C-Methylated Flavonoids from *Cleistocalyx operculatus* and Their Inhibitory Effects on Novel Influenza A (H1N1) Neuraminidase

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As part of an ongoing study focused on the discovery of anti-influenza agents from plants, four new (**1**−**4**) and 10 known (**5**−**14**) C-methylated flavonoids were isolated from a methanol extract of *Cleistocalyx operculatus* buds using an influenza H1N1 neuraminidase inhibition assay. Compounds **4**, **7**, **8**, and **14**, with a chalcone skeleton, showed significant inhibitory effects on the viral neuraminidases from two influenza viral strains, H1N1 and H9N2. Compound **4** showed the strongest inhibitory activity against the neuraminidases from novel influenza H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y mutant) expressed in 293T cells with IC_{50} values of 8.15 ± 1.05 and 3.31 ± 1.34 μM, respectively. Compounds **4**, **7**, **8**, and **14** behaved as noncompetitive inhibitors in the kinetic studies. These results indicate that C-methylated flavonoids from *C. operculatus* have the potential to be developed as neuraminidase inhibitors for novel influenza H1N1.

Cleistocalyx operculatus (Roxb.) Merr and Perry (Myrtaceae) is a medium-sized tree (6−12 m in height) that is distributed widely in tropical Asia. Its leaves have been used in traditional medicine to treat gastric ailments, while the buds, commonly called "Nu Voi", have been used since ancient times to produce various beverages and as an antiseptic agent and a tonic drink for fever patients in Vietnam and Southern China.[1] *C. operculatus* extract exhibits strong protective effects on lipid peroxidation in rat liver microsomes[2] and is considered a promising material for preventing and treating diabetes.[3] Previous phytochemical studies led to the characterization of some triterpenoids, flavonoids, and essential oils from the buds, which have antioxidant and anti-inflammatory activities.[4−7] Recently, the ethanol extract of the buds of this plant exhibited inhibitory activity against a wide range of bacterial strains.[8] However, there are no reports of *C. operculatus* exhibiting influenza neuraminidase inhibitory activity.

Currently, neuraminidase (NA) inhibitors are the mainstay of pharmacological protocols to fight global influenza pandemics.[9] NA, also known as sialidase, is a surface glycoprotein of the influenza A virus, which plays a key role in the release of virions from the infected host cells and in their movement through the upper respiratory tract.[10,11] When influenza viruses show deficient NA activity, particles of progeny viruses aggregate at the surface of an infected cell, which severely impairs their further spread to other cells.[10−12] To date, commercially available anti-influenza drugs include the NA inhibitors oseltamivir, zanamivir, and peramivir, which impair the efficient release of viruses from infected host cells, and amantadine and rimantadine, which specifically inhibit viral proliferation by blocking the M_2 ion channel.[13,14] While antiviral chemotherapy with M_2 inhibitors reduces the duration of symptoms of clinical influenza, there are many side effects.[14] Zanamivir (Relenza) exhibits excellent antiviral activity, but its bioavailability is low by rapid elimination through renal excretion. Adults receiving peramivir and oseltamivir (Tamiflu) have reported nausea and vomiting.[15] Furthermore, the high occurrence of drug resistance is associated with both inhibitors. The situation suddenly changed for the worse in 2009, and an oseltamivir-resistant H1N1 virus, which was conferred by a change in a single amino acid in the viral neuraminidase (H274Y), arose spontaneously and spread globally.[16] For these reasons, more research into new antiviral compounds from natural products is needed to develop new therapeutic agents in the battle against the influenza virus.[17]

During the course of an anti-influenza screening program of natural products, the methanol extract of *C. operculatus* was found to exhibit potential NA inhibitory activity. This prompted us to phytochemically examine the molecular constituents responsible for the NA inhibitory activity using in vitro assays for bioactivity-guided fractionation. This paper reports the isolation, structural elucidation, and antiviral activity of these compounds on NAs from two influenza viral strains, H1N1 and H9N2, as well as from both novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells.

Results and Discussion

A succession of chromatographic procedures (silica gel, Sephadex LH-20, RP-18, and HPLC) of the methanol extract of *C. operculatus* afforded 14 compounds, including four new (**1**−**4**) and 10 known (**5**−**14**) C-methylated flavonoids.

Compound **1** was obtained as a yellow, amorphous powder. A molecular formula of $C_{18}H_{16}O_4$ was determined from the molecular ion peak at *m*/*z* 296.1047 (calcd for $C_{18}H_{16}O_4$, 296.1049), in the HREIMS spectrum. The IR spectrum revealed the presence of hydroxy and conjugated carbonyl groups at 3386 and 1637 cm^{-1}, respectively. The singlet resonance at δ_H 7.88 and five aromatic protons at δ_H 7.34 and 7.51 in the ^1H NMR spectrum of compound **1** suggest it to be an isoflavone with an unsubstituted B ring.[5,18] In addition, the ^1H NMR spectrum (Table 1) indicated the presence of a methoxy group [δ_H 3.81 (3H, s, 5-OMe)] and two methyl groups [δ_H 2.23 (3H, s, 6-Me) and 2.28 (3H, s, 8-Me)]. The position of the methyl groups at C-6 and C-8 on the A ring was established through HMBC correlations between 6-Me/C-5, C-6, and C-7 and 8-Me/C-7, C-8, and C-9. The attachment of the methoxy group to C-5 on the A ring was also determined by an HMBC correlation between 5-OMe (δ_H 3.81) and C-5 (δ_C 156.3) (Figure 1).[19] Therefore, the structure of compound **1** was determined as 7-hydroxy-5-methoxy-6,8-dimethylisoflavone.

Compound **2** was isolated as a brown, amorphous powder with absorption bands at 3423 (OH) and 1639 cm^{-1} (CO) in its IR

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Chart 1

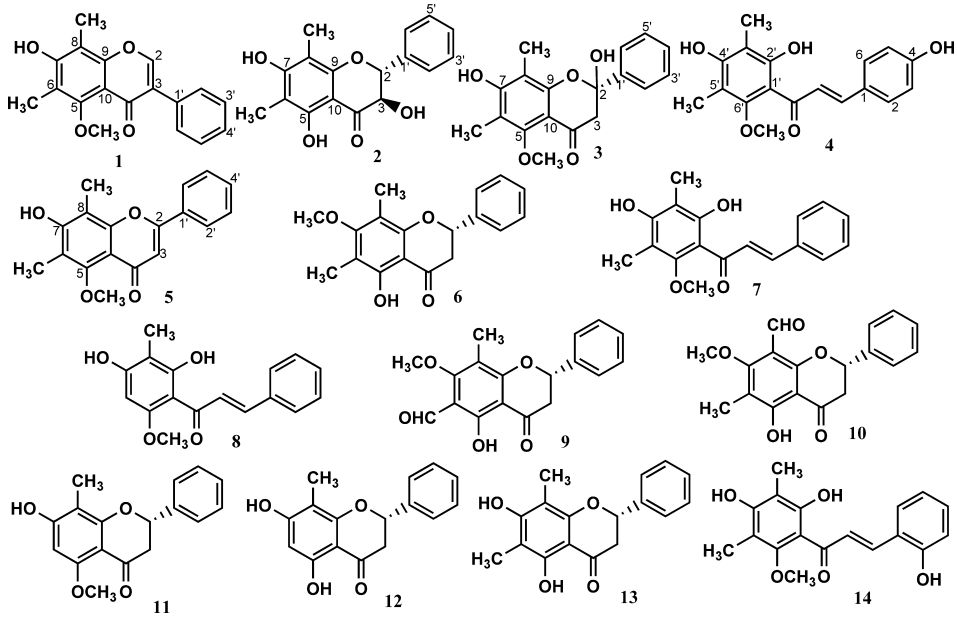

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for Compounds **1**−**5**

	1[a]		**2**[b]		**3**[a]		**4**[c]		**5**[a]	
position	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C
1								128.1		
2	7.88 s	151.0	5.04 d (11.0)	85.0		103.8	7.65 d (8.5)	131.4		160.9
3		125.7	4.51 d (11.0)	74.1	3.16 d (13.5)	42.0	6.94 d (8.5)	116.9	6.71 s	108.2
					3.24 d (13.5)					
4								160.7		
5		156.3		158.9		155.2	6.94 d (8.5)	116.9		155.8
6		115.5		104.5		109.3	7.65 d (8.5)	131.4		115.3
7		156.7		164.7		162.3				156.9
8		106.9		105.5		101.6				107.2
9		154.9		160.3		167.9				154.9
10		113.1		101.8		104.8				112.3
α							7.92 d (15.5)	124.4		
β							7.82 d (15.5)	144.3		
1′		132.2		139.1		132.9		109.2		131.9
2′	7.51 d (8.4)	129.2	7.56 d (8.0)	129.0	7.29 d (8.0)	130.6		162.7	7.90 m	126.0
3′	7.34 m	128.4	7.41 m	129.6	7.25 m	128.3		107.9	7.52 m	129.0
4′	7.34 m	127.9	7.41 m	129.9	7.23 m	127.4		161.4	7.52 m	131.2
5′	7.34 m	128.4	7.41 m	129.6	7.25 m	128.3		110.6	7.52 m	129.0
6′	7.51 d (8.4)	129.2	7.56 d (8.0)	129.0	7.29 d (8.0)	130.6		159.8	7.90 m	126.0
6, 3′-Me	2.23 s	8.2	1.97 s	7.6	2.03 s	7.2	2.09 s	8.3	2.26 s	8.3
8, 5′-Me	2.28 s	8.1	2.02 s	8.1	2.09 s	7.9	2.15 s	9.0	2.44 s	8.5
2′-OH							13.96 s			
5, 6′-OMe	3.81 s	61.7			3.97 s	61.7	3.69 s	62.6	3.87 s	61.8
CO		175.4		198.9		193.7		193.8		177.8

[a] Recorded in $CDCl_3$. [b] Recorded in methanol-d_4. [c] Recorded in acetone-d_6.

spectrum. A molecular formula of $C_{17}H_{16}O_5$ was determined from the molecular ion peak at m/z 300.0999 $[M]^+$ (calcd for $C_{17}H_{16}O_5$, 300.0998), in the HREIMS. The ^{1}H NMR spectrum contained signals for two AB protons at δ_H 4.51 (1H, d, J = 11.0 Hz) and 5.04 (1H, d, J = 11.0 Hz), which are characteristic of the diaxial-oriented H-2 and H-3 of a 2,3-*trans*-dihydroflavonol,[20] and δ_H 7.41 (3H, m) and 7.56 (2H, m), which are indicative of an unsubstituted B ring of the flavonoid,[5] as well as the signals for two methyl groups [δ_H 1.97 (3H, s, 6-Me) and 2.02 (3H, s, 8-Me)]. Consistent with the above ^{1}H NMR analysis, the ^{13}C NMR spectrum of this compound showed signals for all 17 carbons. Fifteen signals were assigned to the flavonoid skeleton. Among them, three signals belonged to aromatic carbons bearing an oxygen atom [δ_C 158.9 (C-5), 160.3 (C-9), and 164.7 (C-7)]. A comparison of this data with those of demethoxymatteucinol[21] indicated compound **2** to be a dihydroflavonol with two hydroxy and two methyl substituents

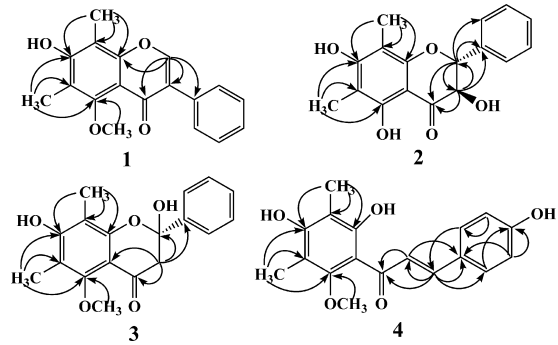

Figure 1. Key HMBC (H → C) correlations for new compounds **1**−**4**.

Table 2. Inhibitory Effects of Compounds **1**−**14** on Neuraminidase Activity

compound	H1N1 $IC_{50}(\mu M)^a$	H9N2 $IC_{50}(\mu M)^a$	H1N1 (WT) $IC_{50}(\mu M)^a$	H1N1 (H274Y) IC_{50} $(\mu M)^a$	H1N1 (H274Y) fold increase vs WT
1	430.51 ± 8.18	391.01 ± 8.85	NT[c]	NT[c]	
2	374.67 ± 7.50	367.87 ± 6.57	NT[c]	NT[c]	
3	282.58 ± 6.59	284.78 ± 8.12	NT[c]	NT[c]	
4	20.45 ± 1.37	18.57 ± 1.37	8.15 ± 1.05	3.31 ± 1.34	2.46
5	414.32 ± 9.36	362.87 ± 7.26	NT[c]	NT[c]	
6	>500	>500	NT[c]	NT[c]	
7	32.48 ± 2.32	21.74 ± 2.35	35.23 ± 2.75	5.03 ± 0.87	7.00
8	85.74 ± 7.25	66.48 ± 8.20	93.77 ± 5.35	26.02 ± 3.03	3.60
9	268.59 ± 9.52	256.25 ± 4.90	NT[c]	NT[c]	
10	289.94 ± 10.10	298.91 ± 6.86	NT[c]	NT[c]	
11	>500	>500	NT[c]	NT[c]	
12	>500	>500	NT[c]	NT[c]	
13	333.59 ± 9.26	319.12 ± 8.56	NT[c]	NT[c]	
14	28.12 ± 3.57	23.12 ± 1.94	10 ± 2.17	2.55 ± 0.29	4.23
oseltamivir[b]	95.22 ± 2.51 (nM)	10.34 ± 2.00 (nM)	51.54 ± 3.32 (nM)	12.29 ± 1.00	

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

on the A ring. The positions of the C-6 and C-8 methyl groups were further confirmed by HMBC correlations between 6-Me/C-5, C-6, and C-7 and 8-Me/C-7, C-8, and C-9 (Figure 1). The absolute configurations at C-2 and C-3 were then determined as 2(*S*) and 3(*S*) by a negative Cotton effect at 304 nm and a positive Cotton effect at 295 nm in the CD spectrum.[22] On the basis of the above data, compound **2** was identified as (2*S*,3*S*)-2,3-*trans*-5,7-dihydroxy-6,8-dimethyldihydroflavonol.

Compound **3** was obtained as a brown, amorphous powder. A molecular formula of $C_{18}H_{18}O_5$ was identified from the molecular ion peak at *m*/*z* 314.1152 $[M]^+$ (calcd for $C_{18}H_{18}O_5$, 314.1154). The ^1H NMR spectrum showed two AB protons [δ_H 3.16 (1H, d, J = 15.0 Hz, H-3ax) and 3.24 (1H, d, J = 15.0 Hz, H-3eq)], signals for a methoxy group [δ_H 3.97 (3H, s, 5-OMe)], and two methyl groups [δ_H 2.03 (3H, s, 6-Me) and 2.09 (3H, s, 8-Me)]. The position of the hydroxy group at C-2 was supported by the presence of two AB protons (H-3) and a hemiacetal-type sp^3 carbon signal at δ_C 103.8 (C-2) in the ^{13}C NMR spectrum.[23] The substitution pattern on the A ring was determined to be 7-hydroxy-5-methoxy-6,8-dimethyl from the $^1H-^{13}C$ HSQC and HMBC correlations between 6-Me/C-5, C-6, and C-7; 8-Me/C-7, C-8, and C-9; and 5-OMe/C-5 (Figure 1). The CD spectrum showed a negative Cotton effect at 305 nm and a positive Cotton effect at 294 nm, which is consistent with the *S* configuration at C-2.[22] Therefore, the structure of compound **3** was determined to be (2*S*)-2,7-dihydroxy-5-methoxy-6,8-dimethylflavanone.

Compound **4** was obtained as a yellow, amorphous powder. The IR spectrum revealed absorption bands at 3385 cm^{-1} for one or more hydroxy groups and 1605 cm^{-1} for a conjugated carbonyl group, respectively. The ^1H NMR spectrum of compound **4** showed signals for a methoxy group δ_H 3.69 (3H, s, 6′-OMe), two *trans*-olefinic protons [δ_H 7.82 (1H, d, J = 15.5 Hz, H-β) and 7.92 (1H, d, J = 15.5 Hz, H-α)], two methyl groups [δ_H 2.09 (3H, s, 3′-Me) and 2.15 (3H, s, 5′-Me)], and an A_2B_2 system of aromatic protons [δ_H 6.94 (2H, d, J = 8.5 Hz, H-3, 5) and 7.65 (2H, d, J = 8.5 Hz, H-2, 6)]. The ^{13}C NMR spectrum displayed signals corresponding to the methoxy group at δ_C 62.6 (6′-OMe), two olefinic carbons [δ_C 124.4 (C-α), 144.3 (C-β)], two methyl groups [δ_C 8.3 (3′-Me) and 9.0 (5′-Me)], a conjugated ketone at δ_C 193.8, and 12 carbons of the two aromatic rings. The 1D NMR data were similar to those of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone except for an additional hydroxy group on the A ring.[24] This was further supported by the molecular ion peak at *m*/*z* 314.1156 $[M]^+$ in the HREIMS, which indicated a molecular formula of $C_{18}H_{18}O_5$. The position of the two methyl groups at C-3′ and C-5′ was established through HMBC correlations between 3′-Me/C-2′, C-3′ and C-4′ and between 5′-Me/C-4′, C-5′, and C-6′. The HMBC correlation between the methoxy singlet at δ_H 3.69 and the C-6′ peak at δ_C 159.8, which was in turn correlated with the 5′-methyl protons at δ_H 2.15, indicated the methoxy group to be located on the B ring. Finally, an additional hydroxy group was found to be attached to C-4 according to the observation of an aromatic A_2B_2 proton system (δ_H 6.94 and 7.65) and their HMBC correlations (Figure 1). Therefore, compound **4** was determined to be (*E*)-4,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone.

Compound **5** was determined to be 7-hydroxy-5-methoxy-6,8-dimethylflavone. This compound has been reported as a semisynthetic product, but only limited spectroscopic data are available (Table 1).[25] The structures of the known compounds **6**, **7**, and **8**−**14** were identified as (2*S*)-5-hydroxy-7-methoxy-6,8-dimethylflavanone (**6**),[26] 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**7**),[24] 2′,4′-dihydroxy-3′-methyl-6′-methoxychalcone (**8**),[27] (2*S*)-6-formyl-8-methyl-7-*O*-methylpinocembrin (**9**),[28] (2*S*)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (**10**),[5] (2*S*)-7-hydroxy-5-methoxy-8-methylflavanone (**11**),[29] (2*S*)-8-methylpinocembrin (**12**),[30] (2*S*)-5,7-dihydroxy-6,8-dimethylflavanone (**13**),[27] and 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**14**)[24] by a comparison of their physicochemical and spectroscopic data with reported data. This is the first report of the seven known compounds (**5**, **6**, **8**, **9**, **11**, **12**, and **14**) being isolated from *C. operculatus*.

All isolates were examined for their inhibitory activity against swine influenza virus (H1N1) NA using oseltamivir phosphate (Hoffman-La Roche Ltd., Basel, Switzerland) as a positive control.[31] All compounds, with the exception of compounds **6**, **11**, and **12**, inhibited NA in a dose-dependent manner. Chalcones **4**, **7**, **8**, and **14** exhibited stronger activity than flavanones, dihydroflavonols, and isoflavones (Table 2). Furthermore, compound **4** (IC_{50} 20.45 ± 1.37 μM), a C-4 hydroxy derivative, exhibited greater activity against NA than chalcones **7** and **14**. Similarly, the presence of a methyl group at C-5′ (or C-6) might increase the NA inhibitory properties, as observed in compounds **7** and **13**, compared to structurally similar compounds **8** and **12**. Interestingly, the inhibitory effects of these isolated compounds are also on the same order of magnitude as in the case of avian influenza virus (H9N2) neuraminidase (Table 2 and Figure 2).

In the next step, we evaluated the inhibitory effect of the isolates against NAs from the wild-type novel swine flu (WT) virus and oseltamivir-resistant virus with a H274Y mutation.[32] The most active compound, **4**, inhibited the NA derived from the wild-type with an IC_{50} value of 8.15 ± 1.05 μM. Interestingly, the tested compounds exhibited stronger activity against the H274Y mutant form than the novel H1N1 form (from 2.46- to 7.00-fold increases in IC_{50} values). While oseltamivir, as the positive control, showed excellent inhibition (IC_{50} 51.54 ± 3.32 nM) of the NA from the wild-type virus, its inhibitory activity on oseltamivir-resistant novel influenza (H274Y) decreased dramatically to an IC_{50} value of 12.29 ± 1.00 μM (Table 2). This strongly suggests that novel H1N1 influenza NA and its oseltamivir-resistant NA (H274Y) expressed

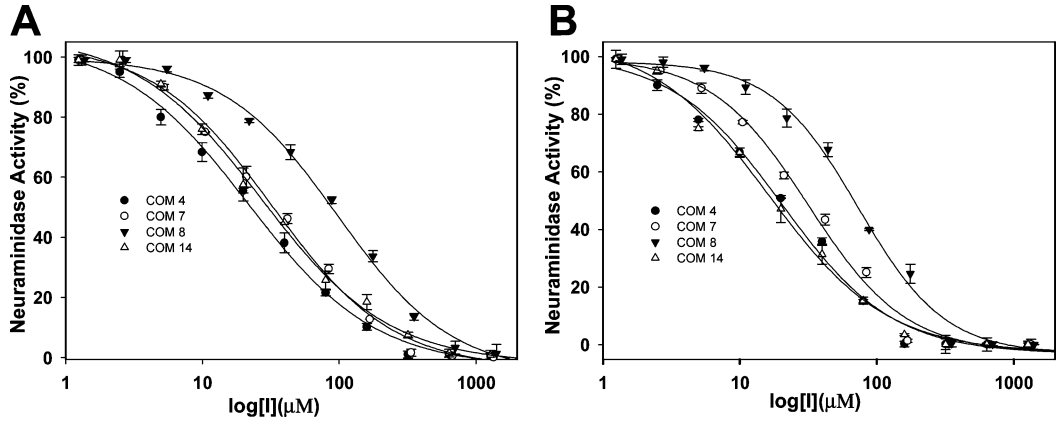

Figure 2. Effects of compounds (**4**, **7**, **8**, and **14**) on the activity of the NAs from influenza viruses H1N1 (A) and H9N2 (B) for the hydrolysis of 4-MU-NANA at 37 °C. The inhibitor concentrations are displayed on a logarithmic scale. The IC_{50} was identified from the midpoint (neuraminidase activity = 50%) of the semilog plot. The data are expressed as the mean of three to five independent experiments.

in 293T cells were inhibited by the oseltamivir treatment as a positive control. Interestingly, although the inhibitory activities of the chalcones on the NAs were weaker than oseltamivir, the inhibitory effect against the virus-resistant NAs did not change in both NAs from novel H1N1 influenza NA and its oseltamivir-resistant NA (H274Y).

The double-reciprocal Lineweaver−Burk and Dixon plots were used to examine the mode of inhibition (Figure 3). All compounds were noncompetitive inhibitors because increasing substrate concentrations resulted in a family of lines that intersected at a nonzero point on the x axis ($-K_i$) (Figure 3A−D). A summary of the K_i values for tested compounds agreed with the IC_{50} values (Table 2 and Figure 3E−H).

During replication, many viruses destroy not only the host cells that they infect but also the neighboring uninfected cells through a cytopathic effect (CPE). The antiviral activity of potential compounds against these viruses can be determined by evaluating the inhibition of this virus-induced cell death. The inhibitory effects of compounds **4**, **7**, **8**, and **14** on replication of influenza A/PR/8/34 (H1N1) virus were determined by CPE assays in MDCK cells. When the inhibitor-free cell cultures were destroyed completely by the virus, the mean percentage of cell viability in each set of three infected wells was quantified by measuring the uptake of neutral red dye. The concentrations of compounds reducing viral CPE by 50% (EC_{50} values) were calculated. A separate assay was performed to determine the cytotoxicity of these compounds, and the 50% cell inhibitory concentrations (CC_{50} values) were then calculated. A selectivity index (SI value, which is equal to EC_{50} divided by CC_{50}) was determined. As shown in Table 3, the tested compounds exhibited dose-dependent anti-influenza virus activity, with SI values ranging from 10.15 ± 0.54 to >24.49. Compound **4**, which was the most effective with EC_{50} values of 4.90 ± 0.35 μM and CC_{50} > 120 μM, showed a high SI value of >24.49. This suggests that compound **4** may be a potent antiviral agent against the influenza virus with a strong protective effect and low toxicity to the host MDCK cells.

The immunofluorescence assay (IFA) is used widely as a sensitive method for diagnosing influenza virus infections in cell cultures. The intracellular green color observed by fluorescence microscopy shows the presence of the virus. As mentioned above, compound **4** is an active compound with the strongest effect on the NAs from four sources of influenza virus (Table 2) and also the highest SI value in the CPE assay. Therefore, compound **4** was used to evaluate the antiviral activity in MDCK cells using an immunofluorescence assay. As shown in Figure S5, treatment of the cells with compound **4** (15.92 and 3.18 μM) or oseltamivir reduced the number of fluorescence-positive cells. This further confirmed the inhibitory effect of compound **4** against viral replication in cell cultures. Therefore, compound **4** may have potential as a future antiviral drug.

The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for novel and effective antiviral agents.[33] This study focused on a phytochemical investigation and pharmacological evaluation of *C. operculatus* buds with the target enzyme NA. Accordingly, four out of 14 C-methylated flavonoids exhibited strong inhibition against the NA of influenza H1N1 virus. Moreover, one of the most active isolates, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**7**), was found to be the major component of the EtOAc-soluble extract of *C. opecurlatus* buds. Therefore, this compound can be used as a marker component for quality control of this antiviral botanical supplement. Some studies have reported the NA inhibitory activity of flavonoids against influenza H1N1 virus.[34,35] The present study showed that the chalcone derivatives have higher inhibitory activity against influenza NAs than the other compounds tested. This finding suggests an association between the chemical structure of flavonoids and their NA inhibitory activity. Nevertheless, further study and optimization of these derivatives may enable the discovery of new drugs to combat this serious disease.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Rudolph Autopol IV polarimeter using a 100 mm glass microcell. UV spectra were recorded in MeOH on a JASCO V-550 UV/vis spectrometer with a 0.5 nm resolution, and IR spectra (KBr) were obtained using a Nicolet 6700 FT-IR (Thermo Electron Corp.). CD spectra were recorded on a JASCO J-710 spectropolarimeter. NMR spectra were obtained on a Varian Unity Inova 500 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). EIMS and HREIMS data were recorded on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63−200 μm particle size), RP-18 (Merck, 40−63 μm particle size), and Sephadex LH-20 were used for column chromatography. TLC was carried out with silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). All solvents used for extraction and isolation were of analytical grade.

Plant Material. The buds of *C. operculatus* were purchased from the Dong Xuan herbal market, Hanoi, Vietnam, in February 2009 and were identified botanically by Dr. Nguyen Bich Thu, National Institute of Medicinal Materials, Hanoi, Vietnam. A voucher specimen (NIMM2009-05) was deposited at the Herbarium of the National Institute of Medicinal Materials, Hanoi, Vietnam.

Extraction and Isolation. The dried buds of *C. operculatus* (1.5 kg) were extracted with MeOH (4 L × 2 times) at room temperature

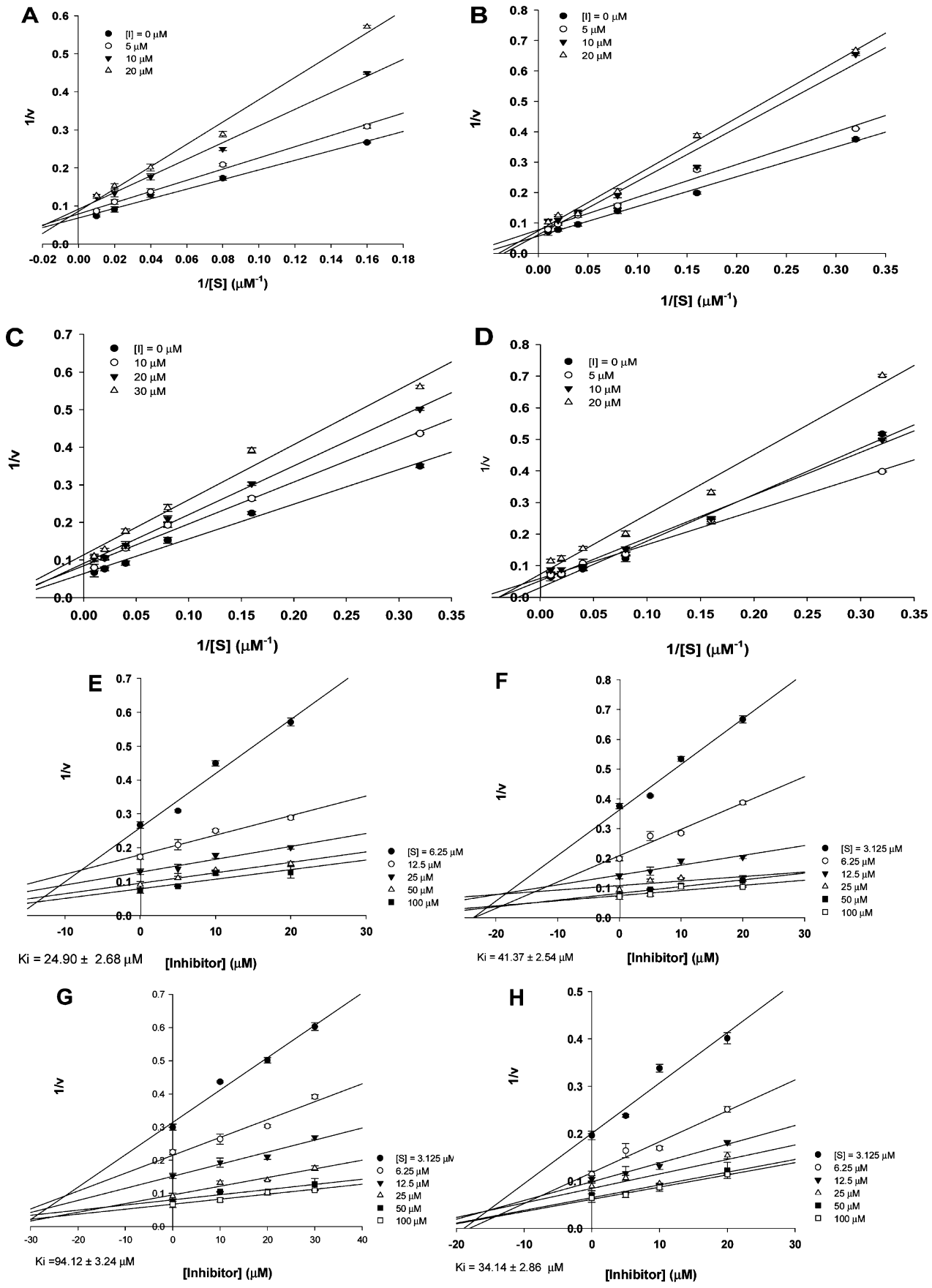

Figure 3. Graphical determination of the type of inhibition for the isolated compounds. (A−D) Lineweaver−Burk plots for the inhibition of compounds **4**, **7**, **8**, and **14** on the NA from influenza A (H1N1) for the hydrolysis of the substrate. The data are expressed as the mean reciprocal of the intensity/min for $n = 3$ replicates at each substrate concentration. (E−H) Dixon plots for compounds **4**, **7**, **8**, and **14** determining the inhibition constant K_i. The K_i value was determined from the negative of the *x*-axis value at the point of the intersection of the three lines. The data are expressed as the mean reciprocal of the intensity/min of $n = 3$ replicates at each substrate concentration.

Table 3. Antiviral Activities of Compound **4**, **7**, **8**, and **14** against A/PR/8/34 (H1N1) in the MDCK Cells Using a CPE Reduction Assay

compound	CC_{50} (μM)[a]	EC_{50} (μM)[b]	SI[c]
4	>120	4.90 ± 0.35	>24.49
7	78.39 ± 4.16	7.72 ± 0.70	10.15 ± 0.54
8	135.00 ± 5.81	8.13 ± 0.81	16.61 ± 0.71
14	>120	8.79 ± 1.05	>13.65
Tamiflu	−[d]	2.24 ± 0.15	

[a] CC_{50}: mean (50%) value of the cytotoxic concentration. [b] EC_{50}: mean (50%) value of the effective concentration. [c] SI: selective index, CC_{50}/EC_{50}. [d] Tamiflu cytoxicity was measured as only 7% inhibition at a concentration of 97.56 μM.

for one week. The combined MeOH extract was concentrated to yield a dry residue (170 g). This crude extract was then suspended in H_2O (2 L) and partitioned successively with *n*-hexane (3 × 1.5 L), EtOAc (3 × 1.5 L), and *n*-BuOH (3 × 1.5 L). The EtOAc fraction (75 g), which exhibited strong influenza NA inhibitory activity, was chromatographed over a silica gel column (10 × 30 cm; 63−200 μm particle size) and eluted with *n*-hexane/EtOAc (19:1, 18:2, 1:19, each 2.5 L) to yield nine fractions (F1: 10.6 g; F2: 3.5 g; F3: 2.5 g; F4: 4.2 g; F5: 5.6 g; F6: 3.6 g; F7: 2.5 g; F8: 5.6 g; F9: 10.6 g). Fraction F3 was chromatographed over a Sephadex LH-20 column (7 × 40 cm) using MeOH as the eluting solvent to yield compound **7** (650 mg). Fraction F4 was applied to an RP-18 column (7 × 30 cm; 40−63 μm particle size) eluting with a stepwise gradient of MeOH/H_2O (2:1 to 10:1) to afford five subfractions (F4.1−F4.5). F4.2 (160 mg) was separated by HPLC [Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% HCO_2H (0−65 min: 63% MeOH, 65−70 min: 63−100% MeOH, 70−80 min: 100% MeOH); flow rate 2 mL/min; UV detection at 205 and 254 nm] to give compounds **1** (t_R = 45 min, 3.5 mg), **5** (t_R = 63 min, 4.5 mg), and **6** (t_R = 49 min, 13.5 mg). Compounds **8** (t_R = 31 min, 19.5 mg), **9** (t_R = 45 min, 9.5 mg), and **10** (t_R = 48 min, 14 mg) were separated from subfraction F4.3 (220 mg) by HPLC (0−55 min: 72% MeOH, 55−60 min: 72−100% MeOH, 60−70 min: 100% MeOH). Fraction F5 was chromatographed over a Sephadex LH-20 column (7 × 30 cm) using MeOH as the eluting solvent to give four subfractions (F5.1−F5.4). F5.3 (2.6 g) was fractionated continuously into five subfractions (F5.3.1−F5.3.5) using an RP-18 column (5 × 30 cm; 40−63 μm particle size) with a stepwise gradient of MeOH/H_2O (1:1 to 10:1). Subfraction F5.3.2 (150 mg) was further separated by HPLC (0−45 min: 58% MeOH, 45−50 min: 58−100% MeOH, 50−60 min: 100% MeOH), which led to the isolation of compounds **2** (t_R = 28 min, 4.5 mg) and **11** (t_R = 40 min, 14 mg). Compounds **3** (t_R = 33 min, 3 mg), **12** (t_R = 42 min, 8.5 mg), and **13** (t_R = 56 min, 15.5 mg) were obtained from F5.3.3 (170 mg) by HPLC (0−65 min: 62% MeOH, 65−70 min: 62−100% MeOH, 70−80 min: 100% MeOH). Finally, compounds **4** (t_R = 29 min, 6.5 mg) and **14** (t_R = 37.5 min, 9.5 mg) were purified by HPLC (0−45 min: 65% MeOH, 45−50 min: 65−100% MeOH, 50−60 min: 100% MeOH) from subfraction F5.3.4 (110 mg).

7-Hydroxy-5-methoxy-6,8-dimethylisoflavone (1): yellow, amorphous powder; UV (MeOH) λ_{max} nm (log ε) 255 (4.32), 298 (3.79); IR (KBr) ν_{max} 3386 (OH), 2926, 1637 (C=O), 1591, 1447, 1230, 1136 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m/z* (rel int) 296 ($[M]^+$, 100), 295 (31), 281 (89), 278 (57), 266 (6), 265 (22), 250 (17), 195 (18), 77 (19); HREIMS *m/z* 296.1047 $[M]^+$ (calcd for $C_{18}H_{16}O_4$, 296.1049).

(2*S*,3*S*)-2,3-*trans*-5,7-Dihydroxy-6,8-dimethyldihydroflavonol (2): brown, amorphous powder; $[\alpha]^{25}_D$ −24 (*c* 0.08, MeOH); UV (MeOH) λ_{max} nm (log ε) 297 (4.45), 340 (3.84); IR (KBr) ν_{max} 3423 (OH), 2927, 1639 (C=O), 1469, 1282, 1123 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 304 (−11.2), 295 (+10.8); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m/z* (rel int) 300 ($[M]^+$, 67), 285 (2), 271 (14), 270 (2), 181 (100), 152 (49), 77 (10); HREIMS *m/z* 300.0999 $[M]^+$ (calcd for $C_{17}H_{16}O_5$, 300.0998).

(2*S*)-2,7-Dihydroxy-5-methoxy-6,8-dimethylflavanone (3): brown, amorphous powder; $[\alpha]^{25}_D$ −7 (*c* 0.02, MeOH); UV (MeOH) λ_{max} nm (log ε) 294 (4.47), 338 (3.87); IR (KBr) ν_{max} 3391 (OH), 2926, 1681 (C=O), 1621, 1410, 1338, 1114 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 305 (−21.7), 294 (+23.4); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m/z* (rel int) 314 ($[M]^+$, 24), 299 (2), 223 (77), 195 (100), 152 (17), 77 (8); HREIMS *m/z* 314.1152 $[M]^+$ (calcd for $C_{18}H_{18}O_5$, 314.1154).

(*E*)-4,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (4): yellow, amorphous powder; UV (MeOH) λ_{max} nm (log ε) 298 (3.91), 362 (4.47); IR (KBr) ν_{max} 3385 (OH), 2931, 1605 (C=O), 1545, 1437, 1229, 1164 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m/z* (rel int) 314 ($[M]^+$, 78), 313 (18), 299 (4), 284 (2), 221 (10), 194 (100), 166 (19), 136 (20); HREIMS *m/z* 314.1156 $[M]^+$ (calcd for $C_{18}H_{18}O_5$, 314.1154).

Cloning of Novel H1N1 Influenza NA. A full-length cDNA encoding the neuraminidase of novel H1N1 influenza [A/California/08/2009 (H1N1)] was constructed using a custom gene synthesis service (Nanomol, South Korea). The synthesized cDNA was subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad) for protein expression. Oseltamivir-resistant neuraminidase (H274Y mutant) was generated using a PCR-mediated site directed mutagenesis method with the forward primer GAA TGC CCC TAA TTA TTA CTA TGA GGA ATG CTC and reverse primer GAG CAT TCC TCA TAG TAA TAA TTA GGG GCA TTC, and the mutant clone was sequenced to confirm the presence of the intended mutation.

Viruses, Cells, and Expression of Neuraminidase. The influenza strains A/Chicken/Korea/O1310/2001 (H9N2) and A/Sw/Kor/CAH1/04 (H1N1, KCTC 11165BP) were used in this study. The 293T cells (human embryonic kidney cells) and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. DMEM containing 0.15 μg/mL trypsin and 5 μg/mL BSA was used as the infection medium for the MDCK cells. The cells were counted and plated in six-well plates at a density of 10^5 cells/well. After 24 h, the plasmid DNA was introduced into each well by lipid-mediated transfection using Welfect-Ex plus transfection reagent (Welgene) according to the manufacturer's manual. Briefly, the cells were incubated in TOM with 1.5 μg of DNA and 7.5 μg of Welfect-Ex plus reagent for 6 h at 37 °C. After transfection, the cultures were maintained in 5% FBS-DMEM medium. At 48 h after post-transfection, the cells were treated with 0.02% EDTA in PBS pH 7.4 and harvested.

Influenza A (H1N1 and H9N2) Neuraminidase Inhibition Assay. The enzyme assay was performed as previously reported with a slight modification.[32] In general, large-scale influenza virus suspensions were prepared from MDCK cells infected with the influenza viruses H1N1 or H9N2. The virus suspensions were treated with formaldehyde at a final concentration of 0.1% at 37 °C for 30 min to inactivate the viral infectivity. The NA activity was measured using 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639) in the enzyme buffer (MES buffer: 32.5 mM 2-(*N*-morpholino)ethanesulfonic acid, 4 mM $CaCl_2$, pH 6.5) as the substrate. All compounds were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer (32.5 mM 2-(*N*-morpholino)-ethanesulfonic acid, 4 mM $CaCl_2$, pH 6.5). An enzyme inhibitory assay was carried out in 96-well plates containing 10 μL of the diluted virus supernatant (containing active influenza NA) and 10 μL of isolated compound in the enzyme buffer. The mixture was incubated for 30 min at 37 °C, and 30 μL of 4-MU-NANA substrate per well in enzyme buffer was added. The enzymatic reactions were carried out for 2 h at 37 °C and quenched by adding 150 μL of the stop solution (25% EtOH, 0.1 M glycine, pH 10.7). The fluorescence intensity of the product (4-MU) was measured using a Spectramax M2^e spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) with excitation and emission wavelengths of 360 and 440 nm, respectively. The IC_{50} for reducing the NA activity was then determined. The data were analyzed using Sigmaplot 11.0 (SPCC Inc., Chicago, IL, USA). For the enzyme kinetic study, 4-methylumbelliferone was quantified immediately without adding a stop solution.

$$\%\ \text{Inhibtion} = \frac{100}{1 + (IC_{50}/[I])}$$

[I] = concentration of inhibitor (μM); IC_{50} = half-maximal inhibitory concentration (μM).

Novel H1N1 (WT) and Oseltamivir-Resistant Novel H1N1 (H274Y) Neuraminidase Inhibition Assay. The 293T cells transfected with the plasmids were harvested by treatment with 0.02% EDTA in PBS (pH 7.4). After washing with PBS pH 7.4, the cells (approximately 5 × 10^6 cells) were suspended in 250 μL of PBS (pH 7.4) containing 3.5 mM $CaCl_2$. The suspensions were then divided into 50 μL aliquots and stored at −80 °C until used. The NA inhibition assays were

performed using 4-MU-NANA as the fluorescent substrate and dilutions of the samples with an NA activity equivalent to 8−10 × fluorescence units compared to the background. The tested compounds were preincubated with 10 μL of the cell suspensions in an enzyme buffer (32.5 mM MES, 4 mM $CaCl_2$, pH 6.5) at 37 °C in 30 min. After 30 min incubation, the substrate (30 μL) was added and the resulting mixture was incubated for a further 2 h at 37 °C and finally terminated by adding 150 μL of a stop solution (25% EtOH, 0.1 M glycine, pH 10.7). The plates were read in a Spectramax M2^e spectrofluorometer with an excitation and emission wavelength of 360 and 465 nm, respectively.

Cytopathic Effect (CPE) Inhibition Assay. After the near-confluent MDCK cell monolayers (1×10^5 cells/well) were inoculated with the virus for 1 h, the cells were replaced with DMEM containing 10 μg/mL trypsin and several compounds at different concentrations. The cells were incubated for 3−4 days at 37 °C under a 5% CO_2 atmosphere, and the level of virus inhibition was determined in triplicate by adding 0.034% neutral red to each well followed by incubation for 2 h at 37 °C in the dark. The neutral red solution was removed, and the cells were washed with PBS (pH 7.4). After adding a destaining solution (containing 1% HOAc, 49% H_2O, and 50% EtOH) to each well, the plates were incubated in the dark for 15 min at room temperature. The 50% effective concentration (EC_{50}) was calculated by regression analysis of the absorbance at 540 nm in a microplate reader.

Cytotoxicity Assay. The MDCK cells were grown in 96-well plates at 1×10^5 cells/well for 24 h. The plates were replaced with media containing the serially diluted compounds. After 48 h incubation, a 10 μL Ez-cytox solution (Daeil Lab Service Co.) was added to each well. The cells were incubated for a further 3 h at 37 °C, and the optical density (OD) of the wells was determined at a test wavelength of 450 nm. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

Immunofluorescence Assay. The MDCK cells were grown on eight-well chamber slides (LAB-TEK, NUNC, USA). After incubation for 24 h, the media was removed and 1 mL of infection media (DMEM + 0.5% BSA, 1.5 μg/mL Trypsin, 1% P/S) was added into each well. The cell monolayers were infected with the influenza virus (A/PR/8/34) at 1000 $TCID_{50}$/mL for 2 h. The solution was removed and replaced with the culture media (DMEM, 1% A/A, 5% FBS, and 2 mL of L-glutamine) and treated with the compounds at the corresponding concentration. The cultures were incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. The cells were rinsed (×3) with PBS (pH 7.4) and fixed with a 4% paraformaldehyde solution for 30 min at room temperature. After washing twice with PBS (pH 7.4), 1 mL of 0.2% triton X-100 was added to the cells. After blocking with 1 mL of 1% BSA for 1 h, the cells were incubated with the monoclonal antibodies against the influenza virus M1 protein (Santa Cruz, USA). After washing with PBS (pH 7.4), the cells were incubated with the secondary FITC-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, Inc.) for 1 h. After washing three times with PBS (pH 7.4), the cells were stained with a 500 μL of DAPI solution for 1 min at room temperature. After washing with PBS (pH 7.4), the slides were mounted with a mounting medium for fluorescence (VECTASHIELD, Vector Laboratories Inc.) and observed by fluorescence microscopy (Axiovert 200M, Carl Zeiss, Germany).

Statistical Analysis. A statistical calculation was carried out using Microsoft Excel 2003. The results are expressed as the mean ± SD of three to five independent experiments.

Supporting Information Available: ^1H and ^{13}C NMR, HSQC, and HMBC spectra of compounds **1**−**4**, along with the synergistic effect of compound **4** on NA and the inhibitory activity of oseltamivir, are available free of charge via the Internet at http://pubs.acs.org.

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