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Synthesis and biological evaluation of dihydrofuran-fused perhydrophenanthrenes as a new anti-influenza agent having novel structural characteristic

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Abstract—Dihydrofuran-fused perhydrophenanthrenes were synthesized by means of *o*-quinodimethane chemistry with high generality and stereoselectivity, and found to exhibit potent anti-influenza activity. These compounds exerted an inhibitory effect on various strains of influenza virus growth, including influenza A and B, with a concentration dependent manner, and direct cytotoxicity was low. Several biological experiments suggested that these new drugs affected a virus replication process before mRNA synthesis stage. Novel rigid cage-type of structural characteristic of the compounds has not been found in hitherto anti-influenza drugs, and will provide new basis and motif for exploring promising and unprecedented anti-influenza agents.

1. Introduction

Influenza has been a contagious respiratory disease typically caused by influenza A and B viruses. These respiratory tract infections have been a major cause of pandemic mortality and morbidity all over the world, being a significant medical problem.[1] In addition to vaccination as a prophylactic method, several therapeutic treatments have been employed for decades, representatively including amantadine (**1**) and rimantadine (**2**).[2] These agents block an ion channel of the viral M2 protein, which has a vital role for the replication process; however, lack of the M2 protein in the influenza B virus limits their utility to treating influenza A virus infections.[3] Recent advances in the understanding of the replication mechanism of the influenza viruses have resulted in the identification of potential molecular targets for pharmaceutical intervention. For example, endonuclease[4] and neuraminidase[5] inhibitors have been newly developed and have become available such as zanamivir (**3**)[6] and oseltamivir (**4**).[7] Despite such continuous efforts for new anti-influenza drug discovery, there remains a considerable and urgent demand for improved therapeutic agents having different mechanisms of action, due to rapid emergence of mutant viral antigens and drug resistance as well as undesirable adverse effects of the present treatment options.

In conjunction with our recent research program directed toward the development of new anti-viral agents, we have reported that the dihydrofuran-fused tetracyclic compounds (**5**) exhibit a notable growth inhibitory activity against hemagglutinating virus of Japan (HVJ) in rhesus monkey kidney (LLC-MK2) cells.[8] These compounds have an interesting structural feature characterized by a highly rigid cage-type conformation (Fig. 1), which is suggested to play a crucial role for showing the anti-viral activity by a preliminary survey using the hemagglutinin aggregation (HA) assay method. These results prompted us to investigate a possibility of the dihydrofuran-fused compounds as a new class of anti-influenza agents possessing a novel structural characteristic. Here, we describe the synthesis and biological evaluation of variously substituted derivatives, containing the common dihydrofuran-fused perhydrophenanthrene core structure, as new potential influenza therapeutic agents.

Keywords: Perhydrophenanthrenes; Anti-influenza agents; Cage structure; mRNA.
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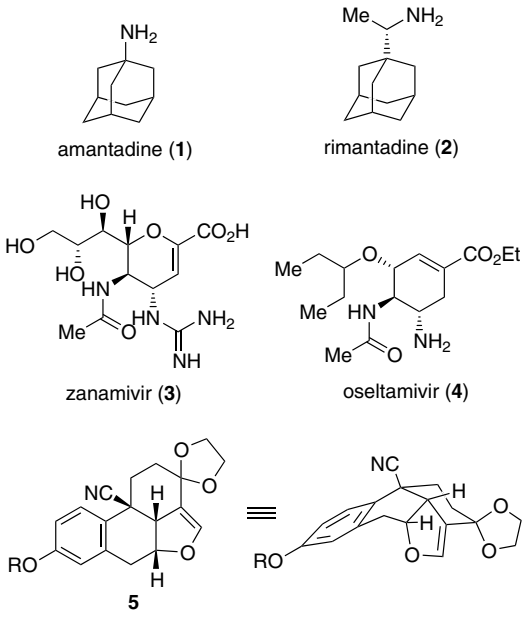


Figure 1. Structures of representative known anti-influenza agents (**1**–**4**) and dihydrofuran-fused perhydrophenanthrenes (**5**) having rigid cage-type conformation.

2. Results and discussion

2.1. Chemistry

In the course of our research on the *o*-quinodimethane chemistry,[9] we have established a concise and efficient synthetic method of the tetracyclic compounds (**5**) with a high stereoselectivity.[8,10] Among target compounds in this study, listed in Figure 2, the compounds **6**, **7**, and **8** have been synthesized and reported in our previous paper,[8] taking advantage of a high generality of the above method based on successive thermal electrocyclic reactions of benzocyclobutenes via *o*-quinodimethanes. Biological examination of this series of compounds has revealed that the TIPS (triisopropylsilyl) derivative (**8**) exhibits the most potent anti-viral activity, at least against HVJ growth.[8] Therefore, a simple regioisomer (**9**) of the compound **8** was synthesized according to Scheme 1, which presents good exemplification of a general synthetic scheme for the series of dihydrofuran-fused tetracyclic compounds.

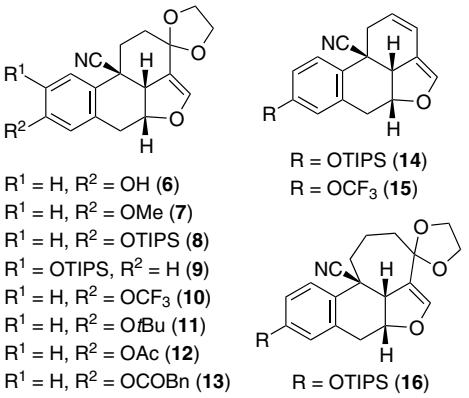
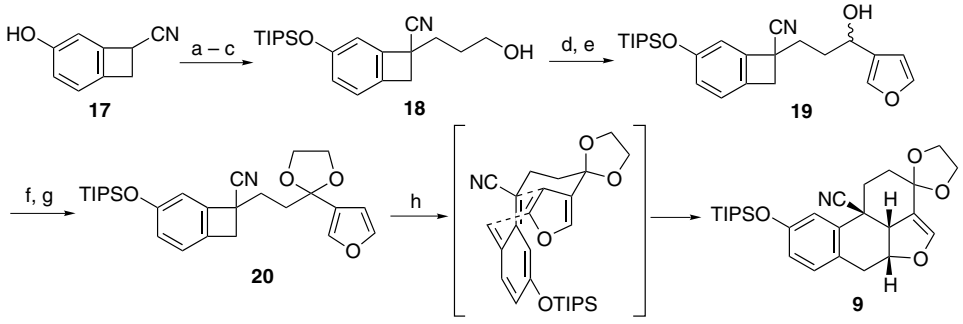


Figure 2. Chemical structures of dihydrofuran-fused perhydrophenanthrenes investigated in this study.

Readily available benzocyclobutene derivative **17**[11] was subjected to alkylation, introduction of a TIPS group, and then deprotection to give an alcohol **18**, which was transformed into a furan-containing derivative **19** by an oxidation-addition sequence. Re-oxidation of the alcohol **19** followed by ketalization afforded a compound **20**, a substrate for the key thermal electrocyclic reactions. This compound was heated in refluxing *o*-dichlorobenzene to form an *o*-quinodimethane intermediate, which subsequently participated in [4 + 2] cycloaddition with the furan moiety in an intramolecular fashion with exclusive endo selectivity to furnish the tetracyclic compound **9** (Scheme 1).

To facilitate the consideration of the effects of substituents on the aromatic ring to the bioactivities, compounds **10**–**13** were synthesized through simple derivatizations of the phenolic compound **6**. Among them, for instance, trifluoromethyl and *tert*-butyl derivatives (**10** and **11**) are imitative compounds of the TIPS derivative **8** with regard to the lipophilic and sterically congested nature, respectively. Synthetic methods of these compounds are presented in Section 4, and briefly, reagents and conditions are as follows; for **10**: *S*-(trifluoromethyl)dibenzothiophenium triflate, K_2CO_3 (54%); for **11**: *t*-BuOC(=NH)CCl_3, BF_3.OEt_2 (47%); for **12**: Ac_2O, pyridine (79%); for **13**: $PhCH_2COCl$, Et_3N, DMAP (61%).

Two congeners (**14** and **15**) having a double bond instead of the ethylene ketal structure were also synthesized, taking our previous finding into account that



Scheme 1. Synthesis of compound **9**. Reagents and conditions: (a) LDA, then $Br(CH_2)_3OTHP$ (82%); (b) TIPSOTf (91%); (c) PPTs (78%); (d) TPAP, NMO (62%); (e) 3-lithiofuran (73%); (f) TPAP, NMO (68%); (g) $TMSO(CH_2)_2OTMS$, TMSOTf (88%); (h) *o*-dichlorobenzene, reflux (59%).

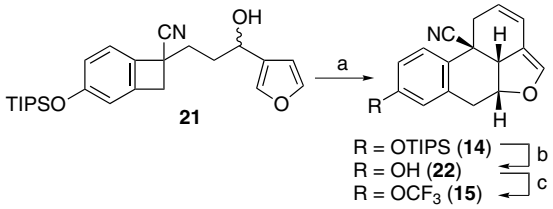
such type of compound showed a relatively high inhibitory activity against HVJ growth.[8] The compound **21**, which was prepared by the same synthetic procedure for **19**, was subjected to the thermal reaction to afford the compound **14** as a result of β-elimination of the hydroxyl group after the cyclization.[8] This compound was further derivatized to the compound **15** in two steps (Scheme 2). Additionally, a ring-expanded derivative (**16**) was prepared for the comparison of the bioactivities, the synthesis of which had already been reported.[10]

2.2. Biology

Initially, we examined the inhibitory activity of the test compounds (**6**–**16**, Fig. 2) against the virus growth in Madin-Darby canine kidney (MDCK) cells using influenza A/Aichi/2/68 (H3N2 subtype) virus strain at 10 μM drug concentration. The virus yields as a percent of control were estimated by a plaque titration method,[12] and the results are shown in Figure 3, including amantadine as a positive control (PC). This survey disclosed that several compounds inhibited the virus growth and could have potential as a new anti-influenza agent. In particular, the compounds **10**, **14**, and **16** exhibited a potent activity, suppressing the virus proliferation up to ca. 30% of control, and consequently, these three compounds were subjected to the examinations of dose-dependence and cytotoxicity.

Figure 4 clearly shows that these compounds influenced the influenza A/Aichi/2/68 virus yields at 72-h postinfection in a concentration-dependent manner. The cytotoxic activity was evaluated by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method,[13] and the OD (optical density) values for 24 h cultured MDCK cells after treatment with 100 μM or 200 μM of the test compounds are summarized in Table 1. These results indicated that these compounds did not exhibit any direct cytotoxicity, at least at 100 μM drug concentration, and were expected to have good safety indexes.

The scope of applicability of the compounds was investigated using a variety of influenza virus strains, including A/PR/8/34 (H1N1), A/USSR/92/77 (H1N1), A/NWS/33 (H1N1), B/Lee, and B/Singapore/222/79. As shown in Figure 5, it was found that these compounds were effective for both influenza A and B type viruses, implying that the mechanism of action differs from that of amantadine. These data suggest that a series of dihydrofuran-fused perhydrophenanthrenes investigated in this study are likely to have a broad spectrum



Scheme 2. Synthesis of compounds **14**, **15**, and **22.** Reagents and conditions: (a) *o*-dichlorobenzene, reflux (30%); (b) TBAF, THF (94%); (c) *S*-(trifluoromethyl)dibenzothiophenium triflate, K_2CO_3 (43%).

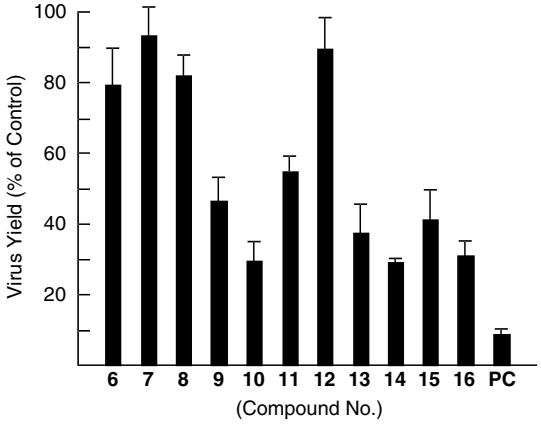


Figure 3. Inhibitory effect of the test compounds **6**–**16** on the growth of influenza A/Aichi/2/68 virus in MDCK cells at 10 μM drug concentration. Amantadine was used as a positive control (PC). Data are expressed as means ± SD of three experiments (percent of control yielding 2.77×10^4 plaque forming unit (PFU)/mL).

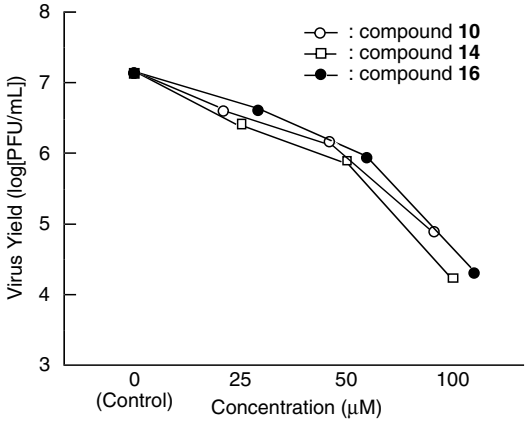


Figure 4. Examination of concentration-dependence of the compounds **10**, **14**, and **16** on the growth of influenza A/Aichi/2/68 virus in MDCK cells. Data are expressed as log of PFU/mL (means of three experiments). For clarity of the figure, error bars are omitted, which are presented in Supplementary material in tabular form.

Table 1. MTT assay for evaluation of the direct cytotoxicity of the compounds **10**, **14**, and **16**[a]

Compound	OD value	
	100 μM	200 μM
10	1.018 ± 0.044	1.062 ± 0.029
14	1.035 ± 0.054	1.085 ± 0.065
16	1.010 ± 0.066	0.722 ± 0.018
Control	1.071 ± 0.050	

[a] Optical density (OD) for cultured MDCK cells after treatment with the test compound and then MTT solution was measured at 570 nm, and expressed as mean ± SD of three measurements.

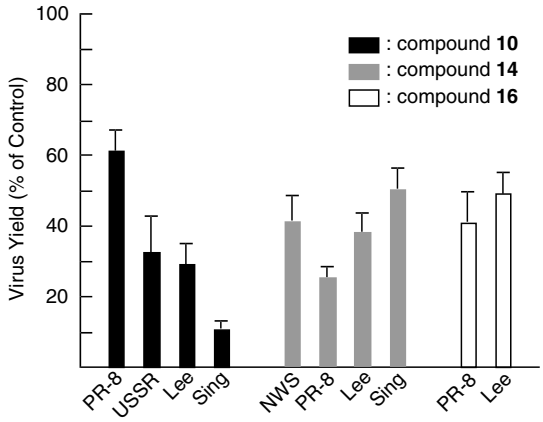


Figure 5. Examination of anti-viral activity of the compounds **10**, **14**, and **16** against various strains of influenza A and B viruses. Data are expressed as means ± SD of three experiments (percent of control) at 100 μM drug concentration. Viruses used are A/PR/8/34 (PR-8), A/USSR/92/77 (USSR), A/NWS/33 (NWS), B/Lee (Lee), and B/Singapore/222/79 (Sing).

of anti-influenza activity and may be useful for the management of outbreaks with pandemic potential.

In conjunction with a mode of action, several experiments were performed using the compound **10** as a representative. At first, time-related effect of the test compound was investigated, which includes a comparison among the drug treatments initiated at various times postinfection. The results, shown in Figure 6, indicated that the anti-influenza effect was observed only when the drug treatment was initiated within 1-h postinfection, and the virus yields were comparable to that of control culture in the cases of 2 h or later after the virus infection. These facts suggest that the drug affects the influenza virus growth at a relatively early stage in the replication process.

The cycle of influenza infection is initiated by binding of virus particles to host cell surface receptors followed by

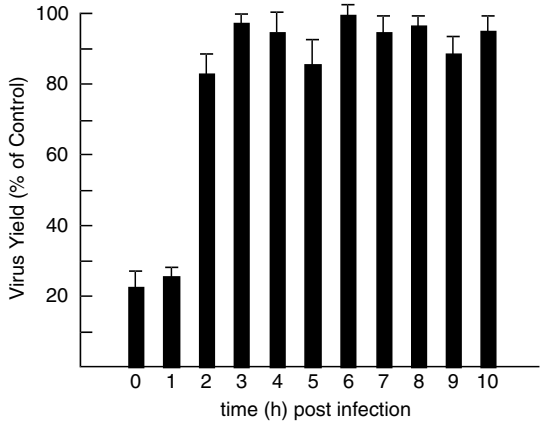


Figure 6. Time-related effect of the compound **10** on the growth of influenza A/Aichi/2/68 virus in MDCK cells at 100 μM drug concentration. After infection under drug-free condition, the cells were treated after various delays of 0–10 h at 37 °C. After a total of 24 h incubation, data are expressed as means (% of control) ± SD of three experiments.

Figure 7. Electrophoresis of RNA derived from MDCK cells infected with influenza A/Aichi/2/68 virus (lane 1: no drug treatment, lane 2: 100 μM drug **10** treatment) or without infection (lane 3). After isolation of total RNA, reverse transcription, and PCR, the mRNA preparations were electrophoresed in 1.6% agarose gel.

endocytosis, whose processes are mediated by one of the viral glycoproteins, hemagglutinin. After sequential fusion process between the viral envelope and the endosome constituting the cell membrane, and uncoating process, synthesis of virus mRNA occurs in the nucleus, which is catalyzed by a virally encoded polymerase. After replication, progeny viruses can be released from the infected cell with the aid of another glycoprotein, neuraminidase, and spread the infection. In the light of the time-related effect above described, we investigated whether mRNA synthesis occurred after the drug treatment. The experiments were performed by isolation of RNA from MDCK cells infected with influenza A/Aichi/2/68 virus, reverse transcription, followed by PCR and electrophoresis, whose results are shown in Figure 7. Lanes 1 and 2 represent RNA derived from the infected cells without drug treatment and with drug treatment (**10**, 100 μM), respectively, and lane 3 represents RNA derived from non-infected cells. The virus mRNA band found in lane 1 obviously disappeared in lane 2, indicating that the drug affected virus proliferation at the mRNA synthesis stage or before it.

Low pH environments in endosomes and lysosomes have been known to play an important role for uncoating process of the viral RNA during influenza infection.[14] For example, bafilomycin A1 has been reported to exert an inhibitory effect on the influenza virus growth through a specific inhibition of vacuolar-type proton pump to raise the pH in endosomes and lysosomes.[15] To examine whether the drug **10** could exert a similar effect, acidification of intracellular compartments under influence of the drug was monitored using a vital fluorescence microscope with acridine orange.[12b] This examination revealed that the amount and intensity of fluorescence in the drug-treated cells were almost identical with the control cells, implying that the drug did not affect the acidic environment of intracellular compartments, which causes the uncoating process. Although further studies may be necessary to elucidate a conclusive mechanism of action, we overall consider that the dihydrofuran-fused perhydrophenanthrenes investigated in this study exhibit the anti-influenza activity by affecting a process before mRNA synthesis.

3. Conclusion

In this paper, we disclosed that dihydrofuran-fused perhydrophenanthrenes could have potential for new type

of an anti-influenza agent. Novel structural features of these compounds may serve for a new therapeutic option against influenza infections. Broad generality of the synthetic method to the core structure by means of the *o*-quinodimethane chemistry will facilitate preparation of a wide variety of analogous derivatives, which can contribute further in-depth SAR considerations.

4. Experimental

4.1. Chemistry

Reagents were purchased from commercial sources and used as received. Anhydrous solvents were obtained from commercial sources or prepared by distillation over CaH_2 or P_2O_5. ^1H and ^{13}C NMR spectra were obtained on a Varian Gemini 300 (300 MHz for ^1H and 75.46 MHz for ^{13}C), using chloroform as an internal reference. Mass spectra were measured on a JEOL D-200 or a JEOL AX 505 mass spectrometer, and the ionization method was electron impact (EI, 70 eV). IR spectra were recorded on a Perkin-Elmer 1600 spectrometer. Column chromatography was carried out by employing Cica Silica Gel 60 N (spherical, neutral, 40–50 μm). Synthetic procedures for the compounds **6**–**8**,[8] **16**,[10c], and **21**[10c] have already been reported previously. NMR charts of the final compounds subjected to biological examinations are provided as Supplementary material.

4.1.1. 5-Hydroxybenzocyclobutene-1-carbonitrile (17). To a solution of 5-methoxybenzocyclobutene-1-carbonitrile[11] (2.0 g, 12.6 mmol) in MeCN (38 mL) was added TMSI (7.2 mL, 50.3 mmol) at room temperature. After stirring for 5 min, the mixture was refluxed for 18 h. The reaction mixture was cooled, and then diluted with H_2O, and the mixture was extracted with AcOEt. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography to afford **17** (1.6 g, 86%) as a colorless solid. Mp 84–85 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 3.43 (dd, 1 H, J = 14, 2.8 Hz), 3.57 (dd, 1H, J = 14, 5.5 Hz), 4.17 (dd, 1H, J = 5.5, 2.8 Hz), 6.73 (s, 1H), 6.75 (s, 1H), 6.82 (d, 1H, J = 7.9 Hz), 6.96 (d, 1H, J = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.2, 35.5, 109.9, 117.5, 119.9, 124.7, 134.0, 138.9, 156.4; IR (KBr) cm^{-1}: 3382, 2245; MS *m*/*z* 145 (M^+); HRMS Calcd for C_9H_7NO: 145.0528 (M^+) Found: 145.0531.

4.1.2. 1-(3-Hydroxyprop-1-yl)-5-(triisopropylsilyloxy)-benzocyclobutene-1-carbonitrile (18). To a solution of **17** (500 mg, 3.14 mmol) in dry THF (6.3 mL) was added LDA (1.0 M THF solution, 6.91 mL, 6.91 mmol) at −78 °C. After stirring for 10 min, a solution of $Br(CH_2)_3OTHP$ (770 mg, 3.45 mmol) in dry THF (4 mL) was added dropwise to the mixture, which was then stirred for 30 min at the same temperature. The reaction was quenched with H_2O at −78 °C and extracted with Et_2O. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was subjected to column chromatography to afford C-alkylated compound (740 mg, 82%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.53–2.19 (m, 10H), 3.17 (d, 1H, J = 14 Hz), 3.44–3.54 (m, 2H), 3.60 (d, 1H, J = 14 Hz), 3.79–3.87 (m, 2H), 4.59 (br, 1H), 6.69 (s, 1H), 6.78 (d, 1H, J = 8.0 Hz), 6.96 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 19.8, 25.5, 30.9, 34.3, 42.1, 62.8, 67.0, 99.3, 109.0, 117.4, 121.8, 125.2, 132.0, 143.9, 156.5; IR (neat) cm^{-1}: 3375, 2235; MS *m*/*z* 287 (M^+); HRMS Calcd for $C_{17}H_{21}NO_3$: 287.1521 (M^+). Found: 287.1523.

To a solution of this compound (700 mg, 2.44 mmol) and triethylamine (407 μL, 2.92 mmol) in dry CH_2Cl_2 (8 mL) was added TIPSOTf (938 μL, 2.68 mmol) at 0 °C, and the mixture was stirred at the same temperature for 45 min. The reaction mixture was diluted with H_2O at 0 °C, and extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography to afford TIPS derivative (985 mg, 91%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.02–1.10 (m, 18H), 1.22–1.29 (m, 3H), 1.52–2.06 (m, 10H), 3.19 (d, 1H, J = 14 Hz), 3.42–3.51 (m, 2H), 3.62 (d, 1H, J = 14 Hz), 3.76–3.86 (m, 2H), 4.57 (br, 1H), 6.75 (s, 1H), 6.82 (d, 1H, J = 8.0 Hz), 6.97 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.2, 19.9, 25.7, 26.9, 30.9, 34.5, 41.9, 42.1, 62.6, 66.8, 99.0, 113.4, 121.9, 125.0, 133.0, 144.1, 156.2; IR (neat) cm^{-1}: 2236; MS *m*/*z* 443 (M^+); HRMS Calcd for $C_{26}H_{41}NO_3Si$: 443.2856 (M^+). Found: 443.2853.

To a solution of the TIPS derivative (900 mg, 2.03 mmol) in dry EtOH (5 mL) was added PPTs (102 mg, 0.41 mmol) at room temperature, and the mixture was refluxed for 3 h. The reaction mixture was diluted with H_2O and then extracted with Et_2sO. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography to afford **18** (569 mg, 78%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.06–1.08 (m, 18H), 1.18–1.26 (m, 3H), 1.74–1.81 (m, 2H), 1.97–2.02 (m, 2H), 2.70 (s, 1H), 3.15 (d, 1H, J = 14 Hz), 3.58 (d, 1H, J = 14 Hz), 3.63 (t, 1H, J = 6.0 Hz), 6.73 (d, 1H, J = 1.7 Hz), 6.82 (dd, 1H, J = 8.0, 1.7 Hz), 6.95 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.2, 29.6, 34.1, 42.0, 42.2, 62.3, 113.4, 121.9, 122.0, 125.1, 133.0, 144.0, 156.3; IR (neat) cm^{-1}: 3433, 2235; MS *m*/*z* 359 (M^+); HRMS Calcd for $C_{21}H_{33}NO_2Si$: 359.2281 (M^+). Found: 359.2290.

4.1.3. 1-[3-(Furan-3-yl)-3-hydroxyprop-1-yl]-5-(triisopropylsilyloxy)benzocyclobutene-1-carbonitrile (19). To a solution of **18** (300 mg, 0.83 mmol) and NMO (117 mg, 1.00 mmol) in dry CH_2Cl_2 (5 mL) was added TPAP (catalytic amount) at room temperature. After stirring for 10 min, the solution was filtered through Celite, and the solvent was evaporated off to leave a residue, which was subjected to column chromatography to give a corresponding aldehyde (185 mg, 62%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.04–1.10 (m, 18H), 1.15–1.25 (m, 3H), 2.18–2.29 (m, 2H), 2.68–2.76 (m, 2H), 3.14 (d, 1H, J = 14 Hz), 3.58 (d, 1H, J = 14 Hz), 6.70 (d, 1H, J = 1.6 Hz), 6.80 (dd, 1H, J = 7.9, 1.6 Hz), 6.91 (d, 1H, J = 7.9 Hz).

To a solution of the aldehyde (202 mg, 0.56 mmol) in dry THF (5 mL) were added 3-lithiofuran (1.0 M THF solution, 0.62 mL, 0.62 mmol, prepared from 3-bromofuran and *n*-butyllithium) at −78 °C, and the mixture was stirred for 1 h. The reaction was quenched with H_2O, and the aqueous mixture was extracted with Et_2O. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography to afford **19** (174 mg, 73%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.08–1.10 (m, 18H), 1.20–1.30 (m, 3H), 1.85–2.13 (m, 5H), 3.16 (d, 1H, J = 14 Hz), 3.62 (d, 1H, J = 14 Hz), 4.73 (t, 1H, J = 5.8 Hz), 6.39–6.40 (m, 1H), 6.74 (d, 1H, J = 1.9 Hz), 6.83 (dd, 1H, J = 8.0, 1.9 Hz), 6.97 (d, 1H, J = 8.0 Hz), 7.38–7.40 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.2, 33.6, 34.5, 41.9, 42.2, 66.5, 108.3, 113.4, 121.8, 122.0, 125.1, 128.5, 133.0, 139.2, 143.7, 143.9, 156.3; IR (neat) cm^{-1}: 3434, 2234; MS *m*/*z* 425 (M^+); HRMS Calcd for $C_{25}H_{35}NO_3Si$: 425.2386 (M^+). Found: 425.2389.

4.1.4. 1-[3-(Furan-3-yl)-3-oxo-prop-1-yl]-5-(triisopropylsilyloxy)benzocyclobutene-1-carbonitrile ethylene ketal (20). To a solution of **19** (100 mg, 0.23 mmol) and NMO (33 mg, 0.28 mmol) in dry CH_2Cl_2 (5 mL) was added TPAP (catalytic amount) at room temperature. According to the work-up procedure mentioned above, a corresponding ketone (66 mg, 68%) was obtained as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.06–1.10 (m, 18H), 1.19–1.27 (m, 3H), 2.35–2.40 (m, 2H), 3.02–3.08 (m, 2H), 3.22 (d, 1H, J = 14 Hz), 3.65 (d, 1H, J = 14 Hz), 6.73–6.77 (m, 2H), 6.85 (d, 1H, J = 8.2 Hz), 6.99 (d, 1H, J = 8.2 Hz), 7.44–7.46 (m, 1H), 8.04–8.09 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.1, 31.5, 40.0, 41.4, 42.0, 108.6, 113.3, 121.5, 122.2, 125.2, 127.4, 132.8, 143.3, 144.4, 147.3, 156.4, 192.5; IR (neat) cm^{-1}: 2233, 1682; MS *m*/*z* 423 (M^+); HRMS Calcd for $C_{25}H_{33}NO_3Si$: 423.2230 (M^+). Found: 423.2229.

To a solution of the ketone (60 mg, 0.14 mmol) and ethylenedioxybis(trimethylsilane) (42 μL, 0.17 mmol) in dry CH_2Cl_2 (2 mL) was added trimethylsilyl trifluoromethanesulfonate (catalytic amount) at 0 °C, and the mixture was stirred at the same temperature for 2 h. The solvent was evaporated off, and the residue was purified by column chromatography to afford **20** (58 mg, 88%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.07–1.10 (m, 18H), 1.20–1.29 (m, 3H), 2.00–2.06 (m, 2H), 2.11–2.21 (m, 2H), 3.15 (d, 1H, J = 14 Hz), 3.60 (d, 1H, J = 14 Hz), 3.87–3.92 (m, 2H), 3.97–4.01 (m, 2H), 6.31–6.33 (m, 1H), 6.72 (d, 1H, J = 2.2 Hz), 6.82 (dd, 1H, J = 8.0, 2.2 Hz), 6.96 (d, 1H, J = 8.0 Hz), 7.37–7.40 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.2, 31.7, 36.3, 41.7, 42.1, 65.0, 106.9, 108.6, 113.4, 122.0, 125.1, 127.3, 133.0, 140.1, 143.6, 143.9, 156.3; IR (neat) cm^{-1}: 2233; MS *m*/*z* 467 (M^+); HRMS Calcd for $C_{27}H_{37}NO_4Si$: 467.2492 (M^+). Found: 467.2493.

4.1.5. 2,3,5a,10c-Tetrahydro-1*H*,6*H*-5-oxa-3-oxo-9-(triisopropylsilyloxy)acephenanthrylene-10b-carbonitrile ethylene ketal (9). A solution of **20** (40 mg, 0.09 mmol) in *o*-dichlorobenzene (3 mL) was refluxed for 2 h. The solvent was evaporated to leave a residue, which was purified by column chromatography to afford **9** (24 mg, 59%) as a colorless solid. Mp 62–64 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.02–1.10 (m, 18H), 1.17–1.29 (m, 3H), 1.83–1.92 (m, 2H), 2.47–2.58 (m, 1H), 2.78–2.82 (m, 1H), 3.10 (dd, 1H, J = 16, 2.7 Hz), 3.20 (dd, 1H, J = 16, 2.5 Hz), 3.86–3.96 (m, 4H), 4.02 (d, 1H, J = 10 Hz), 5.28 (ddd, 1H, J = 10, 2.7, 2.5 Hz), 6.00 (s, 1H), 6.81 (d, 1H, J = 8.2 Hz), 6.84 (s, 1H), 7.12 (d, 1H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.2, 30.0, 31.6, 31.9, 33.2, 35.9, 51.2, 63.9, 65.5, 80.0, 104.8, 110.5, 117.6, 120.0, 127.5, 131.3, 131.8, 143.1, 155.5; IR (KBr) cm^{-1}: 2364; MS *m*/*z* 467 (M^+); HRMS Calcd for $C_{27}H_{37}NO_4Si$: 467.2492 (M^+). Found: 467.2493.

4.1.6. 2,3,5a,10c-Tetrahydro-1*H*,6*H*-5-oxa-3-oxo-8-(trifluoromethoxy)acephenanthrylene-10b-carbonitrile ethylene ketal (10). To a solution of **6** (50 mg, 0.16 mmol) in dry DMF (1 mL) were added K_2CO_3 (27 mg, 0.19 mmol) and *S*-(trifluoromethyl)dibenzothiophenium trifluoromethanesulfonate (71 mg, 0.18 mmol) at room temperature, and the mixture was stirred for 14 h. The reaction mixture was diluted with H_2O and then extracted with AcOEt. The combined organic layer was dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography to afford **10** (33 mg, 54%) as a colorless solid. Mp 176–178 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.79–1.94 (m, 2H), 2.45–2.63 (m, 1H), 2.81–2.90 (m, 1H), 3.07 (dd, 1H, J = 16, 3.2 Hz), 3.25 (dd, 1H, J = 16, 2.9 Hz), 3.82–3.97 (m, 4H), 3.99–4.04 (m, 1H), 5.28–5.29 (m, 1H), 6.02 (s, 1H), 6.75–6.93 (m, 2H), 7.19 (d, 1H, J = 8.8 Hz); ^{13}C NMR (acetone-d_6, 75 MHz): δ 30.5, 32.2, 34.5, 36.0, 51.9, 65.7, 80.2, 101.2, 105.1, 111.4, 114.7, 117.8, 122.8, 123.0, 127.7, 137.8, 143.4, 146.8, 158.1; IR (KBr) cm^{-1}: 2269; MS *m*/*z* 379 (M^+); HRMS Calcd for $C_{19}H_{16}F_3NO_4$: 379.1031 (M^+). Found: 379.1043; Anal. Calcd for $C_{19}H_{16}F_3NO_4$: C, 60.16; H, 4.25; N, 3.69. Found: C, 60.29; H, 4.32; N, 3.72.

4.1.7. 8-*tert*-Butoxy-2,3,5a,10c-tetrahydro-1*H*,6*H*-5-oxa-3-oxo-acephenanthrylene-10b-carbonitrile ethylene ketal (11). To a solution of **6** (100 mg, 0.32 mmol) in dry hexane (1.3 mL) and dry CH_2Cl_2 (0.3 mL) were added BF_3 etherate (catalytic amount) and *tert*-butyl trichloroacetimidate (187 μL, 0.96 mmol) at room temperature, and the mixture was stirred for 20 h. After addition of H_2O, the mixture was extracted with AcOEt. The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography to afford **11** (55 mg, 47%) as a colorless solid. Mp 191–193 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.38 (s, 9H), 1.80–1.97 (m, 2H), 2.44–2.60 (m, 1H), 2.81–2.92 (m, 1H), 3.10 (dd, 1H, J = 16, 3.2 Hz), 3.24 (dd, 1H, J = 16, 2.9 Hz), 3.83–3.95 (m, 4H), 3.98–4.03 (m, 1H), 5.30 (ddd, 1H, J = 10, 3.2, 2.9 Hz), 6.00 (s, 1H), 6.88–6.95 (m, 2H), 7.19 (d, 1H, J = 9.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 29.2, 30.0, 31.6, 34.2, 35.6, 51.4, 60.6, 63.9, 65.5, 79.0, 79.9, 104.8, 110.3, 122.3, 122.4, 125.2, 125.7, 126.3, 128.4, 136.2, 143.1, 155.7; IR (KBr) cm^{-1}: 2235; MS *m*/*z* 367 (M^+); HRMS Calcd for $C_{22}H_{25}NO_4$: 367.1784 (M^+), found: 367.1786; Anal.

Calcd for $C_{22}H_{25}NO_4$: C, 71.91; H, 6.86; N, 3.81. Found: C, 71.81; H, 6.99; N, 3.70.

4.1.8. 8-Acetoxy-2,3,5a,10c-tetrahydro-1*H*,6*H*-5-oxa-3-oxo-acephenanthrylene-10b-carbonitrile ethylene ketal (12). To a solution of **6** (100 mg, 0.32 mmol) in dry pyridine (2 mL) was added acetic anhydride (33 μL, 0.35 mmol) at room temperature, and the mixture was stirred for 12 h. The reaction mixture was diluted with H_2O and extracted with AcOEt. The combined organic layer was washed with 10% HCl and saturated $NaHCO_3$, successively, then dried over $MgSO_4$. Evaporation of the solvent gave a residue, which was purified by column chromatography to afford **12** (96 mg, 79%) as a colorless solid. Mp 121–123 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.86 (ddd, 1H, J = 12, 6.5, 3.2 Hz), 1.90 (ddd, 1H, J = 12, 3.5, 3.0 Hz), 2.29 (s, 3H), 2.56 (ddd, 1H, J = 15, 6.5, 3.0 Hz), 2.88 (ddd, 1H, J = 15, 3.5, 3.2 Hz), 3.14 (dd, 1H, J = 16, 3.5 Hz), 3.29 (dd, 1H, J = 16, 2.4 Hz), 3.86–3.96 (m, 4H), 4.02 (d, 1H, J = 11 Hz), 5.30 (ddd, 1H, J = 11, 3.5, 2.4 Hz), 6.01 (s, 1H), 7.05 (s, 1H), 7.08 (d, 1H, J = 9.3 Hz), 7.32 (d, 1H, J = 9.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.4, 29.9, 31.5, 34.1, 35.6, 51.3, 63.9, 65.5, 79.6, 104.7, 110.3, 120.5, 121.8, 123.5, 126.9, 128.4, 137.2, 143.2, 150.7, 169.1; IR (KBr) cm^{-1}: 2230, 1760; MS m/z 353 (M^+); HRMS Calcd for $C_{20}H_{19}NO_5$: 353.1263 (M^+). Found: 353.1253; Anal. Calcd for $C_{20}H_{19}NO_5$: C, 67.98; H, 5.42; N, 3.96. Found: C, 67.99; H, 5.42; N, 3.98.

4.1.9. 2,3,5a,10c-Tetrahydro-1*H*,6*H*-5-oxa-3-oxo-8-(phenylacetoxy)acephenanthrylene-10b-carbonitrile ethylene ketal (13). To a solution of **6** (50 mg, 0.16 mmol) in dry CH_2Cl_2 (1 mL) were added phenylacetyl chloride (23 μL, 0.18 mmol) and DMAP (catalytic amount) at room temperature, and the mixture was stirred for 2 h. According to the work-up procedure just mentioned above, the compound **13** (42 mg, 61%) was obtained as a colorless solid. Mp 72–74 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.83 (ddd, 1H, J = 11, 4.3, 3.5 Hz), 1.88 (ddd, 1H, J = 11, 7.9, 3.2 Hz), 2.17 (s, 2H), 2.55 (ddd, 1H, J = 15, 7.9, 4.3 Hz), 2.88 (ddd, 1H, J = 15, 3.5, 3.2 Hz), 3.12 (dd, 1H, J = 16, 2.1 Hz), 3.26 (dd, 1H, J = 16, 3.5 Hz), 3.82–3.95 (m, 4H), 3.99–4.03 (m, 1H), 5.29 (ddd, 1H, J = 10, 3.5, 2.1 Hz), 5.99 (s, 1H), 7.03–7.06 (m, 2H), 7.28–7.38 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 29.9, 31.5, 34.1, 35.6, 41.6, 51.3, 63.9, 65.5, 79.6, 104.6, 110.3, 120.4, 121.8, 123.4, 126.9, 127.5, 128.8, 129.3, 133.3, 137.2, 143.2, 150.7, 169.6; IR (KBr) cm^{-1}: 2229, 1755; MS m/z 429 (M^+); HRMS Calcd for $C_{26}H_{23}NO_5$: 429.1576 (M^+). Found: 429.1565; Anal. Calcd for $C_{26}H_{23}NO_5$: C, 72.71; H, 5.40; N, 3.26. Found: C, 72.77; H, 5.60; N, 3.29.

4.1.10. 5a,10c-Dihydro-1*H*,6*H*-5-oxa-8-(triisopropylsilyloxy)acephenanthrylene-10b-carbonitrile (14). A solution of **21**[10c] (104 mg, 0.24 mmol) in *o*-dichlorobenzene (3 mL) was refluxed for 2 h. The solvent was evaporated to leave a residue, which was chromatographed to afford **14** (30 mg, 30%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.01–1.11 (m, 18H), 1.19–1.29 (m, 3H), 2.89–3.00 (m, 2H), 3.18–3.32 (m, 2H), 3.89 (d, 1H, J = 11 Hz), 5.24–5.36 (m, 1H), 5.40–5.45 (m, 1H), 5.98 (dd, 1H, J = 8.8, 2.1 Hz), 6.03 (s, 1H), 6.69 (dd, 1H, J = 8.5, 2.0 Hz), 6.78 (s, 1H), 6.85 (d, 1H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.9, 18.1, 32.1, 34.5, 35.4, 49.1, 55.7, 109.7, 112.1, 115.0, 118.4, 121.0, 122.9, 123.9, 126.4, 136.7, 142.4, 159.1; IR (neat) cm^{-1}: 2241; MS m/z 407 (M^+); HRMS Calcd for $C_{25}H_{33}NO_2Si$: 407.2281 (M^+). Found: 407.2284.

4.1.11. 5a,10c-Dihydro-1*H*,6*H*-8-hydroxy-5-oxa-acephenanthrylene-10b-carbonitrile (22). To a solution of **14** (30 mg, 0.07 mmol) in CH_2Cl_2 (1 mL) was added TBAF (1.0 M THF solution, 80 μL, 0.08 mmol) at 0 °C, and the mixture was stirred for 0.5 h at room temperature. The reaction mixture was diluted with H_2O, extracted with Et_2O, and then dried over $MgSO_4$. Evaporation of the solvent followed by column chromatography afforded **22** (17 mg, 94%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.91–3.02 (m, 2H), 3.20–3.31 (m, 2H), 3.88 (d, 1H, J = 11 Hz), 4.90–5.01 (m, 1H), 5.25–5.38 (m, 1H), 5.42–5.49 (m, 1H), 6.01 (dd, 1H, J = 8.8, 1.9 Hz), 6.12 (s, 1H), 6.64 (dd, 1H, J = 8.7, 2.5 Hz), 6.77 (s, 1H), 6.98 (d, 1H, J = 8.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 32.0, 34.5, 35.6, 49.2, 55.2, 109.7, 112.9, 115.1, 118.4, 120.8, 122.9, 124.0, 126.5, 136.3, 142.4, 159.0; IR (neat) cm^{-1}: 2232; MS m/z 251 (M^+); HRMS Calcd for $C_{16}H_{13}NO_2$: 251.0946 (M^+). Found: 251.0958.

4.1.12. 5a,10c-Dihydro-1*H*,6*H*-5-oxa-8-(trifluoromethoxy)acephenanthrylene-10b-carbonitrile (15). According to the synthetic procedure for **10**, the compound **22** (17 mg, 0.07 mmol) afforded **15** (9 mg, 43%) as a colorless solid. Mp 167–169 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.84–2.98 (m, 2H), 3.17–3.27 (m, 2H), 3.83 (d, 1H, J = 11 Hz), 5.23–5.32 (m, 1H), 5.39–5.46 (m, 1H), 5.97 (dd, 1H, J = 8.8, 2.2 Hz), 6.08 (s, 1H), 6.63 (dd, 1H, J = 8.7, 2.4 Hz), 6.77 (s, 1H), 6.94 (d, 1H, J = 9.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 32.1, 34.6, 35.5, 49.2, 55.1, 109.7, 112.8, 115.3, 118.3, 120.8, 122.7, 124.5, 126.1, 136.3, 142.2, 147.0, 158.9; IR (KBr) cm^{-1}: 2243; MS m/z 319 (M^+); HRMS Calcd for $C_{17}H_{12}F_3NO_2$: 319.0820 (M^+). Found: 319.0828; Anal. Calcd for $C_{17}H_{12}F_3NO_2$: C, 63.95; H, 3.79; N, 4.39. Found: C, 63.82; H, 3.71; N, 4.50.

4.2. Biology

4.2.1. Drugs. The test compounds were dissolved in dimethylsulfoxide (DMSO) and then diluted with suitable medium to become a final concentration of DMSO to less than 1%.

4.2.2. Virus. Influenza virus was propagated for 3 days at 35 °C in chorioallantoic cavities of 10-day-old embryonated hen eggs. The infected allantoic fluids were clarified by centrifugation at 1000g for 20 min and stored in small portions at −80 °C as a virus stock solution.

4.2.3. Cells. Madin-Darby canine kidney (MDCK) cells were cultured as monolayers in Eagle's minimum essential medium (MEM) supplemented with 8% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C.

4.2.4. Virus growth assay. A confluent monolayer of MDCK cells in a 24-well plate was washed once with phosphate-buffered saline (PBS) and then infected with influenza A (Aichi/2/68) virus at a multiplicity of infection (MOI) of 5 plaque forming unit (PFU)/cell (for experiments for Figures 3 and 6) or 5×10^{-4} PFU/cell (for experiments for Fig. 4) for 45 min at room temperature under a drug-free condition. After adsorption, the cells were washed three times with PBS and then cultured in serum free MEM (supplemented with 2.5×10^{-3}% trypsin for Fig. 4) with various drugs or 1% DMSO (as a control) at 37 °C. At 24 h (for Figs. 3 and 6) or 72 h (for Fig. 4) postinfection, the culture fluids were collected and centrifuged at 500*g* for 5 min. The virus yield in the supernatants was assayed by plaque titration on MDCK cells.[12] Reasons for employing different growth conditions between the experiments of Figures 4 and 6 are as follows. In the case of influenza virus, proliferation stages, including early and late periods, are proceeding by hour order and at least 8–12 h is required to accomplish one-step growth cycle. Therefore, we adopted 1-h drug treatment to clarify the target stages of the tested drugs under one-step growth condition using a relatively higher MOI (5 PFU/cell) as shown in Figure 6. By using such higher MOI, all cells in a dish receive virus infection and virus growth cycle is proceeding synchronously in the cells convenient to examine the target stages in virus growth cycle. On the other hand, the multiple growth cycle condition using the relatively lower MOI is convenient to examine whether virus could grow or not under a specific condition such as treatment with drugs. Therefore, the dose-dependency in Figure 4 was examined under a multiple growth cycle condition using 5×10^{-4} PFU/cell (compare with 5 PFU/cell in Figure 6) and virus yields were assayed at 72-h postinfection at which several virus growth cycles should be accomplished.

For experiments for Figure 5: a confluent monolayer of MDCK cells in a 6-cm dish was washed once with PBS and then inoculated with 200 μL of virus solution (in serum free MEM). After adsorption at room temperature for 45 min, the cells were cultured in the agar media with drug (100 μM) or 1% DMSO (as a control). Three days later, the cells were fixed with 5% formalin and stained with 0.01%. Crystal violet to visualize plaques.

4.2.5. Cytotoxicity assay. The subconfluent cells (10^4 cell/well in a 96-well plate) were treated with 150 μL MEM supplemented with 2% FBS containing drug or 1% DMSO (as a control). After 24 h culture, the cells were processed to MTT assay, according to the reported procedure.[13]

4.2.6. Evaluation of mRNA synthesis. A confluent monolayer of MDCK cells in a 6-cm dish was washed once with PBS and then inoculated with 200 μL of virus solution (serum free MEM) for 45 min. After infection, the cells were washed three times with PBS and then cultured in serum free MEM with the compound **10** (100 μM) or 1% DMSO (as a control) at 37 °C. At 3-h postinfection, the cells were washed with serum free MEM and collected to Eppendorf tubes. Total RNA was isolated from collected cells by RNA-Bee Kit (Tel-Test INC. Texas). Reverse transcription of RNA was carried out in 20 μL reaction mixture containing 25 mM $MgCl_2$ (2.8 μL), PCR Buffer (Mg^{2+} free, 2.0 μL), dNTP Mix (4.0 μL), isolated RNA (1 μg) in DEPC-H_2O (8.2 μL), RNase inhibitor (1.0 μL), reverse transcriptase (1.0 μL), and oligo dT16 (50 pM, 1.0 μL). Reaction was performed for 60 min at 37 °C, 5 min at 99 °C, and 10 min at 4 °C. Subsequently, PCR was carried out in 30 μL reaction mixture containing reverse transcription product (1.0 μL), 25 mM $MgCl_2$ (1.66 μL), PCR Buffer (Mg^{2+} free, 3.0 μL), DEPC-H_2O (18.84 μL), sense primer (5′-AATTTTGATGCCT-GAAACCGT-3′, 1.0 μL), antisense primer (5′-GCTCTGTCCATGTTATTTGGATC-3′, 1.0 μL), dNTP Mix (3.0 μL), and Taq DNA polymerase (0.5 μL). The mixture was denatured initially for 3 min at 94 °C, followed by 30 cycles consisted of 1 min at 94 °C, 2 min at 55 °C, 3 min at 72 °C, and finally, for 7 min at 72 °C. The products were separated in 1.6% agarose gel electrophoresis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.09.046.

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