Synthesis and Anti-influenza Activities of Novel Baicalein Analogs

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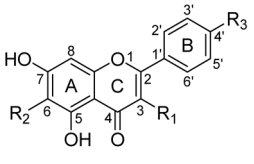
A series of novel flavones derivatives were synthesized based on modification of the active ingredients of a traditional Chinese medicine *Scutellaria baicalensis* Georgi and screened for anti-influenza activity. The synthetic baicalein (flavone) analogs, especially with the B-rings substituted with bromine atoms, were much more potent than oseltamivir or ribavirin against H1N1 Tamiflu-resistant (H1N1 TR) virus and usually with more favorable selectivity. The most promising were 5b, 5c, 6b and 6c, all displaying an 50% effective concentration (EC_{50}) at around 4.0–4.5 μM, and a selective index (SI=50% cytotoxic concentration (CC_{50})/ EC_{50})>70. For seasonal H3N2-infected influenza virus, both 5a and 5b with SI >17.3 indicated superior to ribavirin. The flavonoids having both not-naturally-occurring bromo-substituted B-rings and appropriate hydroxyls positioning on the A-rings might be critical in determining the activity and selectivity against H1N1-Tamiflu-resistant infected influenza viruses.

Key words flavonoid; anti-influenza virus; H1N1 Tamiflu-resistant virus; H3N2 virus

Influenza viruses are the most significant source of viral respiratory infections in humans worldwide, causing recurrent epidemics and global pandemics that bring about severe morbidity and mortality involving millions of people annually.[1)] Influenza virus is an RNA virus of the *Orthomyxoviridae* family and can be classified into three types: A, B, and C.[2)] Unlike influenza B and C viruses, which mainly infect humans, influenza A viruses infect a wide range of hosts, including humans, swine, birds, horses and whales.[3)] H1N1 and H3N2 influenza A viruses have co-circulated since 1977. In the spring of 2009, a new virulent pandemic strain with the H1N1 antigenic subtype appeared and spread globally.[4)]

To date, there have been several antiviral agents approved to treat and prevent influenza virus infection. Amantadine and rimantadine remain important in this regard. However, the rapid development of resistance, the serious toxic effects in the autonomic nervous system in addition to their limited antiviral efficacy other than virus A have made these two agents impractical in clinical use. Two major specific neuraminidase (NA) inhibitors, oseltamivir (Tamiflu) and zanamivir (Relenza), have been successfully prepared by computer-aided drug design and extensively used.[1,5)] The structures of NA inhibitors, in many cases, consist of a transition-state configuration. Therefore, they present their antiviral activity by targeting neuraminidase and thus effectively abolish the proliferation and spreading of viruses. The genesis of oseltamivir and zanamivir represent a new generation of antiviral agents associated with excellent efficacy and specificity. Unfortunately, these two potent NA inhibitors are by no means free from adverse effects.[6)] Moreover, recent studies have indicated that pandemic H1N1 Tamiflu-resistant (H1N1 TR) viral isolates have been progressively rising, which may pose a risk for increasing fatality in human beings.[7,8)] This means that development of new therapeutic agents are urgently needed and prompted us to synthesize some novel flavonoids and explore the possibility as potential anti-influenza agents.

Flavonoids are important polyphenolic constituents of vegetables, fruits, tea and wine,[9,10)] and are well known to exhibit various favorable pharmacological activities, including anti-inflammation, anti-osteoporosis, anticancer, antivirus, anti-bacteria and anti-oxidation.[11–16)] In general, the structures of flavonoids consist of three aromatic rings designated as ring A, B and C based on the sequence of their biogenesis. The plants synthesize a two-ring intermediate chalcone first and then form a bicyclic fused A/C ring system appended with a B-ring. One major class of flavonoids includes baicalein (5,6,7-trihydroxyflavone) (Fig. 1), together with its glycoside baicalin, which are extracted from the root of *Scutellaria baicalensis* Georgi, a traditional Chinese medicine (TCM) having been used for hundreds of years.[14)] It is particularly noteworthy that, in recent years, baicalein and baicalin have been demonstrated anti-influenza virus activity with low toxicity.[16–19)] Recently, in our investigations on the anti-viral screening from traditional Chinese medicines, we had isolated two very potent flavones, kaemferol from *Thesium chinense* Turcz and licoflavonol from *Sophora flavescens* Ait (Fig. 1), and found that both possessed anti-influenza (H1N1 TR) activity with EC_{50} at as low as 10.9 μM and 8.8 μM, respectively. Preliminary data of the molecular modeling study showed that both kaemferol and licoflavonol bind to the same active site of neuraminidase as oseltamivir but somehow in a different



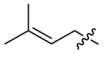
	R_1	R_2	R_3
Baicalein	H	OH	H
Kaempferol	OH	H	OH
Licoflavonol	OH		OH

Fig. 1. The Structures of Baicalein, Kaemferol, and Licoflavonol

The authors declare no conflict of interest.

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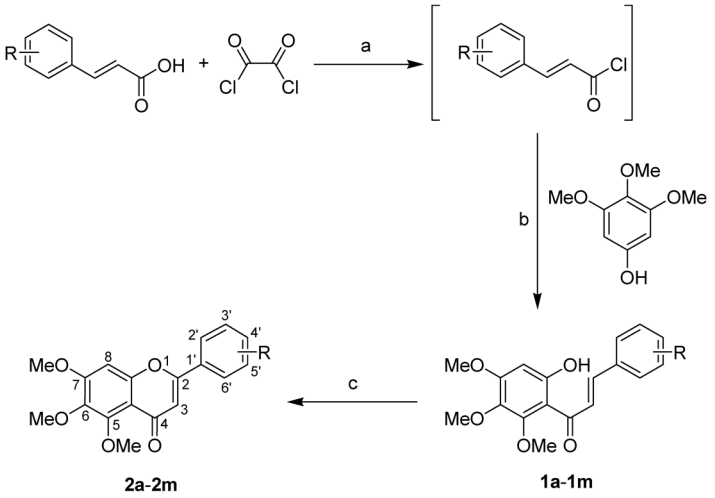
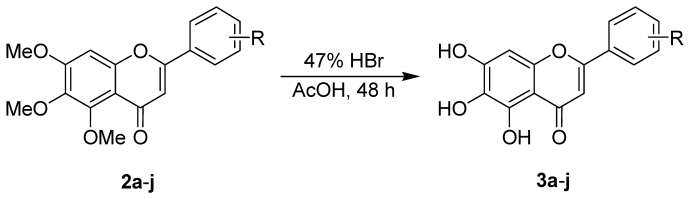


1a, **2a**: R = H
1b, **2b**: R = 2'-Cl
1c, **2c**: R = 3'-Cl
1d, **2d**: R = 4'-Cl
1e, **2e**: R = 2',4'-$(Cl)_2$
1f, **2f**: R = 3',4'-$(Cl)_2$
1g, **2g**: R = 3'-Br, 4'-F
1h, **2h**: R = 4'-COOH
1i, **2i**: R = 4'-Ph
1j, **2j**: R = 4'-NO_2
1k, **2k**: R = 2'-Br
1l, **2l**: R = 3'-Br
1m, **2m**: R = 4'-Br

Reagents and conditions: (a) DMF, DCM, rt; (b) BF_3–Et_2O, reflux; (c) I_2, DMSO, reflux

Chart 1. Synthesis of Compounds **2a**–**m**



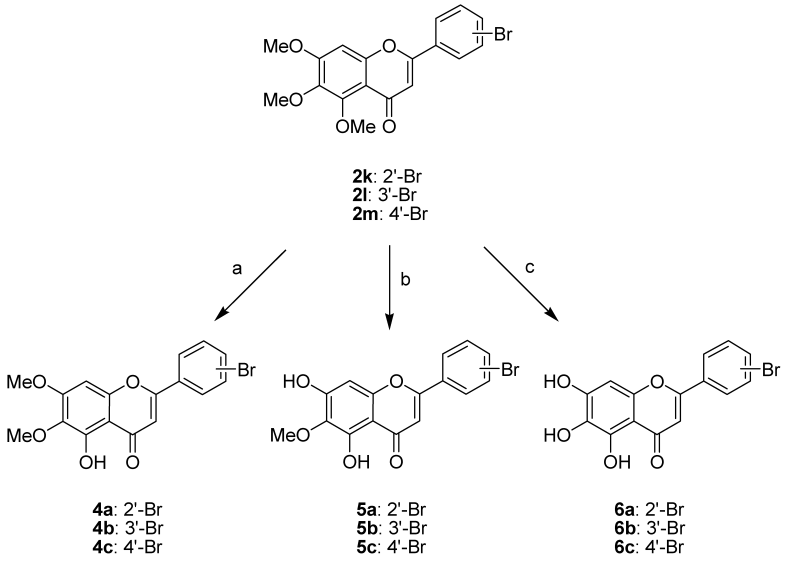
2a, **3a**: R = H
2b, **3b**: R = 2'-Cl
2c, **3c**: R = 3'-Cl
2d, **3d**: R = 4'-Cl
2e, **3e**: R = 2',4'-$(Cl)_2$
2f, **3f**: R = 3',4'-$(Cl)_2$
2g, **3g**: R = 3'-Br,4'-F
2h, **3h**: R = 4'-COOH
2i, **3i**: R = 4'-Ph
2j, **3j**: R = 4'-NO_2

Chart 2. Synthesis of Compounds **3a**–**j**

manner (unpublished result). These might explain the reason why kaemferol and licoflavonol are still active against the H1N1 Tamiflu-resistant strain. We had reported that kaemferol appended with a B-ring bearing properly positioning chlorine atoms could significantly improve their anti-viral activity and selectivity.[20] In this study, based on the structures of the active flavonoids mentioned above, we wish to report our results of synthesis of novel baicalein analogs, especially with B-rings substituted with bromine atoms, and their ability of inhibiting influenza virus. Again, some of the synthetic products showed still superior activity and excellent selectivity, *in vitro*, against H1N1 Tamiflu-resistant virus and seasonal H3N2 virus, respectively. The necessary functional groups and moieties are discussed herein.

Results and Discussion

Chemistry The synthetic approaches and molecular structures are shown in Charts 1–3. In our laboratories various derived flavonoids related to baicalein were readily obtained according to our published procedures with slight modifications.[14,20,21] In this study, we determined the data of spectra and elemental analyses and anti-viral activities of reported **2l**, **2m**, **5b** and **6c**, which have been known to possess activities against inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).[22,23] However, they were published in the form of a letter and none of their physico-chemical properties



Reagents and coditions: (a) BF_3–Et_2O, 45 min; (b) 47% HBr, AcOH, 2.5 h; (c) 47% HBr, AcOH, 48–54 h

Chart 3. Synthesis of Compounds **4a**–**6c**

have been characterized.

Of the synthetic routes toward flavonoids, the most efficient was to direct Fries acylation, catalyzed by BF_3–Et_2O for 10 min under reflux, of trimethoxyphenol with substituted cinnamoyl chlorides, obtainable by *in-situ* treatment of the corresponding cinnamic acids with oxalyl chloride in dichloromethane at room temperature for 2 h, to produce the chalcone intermediates **1a**–**m** in excellent yields. Sequential oxidative cyclization of above-mentioned chalcone with iodine in dimethyl sulfoxide (DMSO) for 3 h under reflux led to the isolation of the desired substituted 5,6,7-trimethoxyflavonids **2a**–**m** in good overall yields which is illustrated in Chart 1. Of the products obtained, compounds **2a**–**j** were subject to exhaustive demethylation. Thus, treatment with 47% HBr in acetic acid (1 : 2, v/v) under reflux for 48 h, cooling, and addition of ice water and filtration gave precipitated crude products **3a**–**j** which were re-crystallized from ethanol to yield very pure compounds (Chart 2). The bromo-substituted **2k**, **2l**, and **2m** were subject to hydrolytic demethylation, dependent upon the meticulously controlled conditions, to yield various functionalized target flavonoids **4a**–**c**, **5a**–**c**, and **6a**–**c**, in excellent yields after purification by column chromatography on silica gel (Chart 3).

In most cases, crystallization or column chromatography was necessary for purification. The structures of synthetic intermediates and end products were established by various spectroscopy and the specific data of high resolution mass spectra.

In-Vitro **Anti-influenza Virus Activity** *In-vitro* anti-influenza virus activity was expressed as selective index (SI) which is the value of 50% cytotoxic concentration (CC_{50}) on Madin-Darby canine kidney (MDCK) cells divided by the 50% effective concentration (EC_{50}) on H1N1virus ($SI=CC_{50}/EC_{50}$). The higher the selective index is, the more potent or less toxic the test compound is.

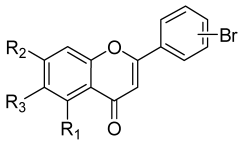
Even though flavonoids are a kind of well-known C6-C3-C6 phenylbenzopyrone scaffold, very few reports have been focused on the anti-influenza virus activities of several flavonoids derived from plants or Chinese medicines in the past years.[16–18]

In our previous report,[20] we have demonstrated that flavonoids with halide-substituents such as Cl on the B-ring consistently possessed stronger anti-influenza activity than baicalein, especially at the 4′-Cl but not at the 2′-Cl. This prompted us to systematically investigate the baicalein analogs with bromo-substituted B-rings, together with the hydroxyl functionality on the A-ring, to treat the infection of specific influenza viruses of H1N1 Tamiflu-resistant and seasonal H3N2 strains. Most compounds displayed salient anti-viral activities (Tables 1, 2).

Table 1 showed that the most potent compounds were **2l**, **4b**, **4c**, **5b**, **5c**, **6b**, and **6c** that revealed an effective anti-H1N1 Tamiflu-resistant virus activity with EC_{50} at around 4.0–4.5 μM and selectivity index >70 except for **2l** and **4b** (SI >8 and 16, respectively). Compounds **2k**, **4a**, **5a**, and **6a**, however, exhibited a slightly less inhibitory activity in the range of 8.6–16.0 μM against H1N1 Tamiflu-resistant virus. Among them, extensive placement of OCH_3 groups onto the A-ring of bromoflavones undesirably led to augmentation in cytotoxicity and reduction in inhibition (**2k**, **2l**, and **2m**). Interestingly, flavonoids with B-rings substituted with either 2′-Br or 3′-Br were not affected. Introduction of OH groups onto the A-ring of bromoflavones dramatically facilitated the inhibitory activity and diminished the cytotoxicity, no matter what positions of the bromine atom are on the B-rings (**5b, 5c, 6b** and **6c**), and thus seemed to be advantageous in general. The presence and appropriately positioning of these hydroxyl residues at C-5, C-7 and/or C-6 of the A-ring appeared to be critical determinants of anti-viral potency as was proved to be true in bromoflavones **4a**–**c**, **5a**–**c**, and **6a**–**c**. Substitution of a bromine atom at the *meta* or *para* position of the B-ring of 5,7-dihydroxy-6-methoxyflavone or 5,6,7-trihydroxyflavone (**5a**, **5b**, **5c**, **6a**, **6b**, and **6c**) resulted in highest *in vitro* anti-influenza virus activity against the H1N1 Tamiflu-resistant strain and selective indexes, even superior to ribavirin.

In the screening test for anti-H3N2 virus activity, all the synthetic flavonoids illustrated less potent. However, introduction of a Br substituent situated at *meta*- or *para*-position of the B-ring of 5,7-dihydroxy-6-methoxyflavone (**5a**, **5b**) result-

Table 1. *In Vitro* Anti-influenza Activities of Flavonoids with Bromo-Substituted B Rings in MDCK Cells Using CPE Assay[a,c]

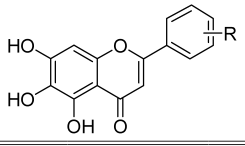


2k, **4a**, **5a**, **6a**: 2′-Br
2l, **4b**, **5b**, **6b**: 3′-Br
2m, **4c**, **5c**, **6c**: 4′-Br

Compd	R_1	R_2	R_3	H1N1 Tamiflu-resistant			H3N2		
				EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
2k	OCH_3	OCH_3	OCH_3	16.0	128.2	8.0	64.1	128.2	2.0
2l	OCH_3	OCH_3	OCH_3	4.0	32.1	>8.0	128.2	64.1	0.5
2m	OCH_3	OCH_3	OCH_3	192.3	128.2	0.7	—[b]	>300	—
4a	OH	OCH_3	OCH_3	16.1	133.0	8.3	133.0	199.5	1.5
4b	OH	OCH_3	OCH_3	4.2	66.5	>15.8	133.0	66.5	0.5
4c	OH	OCH_3	OCH_3	4.2	>300	>71.4	—	>300	—
5a	OH	OH	OCH_3	8.6	>300	>34.9	17.3	>300	>17.3
5b	OH	OH	OCH_3	4.3	>300	>69.8	17.3	>300	>17.3
5c	OH	OH	OCH_3	4.3	>300	>70.0	34.5	>300	>8.7
6a	OH	OH	OH	9.0	>300	>33.3	71.8	>300	>4.2
6b	OH	OH	OH	4.5	>300	>66.7	35.9	>300	>8.4
6c	OH	OH	OH	4.5	>300	>66.7	35.9	>300	>8.4
Baicalein				46.2	>300	>6.5	92.3	>300	>3.3
Baicalin				69.3	>300	>4.3	103.9	>300	>2.9
Ribavirin				25.6	>300	>11.7	25.6	>300	>11.7

a) EC_{50}: 50% effective concentration; CC_{50}: 50% cytotoxic concentration; SI (selective index)=CC_{50}/EC_{50}. *b*) —: cell viability below 50–75%. *c*) *In vitro* anti-H1N1 influenza virus activity of oseltamivir (Tamiflu): EC_{50}=32.0 μM, CC_{50}=320.1 μM, SI=10; oseltamivir is void of activity against H1N1 Tamiflu-resistant virus.

Table 2. *In Vitro* Anti-influenza Activities of Flavonoids **3** and **6** in MDCK Cells Using CPE Assay[a,c]



Compd	R	H1N1 Tamiflu-resistant virus		
		EC_{50} (μM)	CC_{50} (μM)	SI
3a	H	92.3	>300	>3.3
3b	2′-Cl	10.2	>300	>29.4
3c	3′-Cl	50.0	>300	>6.0
3d	4′-Cl	10.2	>300	>29.4
3e	2,′4′-$(Cl)_2$	73.7	>300	>4.1
3f	3,′4′-$(Cl)_2$	—[b]	—	—
3g	3′-Br, 4′-F	204.4	>300	>1.5
3h	4′-COOH	39.7	>300	>7.6
3i	4′-Ph	72.0	>300	>4.2
3j	4′-NO_2	18.1	>300	>16.6
6a	2′-Br	9.0	>300	>33.3
6b	3′-Br	4.5	>300	>66.7
6c	4′-Br	4.5	>300	>66.7
Baicalein	H	92.3	>300	>3.3
Ribavirin		25.6	>300	>11.7

a) EC_{50}: 50% effective concentration; CC_{50}: 50% cell cytotoxic concentration; SI (selective index)=CC_{50}/EC_{50}. *b*) —: cell viability below 50–75%. *c*) *In vitro* anti-H1N1 influenza virus activity of oseltamivir (Tamiflu): EC_{50}=32.0 μM, CC_{50}=320.1 μM, SI=10; oseltamivir is void of activity against H1N1 Tamiflu-resistant virus.

ed in better inhibition (EC_{50}=17.3 μM, SI >17.3) than ribavirin. All these compounds presented higher *in vitro* anti-H1N1 Tamiflu-resistant virus activity than anti-H3N2 virus activity.

At this stage, we turned to focus our effort on the substitution effect on the B-ring of 5,6,7-trihydroxyflavone (Table 2). Various functionalities with different electronic effects were thus introduced and examined their anti-H1N1 Tamiflu-resistant virus activity. Once again, all the flavonoids-displayed high anti-viral potency without significant cellular toxicity. However, their effective concentrations somehow appeared to distribute in a wide range. Particularly noteworthy is that, of the compounds tested, **6a**–**c** with a bromine atom attached on any position of the B-ring would exhibit a significantly higher inhibitory activity, in the range of 4.5–9.0 μM, than the others (**3a**–**j**). It is known that the bromine atom, owing to its intrinsic electronegativity, displays negative inductive effect, and among other things, would manifest a positive resonance effect after conjugation with aromatic rings by increasing the electronic density in the conjugated systems. Our favorable result distinctly revealed the significance of the impact of bromo-substitution in the B-ring, which was never found in the structures of naturally-occurring flavonoids, on the anti-H1N1 Tamiflu-resistant virus. Interestingly, paralleling the bromo-substituted flavonoids (**6a**–**c**), two of the monochloro substituted products (**3b** and **3d**) still demonstrated highly promising in terms of their prominent selective indexes. As mentioned above, introduction of a Br-substituent onto the B-ring of 5,6,7-trihydroxyflavone (**6a**–**c**) exhibited much better inhibition of H1N1 Tamiflu-resistant virus than the corresponding Cl-substituent (**3b**–**d**), especially on the 3′-position of the B-ring. Surprisingly, in the cases of the flavonoids substituted with two halogens on the B-rings (**3e**–**g**), all of them unexpectedly displayed rather poor anti-viral potency, even one of the substituted functionality was deliberately introduced as a bromine atom. Enhancement of the anti-H1N1 Tamiflu-resistant virus activity with *para* substitution on the

B-rings followed the order Br>Cl>NO_2>COOH>Ph. The influence of the electron-releasing or electron-withdrawing groups in the B-rings of the flavonoids is apparent and complex but nevertheless not very clear.

By analyzing the EC_{50} and CC_{50} values shown in the tables, we noticed that there existed two generalizations in the following structure–activity relationships: (1) the bromoflavonoids were generally more active and selective than the other flavonoids, even the corresponding chloro-flavonoids; (2) substitution of a bromine atom at any carbon in the B-rings, together with all free hydroxyls or with C6-OCH_3 in the A-rings, consistently led to tremendous improvements in activity and selectivity in the flavonoids.

Conclusion

We have successfully prepared a novel series of flavonoids, and demonstrated that certain members of the products possessed significant anti-influenza viral activity. It is noteworthy that the synthetic flavonoids with the bromine substituted on the B-ring and hydroxyls on the A-rings, consistently displayed extremely potent and very high selective indexes. This is the first report on systematic investigation about the bromo-bearing flavonids as novel anti-H1N1-TR-infected influenza virus in a MDCK cell line. Among the products obtained, the 3′-Br substituted (**5b**, **6b**) and the 4′-Br substituted (**5c**, **6c**) products have shown the most promising. We believed that the substituted bromine atoms might also contribute to the inhibition of resistance in viruses. Taking their facile syntheses into consideration, they were superior to the licensed oseltamivir (Tamiflu) in every experiment and might be favorable alternatives in the management of H1N1-TR-infected influenza in clinic practice. While the role of the bromine atom in the aromatic B-rings, however, is to be clarified, the results gleaned from this study have provided a basis to facilitate the design and further development of new agents for the treatment (prophylaxis and/or therapy) of influenza. We have chosen these four compounds (**5b**, **5c**, **6b**, **6c**) for further pharmacokinetic investigations and their preliminary results were also satisfactory. Detailed pharmacokinetic, pharmacological and toxicological studies are in progress and will be reported subsequently.

Experimental Section

Chemistry All the chemicals were purchased from Aldrich-Sigma Chemical Company (St. Louis, MO, U.S.A.), and Alfa-Aesar Chemical Company (Heysham, LA32XY, England) and used without further purification. All reactions were routinely monitored by TLC on Merck F254 silica gel plates. Merck silica gel (70–230 mesh) was used for chromatography. Melting points were measured on a Büchi-530 melting point apparatus. UV-VIS spectra were recorded on a Shimazu UV-160 A UV-Visble recording spectrophotometer. IR spectra were registered on a Perkin-Elmer FTIR 1610 series infrared spectrophotometer in KBr discs. The ^{1}H- and ^{13}C-NMR spectra were determined on a Varian Gemini-300 NMR instrument in DMSO-d_6 unless otherwise noted. Chemical shifts (δ) were reported as parts per million (ppm) downfield from tetramethylsilane (TMS) as the internal standard (σ 0.00), and coupling constants (J) were given in hertz (Hz). High resolution mass spectra (HR-MS) were performed in the Instrument Center of the National Science Counsel at the National Tsing-Hua University, Taiwan, using a Finnigan MAT-95XL. All the solvents and reagents were obtained from commercial sources and purified before use if necessary.

General Procedure for the Synthesis of Compounds 2a–m To a solution of cinnamic acid (10 mmol) in dichloromethane (30 mL) in an ice bath was added a mixture of oxalyl chloride (1.5 mL, 17.5 mmol) and dimethylformamide (2 drops). After the mixture was stirred for 2 h at room temperature, the solvent was removed under reduced pressure to give cinnamoyl chloride which was mixed with 3,4,5-trimethoxyphenol (1.8 g, 10 mmol) and then dissolved in boron trifluoride etherate (10 mL) and heated to reflux for 10 min. After cooling, the mixture was poured into ice water and then the precipitate was filtered and washed with water until free from acid. The resulting precipitate was washed with hexane and then with ether to obtain the corresponding chalcones **1a**–**m** (89–97%) as orange-yellow powders. A mixture of chalcones **1a**–**m** (10 mmol) and iodine (1.0 eq) in DMSO (25 mL) was heated to reflux for 3 h. After cooling, the mixture was poured into ice water. The precipitate was filtered and washed with saturated sodium thiosulfate solution. The residue was chromatographed on a silica gel column with hexane/EtOAc (2 : 1) as the eluent to give pure **2a**–**m** as pale yellow solids. The preparation of **3a**–**j** (Chart 2) were essentially according to our published procedure.[20] All the physicochemical and spectroscopic characteristics of products **2a**–**j** and **3a**–**j** are in agreement with those reported.[20]

2-(2-Bromophenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one (2k) Yield: 61%. mp: 122–123°C. UV λ_{max} (MeOH) nm ($\log\varepsilon$): 215 (4.56). IR (KBr) ν cm^{-1}: 2994, 2934, 2346, 1648, 1604. HR-electron impact (EI)-MS m/z: 390.0110 (M^+), Calcd for $C_{18}H_{15}BrO_5$: 390.0103. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.77 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.34 (1H, s, ArH), 7.07 (1H, s, CHCO), 7.48–7.59 (2H, m, ArH), 7.71 (1H, dd, J=9.5, 5.7 Hz, ArH), 7.82 (1H, dd, J=9.5, 5.7 Hz, ArH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.4, 60.9, 61.7, 97.1, 112.1, 112.7, 121.1, 128.1, 131.2, 132.4, 133.2, 133.4, 140.1, 151.8, 154.4, 157.9, 161.2, 175.3.

2-(3-Bromophenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one (2l) Yield: 59%. mp: 190–191°C. UV λ_{max} (MeOH) nm ($\log\varepsilon$): 265 (4.24), 216 (4.48). IR (KBr) ν cm^{-1}: 3088, 2941, 2834, 1654, 1604. HR-EI-MS m/z: 390.0095 (M^+), Calcd for $C_{18}H_{15}BrO_5$: 390.0103. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.77 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 6.89 (1H, s, ArH), 7.30 (1H, s, CHCO), 7.51 (1H, t, J=8.3 Hz, ArH), 7.77 (1H, dd, J=8.3 Hz, 1.2 Hz, ArH), 8.07 (1H, d, J=8.3 Hz, ArH), 8.27 (1H, t, J=2.0 Hz, ArH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.4, 60.8, 61.6, 97.4, 108.3, 112.1, 122.4, 125.0, 128.4, 131.0, 133.3, 134.0, 140.0, 151.5, 153.93, 157.7, 158.5, 175.5.

2-(4-Bromophenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one (2m) Yield: 67%. mp: 165–166°C. UV λ_{max} (MeOH) nm ($\log\varepsilon$): 310 (4.34), 268 (3.32), 216 (4.50). IR (KBr) ν cm^{-1}: 3084, 2945, 2841, 1656, 1604. HR-EI-MS m/z: 390.0110 (M^+), Calcd for $C_{18}H_{15}BrO_5$: 390.0103. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.76 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 6.86 (1H, s, ArH), 7.23 (1H, s, CHCO), 7.76 (2H, d, J=8.6 Hz, ArH), 8.01 (2H, d, J=8.6 Hz, ArH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.3, 60.8, 61.6, 97.2, 107.8, 112.1, 125.0, 127.9, 130.2, 132.0, 134.0, 151.6, 153.9, 157.7, 159.2, 175.6.

General Procedure for the Synthesis of Compounds 4a–c Flavones **2k–m** (1.0 mmol) were placed in BF_3–Et_2O (3 mL) and then heated to reflux for 45 min, respectively. After cooling, the mixture was poured into ice water. The precipitate was filtered and washed with water. The residue was chromatographed on a silica gel column with hexane/EtOAc (3 : 1) as the eluent to give pure **4a–c** as pale yellow solids.

2-(2-Bromophenyl)-5-hydroxy-6,7-dimethoxy-4*H*-chromen-4-one (4a) Yield: 69%. mp: 221–222°C. UV λ_{max} (MeOH) nm (logε): 211 (4.50). IR (KBr) ν cm^{-1}: 3092, 3023, 2945, 1645. HR-EI-MS *m/z*: 375.9946 (M^+), Calcd for $C_{17}H_{13}BrO_5$: 375.9946. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.74 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 6.59 (1H, s, ArH), 6.85 (1H, s, CHCO), 7.50–7.60 (2H, m, ArH), 7.73 (1H, dd, *J*=9.2, 5.9 Hz, ArH), 7.84 (1H, dd, *J*=9.2, 5.9 Hz, ArH), 12.61 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.4, 59.9, 91.6, 105.3, 110.2, 120.9, 128.1, 131.4, 132.4, 132.7, 133.0, 133.5, 152.1, 153.2, 159.2, 164.4, 182.0.

2-(3-Bromophenyl)-5-hydroxy-6,7-dimethoxy-4*H*-chromen-4-one (4b) Yield: 64%. mp: 188–189°C. UV λ_{max} (MeOH) nm (logε): 272 (4.26), 215 (4.41). IR (KBr) ν cm^{-1}: 3071, 2931, 2836, 1654, 1622. HR-EI-MS *m/z*: 375.9933 (M^+), Calcd for $C_{17}H_{13}BrO_5$: 375.9946. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.73 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 7.03 (1H, s, ArH), 7.11 (1H, s, CHCO), 7.52 (1H, t, *J*=8.1 Hz, ArH), 7.80 (1H, dd, *J*=8.1 Hz, 1.5 Hz, ArH), 8.10 (1H, d, *J*=8.1 Hz, ArH), 8.29 (1H, t, *J*=1.8 Hz, ArH), 12.66 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.4, 59.9, 91.8, 105.4, 105.8, 122.4, 125.4, 128.8, 131.1, 132.2, 133.0, 134.6, 152.0, 152.7, 159.0, 161.7, 182.3.

2-(4-Bromophenyl)-5-hydroxy-6,7-dimethoxy-4*H*-chromen-4-one (4c) Yield: 72%. mp: 206–208°C. UV λ_{max} (MeOH) nm (logε): 278 (4.19), 215 (4.30). IR (KBr) ν cm^{-1}: 2919, 2852, 1670, 1624. HR-EI-MS *m/z*: 375.9929 (M^+), Calcd for $C_{17}H_{13}BrO_5$: 375.9946. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.73 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 6.99 (1H, s, ArH), 7.08 (1H, s, CHCO), 7.79 (2H, d, *J*=8.6 Hz, ArH), 8.05 (2H, d, *J*=8.6 Hz, ArH), 12.70 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.4, 59.9, 91.7, 105.3, 105.4, 125.8, 128.3, 129.6, 129.9, 132.1, 152.0, 152.8, 159.0, 162.5, 182.3.

General Procedure for the Synthesis of Compounds 5a–c To a solution of flavones **2k–m** (1.0 mmol) in glacial acetic acid (20 mL) in the ice bath was added 47% hydrobromic acid (10 mL). The mixture was heated to reflux for 2.5 h. After cooling, the mixture was poured into ice water. The resulting precipitate was filtered and washed with water until free from acid as monitored by the pH paper. Re-crystallization from ethanol afforded pure **5a–c** as yellow solids.

2-(2-Bromophenyl)-5,7-dihydroxy-6-methoxy-4*H*-chromen-4-one (5a) Yield: 77%. mp: 161–162°C. UV λ_{max} (MeOH) nm (logε): 211 (4.54). IR (KBr) ν cm^{-1}: 3092, 3066, 3005, 1680, 1622. HR-EI-MS *m/z*: 361.9791 (M^+), Calcd for $C_{16}H_{11}BrO_5$: 361.9790. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.89 (3H, s, OCH_3), 6.53 (1H, s, ArH), 6.83 (1H, s, CHCO), 7.51–7.58 (2H, m, ArH), 7.73 (1H, dd, *J*=6.3, 1.8 Hz, ArH), 7.83 (1H, dd, *J*=5.4 Hz, 1.5 Hz, ArH), 8.83 (1H, s, OH), 12.34 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.3, 91.2, 105.2, 109.9, 121.0, 128.1, 130.4, 131.3, 132.6, 133.2, 133.5, 146.3, 150.2, 154.9, 164.1, 182.0.

2-(3-Bromophenyl)-5,7-dihydroxy-6-methoxy-4*H*-chromen-4-one (5b) Yield: 72%. mp: 249–250°C. UV λ_{max} (MeOH) nm (logε): 276 (4.33), 216 (4.46). IR (KBr) ν cm^{-1}: 3588, 3061, 2363, 1670, 1615. HR-EI-MS *m/z*: 361.9789 (M^+), Calcd for $C_{16}H_{11}BrO_5$: 361.9790. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.92 (3H, s, OCH_3), 7.02 (1H, s, ArH), 7.07 (1H, s, CHCO), 7.52 (1H, t, *J*=8.0 Hz, ArH), 7.79 (1H, dd, *J*=9.3, 6.9 Hz, ArH), 8.10 (1H, d, *J*=8.1 Hz, ArH), 8.29 (1H, t, *J*=1.8 Hz, ArH), 8.77 (1H, s, OH), 12.40 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.2, 91.4, 105.4, 105.5, 122.4, 125.3, 128.7, 130.2, 131.1, 133.2, 134.4, 146.2, 149.8, 154.8, 161.4, 182.2.

2-(4-Bromophenyl)-5,7-dihydroxy-6-methoxy-4*H*-chromen-4-one (5c) Yield: 85%. mp: 275–276°C. UV λ_{max} (MeOH) nm (logε): 282 (4.16), 218 (4.08). IR (KBr) ν cm^{-1}: 3370, 3084, 2364, 2321, 1669, 1609. HR-EI-MS *m/z*: 361.9792 (M^+), Calcd for $C_{16}H_{11}BrO_5$: 361.9790. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.91 (3H, s, OCH_3), 6.96 (1H, s, ArH), 7.04 (1H, s, CHCO), 7.79 (2H, d, *J*=8.9 Hz, ArH), 8.04 (2H, d, *J*=8.9 Hz, ArH), 8.79 (1H, s, OH), 12.70 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 56.2, 91.3, 105.0, 105.3, 125.6, 128.2, 130.2, 130.2, 132.1, 146.2, 149.8, 154.8, 162.1, 182.3.

General Procedure for the Synthesis of Compounds 6a–c To a solution of flavones **2k–m** (1.0 mmol) in glacial acetic acid (20 mL) in the ice bath was added 47% hydrobromic acid (10 mL). The mixture was heated to reflux for 48–54 h. After cooling, the mixture was poured into ice water. The resulting precipitate was filtered and washed with water until free from acid as monitored by the pH paper. Re-crystallization from ethanol afforded pure **6a–c** as yellow-brown solids.

2-(2-Bromophenyl)-5,6,7-trihydroxy-4*H*-chromen-4-one (6a) Yield: 75%. mp: 249–251°C. UV λ_{max} (MeOH) nm (logε): 213 (4.55). IR (KBr) ν cm^{-1}: 3416, 3378, 3053, 1665, 1618. HR-EI-MS *m/z*: 347.9637 (M^+), Calcd for $C_{15}H_9BrO_5$: 347.9633. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.46 (1H, s, ArH), 6.50 (1H, s, CHCO), 7.48–7.59 (2H, m, ArH), 7.71 (1H, dd, *J*=9.0, 5.7 Hz, ArH), 7.82 (1H, sd, *J*=8.3 Hz, ArH), 8.82 (1H, s, OH), 10.59 (1H, s, OH), 12.48 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 93.9, 104.2, 109.6, 121.0, 128.1, 129.5, 131.3, 132.6, 133.3, 133.5, 147.1, 150.2, 153.9, 163.8, 181.8.

2-(3-Bromophenyl)-5,6,7-trihydroxy-4*H*-chromen-4-one (6b) Yield: 72%. mp: 255–256°C. UV λ_{max} (MeOH) nm (logε): 276 (4.23), 211 (4.41). IR (KBr) ν cm^{-1}: 3358, 3100, 2893.0, 1654, 1618. HR-EI-MS *m/z*: 347.9631 (M^+), Calcd for $C_{15}H_9BrO_5$: 347.9633. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.65 (1H, s, ArH), 7.00 (1H, s, CHCO), 7.51 (1H, t, *J*=8.1 Hz, ArH), 7.78 (1H, dd, *J*=8.1, 1.5 Hz, ArH), 8.06 (1H, dd, *J*=8.1, 1.5 Hz, ArH), 8.24 (1H, t, *J*=1.5 Hz, ArH), 8.83 (1H, s, OH), 10.59 (1H, s, OH), 12.56 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 94.1, 104.3, 105.4, 122.4, 125.3, 128.7, 129.4, 131.1, 133.4, 134.3, 147.0, 149.9, 153.8, 161.2, 182.0.

2-(4-Bromophenyl)-5,6,7-trihydroxy-4*H*-chromen-4-one (6c) Yield: 80%. mp: 301–303°C. UV λ_{max} (MeOH) nm (logε): 265 (4.19), 216 (4.27). IR (KBr) ν cm^{-1}: 3412, 3102, 1654, 1618. HR-EI-MS *m/z*: 347.9642 (M^+), Calcd for $C_{15}H_9BrO_5$: 347.9633. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.61 (1H, s, ArH), 6.96 (1H, s, CHCO), 7.76 (2H, dd, *J*=7.8 Hz, 1.8 Hz, ArH), 8.01 (2H, dd, *J*=6.9, 2.1 Hz, ArH), 8.80 (1H, s, OH), 10.57 (1H, s, OH), 12.58 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 94.0, 104.4, 104.8, 125.5, 128.2, 129.0, 129.4, 130.3, 131.4, 132.1, 147.1, 149.9, 153.8, 161.9, 182.1.

Assay of Cytopathic Effect (CPE) The anti-viral activity of flavonoids was measured by the CPE assay and using ribavirin as a positive control. The CPE inhibition assays used

in this study were performed as described previously.[24] Two influenza virus strains, namely Tamiflu-resistant 2009 pandemic influenza A (H1N1) virus, which detected the H275Y mutation (N1 numbering) in neuraminidase, and influenza A/New York/469/2004-like flu (H3N2) virus, were provided by Centers for Disease Control (CDC), Taiwan, and adapted for evaluating the *in vitro* anti-viral activities of the synthetic flavonoids.

In brief, virus at 100 tissue culture infectious dose ($TCID_{50}$) were inoculated onto near confluent MDCK cell monolayers (1×10^5 cells/well) for 1 h. After being incubated at 37°C for 2 h, the virus solution was removed, and 100 μL sequential 2-fold serial dilutions of the respective flavonoids and reference compound ribavirin were added to each well of the 96-well culture plates, using the maximal non-cytotoxic concentration (MNCC, *i.e.*, 90% viable cells) as the highest concentration. An infection control without flavonoids was also included. The plates were incubated at 37°C in a 75% humidity of 5% CO_2 atmosphere for 24 h, and then the CPE was observed. The virus-induced CPE was scored as follows: scores: 0=0% CPE, 1=0–25% CPE, 2=25–50% CPE, 3=50–75% CPE, and 4=75–100% CPE. The reduction in virus multiplication was calculated as a percentage of the virus control (% virus control=CPEexp/CPEvirus control×100). The IC_{50} of the CPE with respect to virus control was estimated using the Reed–Muench method and was expressed in μM. The selectivity index (SI) was calculated from the ratio CC_{50}/IC_{50}.

Statistical Analysis

Statistical calculations were carried out with Microsoft Excel 2007 version. Results are expressed as the means±S.D. of six independent experiments.

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