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Curcuminoids from *Curcuma longa* and their inhibitory activities on influenza A neuraminidases

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ABSTRACT

The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for new and effective antiviral agents. In this study, we describe the isolation of 3 new (**1**–**3**) and 10 known (**4**–**13**) curcuminoids from a methanol extract of *Curcuma longa* L. All compounds had strong inhibitory effects on the neuraminidases from two influenza viral strains, H1N1 and H9N2, as noncompetitive inhibitors with IC_{50} values ranging from 6.18 ± 0.64 to 40.17 ± 0.79 μg/ml and 3.77 ± 0.75 to 31.82 ± 1.33 μg/ml, respectively. Compounds **4**, **5**, and **13** also exhibited significant inhibitory activity against the neuraminidases from novel influenza H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y mutant) expressed in 293T cells. Our results suggest that the curcuminoids from *C. longa* may be potential supplemental molecules in the prevention and treatment of disease by influenza viruses.

1. Introduction

Influenza viruses (Orthomyxoviridae) cause annual epidemics and occasional pandemics that have claimed the lives of millions. The top of pharmacological strategies for dealing with influenza pandemic is now based on antiviral drugs, in which neuraminidase (NA) inhibitors are the most important (Colman, 2009; Xie, Gong, Li, Fang, & Xu, 2011). NA, also known as sialidase, is a surface glycoprotein of the influenza A virus, that plays a key role in not only in the release of virions from infected host cells but also in their movement through the upper respiratory tract (Beigel & Bray, 2008). When influenza viruses show deficient NA activity, particles of progeny viruses aggregate at the surface of infected cells, which severely impairs the further spread of viruses to other cells (Beigel & Bray, 2008; Zhang, Yu, Zhu, & Jiang, 2006).

To date, there are five well established anti-influenza drugs commercially available. These include NA inhibitors, oseltamivir, zanamivir and peramivir, which impair the efficient release of viruses from an infected host cell, and amantadine and rimantadine, which specifically inhibit viral proliferation by blocking the M_2 ion channel (Regoes & Bonhoeffer, 2006; Smee et al., 2010). Although antiviral chemotherapy with M_2 inhibitors reduces the duration of symptoms of clinical influenza, many side effects have been reported (Regoes & Bonhoeffer, 2006). While zanamivir (relenza) exhibits excellent antiviral activity, its bioavailability is low due to rapidly elimination by renal excretion. Some adults receiving oseltamivir (tamiflu) have also reported nausea and vomiting (Ryan, Ticehurst, & Dempsey, 1995). Furthermore, high-level drug resistance is associated with both inhibitors. In 2009, oseltamivir-resistant H1N1 viruses arose spontaneously and spread globally. This resistance was conferred by a single amino acid change in the viral neuraminidase (H274Y) (Hurt, Holien, Parker, & Barr, 2009). Therefore, continuous research for new antiviral compounds from natural products is needed to develop new therapeutic agents in the battle against the influenza viruses (De Clercq, 2006).

Curcuma longa is a plant belonging to Zingiberaceae family, which is found in south and southeast tropical Asia (Ammon & Wahl, 1991). Its rhizome is used as a spice (main ingredient of curry), a pigment dye of textiles, and in traditional medicine. Two fundamental groups of compounds from this plant are curcuminoids and sesquiterpenes (Nishiyama et al., 2005), in which curcuminoids with the pharmacological activities including cardiovascular protection, antitumour, antioxidant, anti-inflammatory, anti-Alzheimer, anti-hepatotoxic, and antiviral have been reported (Chen et al., 2010; Masuda, Jitoe, Isobe, Nakatani, & Yonemori, 1993; Miriyala, Panchatcharam, & Rengarajulu, 2007; Park & Kim, 2002; Ravindran, Prasad, & Aggarwal, 2009; Simon et al., 1998).

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As part of an ongoing anti-influenza screening programme from natural products (Dao et al., 2010; Nguyen et al., 2011), a methanol extract of *C. longa* was found to exhibit potential NA inhibitory properties. Although the antiviral activity of curcumin has been reported (Chen et al., 2010), the detailed relationships of structure and activity by curcuminoids are unclear. Moreover, there are no reports of the antiviral activities of *C. longa* on novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells. This prompted us to identify the active principles with the NA inhibitory activity of *C. longa* by bioactivity-guided fractionation. This paper describes 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.

2. Materials and methods

2.1. Plant material

The rhizomes of *C. longa* were collected in August 2009 at Jindo, Jeonnam province, Republic of Korea. Plant sample was identified botanically by Prof. Y.H. Moon. A voucher specimen (CU2009-06) was deposited at the Herbarium of Chosun University, Gwangju, Republic of Korea.

2.2. General experimental procedures

The UV spectra were recorded in MeOH on a JASCO V-550 UV/VIS spectrometer. The IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR (Thermo electron Corp.). The NMR spectra were obtained on a Varian Inova 600 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The EIMS and HREIMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63–200 μm particle size), silica gel (Merck, 40–63 μm particle size), and Sephadex LH-20 were used for column chromatography. TLC was carried out using silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and an 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.

2.3. Extraction and isolation

The dried rhizomes of *C. longa* (3 kg) were extracted with MeOH (15 l× 3 times) at room temperature for a week. The combined methanol extract was then concentrated to yield a dry residue (380 g). This crude extract was suspended in distilled water (2.5 l) and partitioned successively with *n*-hexane (3 × 2 l), ethyl acetate (3 × 2 l), and butanol (3 × 2 l). The ethyl acetate and butanol fractions, which showed strong influenza NA inhibitory activity (Table S.1 in Supplementary data), were combined (130 g) and chromatographed over a silica gel column (10 × 30 cm; 63–200 μm particle size) eluting with gradient solvent $CHCl_3$/acetone (19:1,18:2,...,1:19, each 2.5 l), to yield six fractions (F1: 17.6 g; F2: 8.5 g; F3: 5.6 g; F4: 9.8 g; F5: 10.5 g; F6: 12.6 g; F7: 22.4 mg). The crystallisation of fraction F3 from n-hexane/EtOAc afforded compound **13** (curcumin, 4.2 g). Fraction F4 was further applied to a normal-phase silica gel column (5 × 40 cm; 40–63 μm particle size) with a stepwise gradient of $CHCl_3$/MeCN (9:1,8:2,...,1:9, each 2 l) to afford five subfractions (F4.1–F4.5). Crystallisation of subfractions F4.2 and F4.3 from $CHCl_3$ gave compounds **4** (demethoxycurcumin, 1.3 g) and **5** (bisdemethoxycurcumin, 2.5 g), respectively. Fraction F4.4 (90 mg) was separated by HPLC [Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeCN in H_2O containing 0.1% HCO_2H (0–40 min: 45% MeCN, 40–45 min: 100% MeCN, 45–55 min: 100% MeCN); flow rate 2 ml/min; UV detection at 205 and 254 nm] to give compounds **8** (t_R = 30.0 min, 5.5 mg) and **10** (t_R = 34.0 min, 14.0 mg).

Fraction F5 was chromatographed over a Sephadex LH-20 column (7 × 40 cm) using MeOH as the eluting solvent to afford three subfractions (F5.1–F5.3). Subfraction F5.2 (3.1 g) was further chromatographed over a silica gel column (5 × 40 cm; 40–63 μm particle size) with a gradient solvent of $CHCl_3$/MeCN (9:1,8:2,...,1:9, each 2.5 l) to yield five fractions (F5.2.1–F5.5.5). Subfraction F5.2.2 (150 mg) was purified by HPLC (0–45 min: 60% MeCN, 45–50 min: 100% MeCN) to yield compounds **9** (t_R = 35.0 min, 10.5 mg) and **11** (t_R = 38.5 min, 13.0 mg). Further separation of F5.2.3 (110 mg) by HPLC (0–65 min: 57% MeCN, 65–70 min: 100% MeCN) resulted in the isolation of compounds **6** (t_R = 42.0 min, 10.5 mg), **7** (t_R = 58.0 min, 7.5 mg), and **3** (t_R = 61.0 min, 6.0 mg). Finally, compounds **1** (t_R = 46.0 min, 4.5 mg), **2** (t_R = 48.5 min, 3.5 mg), and **12** (t_R = 52.0 min, 6.5 mg) were obtained from subfraction F5.2.4 (95 mg) by HPLC (0–60 min: 35% MeCN, 65 min: 100% MeCN).

2.4. Viruses, cells, cloning, and expression of neuraminidase

A full length cDNA encoding the neuraminidase of novel H1N1 influenza (A/California/08/2009(H1N1)) and oseltamivir-resistant neuraminidase (H274Y mutant) were constructed as previously reported method (Dao et al., 2010). The influenza strains A/Chicken/Korea/O1310/2001 (H9N2) and A/Sw/Kor/CAH1/04 (H1N1, KCTC 11165BP) were used in this study. 293T cells (human embryonic kidney cells) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. The 293T cells were counted and plated in 6-well plates at a density of 10^5 cells/well. After 24 h, the cells were transfected with the plasmids containing the cDNAs using a commercial transfection kit (Welfect EX-plus, Welgene, Daegu, Korea), according to the manufacturer's instructions.

2.5. Influenza A (H1N1 and H9N2) neuraminidase inhibition assays

The enzyme assay was performed as previously reported with a slight modification (Dao et al., 2010; Hung et al., 2009). Large-scale influenza virus suspension was prepared from the MDCK cells infected with the influenza viruses, H1N1 and H9N2. The virus suspensions were treated with formaldehyde at a final concentration of 0.01% 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 an acetate buffer as the substrate.

2.6. Novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) neuraminidase inhibition assays

The 293T cells transfected with the plasmids were harvested by treatment with 0.02% EDTA in PBS. After washing with PBS, the cells (approximately 5 × 10^6 cells) were suspended in 250 μl PBS containing 3.5 mM $CaCl_2$. The suspensions were then divided into 50 μl aliquots and stored at −80 °C until needed. The NA inhibition assays were performed using 4-MU-NANA as the fluorescent substrate and dilutions of the samples with NA activity equivalent to 8–10 × fluorescence units compared to the background. The tested compounds were pre-incubated with 10 μl cell suspensions in 32.5 mM MES buffer (containing 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 assays were incubated for a further 2 h at 37 °C, and finally

terminated by adding 150 μl of the stop solution (25% EtOH, 0.1 M glycine, pH 10.7). The plates were read in a Spectramax M2[e] spectrofluorometer with excitation and emission wavelength of 360 and 465 nm, respectively.

2.7. 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.

3. Results and discussion

In order to isolate the compounds with inhibitory activity against influenza neuraminidase, the methanol extract of *C. longa* was subjected to a succession of chromatographic procedures including silica gel chromatography, Shephadex LH-20, RP-C18, and HPLC to afford thirteen compounds, including 3 new (**1**–**3**) and 10 known (**4**–**13**) curcumin derivatives (Fig. 1).

3.1. Structure determination and identification of curcumin derivatives isolated from C. longa

Compound **1** was obtained as a yellow amorphous powder. The ^{1}H NMR spectrum (Table 1) showed a 1,3,4,5-tetrasubstituted benzene ring [δ_H 7.23 (1H, d, J = 1.8 Hz, H-2′) and 7.21 (1H, d, J = 1.8 Hz, H-6′)], a set of A_2B_2 aromatic protons [δ_H 7.63 (2H, d, J = 8.4 Hz, H-2″, H-6″) and 6.94 (2H, d, J = 8.4 Hz, H-3″, H-5″)], a pair of *trans*-olefinic protons [δ_H 7.67 (1H, d, J = 15.6 Hz, H-7″) and 7.08 (1H, d, J = 15.6 Hz, H-8″)], a methoxy group [δ_H 3.99 (3H, s, 3′-OCH_3)], an

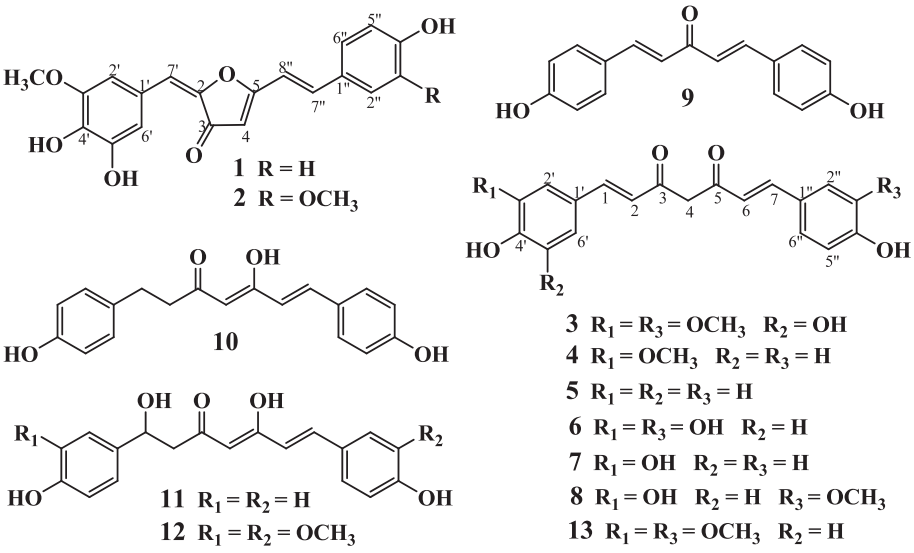

Fig. 1. Chemical structure of compounds **1**–**13** isolated from the rhizomes of *C. longa*.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **1**–**3**.

Position	**1**		**2**		**3**	
	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					7.53 d (15.6)	141.8
2		146.7		146.7	6.67 d (15.6)	122.5
3		186.6		186.6		184.4
4	5.92 s	105.4	5.91 s	105.3	5.98 s	101.8
5		176.1		176.1		184.4
6					6.72 d (15.6)	122.4
7					7.60 d(15.6)	141.5
1′		124.4		124.4		127.3
2′	7.23 d (1.8)	107.9	7.23 d (1.8)	107.9	6.90 s	104.7
3′		149.2		149.0		149.2
4′		137.5		137.4		137.5
5′		146.5		146.5		146.5
6′	7.21 d (1.8)	113.6	7.21 d (1.8)	113.6	6.87 s	110.3
7′	6.52 s	112.2	6.52 s	112.2		
1″		127.7		128.2		128.2
2″	7.63 d (8.4)	131.2	7.43 d (1.8)	111.0	7.34 s	111.5
3″	6.94 d (8.4)	117.0		149.2		148.8
4″		160.8		150.4		150.0
5″	6.94 d (8.4)	117.0	6.91 d (8.4)	116.4	6.88 d (8.4)	116.3
6″	7.63 d (8.4)	131.2	7.20 dd (8.4, 1.8)	124.6	7.18 d (8.4)	123.9
7″	7.67 d (15.6)	139.3	7.67 d (15.6)	139.7		
8″	7.08 d (15.6)	113.3	7.14 d (15.6)	113.5		
3′-OCH_3	3.99 s	56.5	3.94 s	56.4	3.89 s	56.6
3″-OCH_3			3.99 s	56.5	3.90 s	56.3

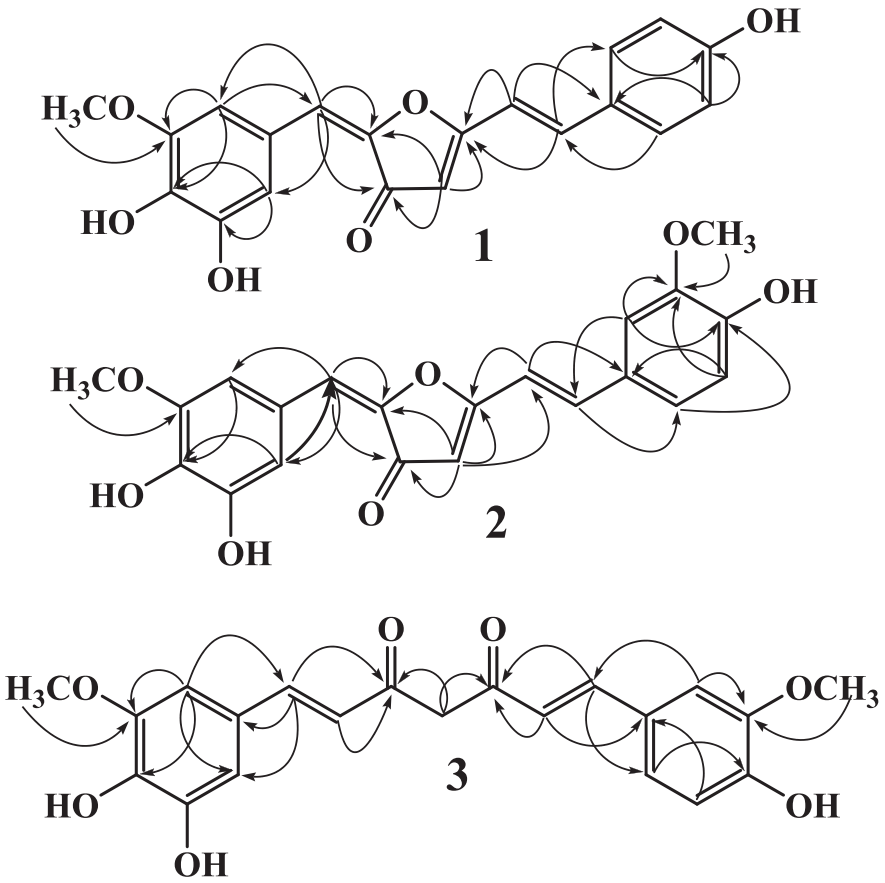

Fig. 2. Key HMBC (H → C) correlations for new compounds **1**–**3**.

alkene proton [δ_H 6.52 (1H, s, H-7′)], and a proton on the central carbon of β-diketone in their enol form δ_H 5.92 (1H, s, H-4). Consistent with the above ^{1}H NMR analysis, the ^{13}C NMR spectrum displayed signals corresponding to the methoxy group at δ_C 56.5, four olefinic carbons at δ_C 146.7, 139.3, 113.3 and 112.2, a conjugated ketone at δ_C 186.6, a hydroxylated olefinic carbon at δ_C 176.1, the central carbon of β-diketone at δ_C 105.4, and 12 carbons of two aromatic rings. These protonated carbons and their bonded protons, which were determined unambiguously by the HMQC experiment, showed the presence of 20 carbons of a curcuminoid skeleton (Li et al., 2009). In addition, the deshielding of both the two olefinic carbons at δ_C 146.7 (C-2) and 176.1 (C-5) indicated their linkage to an oxygen atom. These observations with the presence of characteristic absorption bands in the IR (1665, 1598 cm^{-1}) and UV (398, 295 nm) spectra, suggested the existence of a 3(2*H*)-furanone moiety instead of a linkage fragment (—C═CH—CO—CH═C(OH)—CH═CH—) like in curcuminoid derivatives (Comte, Allais, Chulia, Vercauteren, & Delage, 1996; Gershenzon & Mabry, 1984). This was confirmed by HMBC correlations between H-4/C-2, C-3 and C-5 (Fig. 2), and further supported by the quasi-molecular ion peak at *m*/*z* 353.1022 [M+H] in the HRFABMS, which indicated the molecular formula of $C_{20}H_{16}O_6$. The HMBC correlations from H-7′ to C-2, C-3, C-2′, and C-6′, and from H-2′ and H-6′ to C-7′ suggested that the 1,3,4,5-tetrasubstituted aromatic ring was linked to C-2 of the furanone ring through olefinic carbon C-7′. Finally, the substituted pattern of two benzene rings at C-7′ and C-7″ were deduced from full assignments based on HMQC and key HMBC correlations as shown in Fig. 2. Thus, the structure of compound **1** was identified as 2-(4,5-dihydroxy-3-methoxyphenyl)methenyl-5-(4-hydroxyphenyl)ethenyl-3(2*H*)-furanone, and it was named as curcumalongin A.

Compound **2** was obtained as a yellow amorphous powder. A molecular formula of $C_{21}H_{18}O_7$ was determined from the molecular ion peak at *m*/*z* 382.1051 [M]$^+$ (calcd for $C_{21}H_{18}O_7$, 382.1053) in HREIMS. Its IR spectrum revealed the presence of a hydroxy group (3416 cm^{-1}), conjugated carbonyl group (1665 cm^{-1}), and aromatic ring (1588, 1547, 1512, and 1490 cm^{-1}). The ^{1}H and ^{13}C NMR spectra data of **2** (Table 1) were similar to those of **1**, except for signals due to an additional methoxy group at δ_H 3.99 (3H, s, 3″-OCH_3) and an ABX spin system [δ_H 7.43 (1H, d, *J* = 1.8 Hz, H-2″), 7.20 (1H, dd, *J* = 8.4, 1.8 Hz, H-6″) and 6.91 (1H, d, *J* = 8.4 Hz, H-5″)] instead of the A_2B_2 aromatic protons on benzene ring of **1**. The HMBC correlations from the methoxy proton and aromatic protons (H-2″ and 5″) to carbon C-3″ (δ_C 149.2) indicated that the additional methoxy group was attached to C-3″. Therefore, the structure of compound **2**, curcumalongin B, was determined to be 2-(4,5-dihydroxy-3-methoxyphenyl)methenyl-5-(4-hydroxy-3-methoxyphenyl)ethenyl-3(2*H*)-furanone.

Compound **3** was obtained as a yellow amorphous powder. The IR spectrum showed absorption bands for the hydroxy group (3415 cm^{-1}), carbonyl groups (1737 and 1626 cm^{-1}), and aromatic ring (1599, 1513, and 1467 cm^{-1}). The ^{1}H NMR spectrum (Table 1) showed signals for a 1,3,4-trisubstituted benzene ring [δ_H 7.34 (1H, s, H-2″), 7.18 (1H, d, *J* = 8.4 Hz, H-6″), and 6.88 (1H, d, *J* = 8.4 Hz, H-5″], a 1,3,4,5-tetrasubstituted benzene ring [δ_H 6.90 (1H, s, H-2′) and 6.87 (1H, s, H-6′)], two pair of *trans*-olefinic protons [δ_H 7.60 (1H, d, *J* = 15.6 Hz, H-7), 6.72 (1H, d, *J* = 15.6 Hz, H-6), and 7.53 (1H, d, *J* = 15.6 Hz, H-1), 6.67 (1H, d, *J* = 15.6 Hz, H-2)], two methoxy groups [δ_H 3.90 (3H, s, 3″-OCH_3) and 3.89 (3H, s, 3′-OCH_3)] and the proton on the central carbon of a β-diketone at δ_H 5.98 (2H, s, H-4). The ^{1}H and ^{13}C NMR spectra of compound **3** were similar to those of curcumin (Jayaprakasha, Rao, & Sakariah, 2002)

Table 2
Inhibitory effects of compounds **1**–**13** on neuraminidase activity.

Compound	H1N1 IC_{50} (μg/ml)[a]	H9N2 IC_{50} (μg/ml)[a]	H1N1 (WT) IC_{50} (μg/ml)[a]	H1N1 (H274Y)	
				IC_{50} (μg/ml)[a]	Fold increase vs. WT
1	15.41 ± 0.85	15.59 ± 1.38	NT[c]	NT[c]	
2	14.73 ± 1.42	14.26 ± 1.77	NT[c]	NT[c]	
3	17.22 ± 1.32	18.25 ± 1.55	NT[c]	NT[c]	
4	10.25 ± 1.43	7.07 ± 0.82	4.36 ± 0.57	11.29 ± 0.55	2.59x
5	11.39 ± 0.84	9.86 ± 0.96	6.95 ± 0.92	13.74 ± 1.64	1.98x
6	21.69 ± 1.18	20.84 ± 1.81	NT[c]	NT[c]	
7	20.79 ± 1.37	25.71 ± 1.46	NT[c]	NT[c]	
8	16.59 ± 0.98	15.80 ± 0.92	NT[c]	NT[c]	
9	6.18 ± 0.64	3.77 ± 0.75	NT[c]	NT[c]	
10	20.47 ± 0.88	11.87 ± 0.94	NT[c]	NT[c]	
11	23.97 ± 1.20	18.69 ± 1.81	NT[c]	NT[c]	
12	40.17 ± 0.79	31.82 ± 1.33	NT[c]	NT[c]	
13	8.22 ± 0.87	6.17 ± 0.72	3.46 ± 0.27	6.50 ± 0.53	1.88x
Oseltamivir[b]	0.04[d]	0.004[e]	0.02[f]	5.13 ± 0.23	243.24x

[a] All compounds were examined in a set of triplicate experiments.
[b] The compound was used as the positive control.
[c] NT: not tested.
[d] IC_{50} (40.47 ± 1.29 μg/ml).
[e] IC_{50} (4.29 ± 0.74 μg/ml).
[f] IC_{50} (21.09 ± 1.19 μg/ml).

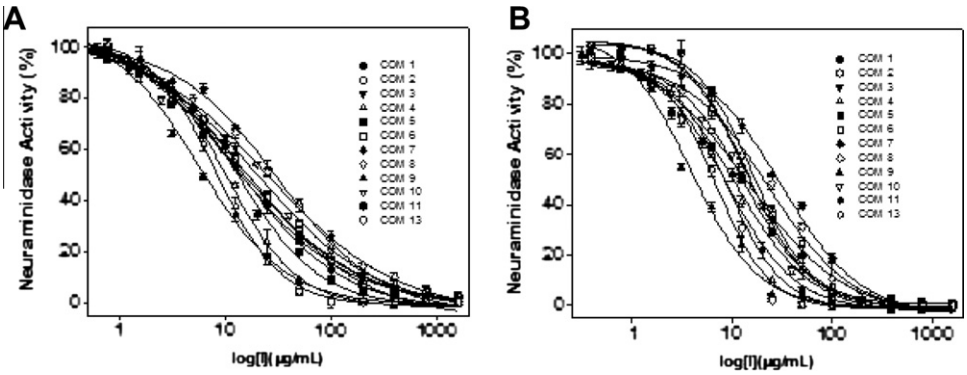

Fig. 3. Effects of compounds (1–**13**) on the activity of the NAs from influenza A H1N1 (A) and H9N2 (B) for the hydrolysis of 4-MU-NANA at 37 °C. The inhibitor concentrations are displayed on logarithmic scales. The IC_{50} was identified from the midpoint (NA activity = 50%) of the semilog plot.

with the exception of an additional hydroxy group attached to carbon C-5′ of the aromatic ring. This was further supported by the molecular ion peak at *m*/*z* 384.1213 $[M]^+$ in the HREIMS which indicated the molecular formula of $C_{21}H_{20}O_7$ for **3**. The HMBC correlations from H-2″ and H-6″/C-4″, H-2″ and H-5″/C-3″, H-2′/C-3′, C-4′, and C-6′, 3′-OCH_3/C-3′, and 3″-OCH_3/C-3″ suggested the location of each substituted groups on the two aromatic rings. The connections of seven-carbons in the diarylheptanoid skeleton were confirmed by the HMBC correlations between H-7/C-1″, C-2″, and C-6″, H-2″ and H-6″/C-7, and H-2″, H-5″/C-1″ (Fig. 2). Therefore, the structure of compound **3** (curcumalongin C) was determined as 1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione.

The structures of the known compounds **4**–**13** were identified as demethoxy curcumin (**4**) (Jayaprakasha et al., 2002), bisdemethoxy curcumin (**5**) (Jayaprakasha et al., 2002), 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**6**) (Venkateswarlu, Ramachandra, & Subbaraju, 2005), 1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl) -1,6-heptadiene-3,5-dione (**7**) (Li et al., 2009), 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-1,6-heptadiene-3, 5-dione (**8**) (Li et al., 2009), 1,5-bis(4-hydroxyphenyl)-1,4-pentadiene-3-one (**9**) (Masuda et al., 1993), 5-hydroxy-1,7-bis(4-hydroxyphenyl)-4,6-heptadiene-3-one (**10**) (Li et al., 2009), 1,5-dihydroxy-1,7-bis(4-hydroxyphenyl)-4,6-heptadiene-3-one (**11**) (Li et al., 2009), 1,5-dihydroxy-1,7-bis(4-hydroxyl-3-methoxyphenyl)-4,6-heptadiene-3-one (**12**) (Li et al., 2009) and curcumin (**13**) (Jayaprakasha et al., 2002) by a comparison of their physicochemical and spectroscopic data with reported data.

3.2. Characteristic data of new compounds

Curcumalongin A (***1***)*:* yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 295 (3.53), 398 (4.06); IR (KBr) ν_{max} 3415 (OH), 1665 (C=O), 1598, 1547, 1511, 1489, 1273 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* (rel. int.): 352 ($[M]^+$, 100), 322 (59), 278 (22), 267 (20), 191 (52), 180 (28), 150 (27); HRFABMS *m*/*z* 353.1022 $[M+H]^+$ (calcd for $C_{20}H_{17}O_6$, 353.1025).

Curcumalongin B (***2***)*:* yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 290 (3.76), 410 (4.12); IR (KBr) ν_{max} 3416 (OH), 1665 (C=O), 1588, 1547, 1512, 1490, 1284 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* (rel. int.): 382 ($[M]^+$, 100), 352 (20), 322 (8), 308 (10), 278 (6), 267 (9), 245 (12), 191 (10), 180 (24); HREIMS *m*/*z* 382.1054 $[M]^+$ (calcd for $C_{21}H_{18}O_7$, 382.1053).

Curcumalongin C (***3***)*:* yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 264 (3.74), 422 (4.27); IR (KBr) ν_{max} 3415 (OH), 2917, 1737 (C=O), 1626, 1599, 1513, 1467, 1272 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* (rel. int.): 384 ($[M]^+$, 100), 366 (47), 284 (51), 267 (55), 208 (32), 192 (53), 177 (97); HREIMS *m*/*z* 384.1213 $[M]^+$ (calcd for $C_{21}H_{20}O_7$, 384.1209).

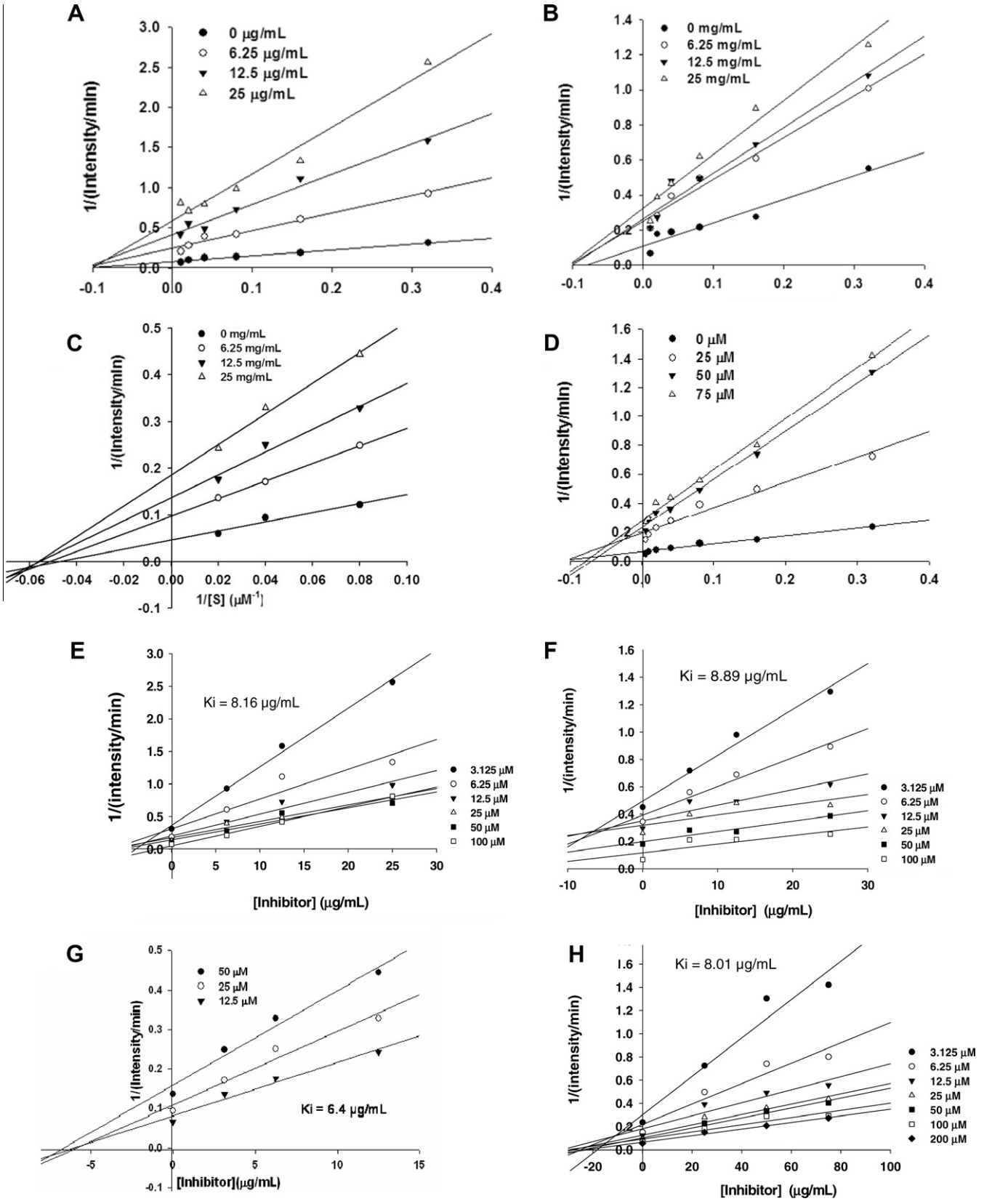

Fig. 4. Graphical determination of the inhibition type for isolated compounds. (A–D) Lineweaver-Burk plots for the inhibition of compounds **4**, **5**, **9**, and **13** on NA from influenza A (H1N1) for the hydrolysis of the substrate. The data is expressed as the mean reciprocal of intensity/min for $n = 3$ replicates at each substrate concentration. (E–H) Dixon plots for compounds **4**, **5**, **9**, and **13** to determine the inhibition constant, K_i. The K_i value was determined from the negative of the x-axis value at the point of intersection of the three lines. The data is expressed as the mean reciprocal of intensity/min for $n = 3$ replicates at each substrate concentration.

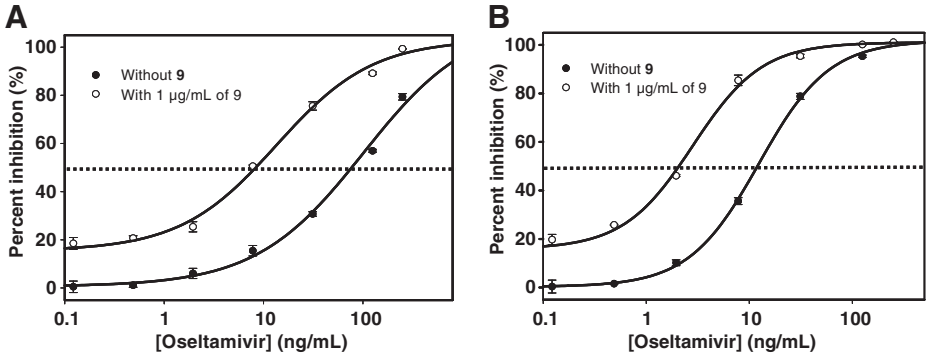

Fig. 5. (A–B) Inhibition of NAs from influenza A (H1N1 and H9N2) by oseltamivir in the presence or absence of compound **9**.

3.3. Effects of isolated compounds on the activity of neuraminidases (NAs) from influenzas A/PR/8/34 (H1N1), A/chicken/Korea/01210/2001 (H9N2), novel H1N1 (WT), and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells.

Inhibitory activities of compounds **1**–**13** were assessed against NAs from influenzas A/PR/8/34 (H1N1) and A/chicken/Korea/01210/2001 (H9N2) using oseltamivir phosphate (Hoffman-La Roche Ltd., Basel, Switzerland) as a positive control. All compounds inhibited NAs in a dose-dependent manner (Table 2 and Fig. 3A and B). The oxyaryl compounds with the unsaturated —C=C—CO— unit showed significant activity towards NAs as in the most active compound **9** (IC_{50} 6.18 ± 0.64 μg/ml). In contrast, the presence of saturated carbons of the C_7 linker as in compounds **10**–**12** might be responsible for the decreased activity compared to the others, e.g., compounds **5**, **9**, and **13**. Additionally, the methoxy substitution of C-3 in the aryl rings might increase the NA inhibitory properties, as observed in compounds **4**, **5**, and **13**, compared to the structurally similar compounds **6**, **7**, and **8**. In generally, inhibitory effects (IC_{50} values of these curcuminoids) on neuraminidase of avian influenza virus (H9N2) were similar to swine influenza (H1N1) (Table 2).

The major compounds **4**, **5**, and **13** were further examined to determine whether they are also effective in inhibiting the NAs from the wild-type novel swine flu (WT) virus and oseltamivir-resistant virus with a H274Y mutation or not (Dao et al., 2010). As shown in Table 2, compounds **4**, **5**, and **13** inhibited the NA derived from the wild-type with an IC_{50} of 4.36 ± 0.57, 6.95 ± 0.92, and 3.46 ± 0.27 μg/ml, respectively. Interestingly, while these compounds showed inhibitory activity against the recombinant NA of oseltamivir-resistant virus (slight decrease of only 1.88- to 2.59-fold at IC_{50}), the inhibitory activity against H274Y of oseltamivir as the positive control was decreased notably (decrease of 243.24-fold at IC_{50} value in comparison to novel H1N1). These results suggest that although inhibitory activities of curcuminoids on influenza NAs were weaker than those of oseltamivir, their inhibitory effects did not change in both NAs from novel H1N1 influenza and its oseltamivir-resistant (H274Y).

3.4. Determination of the inhibition type of compounds on neuraminidase

The inhibition mechanism of the enzyme by compounds was determined from the relative affinity for influenza virus (H1N1) NA (Fig. S.4 in Supplementary data). The inhibition of the tested compound was reversible because increasing of the inhibitor concentration rapidly decreased enzyme activity (Dao et al., 2010). The double reciprocal Lineweaver-Burk and Dixon plots were used to further study on inhibition mode (Fig. 4). All compounds were displayed as noncompetitive inhibitors because increasing of the substrate concentrations resulted in a family of lines that intersected at a non-zero point on the x axis ($-K_i$) (Fig. 4A–D). A summary of the K_i values for the tested compounds was concurred with IC_{50} values (Table 2 and Fig. 4E–H). These results are somewhat congruent with those of reported results for antiviral activity of other diarylheptanoids (Grienke et al., 2010; Tung et al., 2010) as well as a recent report indicating that curcumin might be beneficial for the treatment of an influenza virus infection by inhibiting haemagglutinin (HA) protein (Chen et al., 2010).

*3.5. Synergistic effect of compound **9** on NA inhibitory activity of oseltamivir*

The noncompetitive mechanism of these compounds on NA prompted an investigation into the inhibitory effect of the combination of oseltamivir, a known competitive inhibitor, together with compound **9**. As a result, the NA inhibitory activity of oseltamivir was increased considerably in the presence of compound **9** (at 1 μg/ml or 3.76 μM) (Fig. 5). The oseltamivir activity was increased on H9N2 and H1N1 with IC_{50} values from 6.77; 48.57 to 1.58; 18.21 ng/ml, respectively. These results suggest that oseltamivir and compound **9** inhibit synergistically the NA activity by binding of compound **9** to different sites of oseltamivir-bound enzyme.

Curcumin has been found to exhibit various biological and pharmacological activities including anti-inflammatory, antioxidant, antimicrobial, antiviral, chemopreventive, antiangiogenic, and anticancer activities (Chen et al., 2010; Masuda et al., 1993; Miriyala et al., 2007; Park & Kim, 2002; Ravindran et al., 2009; Simon et al., 1998; Tung et al., 2010; Yamakoshi et al., 2010). This study describes curcuminoids from *C. longa* as active principles against the NAs from influenza A/PR/8/34 (H1N1), avian influenza (H9N2), novel H1N1, and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells. Furthermore, compound **9** with the strongest inhibitory activity showed evidence for synergy on NA inhibition with oseltamivir. These results suggest that curcuminoids could be used as supplementary materials for battle of influenza virus as neuraminidase inhibitors of influenza A.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.02.015.

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