3.87 s	56.0 q	C-2
C=O		188.5 s	

[a] Compound was measured at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in CD_3COCD_3.

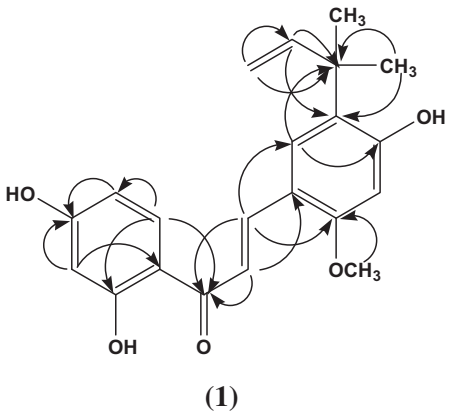

Figure 2. Key HMBC (H→C) correlations for new compound **1**.

Table 2
Inhibitory effects of compounds **1**–**8** on neuraminidase activity

Compound	IC_{50}[a] (μg/mL)				Inhibition type (H1N1, K_i, μg/mL)
	H1N1	H9N2	H1N1 (WT)	H1N1 (H274Y)	
1	37.68 ± 2.17	42.11 ± 2.12	NT[c]	NT[c]	Noncompetitive (41.53 ± 1.30)
2	19.09 ± 1.10	17.98 ± 0.97	5.42 ± 0.40	4.20 ± 0.57	Noncompetitive (18.23 ± 1.14)
3	5.80 ± 0.30	5.70 ± 0.55	2.49 ± 0.14	2.19 ± 0.06	Noncompetitive (8.23 ± 1.32)
4	25.87 ± 2.03	35.50 ± 1.43	NT[c]	NT[c]	Noncompetitive (32.78 ± 2.68)
5	28.62 ± 1.67	35.21 ± 3.10	NT[c]	NT[c]	Noncompetitive (36.52 ± 2.11)
6	8.41 ± 0.39	9.69 ± 0.37	3.48 ± 0.19	3.42 ± 0.12	Noncompetitive (10.56 ± 1.22)
7	51.59 ± 2.77	56.92 ± 2.15	NT[c]	NT[c]	Noncompetitive (60.12 ± 2.15)
8	75.38 ± 2.47	52.96 ± 1.33	NT[c]	NT[c]	Noncompetitive (83.14 ± 2.86)
Oseltamivir[b]	39.74 ± 1.54 (ng/mL)	4.94 ± 0.56 (ng/mL)	21.09 ± 1.19 (ng/mL)	5.13 ± 0.23	NT[c]

[a] All compounds were examined in a set of triplicated experiments.
[b] The compound was used as the positive control.
[c] NT: not tested.

the A ring from the aromatic ABX proton system.[8] This was further supported by the molecular ion peak at *m/z* 354.1465 $[M]^+$ in the HREIMS, which indicated the molecular formula of $C_{21}H_{22}O_5$ for **1**. The position of the methoxy group was inferred as C-2 by the HMBC correlation from methoxy proton (δ_H 3.87) to C-2 (δ_C 159.8), and a unique fragment peak at *m/z* 323 ($[M-31]^+$) in the EIMS.[8b] According to the HMBC correlations between H-6/C-1″; H-2″, H-4″, and H-5″/C-5, the location of 1,1-dimethylallyl group at C-5 was confirmed. Finally, the additional hydroxy group was determined to attach to C-2′ by the observation of the aromatic ABX proton system (δ_H 6.94, 7.56, and 7.60) and their HMBC correlations (Fig. 2). Thus, the new compound **1**, named licochalcone G, was characterized as 2-methoxy-5-(1,1-dimethylallyl)-4,2′, 4′-trihydroxychalcone.

All isolated compounds with the purity of more than 98%, as assessed by HPLC and NMR spectroscopic data, were evaluated for

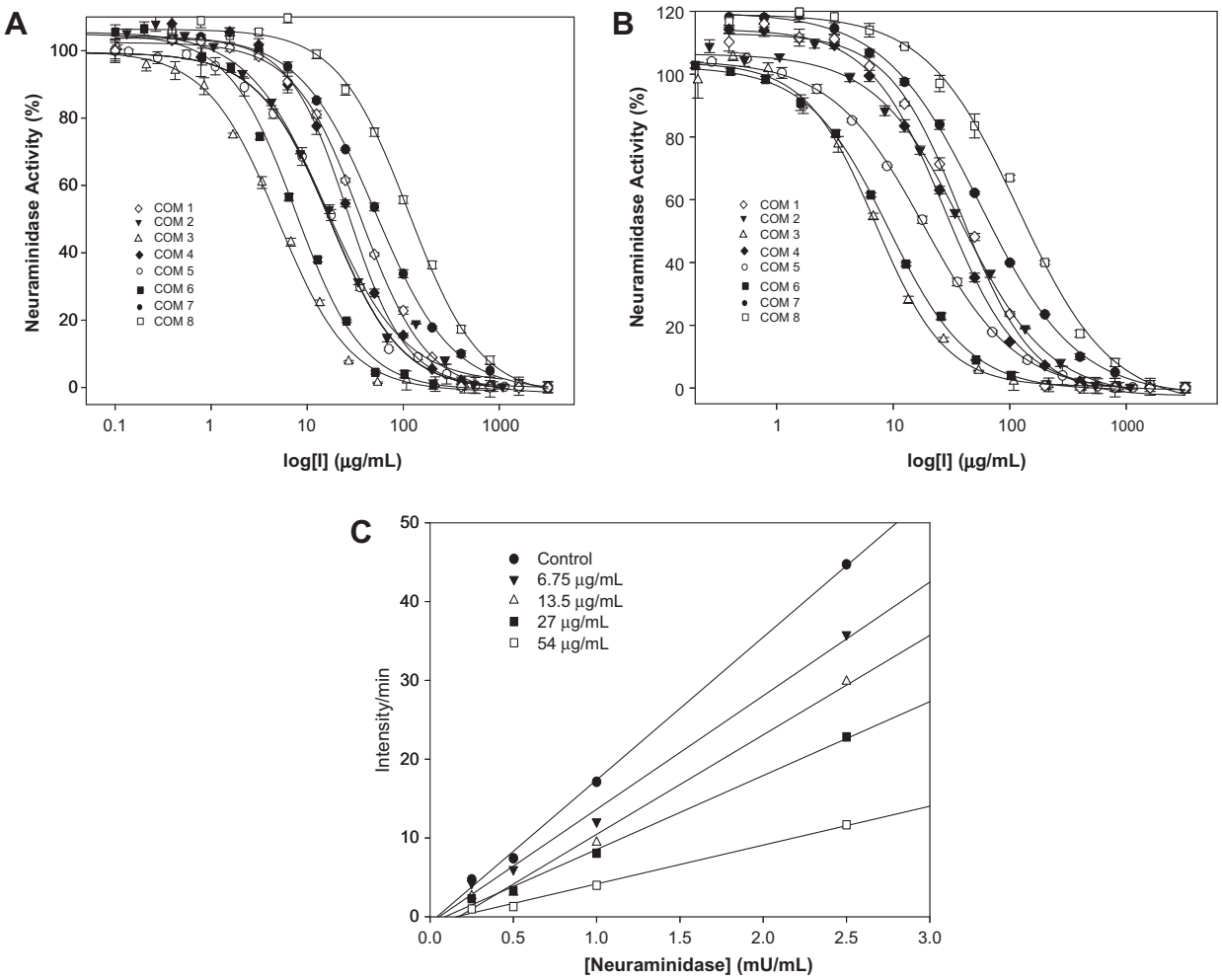

Figure 3. (A and B) Effects of compounds **1**–**8** on the activity of NAs from influenza A (H1N1 and H9N2) for the hydrolysis of 4-MU-NANA at 37 °C. Inhibitor concentrations are displayed on logarithmic scales. (C) Relationship between hydrolytic activities of NA with enzyme concentration at different concentrations of compound **3**. Concentration of compound **3** for curves from top to bottom: 0, 6.75, 13.5, 27.0, and 54.0 μg/mL, respectively.

their inhibitory activity against NAs from influenza viruses. A known NA inhibitor, oseltamivir phosphate (Hoffman-La Roche Ltd, Basel, Switzerland) was used as a positive control. The NA assay was performed as previously reported with a slight modification[13a,b] by following the hydrolysis of 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639) via fluorescence. As shown in Table 2 and Figure 3A–B, the non-prenylated chalcones **3** and **6** (IC_{50} 5.80 ± 0.30 and 8.41 ± 0.39 μg/mL, respectively) exhibited higher activity than the prenylated compounds. Compound **4**, the C-5 hydroxy derivative of **7**, showed a double activity towards the NA (IC_{50} 25.87 ± 2.03 μg/mL). In contrast, the presence of a C-2′ hydroxy group as in compounds **1** and **6** might be responsible for the decrease in activity compared with structurally similar compounds **2** and **3**. The inhibitory effects of these chalcones are also of the same order of magnitude in case of avian influenza virus (H9N2) neuraminidase.

We next examined whether the isolates are effective in inhibiting NAs from the wild-type novel swine flu (WT) virus and the oseltamivir-resistant virus with a H274Y mutation.[14] As the result, the most active compound **3** inhibited the NA derived from the novel H1N1 influenza with an IC_{50} of 2.49 ± 0.14 μg/mL. More interestingly, compound **3** remained equally potent in inhibiting the activity of the H274Y mutant form with an IC_{50} of 2.19 ± 0.06 μg/mL. While oseltamivir as the positive control displayed an excellent inhibition (IC_{50} 21.09 ± 1.19 ng/mL) on NA of novel H1N1, its inhibitory activity on oseltamivir-resistant novel influenza (H274Y) decreased dramatically (more than 240 times to the IC_{50} of 5.13 ± 0.23 μg/mL) (Table 2).

The inhibition pattern by the compounds was determined to evaluate the relative affinity for NA from H1N1 influenza virus. As shown in Figure 3C, the inhibition of the tested compounds was reversible because increasing the inhibitor concentration rapidly decreased enzyme activity [line gradient decreased (for compound **3** representatively)].[15a] To study further about the mode of inhibition, we used both the double reciprocal Lineweaver–Burk and Dixon plots (Supplementary data in Fig. S5). All compounds displayed as noncompetitive inhibitors because increasing concentrations of substrate resulted in a family of lines, which intersected at a non-zero point on the *x* axis ($-K_i$) (Supplementary data in Fig. S5). A summary of the K_i values for compounds **1**–**8** showed that these results agreed with those of IC_{50} (Table 2).

The noncompetitive mechanism of the compounds on NA prompted an investigation of the inhibitory effect of the combination of oseltamivir, a known competitive inhibitor, together with compound **3**. The inhibitory activity of oseltamivir in the presence of **3** (at 1.35 μg/mL or 5 μM) was enhanced notablely on NAs of H9N2 (3.6-fold), H1N1 (7.0-fold), novel flu (WT) (3.7-fold), and tamiflu-resistant novel flu (H274Y) (52.6-fold) with IC_{50} values from 4.94, 39.74, 21.09, and 5132.85 ng/mL to 1.39, 5.69, 1.96, and 97.67 ng/mL, respectively (Fig. 4). Therefore, it is evident that oseltamivir and compound **3** might act through different inhibitory mechanisms and synergistically inhibit the NA activity by binding to different acting sites of both the free enzyme and product-bound enzyme. A therapy with synergistically active anti-influenza A drugs has targeted different viral proteins such as the co-treatment of one NA inhibitor (oseltamivir and zanamivir) with another adamantanes (amantadine and rimantadine).[16] Here, we showed the

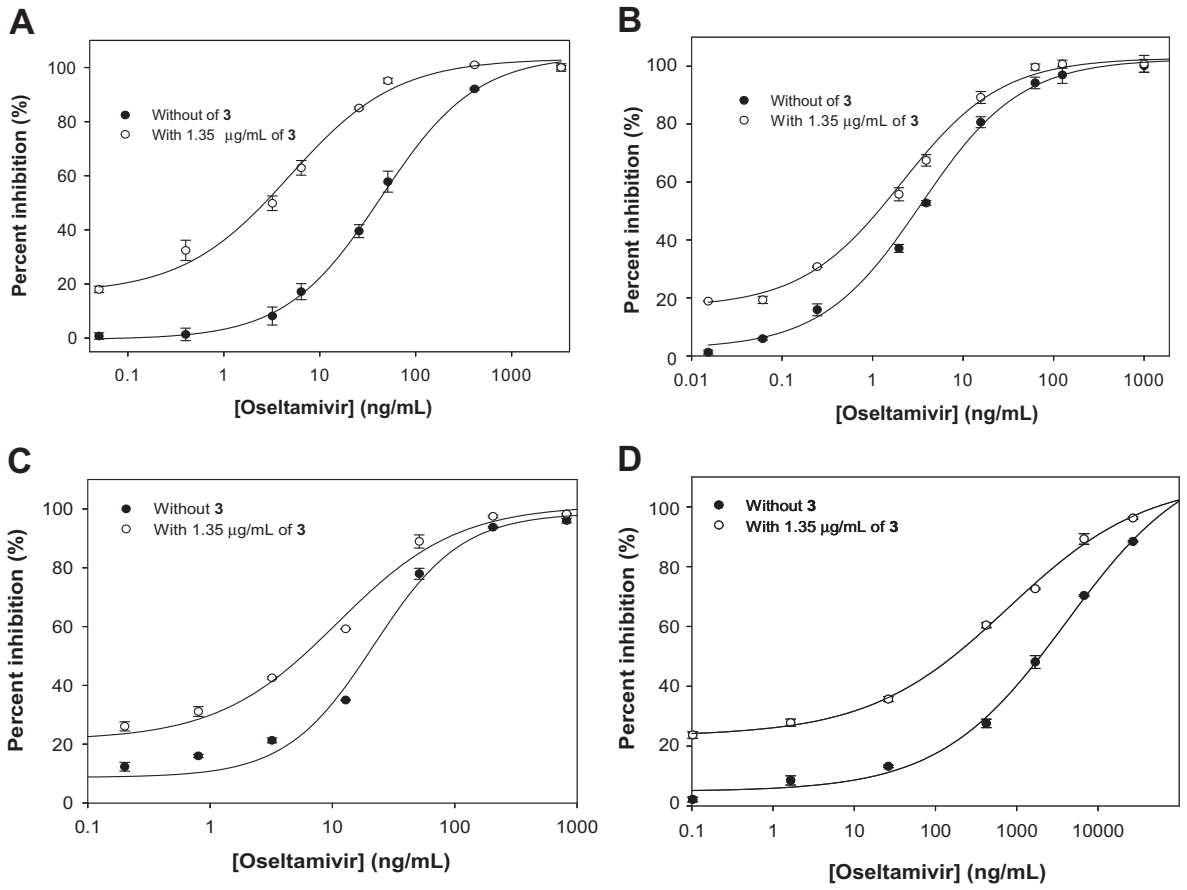

Figure 4. (A–D) Inhibition of NAs from influenza A [H1N1, H9N2, novel H1N1, and oseltamivir-resistant novel H1N1 (H274Y)] by oseltamivir in the presence or absence of compound **3**.

synergistic effect of the combination of two NA inhibitors with different mechanisms of action.

So far, some studies have reported NA inhibitory activity of flavonoids on bacteria,[15] and the prenylated flavonoids on the A and B ring were believed to increase inhibitory activities[15a] (see the data displaying effects of compounds **1**–**8** on the activity of NA from *Clostridium perfringens* in Supplementary data in Table S1). However, this result is reversed on NA of influenza A viruses, in which prenylated chalcone derivatives showed a weak activity on influenza (H1N1 and H9N2) NAs, in comparison to the non-prenylated compounds. This finding may provide some important information relating the chemical structure of the compounds to their NA inhibitory activity, and facilitate the design of chemical compounds with higher potency effects against influenza NA. In conclusion, although structure–activity relationships of these compounds were not thoroughly investigated, this study has suggested that chalcones from *G. inflata* can serve as neuraminidase inhibitors of influenza A. Synthesis of these naturally occurring compounds and their analogs may provide as a lead for the development of new drugs to combat this serious disease.

Acknowledgments

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Supplementary data

Supplementary data (1D (^{1}H and ^{13}C) and 2D (HSQC and HMBC) NMR spectra of new compound **1**, kinetic studies of isolates (**1**–**8**), and assay methods of influenza A viruses) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.016.

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6. Bioassay-guided isolation of the isolated compounds **1**–**8**: The dried root of *Glycyrrhiza inflata* was purchased from Kangwon Herbal Medicne Company (Kangwon-do, Republic of Korea), in November 2008 and was botanically identified by Professor YH Moon. A voucher specimen (CU2008–11) has been deposited at the Herbarium of Chosun University, Gwangju, Republic of Korea. The dried root of *G. inflata* (1 kg) was extracted with acetone (4.0 L × 2 times) at room temperature for a week. The combined acetone extract was then concentrated to yield a dry residue (65.0 g). The crude extract was suspended in water (2.0 L) and partitioned with chloroform (3 × 1.5 L), ethyl acetate (3 × 1.5 L), and *n*-butanol (3 × 1.5 L), successively. The chloroform fraction (29.0 g), which showed strong influenza NA inhibitory activity, was chromatographed over a silica gel column (10 × 30 cm; 63–200 μm particle size) eluting with gradient solvent *n*-hexane/EtOAc (6:1, 5:2···1:6, each 2.5 L), to yield six fractions (F1: 7.6 g; F2: 2.5 g; F3: 3.5 g; F4: 3.6 g; F5: 5.55 g; F6: 4.6 g) based on TLC profile. The active fractions, F2 and F3, were subjected to additional chromatography. Fraction F2 was chromatographed over a Sephadex LH-20 column (7 × 30 cm) using MeOH as eluting solvent to yield licochalcone A (**2**) (800.0 mg). Fraction F3 was applied to a RP-18 column (7 × 30 cm; 40–63 μm particle size) eluting with a stepwise gradient of MeOH/H_2O (50:50 to 100:0) to afford eight subfractions (F3.1–F3.8). Further separation of F3.2 (120.0 mg) by HPLC [using an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% formic acid (0–55 min: 62% MeOH, 55–60 min: 100% MeOH, 60–70 min: 100% MeOH); flow rate 2 mL/min; UV detection at 254 and 320 nm] resulted in the isolation of echinantin (**3**) (t_R = 25.0 min, 20.0 mg) and 5-prenylbutein (**4**) (t_R = 50.0 min, 3.5 mg). From subfraction F3.3 (150.0 mg), licochalcone D (**5**) (t_R = 42.0 min, 17.0 mg) and the new compound (**1**) (t_R = 50.0 min, 5.0 mg) were afforded by HPLC (0–55 min: 65% MeOH, 60 min: 100% MeOH). Subfraction F3.4 (130.0 mg) was separated by HPLC (0–50 min: 72% MeOH, 55 min: 100% MeOH) leading to the isolation of isoliquiritigenin (**6**) (t_R = 41.0 min, 18.0 mg) and licoagrochalcone A (**7**) (t_R = 47.0 min, 4.0 mg). Purification of fraction F3.6 (100.0 mg) on HPLC (0–65 min: 86% MeOH, 70 min: 100% MeOH) yielded kanzonol C (**8**) (t_R = 60.0 min, 8.0 mg).
7. Physical and spectroscopic data of licochalcone G (**1**): Yellowish amorphous powder; mp 122–124 °C; UV (MeOH) λ_{max} nm (log ε) 258 (3.76), 380 (4.09); IR (KBr) ν_{max} 3420 (OH), 2965, 1699 (C=O), 1557, 1445, 1285, 1166 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* (rel. int.): 354 ([M]$^+$, 27), 349 (10), 324 (23), 323 ([M−OMe]$^+$, 100), 137 (42); HREIMS *m*/*z* 354.1465 [M]$^+$ (calcd for $C_{21}H_{22}O_5$, 354.1467).
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