Anti-Influenza Drug Discovery: Structure—Activity Relationship and Mechanistic Insight into Novel Angelicin Derivatives

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By using a cell-based high throughput screening campaign, a novel angelicin derivative 6a was identified to inhibit influenza A (H1N1) virus induced cytopathic effect in Madin—Darby canine kidney cell culture in low micromolar range. Detailed structure—activity relationship studies of 6a revealed that the angelicin scaffold is essential for activity in pharmacophore B, while meta-substituted phenyl/2-thiophene rings are optimal in pharmacophore A and C. The optimized lead 4-methyl-9-phenyl-8-(thiophene-2-carbonyl)-furo[2,3-h]chromen-2-one (8g, I C50 = 70 nM) showed 64-fold enhanced activity compared to the high throughput screening (HTS) hit 6a. Also, 8g was found effective in case of influenza A (H3N2) and influenza B virus strains similar to approved anti-influenza drug zanamivir (4). Preliminary mechanistic studies suggest that these compounds act as anti-influenza agents by inhibiting ribonucleoprotein (RNP) complex associated activity and have the potential to be developed further, which could form the basis for developing additional defense against influenza pandemics.

Introduction

Influenza, usually known as flu, is an infectious disease of birds and mammals caused by ribonucleic acid (RNA) viruses of the family Orthomyxoviridae. It is a contagious, acute, febrile, respiratory disease, which occurs seasonally in epidemics and sometimes in pandemic proportions. On the basis of the serological subtyping, three distinct types A, B, and C are present, of which influenza A and B are of much concern as human pathogens. Further, on the basis of the antigenicity of the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), a total of 16 HA (H1—H16) and 9 NA (N1—N9) subtypes are known, resulting in the possibility of distinct strains of influenza virus such as H1N1, H1N2,..., H2N2,..., and so on. 1,2 Influenza epidemic and pandemics in the past century had caused serious impact on global morbidity, mortality, and economy. For example, the “Spanish flu” (H1N1) outbreak of 1918–1919 resulted in the death of at least 20 million people, and the Asian influenza (H2N2) in 1957 had caused the death of 1 million people worldwide. Besides, Hong Kong influenza (H3N2) in 1968 had reached pandemic proportions. 3–5 The recent repeated lethal outbreaks (2007) of the virulent H5N1 avian influenza strains in humans has caused global health threat, as they may adapt to become easily transmissible from human to human, which could cause serious pandemics. 6–8 Currently (2009), swine influenza A (H1N1) outbreak in Mexico and other parts of the world has led to issuances of pandemic alertness by the WHO. 9 As the impact of influenza pandemic is enormous, a renewed drug discovery effort worldwide is essential to counteract the disease more efficiently.

Presently vaccination is the mainstay, along with anti-influenza drugs, to control the spread and treatment of the disease. Because influenza virus continuously evolves to avoid host detection systems by changing its antigenicity through mutation of its surface glycoprotein (NA, HA) genes by a process called “antigenic drift”, it could reinfect the same host detection systems by changing its antigenicity through mutation of its surface glycoprotein (NA, HA) genes by a process called “antigenic drift”, it could reinfect the same
However, as there is a time gap of 9–12 months from the recommendation to the actual use of vaccine, this could still result in mismatch between the virus and the vaccine, which could result in ineffectiveness. Because of these and other complicating factors influencing the development of an all effective flu vaccine, additional weapons in the form of effective anti-influenza agents are a must in our fight against seasonal or pandemic influenza that may arise in the future. Adamantane derivatives (amantadine) and (rimantadine) are the first two antiviral drugs used for the treatment of influenza, which target the M2 ion channels (Figure 1). Recently, orally administered (oseltamivir) and inhaled (zanamivir) are approved for use as anti-influenza treatments, while (peramivir) is under clinical investigation; all the three compounds target the neuraminidase (NA). However, alternative therapeutics are needed because the development of resistance strains to these drugs targeting NA enzyme, particularly 3, has been reported as an increasing clinical problem. Thus there is an urgent need to identify novel compounds with inhibitory activity against influenza virus, which is the aim of this study.

Using a cell-based assay, high throughput screening (HTS) was carried out to identify novel compounds as anti-influenza agents. The HTS assay measured the ability of the test compounds to inhibit the virus-induced cytopathic effect (CPE) on MDCK (Madin–Darby canine kidney) cells and the results expressed as IC₅₀ (concentration required to reduce CPE by 50% relative to the virus control). Also, a concurrent assay to determine the general cytotoxicity of the compounds on MDCK cells were performed without virus infection and the results expressed as CC₅₀ (concentration required to produce 50% cell death). Cytotoxicity measurement was performed to identify compounds which selectively inhibit virus replication without killing infected cells. The ratio between CC₅₀ and IC₅₀ is an indicator of toxicity potential of a compound and could guide in selecting appropriate compounds for further development. Through the HTS screening of about 20000 compounds, we have discovered a novel angular coumarin derivative 6a (Figure 1) to inhibit influenza virus, with an IC₅₀ of 4.5 μM and a CC₅₀ > 25 μM. Literature survey revealed that linear furanocoumarin, in which the furan ring is attached to the 6,7 positions of the coumarin ring, such as psoralen, is highly phototoxic to many organisms due to their ability to cross-link deoxyribonucleic acid (DNA) after photoactivation. An angular furanocoumarin such as angelicin, in which the furan ring is attached to the 7,8 positions of the coumarin ring, because of their geometry cannot cross-link with DNA, is less phototoxic. To our knowledge, so far angelicin derivatives closely related to 6a are not reported as antiviral agents, let alone anti-influenza agents. Moreover, only a few reports in the literature report the synthesis of 6a and related compounds with angelicin scaffold, making the synthetic exploration interesting. On the basis of structural novelty and the necessity to develop anti-influenza agents to combat any future influenza pandemic, we started a MedChem program to investigate the potential of this novel hit 6a, the results of which are described herein.

Chemistry

For the construction of the most of the desired angelicin derivatives 6–10, appropriately substituted 7-hydroxycoumarin 11 (commercial source or synthesized by Pechmann condensation of meta-hydroxy phenol with β-ketoesters under acidic condition as reported by Lee et al.) was O-acylated with acid chloride (R₄COCl) to give 12. Fries rearrangement of 12 under acidic condition using anhydrous AlCl₃ at elevated temperature gave the key intermediate 8-acyl substituted coumarins 13. The desired angelicin derivatives 6–10 were constructed from 13 and appropriate 2-bromo ketones (R₅COCH₂Br) via a single step O-alkylation–cyclization–elimination process under basic condition with an overall yield of 15–44% over three steps (Scheme 1).

For the preparation of 6b, coumarin 11a was first iodinated and then reacted with 1-phenyl-2-propyn-1-ol using copper mediated ring annulation to construct the furan ring of 15. Oxidation of the secondary alcohol group of 15 using Jones reagent provided the desired angelicin derivative 6b in 37% yield over three steps. For the construction of compounds 8c and 8d without the ketone group, coumarin 11a

Figure 1. Inhibitors of influenza virus: M2 ion channel inhibitors (1 and 2), NA inhibitors (3–5), and HTS angelicin hit 6a.

Scheme 1. General Synthetic Method for the Preparation of Angelicin Derivatives 6–10

![Scheme 1](image-url)
was alkylated with either 2-bromoacetophenone or 2-bromo-2-phenylacetophenone to give the intermediates 16a and 16b, respectively. Cyclization under acidic conditions afforded the desired products 8c, d with a yield of 18–21% over two steps (8c from 16a; 8d from 16b).

For the preparation of lactone ring opened analogue 7a, hydrogen abstraction by n-BuLi from MOM protected 3-hydroxy anisole22 (17), followed by acylation with benzoyl chloride, and then MOM deprotection in acidic conditions gave 18a. Condensation of 2-bromoacetophenone with 18a under basic conditions afforded 19. Demethylation using BBr₃ to give 20, followed by acylation with crotonic anhydride, gave the desired 7a. For the preparation compound 7b without the lactone ring, commercially available 18b was condensed with 2-bromoacetophenone to afford 7b in 93% yield (Scheme 3).

Preparation of 7c without the lactone ring was carried out as shown in Scheme 4. 2-Naphthol (21) was the first methylated using MeI to give 22, then Fridel–Crafts acylation with benzoyl chloride gave 23. Without purification, 23 was cyclized with 2-bromoacetophenone in basic conditions to afford 7b in 8% yield over three steps (Scheme 4). For the preparation of bioisosteric thienocoumarin derivative 7l, Newman–Kwart thermal rearrangement was utilized to obtain the thiophenol intermediate 26 from phenol 13a. Condensation of 26 with 2-bromoacetophenone afforded the desired compound 7l, in an overall yield of 22% in four steps starting from 13a (Scheme 5).

Reduction of the ketone group of 6f by sodium borohydride in THF gave the secondary alcohol 8a. Alternatively, when the reduction process was carried out in the presence of TFA, complete reduction of the keto function to the methylene group was achieved to get the compound 8b (Scheme 6). The phenol 8u was obtained from the methoxy derivative 8p by demethylation using BBr₃. The analogues 9a–f were synthesized by alklylation of the phenol 8u under basic conditions using the required bromo compound (R²Br). In the case of compounds 9g,h, TBDMS protected bromoalkanol (R²Br) was used and deprotection using TBAF in the final step unmasked the OH group (Scheme 7).

Results and Discussion

We initiated structure–activity relationship (SAR) studies to explore the pharmacophore (molecular framework responsible for a drug’s biological activity) requirements for antinfluenza activity based on the HTS hit 6a. For convenience of SAR discussion, the lead molecule 6a is divided into three pharmacophore parts: A (3-methyl group on furan ring), B (angelicin core), and C (2-benzoyl group on furan ring) (Figure 1). In the pharmacophore A (Table 1), removal of the methyl group led to slightly better activity for 6b than the parent compound 6a. However, increasing the alkyl chain

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**Scheme 2. Synthesis of Angelicin Derivatives 6b and 8c,d**

\[ \text{(a) I}_{2}, \text{HIO}_{4} / \text{EtOH}, \text{2 h, rt, 76\%; (b) 1-phenyl-2-propyn-1-ol, Cul, Et}_{3} \text{N, DMF, 60 °C, 24 h, 51\%; (c) Jones reagent, acetone, 0 °C \rightarrow \text{rt, 0.5 h, 95\%; (d) K}_{2} \text{CO}_{3}, \text{CH}_{3} \text{CN, reflux, 16 h; (e) H}_{3} \text{PO}_{4}, \text{xylene, reflux, 16 h, 18–21\% over two steps (8c from 16a; 8d from 16b).} } \]

**Scheme 3. Synthesis of Derivatives 7a,b**

\[ \text{(a) (i) n-BuLi, PhCOCl, THF, 0 °C \rightarrow \text{rt, 2 h; (ii) MeOH, HCl, reflux, 0.5 h, 75\%; (b) PhCOCH}_{2} \text{Br, K}_{2} \text{CO}_{3}, \text{CH}_{3} \text{CN, reflux, 16 h, 72\% for 19 from 18a, 93\% for 7b from 18b. (c) BBr}_{3}, \text{CH}_{2} \text{Cl}_{2}, 0 °C \rightarrow \text{rt, 12 h, 80\%. (d) Crotonic anhydride, pyridine, imidazole, rt, 2 h, 81\%).} ] \]

**Scheme 4. Synthesis of Derivative 7c**

\[ \text{(a) NaH, MeI, THF, 0 °C \rightarrow \text{rt, 12 h; (b) AlCl}_{3}, \text{CH}_{2} \text{Cl}_{2}, \text{PhCOCl, 16 h; (c) PhCOCH}_{2} \text{Br, K}_{2} \text{CO}_{3}, \text{CH}_{3} \text{CN, reflux, 16 h, 8\% over three steps.} } \]
length resulted in significant improvement in activity, with the n-propyl (6c) and n-hexyl (6d) analogues showing nanomolar level activity. However, increasing the length further decreased the activity, with the n-decyl (6e) analogue, showed complete loss of activity. Next we replaced the methyl group of the lead compound 6a with various aromatic and heteroaromatic rings such as phenyl (6f), naphthyl (6g, 6h), thiophene (6i), and furan (6j). All the five analogues showed improved activity compared to the HTS hit 6a, showing that aromatic hydrophobic group at this position is preferred. Of particular interest are the phenyl analogue 6f (IC50 = 140 nM) and the bioisosteric equivalent 2-thiophene analogue 6i (IC50 = 150 nM), both of which showed over 30-fold improved activity compared to the HTS hit 6a.

With the identification of potent compound 6f, we continued the search for optimal phenyl ring substitution. Initial attempts to introduce electron donating CH3/OCH3 (6k,l) or electron withdrawing NO2/Cl/Br groups (6m–o) at the para-position of the phenyl group of 6f, led to the conclusion that the para-Br substitution is better. Furthermore, it was found that when the Br substitution was moved to meta-position in 6p, the activity improved almost 2-fold of that of the unsubstituted compound 6f, with the IC50 reaching a value of 80 nM. However Br substitution in ortho-position (6q) of the phenyl ring led to decreased activity. Moreover, di-Br substituted compound 6r did not show further improvement in activity. Because the meta-Br (6p) compound showed higher potency than para-Br (6o) and ortho-Br (6q) analogues, meta-CH3 (6s), meta-NO2 (6t), meta-Cl (6u), and meta-CN (6v) derivatives were prepared. Even though these meta-substituted analogues (6s–u) are more potent than their

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**Scheme 5. Synthesis of Bioisosteric Thienocoumarin Derivative 71**

(a) Dimethylthiocarbamoyl chloride, NaH, DMAP, THF, rt, 24 h, 84%; (b) mesitylene, 280 °C, sealed tube, 2 h, 65%; (c) NaOCH3, MeOH, reflux, 1 h; (d) PhCOCH2Br, K2CO3, CH3CN, reflux, 16 h, 40% over two steps.

**Scheme 6. Synthesis of Angelicin Derivatives 8a,b**

(a) NaBH4, THF, 0 °C, 16 h, 60%; (b) NaBH4, THF, TFA, 0 °C, 16 h, 72%.

**Scheme 7. Synthesis of Angelicin Derivatives 8u and 9a–h**

(a) BBr3, CH2Cl2, 2 h, rt, 92%; (b) for 9a–f: R’Br (R’ = CH3 or (CH2)nCH;CH3), K2CO3, KI, CH3CN, reflux, 16 h, 25–38%; for 9g–h: (i) R’Br (R’ = TBDMOS(CH2)n), K2CO3, KI, CH3CN, reflux, 16 h; (ii) TBAF, THF, 0 °C, 1.5 h, 15–19%.

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**Table 1. Optimization of Pharmacophore A**

<table>
<thead>
<tr>
<th>compd</th>
<th>R1</th>
<th>IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>CH3</td>
<td>4.51 ± 0.24</td>
</tr>
<tr>
<td>6b</td>
<td>H</td>
<td>2.43 ± 0.31</td>
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<tr>
<td>6c</td>
<td>n-C3H7</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>6d</td>
<td>n-C5H11</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>6e</td>
<td>n-C6H13</td>
<td>&gt;25</td>
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<tr>
<td>6f</td>
<td>Ph</td>
<td>0.14 ± 0.01</td>
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<tr>
<td>6g</td>
<td>1-naphthyl</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>6h</td>
<td>2-naphthyl</td>
<td>1.91 ± 0.23</td>
</tr>
<tr>
<td>6i</td>
<td>2-thienyl</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>6j</td>
<td>2-furan</td>
<td>0.65 ± 0.18</td>
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<tr>
<td>6k</td>
<td>4-CH3-Ph</td>
<td>0.82 ± 0.10</td>
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<tr>
<td>6l</td>
<td>4-CH3O-Ph</td>
<td>10.08 ± 0.47</td>
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<tr>
<td>6m</td>
<td>4-NO2-Ph</td>
<td>0.76 ± 0.12</td>
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<tr>
<td>6n</td>
<td>4-CN-Ph</td>
<td>3.92 ± 1.70</td>
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<tr>
<td>6o</td>
<td>4-Br-Ph</td>
<td>0.46 ± 0.11</td>
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<tr>
<td>6p</td>
<td>3-Br-Ph</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>6q</td>
<td>2-Br-Ph</td>
<td>0.73 ± 0.03</td>
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<tr>
<td>6r</td>
<td>3,5-diBr-Ph</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>6s</td>
<td>3-CH3-Ph</td>
<td>0.10 ± 0.03</td>
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<tr>
<td>6t</td>
<td>3-NO2-Ph</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>6u</td>
<td>3-Cl-Ph</td>
<td>1.41 ± 0.04</td>
</tr>
<tr>
<td>6v</td>
<td>3-CN-Ph</td>
<td>0.53 ± 0.02</td>
</tr>
</tbody>
</table>

*Using influenza A/WSN/33 (H1N1) strain. **All the compounds exhibited CC50 > 25 μM. ***The concentration required for a test compound to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control. Values are expressed as the mean of at least two independent determinations, each experiments performed in triplicates. "Synthesis 6i was not possible by the standard method A reported, due to demethylation of the methoxy group during Fries rearrangement. Hence an alternative synthetic route for this and related compounds were developed, which will be reported separately.
expressed as IC50. Values are expressed as the mean of at least two independent determinations, each experiments performed in triplicates.

These results suggest that substitution in meta position could help in orienting the phenyl ring at an angle to that of the flat furanocoumarin ring, which could result in better interaction with the active site.

For the pharmacophore B (angelicin core) modification (Table 2), we have used compound 6f as our template to which all structural modification were compared. When we attempted to either open (7a), remove (7b), or replace the lactone ring with a phenyl ring (7e), complete loss of activity was observed. This showed the critical requirement of the coumarin ring in maintaining the activity. Next, by retaining the coumarin ring intact, we tried to find the effect of substitution at various positions. Removal of the 4-methyl group (7d) or shifting it to the 3-position (7e) or the 5-position (7f) of the coumarin ring led to decreased activity compared to 6f. Particularly, removal of the 4-position methyl group of 6f had led to drastic loss of activity in 7d. It indicates that the methyl substituent at C-4 position has an essential role in maintaining activity. Next, replacing the methyl group with more bulky ethyl (7g), propyl (7h), and phenyl (7i) groups decreased the activity, with the phenyl substituent 7i being completely inactive, showing that methyl group is optimal for activity at the 4-position of the coumarin ring. By retaining the essential 4-methyl group, we then introduced an additional 3-methyl group (7j) in the coumarin ring, which resulted in retention of activity with only 2-fold loss of activity. However, cyclization of 3- and 4-position of coumarin ring (7k) through a cyclohexyl ring led to complete loss of activity. Also, we investigated the effect of replacing the furan ring with thiophene ring, which led to 5-fold loss of activity for 7l compared to that of 6f. Thus SAR exploration at pharmacophore B clearly shows that the angelicin scaffold is essential and the 4-position methyl group is critical and optimal for activity.

Next, we turned our attention to pharmacophore C (2-benzoyl group on furan ring) modifications (Table 3). To examine the role of ketone group of lead 6f, the ketone group was reduced to a secondary alcohol (8a) or to methylene (8b) group, which led to 2–4-fold loss of activity. However, either removal of 2-benzoyl group completely (8c), attaching the phenyl group directly to the furan ring (8d), or replacement of phenyl group with methyl group (8e), led to complete loss of activity. Compounds 8a–b, which had the phenyl group attached to the furan ring through one-carbon linker showed only a maximum of 2–4-fold loss of activity compared to 6f; while compounds 8c–e, either without the phenyl group or with the phenyl group at different spatial orientation than lead 6f showed complete loss of activity. This clearly shows the importance of 2-benzoyl group attached to the angelicin scaffold for activity.

Further, we attempted to replace the phenyl group of 6f on pharmacophore C with different heteroaromatic groups such as furan (8f), thiophene (8g,8h), and pyridine (8i). 2-Thiophene analogue 8g (IC50 = 70 nM) is of particular interest, which showed a 2-fold improvement in activity than the phenyl analogue 6f. However, substitution on the thiophene
ring of 8g did not further improve the activity (data not shown). Hence, substitution effect on the phenyl ring of pharmacophore C was explored in order to identify optimal substituent in this region. Introduction of electron donating CH3/OCH3 groups in the para-position led to decrease in activity, while introduction of electron withdrawing NO2/Cl (8l,m) groups in the para-position of the phenyl group led to drastic loss of activity compared to the unsubstituted 6f. When the electron donating CH3/OCH3 groups were moved from the para- to either the ortho- or meta-position (8n−q), it led to the identification of a potent meta-OCH3 compound 8p with similar activity to that of unsubstituted 6f. Because moving the substitution from para- to meta-position improved the activity for CH3/OCH3 groups, NO2 (8r), Cl (8s), F (8t), and OH (8u) groups were also introduced at the meta-position of the phenyl ring. Particularly, the meta-Cl (8s) and meta-F (8t) substitution led only to a 2-fold loss of activity compared to 6f, demonstrating that the meta-position could tolerate both electron donating and withdrawing groups much better than the ortho- or para-position of the phenyl ring in pharmacophore C.

With the identification of analogue 8p, further SAR exploration was carried out by increasing the O-alkyl chain length of 8p from one carbon to eight carbons (9a−9e) to identify additional binding elements in this region (Table 4). Up to a five-carbon O-alkyl chain the activity level was retained; particularly, the n-butyl analogue 9c (IC50 = 80 nM) showed a 2-fold improved activity over the lead 6f. Further increasing the chain length to n-octyl (9e) led to drastic loss of activity. However, introduction of hydrophilic groups at the end of the alkyl chain, such as the N(CH3)2 (9f) or OH (9g,h) groups, led to loss of activity. This SAR trend indicates the presence of a tunnel-like hydrophobic cavity with steric limitation at the active site to which pharmacophore C interacts.

Individual optimization of pharmacophores A−C has identified key structural features essential for optimal anti-influenza activity in that region. On the basis of this information, we next synthesized two compounds by bringing together these optimized structural features into a single molecule (Table 5). Thus, the 3-bromo phenyl (6p) was chosen as the pharmacophore A optimized structure, and 3-methoxy phenyl (8p) and 2-thiophene rings (8g) were chosen as the pharmacophore C optimized structures. Results showed that 10a and 10b displayed good anti-influenza activity, with the thiophene derivative 10b being the most potent compound identified in this study. Thus, an overall improvement of ∼75-fold anti-influenza activity was achieved for 10b (IC50 = 60 nM) through systematic SAR study on the HTS hit 6a (IC50 = 4.51 μM).

Having identified potent anti-influenzaangelicin derivatives, we investigated in detail the potential of a representative

Table 4. Optimization of Pharmacophore C: In Vitro Anti-Influenza A Virus Activity of 9a–h

<table>
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<tr>
<th>compd</th>
<th>R</th>
<th>IC50, μM</th>
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<tbody>
<tr>
<td>6f</td>
<td>H</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>8p</td>
<td>OCH3</td>
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</tr>
<tr>
<td>9a</td>
<td>OCH3CH3</td>
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<tr>
<td>9b</td>
<td>O(CH2)3CH3</td>
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<tr>
<td>9c</td>
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<td>9e</td>
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<td>9f</td>
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<td>9h</td>
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<td>2.65±0.39</td>
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* Using influenza A/WSN/33 (H1N1) strain. * All the compounds exhibited CC50 > 25 μM, except 9f (CC50 = 17.65 ± 2.08). * The concentration required for a test compound to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control was expressed as IC50. Values are expressed as the mean of at least two independent determinations.

Table 5. Optimization of Pharmacophore A and C Together: In Vitro Anti-Influenza A Virus Activity of Compounds 10a,b

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<td>Br</td>
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<tr>
<td>8p</td>
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<td>0.11±0.03</td>
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<td>8g</td>
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<tr>
<td>10a</td>
<td>Br</td>
<td>3-OCH2-Ph</td>
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<tr>
<td>10b</td>
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<td>4 (Zanamivir)</td>
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* Using influenza A/WSN/33 (H1N1) strain. * All the compounds exhibited CC50 > 25 μM. * The concentration required for a test compound to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control was expressed as IC50. Values are expressed as the mean of at least two independent determinations, each experiments performed in triplicates.

![Figure 2](image-url) Cytopathic effect (CPE) produced by influenza A/WSN/33 virus strain in MDCK cells and its abrogation on treatment with 8g (1 μM).
The cytopathic effect produced by influenza virus A (WSN/33) on MDCK cells and its abrogation by treatment with 8g is shown in Figure 2. In addition to the standard neutralization assay discussed above, to confirm the anti-influenza activity of 8g, we carried out plaque reduction assay, which measured the ability of the compound in reducing the number of plaques formed by virus infected MDCK cells. In the plaque reduction assay, 8g showed over 50% reduction of plaque formation at a concentration of 20 nM and complete abrogation of plaque formation at a concentration of 100 nM (Figure 3). Further, the anti-influenza activity of 8g was confirmed by determining the viral yield; a reduction in virus yield, a direct indicator of the ability of the compound to decrease the production of next generation influenza (progeny), was observed upon treatment with 8g. Treatment with 100 nM of 8g, during viral adsorption, completely abolished progeny production, as indicated by the viral yield (Figure 4). With the confirmation of the anti-influenza virus A (WSN/33) activity, we were interested to investigate its potential in other strains of the virus. For this purpose, we selected three strains each of influenza A (two H1N1 and one H3N2 strain) and B (Table 6). Influenza inhibition results showed that 8g effectively inhibits all the influenza virus strains tested similar to clinically used anti-influenza medication zanamivir (4), with an IC50 in the range of 40–150 nM. This result suggests that 8g is a broad spectrum anti-influenza agent similar to zanamivir (4) and could provide effective coverage against infection over different strains of influenza.

With the confirmation of the anti-influenza activity, we were interested to identify the molecular target for the novel angelicin derivatives. Initially, we carried out time-of-addition study (Figure 5) to determine the effectiveness of 8g in decreasing the viral yield with regard to the time at which compound was added to the culture medium during the course of infection.
The article discusses the inhibition of influenza RNP activity by compound 8g using reporter assay. Different concentrations of compound 8g were incubated with 293 (human embryonal kidney) cells previously transfected with pPOLI-Fluc and pHW2000 expression vectors for influenza virus PB1, PB2, PA, and NP proteins. Treatment with 8g decreased the ratio of Fluc (firefly luciferase) to Rluc (renilla luciferase) activity, indicating that 8g inhibited RNP complex mediated transcription of pPOLI-Fluc, resulting in decreased Fluc activity without affecting the Rluc activity much. DMSO was included as the mock control, and Rluc expression was used as transfection control.

In addition to the reporter assay, we carried out primer extension assay to see the effect of 8g on viral-like mRNA synthesis in 293 cells previously transfected with pcDNA3-PB2, PB1, PA, NP, and pPOLI-CAT-RT. Transfected cells were treated with different concentration of 8g and the total RNA isolated and analyzed through primer extension assay specific for vRNA (viral RNA) and mRNA through reverse transcription reaction; the results are shown in Figure 7. Treatment with 8g decreased the amount of mRNA production, indicating that the compound targets viral RNP complex, which drives the reporter gene expression; further, we have already shown that another potent compound from this series, 6f, act through interference of viral RNP activity. Further, we also evaluated the ability of the potent compound 8g side-by-side with the inactive compound 7d (as negative control) in plaque formation, viral yield reduction, and viral-like RNA synthesis assay to show that the anti-influenza inhibition is restricted to only potent compound 8g (Figures 3s, 4s, and 5s, Supporting Information).

Finally, we have also carried out immunofluorescence microscopic analysis of MDCK cells infected by influenza virus using anti-dsRNA (double strand RNA), anti-NP, and anti-M1 antibodies to visualize the viral RNA and proteins in the infected MDCK cells. Presence of dsRNA, NP (nucleoprotein), and M1 (matrix protein) is an indicator of ongoing viral replication. Treatment of 8g resulted in decreased amounts of dsRNA, NP, and M1 protein (Figures 6s, 7s, and 8s, Supporting Information), as evidenced by the decreased amount of immunofluorescence signals compared to the control. Thus the preliminary mechanistic studies using 8g and 6f suggest that they act through interference of viral RNA synthesis by targeting RNP complex. It should be noted that there are other agents such as ribavirin, viramidine, and T-705 reported in the literature to possess anti-influenza activity, which act as RNA dependent RNA polymerase inhibitors. Moreover, the novel angelicin derivative 6f also possesses activity against oseltamivir (3) resistant influenza and other RNA viruses such as enterovirus 71, coxsackie B, and human rhinovirus; however, the mechanism for broad antiviral activity needs to be investigated further.
are novel anti-influenza agents with mode of action different from that of the approved influenza therapies in the market.

Conclusion

The threat of a pandemic influenza is real and could be a global disaster in waiting. Considering the multifaceted problem of developing vaccines for millions of people, particularly with the genetics of influenza shifting/drifting, there is a global trend to stockpile effective anti-influenza medication. However, emergence of resistant strains to this medication necessitates the development of newer therapeutic alternatives. A novel series of angelin derivatives identified from the HTS campaign is optimized through detailed SAR exploration. The results suggest that pharmacophore B consisting of the angelin scaffold is very sensitive to structural modification and is essential for maintaining the activity, while pharmacophores A and C are more amenable for structural modification, with the meta-substituted phenyl/2-thiophene rings being optimal for activity in this region. The optimized compound 8g inhibits the activity of both influenza A and B strains in vitro, suggesting that it could act as a lead compound for further anti-influenza drug development. As preliminary mechanistic studies point toward viral RNP as a probable molecular target for these agents, combination therapy with existing anti-influenza drugs which act through the NA target could be used to avoid the development of resistant influenza strains. Moreover, as angelin derivatives are rarely reported as antiviral agents, the leads identified here could open up new opportunities for antiviral drug discovery in general, which need to be explored further to realize the full potential of this novel series. Detailed mechanistic studies and further potential for development are under active pursuit in our laboratories, and the findings will be revealed in due course.

Experimental Section

General Methods. All commercial chemicals and solvents are reagent grade and were used without further treatment unless otherwise noted. All reactions were carried out under an atmosphere of dry nitrogen. Reactions were monitored by TLC using Merck 60 F254 silica gel glass backed plates (5 × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich) followed by heating at 80 °C. Flash column chromatography was done using silica gel (Merck Kieselgel 60, no. 9385, 230–400 mesh ASTM). 1H and 13C NMR spectra were obtained with a Varian Mercury-300 or Varian Mercury-400 or Bruker DMAX-6000 spectrometers. Chemical shifts were recorded in parts per million (ppm, δ) and were reported relative to the solvent peak or TMS. High-resolution mass spectra (HRMS) were measured with a Finnigan (MAT-95XL) electron impact (EI) or using Finnigan/Thermo Quest MAT 95XL FAB mass spectrometer. LCMS data were measured on an Agilent MSD-1100 ESI-MS/MS system. Purity of the final compounds were determined using a Hitachi 2000 series HPLC system using a C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm, 4.6 mm × 150 mm) or a phenyl column (Waters XBridge 5 μm, 4.6 mm × 150 mm) and were found to be >95% unless otherwise stated.

General Synthetic Method for Compounds 6—10 (Method A; Scheme I). Appropriately substituted 7-hydroxy-coumarin 11 (1.0 equiv) was dissolved in THF (40 mL), triethylamine (1.1 equiv) was added, and then the appropriate acyl chloride (R'COCl; 1.2 equiv) and stirred at room temperature for 16 h. The solid product (12) formed was filtered, washed with EtOAc, and the product obtained was used for next step without further purification.

The above obtained 12 (1.0 equiv) was mixed well with finely powdered anhydrous aluminum chloride (3.0 equiv) and heated at 170 °C for 2 h. After cooling, ice and diluted hydrochloric acid (20 mL) were added and stirred for 0.5 h. The EtOAc layer was washed with diluted hydrochloric acid (20 mL × 2) and then with water (20 mL × 2), followed by satd NaHCO3(aq) (30 mL × 2). The combined organic layers were evaporated to give 13 as a solid product, which was used for next step without purification.

A solution of 13 (1.0 equiv), 2-bromo ketone (R'COCH2Br) (1.2 equiv), and K2CO3 (3.0 equiv) in CH3CN (5 mL) was refluxed for 16 h. The reaction mixture was filtered, the solvents from the filtrate were removed under vacuum, and the crude product was purified by silica gel column chromatography (hexane/EtOAc: 3/1 → 1/1) to give the desired products 6—10, with 15—44% yield over three steps.

8-Benzoyl-4,9-dimethyl-furo[2,3-h]chromen-2-one 6a. Method A. Yield 31% over three steps. 1H NMR (300 MHz, CDCl3): δ 8.06—8.01 (m, 2H), 7.67 (d, J = 8.7 Hz, 1H), 7.64—7.60 (m, 1H), 7.56—7.51 (m, 3H), 7.45 (d, J = 8.7 Hz, 1H), 6.29 (d, J = 0.9 Hz, 1H), 2.91 (s, 3H), 2.51 (d, J = 0.9 Hz, 3H). LCMS [M + 1]+: 319.1.

8-Benzoyl-4-methyl-furo[2,3-h]chromen-2-one 6b (Scheme 2). To a solution of 7-hydroxy-4-methylcoumarin 11a (2.00 g, 11.30 mmol) in ethanol (5 mL), was added I2 (2.32 g, 9.10 mmol) and periodic acid (0.53 g, 2.33 mmol) and stirred at room temperature for 2 h. The reaction mixture was diluted with water, and the precipitate formed was collected and recrystallized from ethanol to give light-yellow colored needles of 7-hydroxy-8-iodo-4-methylcoumarin (14, 2.10 g, 76%). 1H NMR (300 MHz, CDCl3): δ 10.77 (s, 1H), 7.43 (d, J = 8.7 Hz, 1H), 6.94 (d, J = 8.7 Hz, 1H), 6.08 (d, J = 1.2 Hz, 1H), 2.41 (d, J = 1.2 Hz, 1H). LCMS [M + 1]+: 302.9.

A solution of 14 (1.01 g, 3.30 mmol), 1-phenyl-2-propyn-1-ol (0.6 mL), CuI (0.01 g, 0.05 mmol), and Et3N (1 mL) in DMF (5 mL) was stirred at 60 °C for 24 h. The reaction mixture was quenched by addition of water and extracted with EtOAc (50 mL × 3). The combined organic liquid was dried over MgSO4, filtered, and evaporated to give the crude product, which was purified by column chromatography over silica gel (hexane/EtOAc: 3/1 → 1/1) to furnish 8-(hydroxy-phenylmethyl)-4-methyl-furo[2,3-h]chromen-2-one (15, 0.52 g, 51%). 1H NMR (300 MHz, CDCl3): δ 7.90—7.19 (m, 6H), 6.81 (s, 1H), 6.13 (d, J = 0.9 Hz, 1H), 3.70 (br, 1H), 2.40 (d, J = 0.9 Hz, 3H). LCMS [M + 1]+: 307.1.

To a solution of 15 (0.03 g, 0.10 mmol) in acetone (5 mL) was added Jones reagent at 0 °C and then stirred at room temperature for 0.5 h. The reaction mixture was evaporated, and the crude product was purified by column chromatography over silica gel (hexane/EtOAc: 3/1 → 1/1) to give 6b (0.03 g, 95%). 1H NMR (300 MHz, CDCl3): δ 8.05—8.01 (m, 2H), 7.83 (s, 1H), 7.83—7.68 (m, 2H), 7.66—7.28 (m, 3H), 6.32 (d, J = 0.9 Hz, 1H), 2.53 (s, 3H). LCMS [M + 1]+: 305.0.

8-Benzoyl-4-methyl-9-propyl-furo[2,3-h]chromen-2-one 6c. Method A. Yield 32% over three steps. 1H NMR (300 MHz, CDCl3): δ 8.06—7.44 (m, 7H), 6.32 (d, J = 1.2 Hz, 1H), 3.37 (t, J = 5.4 Hz, 2H), 2.52 (d, J = 1.2 Hz, 3H), 1.87 (m, 2H), 1.07 (t, J = 7.2 Hz, 3H). LCMS [M + 1]+: 347.2.

8-Benzoyl-9-hexyl-4-methyl-furo[2,3-h]chromen-2-one 6d. Method A. Yield 35% over three steps. 1H NMR (300 MHz, CDCl3): δ 8.06—7.52 (m, 7H), 6.31 (d, J = 1.2 Hz, 1H), 3.38 (t, J = 7.5 Hz, 2H), 2.52 (d, J = 1.2 Hz, 3H), 1.80 (quintet, J = 7.8 Hz, 2H), 1.62—1.26 (m, 6H), 0.87 (t, J = 6.9 Hz, 3H). LCMS [M + 1]+: 389.2. HPLC purity: 91.9%.

8-Benzoyl-9-decy-4-methyl-furo[2,3-h]chromen-2-one 6e. Method A. Yield 39% over three steps. 1H NMR (300 MHz, CDCl3): δ 8.06—7.44 (m, 7H), 6.31 (s, 1H), 3.38 (t, J = 7.5 Hz, 2H), 2.51 (s, 3H), 1.80 (quintet, J = 7.5 Hz, 2H), 1.63 (br, 2H), 1.48 (quintet, J = 7.2 Hz, 2H), 1.43 (br, 10H), 0.86 (t, J = 6.3 Hz, 3H). LCMS [M + 1]+: 445.2.
8-Benzoyl-4-methyl-9-phenyl-furo[2,3-h]chromen-2-one 6f. Method A. Yield 35% over three steps. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 7.79 \sim 7.72 \) (m, 3H), 7.57 (d, \(J = 9.9 \) Hz, 1H), 7.43 \sim 7.40 \(\) (m, 2H), 7.38 \sim 7.30 \(\) (m, 4H), 6.27 (d, \(J = 1.2 \) Hz, 1H), 2.51 (d, \(J = 0.9 \) Hz, 3H). LCMS [M + 1]: \(^{426.1}\) HPLC purity: 99.5%.

8-Benzoyl-4-methyl-9-(4-nitro-phenyl)-furo[2,3-h]chromen-2-one 6m. Method A. Yield 28% over three steps. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 8.27 \sim 8.24 \) (m, 2H), 7.90 \sim 7.80 \(\) (m, 2H), 7.77 \sim 7.70 \(\) (m, 3H), 7.70 \sim 7.53 \(\) (m, 2H), 7.44 \sim 7.27 \(\) (m, 2H), 6.28 (d, \(J = 0.9 \) Hz, 1H), 2.52 (d, \(J = 0.9 \) Hz, 3H). \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 184.6, 159.1, 152.6, 147.9, 136.9, 136.3, 135.3, 131.5, 130.2, 129.8, 129.5, 128.4, 128.2, 126.4, 124.7, 123.0, 115.7, 113.9, 109.1, 19.5. LCMS [M + 1]: \(^{426.1}\) HPLC purity: 99.5%.

(B)-But-2-enio Acid 2-Benzoyl-3-phenyl-benzofuran-4-yl Es- ter 7a (Scheme 3). To a solution of 1-methoxy-3-methoxy-methoxy-benzene\(^{22}\) (17, 0.20 g, 1.19 mmol) in dry THF (10 mL) at \(0 \) °C was added nBuLi (0.96 mL, 1.6 mol in hexane) dropwise. After addition was completed, the mixture was stirred at same temperature for 10 min. Benzoic chloride (0.17 mL, 1.45 mmol) was then added dropwise to the solution. The mixture was stirred for 2 h at room temperature. The reaction was quenched by addition of water, extracted with EtOAc, dried over MgSO\(_4\) filtered, and concentrated. The residue was diluted with MeOH (15 mL), and 2 drops of 13N HCl were added and then heated at reflux temperature for 0.5 h. The solvent was then removed under reduced pressure, and water was added to the residue and extracted with EtOAc. The combined organic layers were dried over MgSO\(_4\) concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (hexane:EtOAc: 10/1) to give (2-hydroxy-6-methoxy-phenyl)-phenyl-methanone (18a, 0.20 g, 75%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 10.72 \) (s, 1H), 7.61 \sim 7.59 \(\) (m, 2H), 7.51 \sim 7.46 \(\) (m, 1H), 7.41 \sim 7.35 \(\) (m, 3H), 6.65 (d, \(J = 0.8 \) Hz, 1H), 6.40 (d, \(J = 8.4 \) Hz, 1H), 3.49 (s, 3H). HRMS (FAB) calc'd for C\(_{17}\)H\(_{18}\)O\(_2\): 228.0786, found 228.0782. A mixture of 18a (0.30 g, 1.31 mmol), potassium carbonate (0.40 g, 2.89 mmol), 2-bromocacetophenone (0.31 g, 1.57 mmol), and 20 mL of acetonitrile was heated at reflux temperature for 16 h. The solution was cooled to room temperature, acetonitrile was removed under reduced pressure, water added to the residue, and extracted with EtOAc (20 mL \times 3). The combined organic layers were dried over MgSO\(_4\) and concentrated under reduced pressure. Purification of the residue by column chromatography...
on silica gel (hexane/EtOAc: 10/1) furnished (4-methoxy-3-phenyl-1-benzofuran-2-yl)(phenyl)methanone (19, 0.31 g, 72%) as a yellow viscous liquid. 1H NMR (400 MHz, CDCl3): δ 7.79–7.85 (m, 2H), 7.57–7.63 (m, 4H), 7.31–7.37 (m, 6H), 7.29 (d, J = 10.4 Hz, 1H), 7.34 (s, 3H). HRMS calcd for C25H18O4 382.1205, found 382.1196. To a solution of 19 (0.25 g, 0.75 mmol) in dry dichloromethane (15 mL) at 0 °C was added NaH (0.18 g, 4.40 mmol) and benzoyl chloride (0.5 mL, 4.26 mmol). The mixture was stirred at room temperature for 12 h. The reaction was quenched with addition of water, extracted with dichloromethane, dried over MgSO4, filtered, and concentrated. The residue obtained was dissolved in EtOAc and washed with water, extracted with EtOAc, dried over MgSO4, filtered, and concentrated. Purification of the residue by column chromatography on silica gel (hexane/EtOAc: 1/2) furnished 2-methoxy-naphthalene (20, 0.19 g, 80%) as a yellow viscous liquid. 1H NMR (300 MHz, CDCl3): δ 7.85–7.91 (m, 2H), 7.51–7.58 (m, 2H), 7.19 (d, J = 8.4 Hz, 1H), 6.72 (d, J = 7.8 Hz, 1H), 5.31 (br, 1H). HRMS calcd for C21H14O3 314.0943, found 314.0943.

To a solution of 20 (0.05 g, 0.16 mmol) in dry pyridine (5 mL) was added crotonic anhydride (0.03 g, 0.20 mmol) and imidazole (2 mg, 0.03 mmol). The mixture was stirred at room temperature for 2 h. The reaction was quenched with addition of water, extracted with EtOAc, dried over MgSO4, filtered, and concentrated. Purification of the residue by column chromatography on silica gel (hexane/EtOAc: 1/1) furnished 7a (0.05 g, 81%) yield as a yellow viscous liquid. 1H NMR (150 MHz, CDCl3): δ 7.86 (m, 2H), 7.51–7.57 (m, 3H), 7.28–7.32 (m, 4H), 7.07 (dd, J = 1.6, 7.2 Hz, 1H), 6.63 (dq, J = 14.0, 7.0 Hz, 1H), 5.48 (qd, J = 1.8, 14.0 Hz, 1H), 1.74 (dd, J = 1.8, 7.0 Hz, 3H). 13C NMR (100 MHz, CDCl3): δ 185.2, 164.1, 155.3, 147.6, 146.6, 145.6, 136.9, 132.7, 130.9, 130.1, 130.0, 129.7, 128.3, 128.0, 127.7, 127.5, 121.2, 117.2, 110.2, 17.9. HRMS calcd for C22H16O4 380.1049, found 380.1039.

8-Benzoyl-9-phenyl-furo[2,3-h]chromen-2-one 7d. Method A. Yield 41% over three steps. 1H NMR (400 MHz, CDCl3): δ 7.76–7.79 (m, 2H), 7.57 (d, J = 1.1 Hz, 1H), 7.51–7.47 (m, 5H), 7.31–7.26 (m, 5H), 2.15 (s, 3H). 13C NMR (150 MHz, CDCl3): δ 185.5, 159.8, 157.8, 156.3, 150.1, 148.1, 136.6, 132.8, 130.6, 129.6, 128.7, 128.0, 127.7, 126.8, 124.1, 116.1, 114.9, 109.1, 17.7. HRMS (M+): calcd for C26H18O4 394.1205, found 394.1203.

8-Benzoyl-9-pentyl-4-ethyl-9-phenyl-furo[2,3-h]chromen-2-one 7e. Method A. Yield 25% over three steps. 1H NMR (600 MHz, CDCl3): δ 7.76–7.79 (m, 2H), 7.57 (d, J = 8.9 Hz, 1H), 7.45–7.42 (m, 3H), 7.30–7.27 (m, 5H), 6.43 (q, J = 2.0 Hz, 1H), 2.85 (qd, J = 7.4, 2.0 Hz, 1H), 2.33 (t, J = 7.4 Hz, 3H). 13C NMR (150 MHz, CDCl3): δ 185.5, 159.8, 157.8, 156.3, 150.1, 148.1, 136.6, 132.8, 130.6, 129.6, 128.7, 128.0, 127.7, 123.8, 116.5, 114.6, 111.5, 108.9, 25.4, 12.2. HRMS (M+): calcd for C27H20O4 394.1205, found 394.1205.
7.42 (d, 1H), 2.76 (d, 1H), 2.83 (br, 6H), 2.45 (d, 1H), 2.73 (dt, 1H), 2.45 (d, 1H), 2.70 (dt, 1H). HRMS calcd for C23H19NO4S: 370.1090, found 370.1096.

In a sealed tube, 24 (0.11 g, 0.29 mmol) and mesitylene (12 mL) were heated at 280 °C for 2 h. The solvent was evaporated and the residue chromatographed on silica gel (hexane/EtOAc 1:1) to afford dimethylthioisocarbamide S-(8-benzoyl-4-methyl-2-oxo-2H-chromen-7-yl) ester (25, 0.07 g, 65%) as a yellow-light yellow solid. 1H NMR (400 MHz, CDCl3): δ 7.79–7.77 (m, 2H), 7.69 (d, J = 8.4 Hz, 1H), 7.57–7.53 (m, 2H), 7.42–7.38 (m, 2H), 6.28 (q, J = 1.2 Hz, 1H), 2.35 (br, 6H), 2.45 (d, J = 1.2 Hz, 3H). HRMS calcd for C23H19NO4S: 370.1090, found 370.1096.

To a solution of 25 (0.14 g, 0.39 mmol) in dry MeOH (20 mL) was added sodium methoxide (0.03 g, 0.57 mmol). The mixture was stirred at reflux temperature for 1 h and then cooled to room temperature and the solvent removed under reduced pressure to give compound 8g. To compound 8g, 0.02 g, 0.06 mmol, of 2-bromoacetophenone was added sodium methoxide (0.03 g, 0.57 mmol). The mixture was cooled to room temperature and the residue purified by silica gel column chromatography (hexane/EtOAc 1:1) to afford 8-hydroxy-4,6-dimethylchro- men-2-one 8h. To a solution of 8h, 0.06 g, 40% yield for three steps. 1H NMR (300 MHz, CDCl3): δ 7.74–7.71 (m, 3H), 7.57 (d, J = 9.0 Hz, 1H), 7.29–7.19 (m, 4H), 6.26 (d, J = 1.2 Hz, 1H), 2.50 (d, J = 0.9 Hz, 3H). LCMS [M + 1]+: 353.1.

8-Acetyl-4-methyl-9-phenyl-furo[2,3-h]chromen-2-one 8c. Method A. Yield 38% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.33–7.31 (m, 1H), 7.38–7.35 (m, 2H), 7.29 (m, 3H), 7.16 (br, 6H), 2.48 (s, 3H), 2.34 (d, J = 0.8 Hz, 3H). 13C NMR (75 MHz, CDCl3): δ 188.7, 159.3, 155.9, 152.7, 150.1, 148.2, 130.3, 130.9, 129.2, 128.4, 128.2, 127.9, 112.6, 117.0, 115.2, 113.5, 108.8, 28.5, 19.4. LCMS [M + 1]+: 319.1. HPLC purity: 91.5%.

8-(Furan-2-carbonyl)-4-methyl-9-phenyl-furo[2,3-h]chromen-2-one 8f. Method A. Yield 35% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.74 (d, J = 8.7 Hz, 1H), 7.62–7.56 (m, 4H), 7.50–7.44 (m, 4H), 6.55 (dd, J = 1.8, 1.8 Hz, 1H), 6.25 (d, J = 1.2 Hz, 1H), 2.50 (d, J = 1.2 Hz, 3H). 13C NMR (75 MHz, CDCl3): δ 171.2, 170.70, 159.4, 156.2, 152.8, 151.0, 149.9, 147.6, 147.1, 130.4, 129.5, 128.8, 127.8, 124.3, 121.2, 116.5, 115.3, 113.6, 111.1058.8, 19.6. LCMS [M + 1]+: 383.0. HPLC purity: 99.9%.

8-Methyl-9-phenyl-8-(thiophene-2-carbonyl)-2,3-hydroxy-3,5-dihydrofuran-2-one 8g. Method A. Yield 44% for three steps. 1H NMR (300 MHz, CDCl3): δ 8.11–8.10 (m, 1H), 7.76–7.71 (m, 2H), 7.63–7.60 (m, 3H), 7.58–7.26 (m, 3H), 7.16–7.14 (m, 1H), 6.25 (d, J = 1.2 Hz, 1H), 2.50 (d, J = 1.2 Hz, 3H). 13C NMR (100 MHz, CDCl3): δ 175.2, 161.69, 160.37, 155.09, 149.83, 149.60, 142.78, 135.99, 135.26, 134.98, 134.85, 129.59, 129.17, 128.66, 128.55, 128.46, 128.02, 113.56, 113.46, 113.01, 108.77, 107.84, 25.46. HRMS (M+): calcd for C17H15NO3S: 386.0613, found 386.0609.

8-Methyl-9-phenyl-8-(thiophene-3-carbonyl)-2,3-hydroxy-3,5-dihydrofuran-2-one 8h. Method A. Yield 39% for three steps. 1H NMR (300 MHz, CDCl3): δ 8.10–8.09 (m, 1H), 7.76–7.71 (m, 2H), 7.63–7.62 (m, 1H), 7.58–7.55 (m, 3H), 7.44–7.42 (m, 3H), 7.27–7.25 (m, 1H), 6.23 (d, J = 1.2 Hz, 1H), 2.48 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 177.3, 159.4, 156.0, 153.0, 152.8, 149.8, 148.2, 140.3, 134.8, 133.3, 130.4, 129.7, 129.2, 128.8, 128.5, 128.2, 127.9, 127.8, 126.8, 126.5, 126.4, 125.6, 124.2, 116.5, 115.3, 113.5, 113.1, 108.8, 19.5. LCMS [M + 1]+: 387.0. HPLC purity: 90.5%.

8-Methyl-9-phenyl-8-(pyridine-2-carbonyl)-2,3-hydroxy-3,5-dihydrofuran-2-one 8i. Method A. Yield 31% for three steps. 1H NMR (300 MHz, CDCl3): δ 8.25–8.24 (m, 1H), 7.80–7.77 (m, 1H), 7.74–7.67 (m, 2H), 7.58 (d, J = 9.0 Hz, 1H), 7.42–7.38 (m, 2H), 7.25–7.20 (m, 4H), 6.24 (d, J = 1.2 Hz, 1H), 2.49 (d, J = 1.2 Hz, 3H). LCMS [M + 1]+: 382.1.

8-Methyl-8-(4-methylbenzyl)-9-phenyl-furo[2,3-h]chromen-2-one 8j. Method A. Yield 25% for three steps. 1H NMR
(300 MHz, CDCl3): δ 7.73–7.69 (m, 3H), 7.55 (d, J = 8.7 Hz, 1H), 7.51–7.47 (m, 2H), 7.36–7.32 (m, 3H), 7.11 (d, J = 8.1 Hz, 2H), 6.24 (d, J = 0.9 Hz, 1H), 4.30 (d, J = 6.0 Hz, 3H), 3.25 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 185.5, 159.5, 159.2, 149.4, 137.8, 130.0, 129.9, 128.5, 128.4, 127.9, 127.1, 124.2, 120.2, 116.6, 115.1, 113.5, 110.5, 109.0, 55.2, 19.5, LCMS [M + 1]+: 411.0. HNMR purity: 90.1%.

8-(3-Methoxy-benzoyl)-4-methyl-9-phenyl-furo[2,3-h]chromene-2-one 8a. Method A. Yield 41% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.79 (d, J = 8.7 Hz, 1H), 7.51–7.47 (m, 2H), 7.36–7.32 (m, 3H), 7.11 (d, J = 8.1 Hz, 2H), 6.24 (d, J = 0.9 Hz, 1H), 4.30 (d, J = 6.0 Hz, 3H), 3.25 (s, 3H). 13C NMR (75 MHz, CDCl3): δ 182.2, 159.4, 156.0, 156.4, 154.8, 154.6, 149.9, 148.7, 130.2, 129.1, 128.1, 127.7, 124.2, 122.5, 119.7, 116.3, 115.3, 113.7, 113.5, 108.9, 55.3, 19.5. HRMS (M+): calcd for C21H19O5N: 395.1. HPLC purity: 91.9%.

8-Methyl-8-(3-nitro-benzoyl)-9-phenyl-furo[2,3-h]chromene-2-one 8b. Method A. Yield 38% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.73 (d, J = 8.7 Hz, 1H), 7.51–7.47 (m, 2H), 7.40–7.28 (m, 5H), 7.02 (t, J = 8.7 Hz, 1H), 6.01–6.97 (m, 1H), 6.24 (d, J = 1.2 Hz, 1H), 3.76 (s, 3H), 2.49 (d, J = 1.2 Hz, 3H). 13C NMR (75 MHz, CDCl3): δ 185.5, 159.5, 159.2, 158.4, 149.4, 137.8, 130.0, 129.9, 128.5, 128.4, 127.9, 127.1, 124.2, 120.2, 116.6, 115.1, 113.5, 110.5, 109.0, 55.2, 19.5. LCMS [M + 1]+: 411.1. HNMR purity: 90.1%.

4-Methyl-8-(3-nitro-benzoyl)-9-phenyl-furo[2,3-h]chromene-2-one 8c. Method A. Yield 28% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.75–7.71 (m, 3H), 7.57 (d, J = 8.7 Hz, 1H), 7.48–7.44 (m, 2H), 7.38–7.32 (m, 3H), 7.29–7.24 (m, 2H), 6.25 (d, J = 1.2 Hz, 1H), 2.50 (d, J = 1.2 Hz, 3H). LCMS purity: 91.9.

8-(4-Methoxy-benzoyl)-4-methyl-9-phenyl-furo[2,3-h]chromene-2-one 8d. Method A. Yield 35% for three steps. 1H NMR (300 MHz, CDCl3): δ 7.73 (d, J = 8.7 Hz, 1H), 7.51–7.47 (m, 2H), 7.36–7.32 (m, 3H), 7.11 (d, J = 8.1 Hz, 2H), 6.24 (d, J = 0.9 Hz, 1H), 4.30 (d, J = 6.0 Hz, 3H), 3.25 (s, 3H), 2.49 (d, J = 1.2 Hz, 3H). 13C NMR (75 MHz, CDCl3): δ 182.2, 159.4, 156.0, 156.4, 154.8, 154.6, 149.9, 148.7, 130.2, 129.1, 128.1, 127.7, 124.2, 122.5, 119.7, 116.3, 115.3, 113.5, 108.9, 55.3, 19.5. LCMS [M + 1]+: 411.0. HNMR purity: 91.9%.

General Method for the Alkylation of 8u with Alkylicloride (RBr) to Give 9a–f (Method B; Scheme 7). A solution of 8u (1.0 equiv) in DCM (5 mL) BBr3 (2.0 equiv) was added and stirred at room temperature for 2 h. Ice water was added to the reaction mixture and extracted with EtOAc (20 mL × 3). The combined organic layer was dried over MgSO4 and evaporated to give crude product, which was purified over silica gel column chromatography (hexane/EtOAc: 1/3 → 1) to give compound 9a–f.
\[ \text{1H NMR (300 MHz, CDCl}_3\text{: } \delta \text{7.62 (d, } J = 9.0 \text{ Hz, 1H), 7.50} \quad \]

\[ \text{7.21 (m, 2H), 7.15 (m, 7H), 7.18 (t, } J = 8.7 \text{ Hz, 1H), 7.00} \quad \]

\[ \text{m, 9H), 6.26 (s, 3H). LCMS [M + 1]^-: 465.0} \quad \]

\[ \text{Virus Source. Influenza A/WSN/33 were generated using the 12-plasmid-based reverse genetics system described by Fodor et al.} \]

\[ \text{Inhibition of Virus Induced CPE on MDCK Cells (IC}_{50} \text{ Determination). This assay measured the ability of a test compound to inhibit the cytopathic effect induced by influenza virus on MDCK cells. The 96-well tissue culture plates were seeded with 200 \mu L of MDCK cells at a concentration of 1.1 \times 10^5 cells/mL in Dulbecco/Vogt modified Eagle’s minimal essential medium (DMEM) with 10% fetal bovine serum (FBS). The plates were incubated for 24-30 h at 37 °C and were used at about 90% confluency. Virus (100 TCID}_{50} \text{ mixed with different concentrations of test compounds was added to the cells and incubated at 37 °C for 1 h. After adsorption, the infected cell plates were overlaid with 50 \mu L of serum free DMEM (E0: DMEM with penicillin [100 U/mL], streptomycin [100 \mu g/mL], l-glutamine [2 mM], nonessential amino acid mixture [0.1 mM], and trypsin [2.5 mg/mL]) and 0.1% DMSO. The plates were incubated at 37 °C for 72 h. At the end of incubation, the plates were fixed by the addition of 100 \mu L of 4% formaldehyde for 1 h at room temperature. After the removal of formaldehyde, the plates were stained with 0.1% crystal violet for 15 min at room temperature. The plates were washed, and the density of the well was measured at 570 nm. The concentration required for a test compound to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control was expressed as IC}_{50}. All assays were performed in triplicate and at least twice.} \]

\[ \text{Cytotoxicity Assay (CC}_{50} \text{ Determination). The 96-well tissue culture plates were seeded with 200 \mu L of MDCK cells at a concentration of 1.1 \times 10^5 cells/mL in DMEM with 10% FBS. The plates were incubated for 24-30 h at 37 °C and were used at about 90% confluency. Test compounds at various concentrations were added to MDCK cells. The plate was incubated at 37 °C for 72 h. At the end of incubation, the plates were fixed by the addition of 100 \mu L of 4% formaldehyde for 1 h at room temperature. After the removal of formaldehyde, the plates were stained with 0.1% crystal violet for 15 min at room temperature. The plates were washed, and the density of the well was measured at 570 nm. The concentration of a test compound required to reduce cell viability to 50% of the tested control culture was expressed as CC}_{50}. All experiments were performed in triplicate and at least twice.} \]

\[ \text{Inhibition of Virus Induced CPE on MDCK Cells by 8g (Figure 2). MDCK cells (2.5 \times 10^5 cells/well) were seeded into a 6 cm tissue culture dish, grown for overnight, and then challenged with virus (multiplicity of infection [MOI] = 0.01). After adsorption of virus for 1 h on ice, the cells were washed with Hanks’ balanced salt solutions (HBSS), after which E0 containing 8g (1 \mu M) was added. After incubation for 36 h, the CPE was evaluated under a microscope.} \]

\[ \text{Plaque Reduction Assay. MDCK cells (5.5 \times 10^5 cells/well) were seeded into six-well tissue culture plates and incubated overnight. The cells were incubated with influenza virus at approximately 125 PFU/well with or without different concentrations of the compound. After adsorption of the virus for 1 h at 37 °C, the viral suspension was removed, and the cells were washed with HBSS. The cells were then overlaid with E0 containing 0.3% agarose with indicated compound concentration. After incubation for 48 h at 37 °C under 5% CO}_2, the cells were fixed with 10% formaldehyde and then stained with 1% crystal violet. The numbers of plaques were counted and the antiviral activity of the compound was calculated with respect to virus control.} \]
**Time of Addition Assay.** MDCK cells (2.4 × 10^5 cells/well) were seeded in six-well tissue culture plates and incubated overnight. The cells were challenged with virus (MOI 0.001) on ice for 1 h. After adsorption of the virus, the viral suspension was removed and the cells were washed with HBSS, then replenished with fresh E medium (pi = 0). The test medium containing 1 μM of 8g was added during the periods 1 to 6 h (adsorption), 0–2, 2–4, 4–6, and 6–8 h. After each incubation period, the monolayer was washed with HBSS and incubated with fresh medium until 12 h postinfection (pi). The supernatant was collected and the viral yield was determined by plaque assay.

**Inhibition of Viral RNP Activity Using Reporter Assay.** Reporter assay was performed in 293 cells transfected with reporter plasmids and pHW2000 expression vectors for influenza virus PB1, PB2, PA, and NP proteins. In a 48-well dish, approximately 5 × 10^3 per well of 293 cells were transfected with pPOLI-Fluc plasmid (0.1 μg), pHW2000-PB1 (0.1 μg), -PB2 (0.1 μg), -PA (0.1 μg), -NP (0.1 μg), and pRL-TK (5 ng, Promega) as a transfection control by using calcium phosphate method. Tenfold serial dilutions of 8g were added 8 h post-transfection, and the cells were incubated for further 20 h. Cells were then harvested for luciferase assay using Dual-Luciferase assay system (Promega, Madison, WI) and a VICTOR3 plate reader (PerkinElmer). Values at each dose were analyzed in triplicate from three experiments. The y-axis is the ratio of Fluc to Rluc controls, normalized to mock treatment (set arbitrarily to 1.0).

**Evaluation of Viral-like RNA Synthesis Inhibition Using Primer Extension Assay.** The production of viral RNAs was measured by plasmid-based reverse genetics system and primer extension assay. 23 One μg of each pHW2000-PB1, PB2, PA, and NP and pPOLI-CAT-RT were cotransfected into 293 cells in a 6-well plate. Compound 8g was added at 8 h post transfection. Total cellular RNA was isolated (RNease Mini kit, Qiagen) 20 h post transfection and hybridized with [γ-32P]-end labeled primers at 50°C for 3 h. This primer extension reaction was carried out using avian myeloblastosis virus reverse transcriptase (primer extension system-AMV reverse transcriptase, Promega) at 42°C for 2 h. The resulting mixtures were terminated by denaturing at 90°C for 10 min and analyzed with 8% polyacrylamide gel containing 7 M urea. The expected sizes of mRNA, cRNA (complementary RNA), and vRNA were 98–106, 89, and 158 bp, respectively.

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**Supporting Information Available:** Hematicagglutination inhibition assay protocol and results, reporter assay (Fluc) results, immunofluorescence microscopic analysis (dsRNA, NP, and M1 protein) protocol and results upon 8g treatment, comparative activity of 8g and 7d in plaque formation, viral yield reduction, and viral-like RNA synthesis assay. This material is available free of charge via the Internet at http://pubs.acs.org.

**References**


