Bioorganic & Medicinal Chemistry 22 (2014) 6647–6654

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Oseltamivir hydroxamate and acyl sulfonamide derivatives as influenza neuraminidase inhibitors

Bei-Tao Hong [a], Chun-Lin Chen [a], Jim-Min Fang [a,b,*], Keng-Chang Tsai [c], Shi-Yun Wang [b], Wen-I Huang [b], Yih-Shyun E. Cheng [b], Chi-Huey Wong [b]

[a] Department of Chemistry, National Taiwan University, Taipei 106, Taiwan
[b] The Genomics Research Center, Academia Sinica, Taipei 115, Taiwan
[c] National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei 112, Taiwan

ARTICLE INFO

Article history:
Received 11 June 2014
Revised 30 September 2014
Accepted 5 October 2014
Available online 25 October 2014

Keywords:
Influenza
Neuraminidase inhibitor
Oseltamivir
Bioisosteres
Hydroxamate
Acyl sulfonamide

ABSTRACT

Tamiflu, the ethyl ester form of oseltamivir carboxylic acid (OC), is the first orally available anti-influenza drug for the front-line therapeutic option. In this study, the OC-hydroxamates, OC-sulfonamides and their guanidino congeners (GOC) were synthesized. Among them, an OC-hydroxamate **7d** bearing an *O*-(2-indolyl)propyl substituent showed potent NA inhibition (IC_{50} = 6.4 nM) and good anti-influenza activity (EC_{50} = 60.1 nM) against the wild-type H1N1 virus. Two GOC-hydroxamates (**9b** and **9d**) and one GOC-sulfonamide (**12a**) were active to the tamiflu-resistant H275Y virus (EC_{50} = 2.3–6.9 μM).

1. Introduction

Influenza often occurs in winter seasons and occasionally causes pandemics due to emergence of virus mutants and unexpected transmission to humans. Influenza has been a long-standing threat to humans' health over centuries. Spanish flu in 1918 has caused more than 20 million deaths because no vaccine or anti-influenza drugs were available.[1] The outbreaks of new type H1N1 pandemic flu[2] in 2009 as well as H5N1 and H7N9 avian flus[3,4] further raise the public concern of possible influenza pandemics and cross-species transmission to humans.

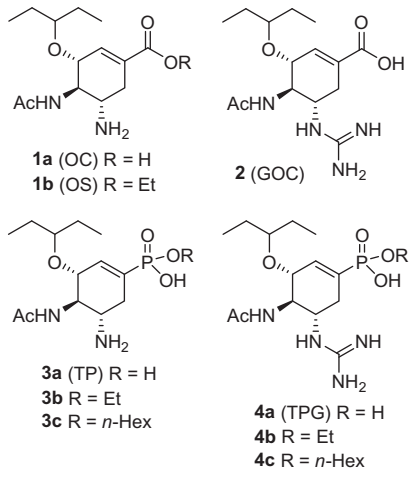
There are two classes of anti-influenza drugs approved to target the virus M2 ion channel and neuraminidase (NA). However, the M2 ion channel inhibitors amantadine[5] and rimantadine[6] are no longer recommended for treatment of influenza infection due to their severe side effects and drug resistance. NA is a viral surface glycoprotein responsible for cutting the connection between the hemagglutinin (HA) and the host cell,[7] an essential function to release the progeny viruses for infection to the surrounding cells. Inhibition of viral NA is a very effective method for influenza therapy because the active site of NA is highly conserved across the influenza A and B viral strains.[8,9] Influenza viral HAs bind the sialo-receptor on host cells. The NA-catalyzed hydrolysis of the terminal sialyl residue involves a cationic intermediate in oxonium-like configuration.[10–12] Oseltamivir carboxylic acid (OC, **1a**) is an NA inhibitor designed to have the cyclohexene scaffold to mimic the sialosyl cationic intermediate.[13,14] Tamiflu™, the phosphate salt of oseltamivir (OS, **1b**), is the first orally available anti-influenza drug. As an ethyl ester, OS is readily hydrolyzed by endogenous esterases to OC as the real active NA inhibitor. The carboxylate group of OC provides strong electrostatic interactions with the three arginine residues (Arg118, Arg292 and Arg371) in the S1 site of influenza NA. OC contains a 3-pentoxy substituent, in lieu of the glycerol side chain in sialic acid, to render hydrophobic interactions with the Glu276, Ala246, Arg224, and Ile222 residues in the NA active site. However, the clinically relevant H275Y OS-resistant virus has evolved over the years because replacement of the histine residue by a bulkier tyrosine will cause collapse of the salt bridge between Glu276 and Arg224 to reduce the affinity with the hydrophobic 3-pentyl moiety in OS.[15,16]

Many OC analogs have been synthesized to test the anti-influenza activities, for example, using lipophilic alkyl and aryl groups other than the 3-pentyl group,[13] using different amido groups at the C-4 position,[17] or relocating the C–C double bond in the cyclohexene scaffold.[18] However, such structural modifications have not led to better anti-influenza activity than OC. In contrast, GOC (**2**) having the amino group at the C-5 position

* Corresponding author. Tel.: +886 2 33661663; fax: +886 2 27325090.
E-mail address: jmfang@ntu.edu.tw (J.-M. Fang).

http://dx.doi.org/10.1016/j.bmc.2014.10.005
0968-0896/

in OC replaced by a more basic gaunidino group does upgrade the NA inhibitory activity.[14] The guanidinium ion is expected to increase the electrostatic interactions with the acidic residues (Glu119, Asp151, and Glu227) in the active site of influenza NA. However, GOC and its ethyl ester have not been developed for therapeutic use because of their poor pharmacokinetic properties. The recent study indicates that *N*-hydroxyguanidine[19] and amidoxime[20] can be utilized as surrogates of the guanidino group to improve bioavailability. The 5-acetamidine analog of OC exhibits high NA inhibition and anti-influenza activities against wild-type and resistant strains.[20] Schade and his co-workers also show that the acetamidoxime analog of OS can act as a prodrug to attain good pharmacokinetic profile with 31% oral bioavailability in rats while the *N*-hydroxyguanidine analog lacks of oral bioavailability ($F = 1.5\%$).[20] In another approach, GOC esters carrying valine or phenylalanine are designed to render better permeability that is facilitated by the peptide transporter–cellular activation.[21]



Bioisosteres are the functional groups through rational modifications of an active composition to mimic the structure with similar chemical, physical, electronic, conformational and biological properties.[22] Many medicinal chemists have taken the advantages of bioisosterism to optimize the pharmacokinetic properties of lead compounds to develop safer and more clinically effective agents. As carboxylic acid is an important functional group in drugs, the bioisosteres of carboxylic acid have been extensively investigated.[23] By replacing the carboxylic group in OC, the phosphonate congener **3a** (tamiphosphor, TP) shows remarkable neuraminidase inhibition and anti-influenza activities in the cell-based and animal experiments.[24,25] The monoesters of TP,[25–28] for example, **3b** and **3c**, are also effective anti-influenza drugs because the singly-charged phosphonate group still retains substantial electrostatic interactions with the three arginine residues (Arg118, Arg292 and Arg371) in the active site of neuraminidase. As expected, guanidino TP (TPG, **4a**) and its monoesters (e.g., **4b** and **4c**) exhibit even better inhibitory activities against avian and human influenza viruses including the drug-resistant H275Y strain.[24,25]

According to molecular modeling experiments, we have previously proposed a 371-cavity near the S1-site of influenza NA to accommodate appropriate alkyl substituents.[28] As a continuing research, we report herein the synthesis and anti-influenza activities of two series of OC bioisosteres: hydroxamates **7a**–**7d** and acyl sulfonamides **10a**–**10d** (Scheme 1). Hydroxamic acid ($pK_a \approx 8.5$) is weaker than carboxylic acid ($pK_a \approx 4.7$), whereas acyl sulfonamide ($pK_a \approx 5$–6) has the acidity close to that of carboxylic acid. Compared with the carboxylic acid group in OC, the hydroxamate groups in **7a**–**7d** and the acyl sulfonamide groups in **10a**–**10d** contain more electronegative atoms and hydrophobic alkyl substituents that may exert additional interactions with influenza NA in the S1 site. Compounds **9b**–**9d** and **12a**–**12d** were also prepared by guanidination of **8b**–**8d** and **10a**–**10d**, respectively, for comparison of the bioactivities.

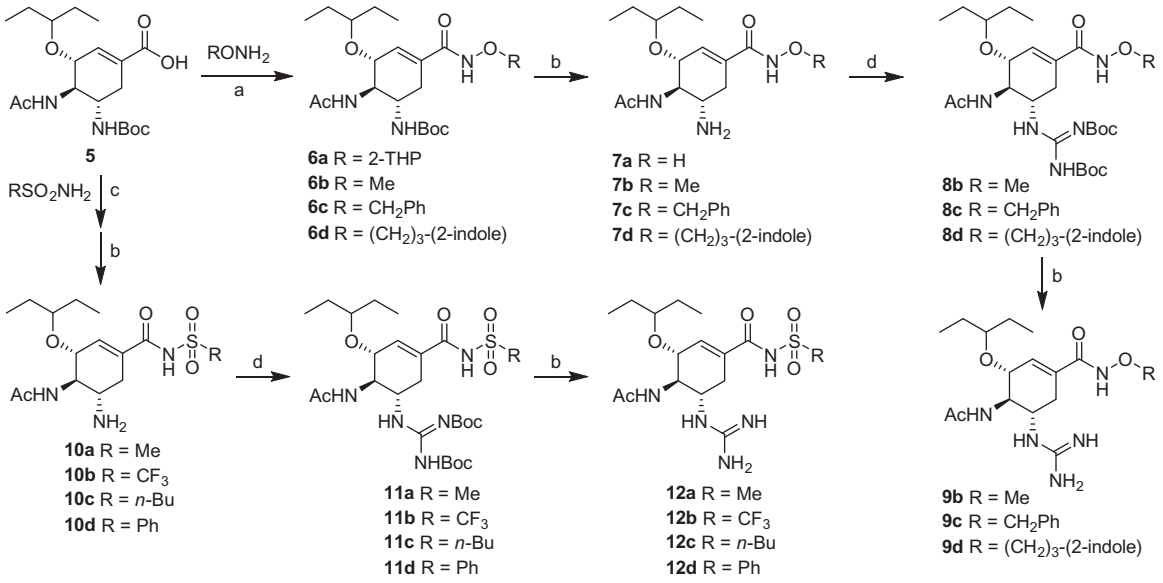
2. Results and discussion

For the synthesis of OC hydroxamate and acyl sulfonamide derivatives, we consider using the Boc-protecting OC (**5**)[28,29] as the starting material to conduct the amide bond forming reactions (Scheme 1). Thus, the commercially available *O*-alkyl hydroxylamines (CH_3ONH_2 and $PhCH_2ONH_2$ as the hydrochloride salts) were subjected to coupling reactions with **5** using diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP) and *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as the combined promoting agents to give **6b** and **6c**, which were subsequently treated with trifluoroacetic acid (TFA) to afford OC-hydroxamates **7b** and **7c**. Attempts for direct coupling reaction of acid **5** with NH_2OH failed, even using various promoting agents such as HBTU, thionyl chloride, ethyl chloroformate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP). Alternatively, NH_2OH was protected as the tetrahydropyran acetal (NH_2OTHP)[30] and proceeded smoothly with an amide forming reaction with **5** in the presence of HBTU, DIPEA and DMAP to give **6a**. Both the Boc and THP protecting groups in **6a** were concomitantly removed by treatment with TFA to give the desired hydroxamic acid **7a**. We also synthesized hydroxamate **7d** containing a 3-(indol-2-yl)propyl substituent to test whether it would provide good NA inhibitory activity. The aralkyl group in **7d** may reside in the hydrophobic 371-cavity that is enclosed by the Arg371, Pro431, Ile427, Lys432 and Trp403 residues near the S1 site to attain additional hydrophobic, π–π and π–cation interactions.[31] For guanidination, **7b**–**7d** were reacted respectively with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine in the presence of Et_3N to give the Boc-protecting compounds **8b**–**8d**, which were subsequently treated with TFA to give the desired products **9b**–**9d**.

By the similar procedure, the coupling reactions of acid **5** with a series of sulfonamides were also carried out, followed by removal of the Boc protecting group with TFA, to afford acyl sulfonamides **10a**–**10d**. The amino group in **10a**–**10d** was further elaborated to guanidino group by treatment with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine to afford GOC-sulfonamides **12a**–**12d** after removal of the Boc groups.

The enzymatic assays for NA inhibition were conducted using a fluorescent substrate, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA), whereas the anti-influenza activities were measured by the cytopathic effect of Madin–Darby canine kidney (MDCK) cells due to infection of influenza viruses. Table 1 shows the IC_{50} and EC_{50} values of OC-hydroxamates **7a**–**7d**, GOC-hydroxamates **9b**–**9d**, OC-sulfonamides **10a**–**10d** and GOC-sulfonamides **12a**–**12d**. In comparison with OC, the NA inhibitory activity of **7a** was much lower, presumably because the hydroxamic acid in **7a** was less acidic to attain strong interactions with the three arginine residues in the S1 site of influenza NA. By incorporation of alkyl substituents at the *O*-atom of hydroxamate moiety, compounds **7b**–**7d** displayed better NA inhibitory and anti-influenza activities than **7a**. In this series, hydroxamate **7d** bearing an (indol-2-yl)propyl substituent was the best inhibitor, showing the IC_{50} value of 6.4 nM and the EC_{50} value of 60.1 nM, close to the activity of OC against the wild-type H1N1 virus.

The molecular modeling of **7d** in the active site of influenza virus NA (Fig. 1) indicates that the hydroxamate moiety exhibits extensive hydrogen bonding interactions (4 pairs ligand–NA



Scheme 1. Synthesis of oseltamivir hydroxamate and acyl sulfonamide derivatives. Reagents and reaction conditions: (a) *i*-Pr_2NEt, DMAP, HBTU, CH_2Cl_2, rt, 10–12 h. (b) CF_3CO_2H, CH_2Cl_2, rt, 1 h. (c) Et_3N, DMAP, HBTU, CH_2Cl_2, rt, 10 h. (d) (BocNH)(TfNH)C=NBoc, Et_3N, CH_2Cl_2, rt, 10–12 h. Overall yields: **7a** (47%), **7b** (51%), **7c** (54%), **7d** (42%), **9b** (41% from **7b**), **9c** (42% from **7c**), and **9d** (37% from **7d**), **10a** (47%), **10b** (51%), **10c** (49%), and **10d** (43%), **12a** (32% from **10a**), **12b** (39% from **10b**), **12c** (36% from **10c**), and **12d** (28% from **10d**). Boc = *tert*-butoxycarbonyl; HBTU = *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DMAP = 4-dimethylaminopyridine; THP = tetrahydropyran; Tf = trifluorosulfonyl.

Table 1
Neuraminidase inhibition and anti-influenza activity

Compd	IC_{50}[a] (nM)		EC_{50}[b] (nM)	
	A/WSN/33[c]	H275Y[c]	A/WSN/33[c]	H275Y[c]
OC	1.78 ± 0.55	260 ± 21	28.2 ± 3.6	11,026 ± 1541
7a	384 ± 14	No effect[d]	359 ± 107	No effect[d]
7b	45.4 ± 11.6	6648 ± 322	127 ± 4	No effect[d]
7c	31.1 ± 6.7	1010 ± 54	72.4 ± 34.9	No effect[d]
7d	6.4 ± 2.0	1108 ± 89	60.1 ± 2.7	No effect[d]
9b	6.82 ± 1.03	781 ± 45	71.7 ± 3.9	2770 ± 1579
9c	8.49 ± 1.61	6946 ± 1971	23.8 ± 11.6	16143 ± 11502
9d	151.2 ± 11.8[e]	1227 ± 51	90.7 ± 49.8	2268 ± 442
10a	67.5 ± 30.6	19,085 ± 4681	401 ± 162	No effect[d]
10b	1203 ± 367	No effect[d]	2223 ± 472	No effect[d]
10c	656 ± 262	No effect[d]	1977 ± 23	No effect[d]
10d	574 ± 105	No effect[d]	7487 ± 2500	No effect[d]
12a	20.3 ± 1.9	19.5 ± 2.2	59.1 ± 0.6	6902 ± 766
12b	94.0 ± 15.9	832 ± 524	484 ± 4	No effect[d]
12c	41.5 ± 8.7	1108 ± 427	261 ± 119	No effect[d]
12d	37.6 ± 9.0	13,399 ± 228	593 ± 34	No effect[d]

[a] A fluorescent substrate, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA), was used to determine the IC_{50} values that are compound concentrations causing 50% inhibition of different influenza neuraminidases. Data are shown as mean ± SD of three experiments.

[b] The anti-influenza activities against different influenza viruses were measured as EC_{50} values that are the compound concentrations for 50% protection of the cytopathic effects due to the infection by different influenza viruses. Data are shown as mean ± SD of three experiments.

[c] A/WSN/33 is human H1N1 virus, and H275Y is an oseltamivir-resistant mutant.

[d] The IC_{50} or EC_{50} values were over 100 μM.

[e] Data for two repeated triplicate experiments.

H-bonds) with three arginine residues (R118, R292 and R371) and the phenol group of Y347 in the NA active site. The oxygen of the acetamido group in **7d** forms a hydrogen bond with the guanidinium group of R152. In addition, the amine group in **7d** forms ionic interactions with residues D151, E119 and E227. The (2-indolyl)propyl substituent in **7d** extends to the 371-cavity to obtain appreciable H-bond, hydrophobic, π–π and π–cation interactions.[28,31]

In the series of OC-sulfonamides, **10a** bearing a methyl substituent in the moiety of acyl sulfonamide showed modest NA inhibitory activity against the wild-type and H275Y H1N1 viruses with IC_{50} values of 67.5 and 401 nM, respectively. However, compound **10b** having an electronegative trifluoromethyl substituent decreased the NA inhibitory activity by 5–17 folds relative to **10a**. Compounds **10c** and **10d** having bulkier butyl and phenyl substituents also caused reduced binding to influenza NA. Though the acidity of acyl sulfonamides is similar to carboxylic acid, OC-sulfonamides **10a**–**10d** did not display good NA inhibition. This result suggested that the acidity of bioisosteres was not a single factor responsible for good anti-influenza activity.

By replacing the amino group in **10a**–**10d** with a more basic guanidino group, compounds **12a**–**12d** did exert much higher inhibitory activities against the wild-type H1N1 virus. However, only compound **12a** showed somewhat better activity than OC against the H275Y mutant of H1N1 virus with IC_{50} value of 19.5 nM and EC_{50} value of 6.9 μM. The guanidino groups in **12a**–**12d** were considered to render stronger interactions with the acidic residues acidic residues of Glu119, Asp151 and Glu227 in the S2 site of NA.[14] The tendency of inhibitory activity in the two acyl-sulfonamide series was similar, i.e. **10a**/**12a** (R = CH_3) > **10d**/**12d** (R = Ph) ≈ **10c**/**12c** (R = *n*-Bu) > **10b**/**12b** (R = CF_3).

The effects of guanidino group in GOC-hydroxamates **9b**–**9d** are discrepant. The *O*-methyl GOC-hydroxamate **9b** showed much better activities than the corresponding OC-hydroxamate **7b** in NA inhibition and cell protection against H1N1 and mutant influenza viruses. In comparison with **7c**, the *O*-benzyl GOC-hydroxamte **9c** also showed better cell protection against H1N1 virus and the H275Y mutant. Compounds **9d** and **7d** showed similar NA inhibition against H275Y mutant, but **9d** exhibited a higher IC_{50} value (151 nM) than **7d** (6.4 nM) against wild-type H1N1 virus for unclear reason. Nonetheless, both compounds displayed equal inhibition activity against the H275Y strain (IC_{50} ≈ 1.2 μM). It was noted that GOC-hydroxamates **9b** and **9d** were effective to protect cells against the infection of H275Y mutant (EC_{50} = 2.3–2.8 μM).

Compounds **9b**, **9d** and **12a** were nontoxic to MDCK and human 293T cells (CC_{50} > 100 μM). Based on the CC_{50} data of the

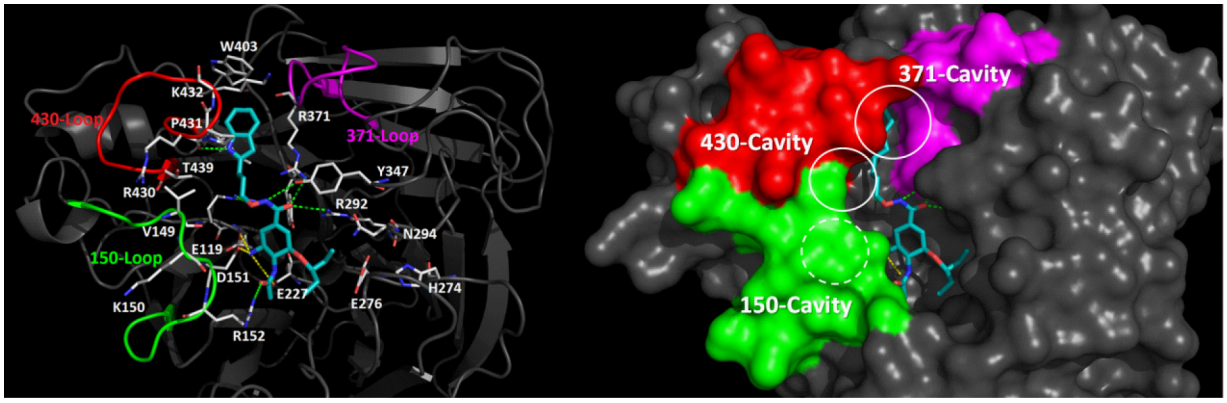


Figure 1. Molecular model of **7d** in the active site of influenza virus neuraminidase (N1 subtype, PDB id: 2HU4). The hydrogen bonds and ionic interactions are shown in green and yellow dotted lines, respectively.

anti-influenza agents, unspecific effects (from cytotoxicity) could be excluded for the entire series of OC derivatives (Table 1).

3. Conclusion

Oseltamivir carboxylic acid (OC) is a potent neuraminidase inhibitor, and the ethyl ester OS is developed as an effective orally available anti-influenza drug. In this study, we first synthesized two series of OC bioisosteres, that is, OC-hydroxamates **7a**–**7d** and OC-sulfonamides **10a**–**10d**, as well as the guanidino congeners **9b**–**9d** and **12a**–**12d**. In the series of OC-hydroxamates, **7d** bearing an *O*-(2-indolyl)propyl substituent showed the best NA inhibitory activity, presumably because the substituent can locate in the 371-cavity near the S1 site of NA to attain additional hydrophobic, π–π and π–cation interactions. In the series of GOC-sulfonamides, **12a** is an effective NA inhibitor with the EC_{50} value slightly lower than OC against the drug-resistant H275Y virus. The GOC-hydroxamates **9b** and **9d** were even better anti-influenza agents against the H275Y mutant ($EC_{50} \approx 2.5$ μM). Our study indicates that the NA inhibitory activity cannot simply be accounted on to the acidity and the numbers of electron-negative atoms in the carboxylate isosteres. So far, only OC phosphonate congeners have been demonstrated to possess enhanced NA inhibition by having higher acidity with complementary topology to interact with the three arginine residues (Arg 181, Arg292 and Arg371) in the NA active site.[24,26]

4. Experimental section

4.1. General

All the reagents were commercially available and used without further purification unless indicated otherwise. All solvents were anhydrous grade unless indicated otherwise. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel. Flash chromatography was performed on silica gel (40–63 μm particle size) and LiChroprep RP-18 (40–63 μm particle size). Yields were reported for spectroscopically pure compounds. Melting points were recorded on a Yanaco melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity Plus-400 (400 MHz) spectrometer. ^{19}F NMR spectra were recorded on Varian Unity Plus-400 (400 MHz) spectrometer. Chemical shifts were given in δ values relative to tetramethylsilane (TMS); coupling constants J were given in Hz. Internal standards were $CDCl_3$ (δH = 7.24), CD_3OD (δH = 3.31) or D_2O (δH = 4.79) for ^{1}H NMR spectra; and $CDCl_3$ (δC = 77.0) or CD_3OD (δC = 49.15) were for ^{13}C NMR spectra. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad) and dd (doublet of doublets). IR spectra were recorded on Varian 640 or Nicolet Magna 500-II. Optical rotations were recorded on digital polarimeter of Japan JASCO Co. DIP-1000. $[\alpha]_D$ values were given in units of 10^{-1} deg cm^2 g^{-1}. ESI mass spectra were recorded on a high-resolution mass spectrometer.

4.2. Materials

Influenza A/WSN/1933 (H1N1) was from Dr. Shin-Ru Shih at Chang Gung University in Taiwan. All viruses were cultured in the allantoic cavities of 10-day-old embryonated chicken eggs for 72 h, and purified by sucrose gradient centrifugation. Madin–Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, VA), and were grown in DMEM (Dulbecco's modified Eagle medium, GibcoBRL) containing 10% fetal bovine serum (GibcoBRL) and penicillin–streptomycin (GibcoBRL) at 37 °C under 5% CO_2.

The oseltamivir-resistant A/WSN H275Y mutant was created using a 12-plasmid system that was based on cotransfection of mammalian cells with 8 plasmids encoding virion sense RNA under the control of a human PolI promoter and 4 plasmids encoding mRNA encoding the RNP complex (PB1, PB2, PA, and nucleoprotein gene products) under the control of a PolII promoter. An H275Y mutation was introduced in the NA gene, and the sequence was confirmed to generate the A/WSN H275Y virus.

4.3. Synthetic procedures and compound characterization

Compound **5** was prepared according to the previously reported method.[28,29]

4.3.1. *N*-Hydroxy (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (7a)

To a solution of the Boc protected oseltamivir acid (**5**, 78 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) were added (tetrahydro-2*H*-pyran-2-oxy)amine[30] (28 mg, 0.24 mmol), HBTU (91 mg, 0.24 mmol), DIPEA (0.1 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The mixture was stirred at room temperature for 10 h, and then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 1.0 M HCl, saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **6a** as pale yellow oil, which was used in the next step without further purification.

A solution of the above-prepared compound **6a** in anhydrous CH_2Cl_2 (1 mL) was cooled to 0 °C in an ice-bath, and

trifluoroacetic acid (0.24 mL, 3.22 mmol) was added. The mixture was stirred and allowed to warm up to room temperature for 1 h, and then concentrated under reduced pressure. The residue was purified on a reversed-phase RP-18 column (MeOH/H_2O = 1:9–1:4) to give the desired product **7a** (28 mg, 47%). $C_{14}H_{25}N_3O_4$; white solid; mp 224–226 °C; $[\alpha]_D^{20}$ −11.5 (*c* 0.5, MeOH); IR (film) 3272, 2968, 2939, 2866, 1673, 1539, 1202, 1139 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 6.50 (1H, t, *J* = 2.0 Hz), 4.40 (1H, t, *J* = 8.8 Hz), 4.18 (1H, dd, *J* = 11.6, 8.8 Hz), 3.76–3.68 (1H, m), 3.67–3.62 (1H, m), 3.00–2.95 (1H, m), 2.71–2.63 (1H, m), 2.20 (3H, s), 1.71–1.54 (4H, m), 1.01–0.94 (6H, m); ^{13}C NMR (100 MHz, D_2O) δ 175.1, 173.9, 133.1, 132.4, 84.2, 75.7, 53.2, 49.7, 29.8, 25.5, 25.2, 22.3, 8.5, 8.4; HRMS calcd for $C_{14}H_{26}N_3O_4$:300.1923, found: *m*/*z* 300.1931 $[M+H]^+$.

4.3.2. *N*-Methoxy (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (7b)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with *O*-methyl hydroxylamine hydrochloride (17 mg, 0.24 mmol) was conducted in the presence of HBTU (91 mg, 0.24 mmol), DIPEA (0.1 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol) to give compound **6b**, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **7b** (32 mg, 51%). The purity of product **7b** was 98.7% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 15.2 min (MeOH/H_2O = 3:7). $C_{15}H_{27}N_3O_4$; hygroscopic solid; $[\alpha]_D^{22}$ −57.5 (*c* 1, MeOH); IR (film) 3475, 3273, 2969, 2940, 2882, 1672, 1548, 1440, 1375, 1303, 1202, 1137, 1078, 1021, 943, 844, 800, 723 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.40 (1H, s), 4.20 (1H, d, *J* = 8.4 Hz), 3.97 (1H, dd, *J* = 11.2, 8.4 Hz), 3.72 (3H, s), 3.54–3.48 (1H, m), 3.42 (1H, quint, *J* = 5.2 Hz), 2.83 (1H, dd, *J* = 18.2, 5.0 Hz), 2.51–2.44 (1H, m), 2.03 (3H, s), 1.59–1.44 (4H, m), 0.93–0.85 (6H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.9, 134.1, 129.6, 84.0, 75.8, 63.4, 54.6, 50.8, 34.1, 29.6, 27.3, 26.7, 23.3, 9.9, 9.7; HRMS calcd for $C_{15}H_{28}N_3O_4$: 314.2080, found: *m*/*z* 314.2080 $[M+H]^+$.

4.3.3. *N*-Benzyloxy (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (7c)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with *O*-benzyl hydroxylamine hydrochloride (38 mg, 0.24 mmol) was conducted in the presence of HBTU (152 mg, 0.24 mmol), DIPEA (0.1 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol) to give compound **6c**, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **7c** (82 mg, 68%). The purity of product **7c** was 96.7% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 12.7 min (MeOH/H_2O = 3:7). $C_{21}H_{31}N_3O_4$; hygroscopic solid; $[\alpha]_D^{23}$ −22.6 (*c* 1.0, MeOH); IR (film) 2967, 2937, 2878, 1673, 1639, 1543, 1526, 1458, 1436, 1374, 1300, 1203, 1184, 1135, 1074, 1019 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 7.60–7.55 (5H, m), 6.24 (1H, t, *J* = 2.0 Hz), 5.05 (2H, s), 4.33 (1H, d, *J* = 9.0 Hz), 4.12 (1H, dd, *J* = 11.8, 9.0 Hz), 3.70–3.63 (1H, m), 3.63–3.56 (1H, m), 2.88 (1H, dd, *J* = 17.0, 5.8 Hz), 2.62–2.55 (1H, m), 2.18 (3H, s), 1.69–1.52 (4H, m), 0.99–0.93 (6H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.9, 137.1, 133.9, 130.6 (2×), 129.8, 129.6 (4×), 84.0, 79.0, 75.9, 54.6, 50.8, 29.7, 27.3, 26.7, 23.3, 9.9, 9.7; HRMS calcd for $C_{21}H_{32}N_3O_4$: 390.2393, found: *m*/*z* 390.2393 $[M+H]^+$.

4.3.4. *N*-[3-(1*H*-Indol-2-yl)propoxy] (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (7d)

The details for preparation of 3-(1*H*-indol-2-yl)propoxylamine is reported in Supplementary data. In brief, 2-iodo-trifluoroacetamide was reacted with 3-butyn-1-ol in the presence of $Pd(OAc)_2$ to give 2-(3-hydroxypropyl)indole,[32] which underwent Mitsunobu reaction with *N*-hydroxyphthalimide in the presence of Ph_3P and diisopropyl azodicarboxylate (DIAD) to afford 3-(1*H*-indol-2-yl) propoxylamine after cleaving the phthalimide moiety with hydrazine.

To a solution of the Boc protected oseltamivir acid (**5**, 78 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) were added 3-(1*H*-indol-2-yl)propoxylamine (as the hydrochloric salt, 137 mg, 0.72 mmol), DIPEA (0.1 mL, 0.6 mmol), HBTU (91 mg, 0.24 mmol) and DMAP (7.4 mg, 0.02 mmol). The mixture was stirred at room temperature for 12 h, and then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 1.0 M HCl, saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/hexane = 3:1) to give **6d** (71 mg, 63%). $C_{30}H_{44}N_4O_6$; pale yellow oil; ^1H NMR (400 MHz, $CDCl_3$) δ 9.84 (1H, br), 9.71 (1H, br), 7.49 (1H, d, *J* = 7.6 Hz), 7.34 (1H, d, *J* = 7.6 Hz), 7.06 (1H, t, *J* = 7.6 Hz), 7.01 (1H, t, *J* = 7.6 Hz), 6.69 (1H, br d, *J* = 6.8 Hz), 6.27 (1H, s), 6.15 (1H, s), 5.52 (1H, d, *J* = 9.2 Hz), 4.08–3.99 (2H, m), 3.86–3.81 (2H, m), 3.32 (1H, t, *J* = 5.6 Hz), 2.85 (2H, t, *J* = 6.6 Hz), 2.69 (1H, dd, *J* = 17.2, 5.4 Hz), 2.31 (1H, dd, *J* = 17.2, 10.2 Hz), 2.03 (3H, s), 1.84 (2H, t, *J* = 6.0 Hz), 1.72–1.13 (14H, m), 0.93–0.82 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 166.9, 156.3, 138.8, 136.1, 132.8, 120.3, 128.5, 120.4, 119.3, 119.0, 110.8, 99.0, 82.9, 79.6, 75.8, 74.8, 55.1, 49.8, 31.0, 28.6, 28.4 (3×), 26.2, 25.7, 24.0, 23.3, 9.8, 9.3; HRMS calcd for $C_{30}H_{44}N_4NaO_6$:579.3159, found: *m*/*z* 579.3149 $[M+Na]^+$.

A solution of the above-prepared compound **6d** (71 mg, 0.12 mmol) in anhydrous CH_2Cl_2 (1 mL) was cooled to 0 °C in an ice-bath, and trifluoroacetic acid (0.24 mL, 3.22 mmol) was added. The mixture was stirred and allowed to warm up to room temperature for 1 h, and then concentrated under reduced pressure. The residue was purified on a reversed-phase RP-18 column (MeOH/H_2O = 1:9–1:4) to give the desired product **7d** (36 mg, 66%). The purity of product **7d** was 97.1% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 14.2 min (MeOH/H_2O = 3:7). $C_{25}H_{36}N_4O_4$; hygroscopic solid; $[\alpha]_D^{20}$ −20.6 (*c* 1.0, MeOH); IR (film) 3422, 2967, 2866, 2844, 1675, 1543, 1433, 1373, 1203, 1139, 1055, 1033, 1015 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 7.64 (1H, d, *J* = 7.6 Hz), 7.50 (1H, d, *J* = 8.4 Hz), 7.24 (1H, t, *J* = 7.4 Hz), 7.17 (1H, t, *J* = 7.4 Hz), 6.40 (1H, s), 4.31 (1H, br d, *J* = 7.6 Hz), 4.13–4.10 (1H, m), 4.07 (2H, t, *J* = 6.2 Hz), 3.68–3.60 (2H, m), 3.55 (1H, t, *J* = 5.4 Hz), 2.98 (2H, t, *J* = 7.2 Hz), 2.96–2.90 (1H, m), 2.67–2.56 (1H, m), 2.18–2.15 (5H, m), 1.66–1.51 (4H, m), 0.99–0.91 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 173.6, 167.4, 138.8, 137.9, 133.0, 129.3, 127.6, 121.6, 118.6, 111.0, 107.0, 82.7, 82.6, 74.5, 63.9, 50.0, 49.5, 39.0, 28.3, 26.0, 25.5, 25.4, 22.0, 8.6, 8.4; HRMS calcd for $C_{25}H_{37}N_4O_4$: 457.2815, found: *m*/*z* 457.2821 $[M+H]^+$.

4.3.5. *N*-Methoxy (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (9b)

NEt_3 (0.041 mL, 0.3 mmol) and 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (47 mg, 0.12 mmol) were added to a solution of compound **7b** (31 mg, 0.1 mmol) in CH_2Cl_2 (1 mL). The mixture was stirred at 25 °C for 10 h, and then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 1.0 M HCl, saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **8b** as colorless oil.

A solution of the above-prepared compound **8b** in anhydrous CH_2Cl_2 (1 mL) was cooled to 0 °C in an ice-bath, and trifluoroacetic acid (0.24 mL, 3.22 mmol) was added. The mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was purified by reversed-phase RP-18

column (MeOH/H_2O = 1:9–1:4) to give the desired product **9b** (15 mg, 41%). The purity of product **9b** was 99.3% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 11.4 min (MeOH/H_2O = 3:7). $C_{16}H_{29}N_5O_4$; hygroscopic solid; $[\alpha]_D^{22}$ −43.9 (*c* 1, MeOH); IR (film) 3416, 3179, 2771, 1643, 1447, 1325, 1268, 1117, 1025, 920 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.41 (1H, t, *J* = 2.4 Hz), 4.20 (1H, br d, *J* = 2.4 Hz), 3.89 (2H, br t, *J* = 3.0 Hz), 3.72 (3H, s), 3.41 (1H, quint, *J* = 5.6 Hz), 2.73 (1H, dd, *J* = 18.4 Hz, 3.2 Hz), 2.36 (1H, br dd, *J* = 11.2 Hz, 6.4 Hz), 1.98 (3H, s), 1.59–1.43 (4H, m), 0.93–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.4, 158.7, 134.3, 130.6, 126.2, 84.0, 76.2, 64.4, 56.2, 51.7, 31.4, 27.3, 26.9, 23.0, 9.90, 9.85; HRMS calcd for $C_{16}H_{30}N_5O_4$: 356.2298, found: *m*/*z* 356.2305 $[M+H]^+$.

4.3.6. *N*-Benzyloxy (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (9c)

By a procedure similar to that for **9b**, guanidination of compound **7c** (32 mg, 0.08 mmol) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (37 mg, 0.096 mmol) was conducted in the presence of NEt_3 (0.033 mL, 0.24 mmol) in CH_2Cl_2 (1 mL). The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **8c** as pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.19 mL, 2.57 mmol) to give the desired product **9c** (14 mg, 42%) after purification on a reversed-phase RP-18 column (MeOH/H_2O = 1:9–1:4). The purity of product **9c** was 96.7% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 17.5 min (MeOH/H_2O = 3:7). $C_{22}H_{33}N_5O_4$; hygroscopic solid; $[\alpha]_D^{23}$ −30.3 (*c* 1, MeOH); IR (film) 3163, 2974, 2378, 1734, 1670, 1635, 1540, 1506, 1472, 1203, 1176, 1136, 1078, 722 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.44–7.41 (2H, m), 7.39–7.32 (3H, m), 6.32 (1H, br d, *J* = 2.0 Hz), 4.16 (1H, br d, *J* = 6.0 Hz), 3.91–3.82 (2H, m), 3.41–3.34 (1H, m), 2.70 (1H, dd, *J* = 16.6, 3.8 Hz), 2.37–2.31 (1H, m), 1.97 (3H, s), 1.56–1.42 (4H, m), 0.92–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.3, 158.8, 137.1, 134.0, 130.7 (2×), 130.6, 129.8 (3×), 129.6, 84.0, 79.0, 76.3, 56.1, 51.7, 31.4, 27.3, 26.9, 23.0, 9.9, 9.8; HRMS calcd for $C_{22}H_{34}N_5O_4$: 432.2611, found: *m*/*z* 432.2618 $[M+H]^+$.

4.3.7. *N*-[3-(1*H*-Indol-2-yl)propoxy] (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (9d)

By a procedure similar to that for **9b**, guanidination of compound **7d** (36 mg, 0.08 mmol) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (37 mg, 0.096 mmol) was conducted in the presence of NEt_3 (0.033 mL, 0.24 mmol) in CH_2Cl_2 (1 mL). The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **8d** as pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.19 mL, 2.57 mmol) to give the desired product **9d** (15 mg, 37%) after purification on a reversed-phase RP-18 column (MeOH/H_2O = 1:9–1:4). The purity of product **9d** was 97.2% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 23.3 min (MeOH/H_2O = 3:7). $C_{26}H_{38}N_6O_4$; hygroscopic solid; $[\alpha]_D^{23}$ −25.7 (*c* 1, MeOH); IR (film) 3273, 3170, 2966, 2925, 2876, 1671, 1548, 1458, 1434, 1377, 1262, 1209, 1136, 1082, 943, 719 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.39 (1H, d, *J* = 7.6 Hz), 7.25 (1H, t, *J* = 4.0 Hz), 7.00 (1H, t, *J* = 7.2 Hz), 6.93 (1H, t, *J* = 7.2 Hz), 6.78 (1H, s), 6.13 (1H, s), 4.24 (2H, t, *J* = 6.4 Hz), 4.15 (1H, d, *J* = 7.6 Hz), 3.90–3.76 (2H, m), 3.37 (1H, quint, *J* = 5.6 Hz), 2.87 (2H, t, *J* = 7.4 Hz), 2.74 (1H, dd, *J* = 17.6, 5.2 Hz), 2.30–2.22 (1H, m), 2.12 (2H, quint, *J* = 6.8 Hz), 1.98 (3H, s), 1.56–1.44 (4H, m), 0.93–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.4, 167.4, 158.7, 140.1, 139.1, 138.1, 130.3, 129.6, 121.6, 120.5, 120.1, 111.6, 100.0, 83.8, 76.1, 65.9, 55.9, 51.6, 31.3, 29.4, 27.4, 27.0, 26.0, 23.0, 10.0, 9.8; HRMS calcd for $C_{26}H_{39}N_6O_4$: 499.3054, found: *m*/*z* 499.3051 $[M+H]^+$.

4.3.8. *N*-Methylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (10a)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with methanesulfonamide (23 mg, 0.24 mmol) was conducted in the presence of HBTU (91 mg, 0.24 mmol), NEt_3 (0.08 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The residues was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **10a** (34 mg, 47%). The purity of product **10a** was 99.2% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 12.5 min (MeOH/H_2O = 3:7). $C_{15}H_{27}N_3O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −52.9 (*c* 1, MeOH); IR (film) 3274, 2965, 2937, 2878, 1658, 1550, 1463, 1373, 1324, 1242, 1100, 1064, 1023, 970, 852, 752 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.68 (1H, s), 4.19 (1H, br d, *J* = 8.0 Hz), 3.96 (1H, dd, *J* = 11.2 Hz, 8.0 Hz), 3.50–3.41 (2H, m), 3.04 (3H, s), 2.90 (1H, br dd, *J* = 17.2 Hz, 8.4 Hz), 2.45–2.39 (1H, m), 2.03 (3H, s), 1.59–1.46 (4H, m), 0.94–0.87 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.9, 174.1, 135.0, 134.4, 83.7, 76.4, 54.7, 51.4, 40.8, 30.4, 27.3, 26.6, 23.4, 10.0, 9.7; HRMS calcd for $C_{15}H_{28}N_3O_5S$: 362.1750, found: *m*/*z* 362.1752 $[M+H]^+$.

4.3.9. *N*-Trifluoroethylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (10b)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with trifluoromethanesulfonamide (36 mg, 0.24 mmol) was conducted in the presence of HBTU (91 mg, 0.24 mmol), NEt_3 (0.08 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The residues was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **10b** (42 mg, 51%). The purity of product **10b** was 97.4% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 7.8 min (MeOH/H_2O = 3:7). $C_{15}H_{24}F_3N_3O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −65.9 (*c* 1, MeOH); IR (film) 3444, 2973, 1652, 1558, 1456, 1374, 1281, 1198, 1096, 845 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.80 (1H, d, *J* = 2.0 Hz), 4.19 (1H, d, *J* = 8.4 Hz), 4.02–3.96 (1H, m), 3.50–3.40 (2H, m), 2.93 (1H, dd, *J* = 17.6, 5.6 Hz), 2.45–2.37 (1H, m), 2.04 (3H, s), 1.59–1.43 (4H, m), 0.94–0.87 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.8, 173.6, 135.6, 134.6, 121.9 (q, J_{C-F} = 321 Hz), 83.7, 76.3, 54.5, 51.5, 30.1, 27.4, 26.8, 23.3, 9.9, 9.7; ^{19}F NMR (396 MHz, CD_3OD) δ −80.26 (s, CF_3); HRMS calcd for $C_{15}H_{25}F_3N_3O_5S$: 416.1467, found: *m*/*z* 416.1468 $[M+H]^+$.

4.3.10. *N*-Butylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (10c)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with *n*-butylsulfonamide (33 mg, 0.24 mmol) was conducted in the presence of HBTU (91 mg, 0.24 mmol), NEt_3 (0.08 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The residues was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **10c** (40 mg, 49%). The purity of product **10c** was 97.8% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 10.5 min (MeOH/H_2O = 3:7). $C_{18}H_{33}N_3O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −52.5 (*c* 1, MeOH); IR (film) 3439, 1643, 1556, 1453, 1377, 1322, 1225, 1094, 921 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.67(1H, s), 4.22 (1H, br d, *J* = 8.2 Hz), 3.97 (1H, br dd, *J* = 10.8, 8.2 Hz), 3.54–3.50 (1H, m), 3.49–3.42 (1H, m), 3.32–3.24 (2H, m), 2.91 (1H, br dd, *J* = 17.0, 4.6 Hz), 2.44 (1H, br dd, *J* = 17.0, 10.2 Hz), 2.04 (3H, s), 1.74 (2H, quint, *J* = 6.8 Hz), 1.60–1.55 (4H, m), 1.54–1.41 (2H, m), 1.00–0.87 (9 H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.9, 172.7,

135.0, 134.2, 83.7, 76.4, 54.6, 53.3, 51.2, 30.2, 27.4, 27.1, 26.7, 23.4, 22.8, 14.2, 10.0, 9.7; HRMS calcd for $C_{18}H_{34}N_3O_5S$: 404.2219, found: m/z 404.2231 $[M+H]^+$.

4.3.11. *N*-Phenylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (10d)

By a procedure similar to that for **7a**, the amide bond forming reaction of acid **5** (78 mg, 0.2 mmol) with benzenesulfonamide (38 mg, 0.24 mmol) was conducted in the presence of HBTU (91 mg, 0.24 mmol), NEt_3 (0.08 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The residues was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.24 mL, 3.22 mmol) to give **10d** (36 mg, 43%). The purity of product **10d** was >95% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 9.0 min (MeOH/H_2O = 3:7). $C_{20}H_{29}N_3O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −61.1 (*c* 1, MeOH); IR (film) 3408, 2360, 1638, 1558, 1382, 1319, 1135, 1082, 839 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.92 (2H, br d, J = 7.2 Hz), 7.51–7.42 (3H, m), 6.66 (1H, s), 4.19 (1H, br), 3.96 (1H, br), 3.45–3.41 (2H, m), 2.86 (1H, br), 2.37 (1H, br), 2.01 (3H, s), 1.57–1.44 (4H, m), 0.92–0.85 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.8, 173.5, 145.1, 135.0, 134.4, 132.5, 129.4 (2×), 128.2 (2×), 83.6, 76.4, 54.5, 51.4, 30.2, 27.3, 26.7, 23.4, 10.0, 9.7; HRMS calcd for $C_{20}H_{30}N_3O_5S$: 424.1906, found: m/z 424.1908 $[M+H]^+$.

4.3.12. *N*-Methylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (12a)

NEt_3 (0.041 mL, 0.3 mmol) and 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (47 mg, 0.12 mmol) were added to a solution of compound **10a** (36 mg, 0.1 mmol) in CH_2Cl_2 (1 mL). The mixture was stirred at 25 °C for 10 h, and then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 1.0 M HCl, saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography CH_2Cl_2/MeOH = 20:1) to give **11a** as pale yellow oil.

A solution of the above-prepared compound **11a** in anhydrous CH_2Cl_2 (1 mL) was cooled to 0 °C in an ice-bath, and trifluoroacetic acid (0.24 mL, 3.22 mmol) was added. The mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was purified by reversed-phase RP-18 column (MeOH/H_2O = 1:9–1:4) to give the desired product **12a** (13 mg, 32%). The purity of product **12a** was 95.3% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 27.4 min (MeOH/H_2O = 3:7). $C_{16}H_{29}N_5O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −35.3 (*c* 0.8, MeOH); IR (film) 3416, 1652, 1437, 1415, 1318, 1017, 925, 720 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.63 (1H, s), 4.27 (1H, br d, J = 7.2 Hz), 3.89 (1H, br t, J = 9.2 Hz), 3.77–3.71 (1H, m), 3.47 (1H, br t, J = 5.4 Hz), 3.07 (3H, s), 2.80 (1H, dd, J = 17.4 Hz, 4.6 Hz), 2.32 (1H, dd, J = 17.4 Hz, 9.4 Hz), 2.00 (3H, s), 1.58–1.43 (4H, m), 0.94–0.85 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.71, 174.65, 158.7, 135.6, 134.7, 84.4, 76.7, 56.3, 52.2, 40.9, 31.9, 27.3, 26.7, 23.1, 9.9 (2×); HRMS calcd for $C_{16}H_{30}N_5O_5S$:404.1968, found: m/z 404.1971 $[M+H]^+$.

4.3.13. *N*-Trifluoromethylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (12b)

By a procedure similar to that for **12a**, guanidination of compound **10b** (42 mg, 0.1 mmol) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (47 mg, 0.12 mmol) was conducted in the presence of NEt_3 (0.041 mL, 0.3 mmol) in CH_2Cl_2 (1 mL). The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **11b** as pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.077 mL, 1.0 mmol) to give **12b** (18 mg, 39%). The purity of product **12b** was 99.6% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 9.4 min (MeOH/H_2O = 3:7). $C_{16}H_{26}F_3N_5O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −19.6 (*c* 1, MeOH); IR (film) 3416, 1640, 1453, 1372, 1293, 1199, 1073, 932 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.79 (1H, s), 4.16 (1H, br t, J = 3.4 Hz), 3.91 (1H, dd, J = 10.0, 8.0 Hz), 3.79–3.73 (1H, m), 3.41 (1H, quint, J = 5.6 Hz), 2.79 (1H, dd, J = 17.8, 5.6 Hz), 2.33–2.25 (1H, m), 1.97 (3H, s), 1.57–1.45(4H, m), 0.94–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.4, 174.2, 158.6, 135.8, 135.5, 121.8 (q, J_{C-F} = 340 Hz)83.9, 76.6, 55.9, 52.1, 31.5, 27.5, 27.0, 23.0, 9.93, 9.91; ^{19}F NMR (396 MHz, CD_3OD) δ −80.10 (s, CF_3); HRMS calcd for $C_{16}H_{27}F_3N_5O_5S$: 458.1685, found: m/z 458.1685 $[M+H]^+$.

4.3.14. *N*-Butylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (12c)

By a procedure similar to that for **12a**, guanidination of compound **10c** (40 mg, 0.1 mmol) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (47 mg, 0.12 mmol) was conducted in the presence of NEt_3 (0.041 mL, 0.3 mmol) in CH_2Cl_2 (1 mL). The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **11c** as pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.077 mL, 1.0 mmol) to give **12c** (16 mg, 36%). The purity of product **12c** was 97.1% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 12.8 min (MeOH/H_2O = 3:7). $C_{19}H_{35}N_5O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −6.7 (*c* 1, MeOH); IR (film) 3423, 2094, 1640, 1545, 1372, 1312, 1220, 1111, 1067 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.69 (1H, s), 4.18 (1H, br), 4.00 (2H, br), 3.44 (1H, quint, J = 5.6 Hz), 3.28–3.16 (2H, m), 2.78 (1H, d, J = 16.0 Hz), 2.42 (1H, d, J = 16.0 Hz), 1.97 (3H, s), 1.78–1.69 (2H, m), 1.60–1.34 (6H, m), 0.96–0.88 (9H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 175.1, 174.2, 158.6, 136.0, 134.6, 83.8, 76.5, 56.5, 53.2, 52.1, 32.1, 27.5, 27.3, 26.9, 23.1, 23.0, 14.3, 10.0, 9.95; HRMS calcd for $C_{19}H_{36}N_5O_5S$: 446.2437, found: m/z 446.2432 $[M+H]^+$.

4.3.15. *N*-Phenylsulfonyl (3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexenecarboxamide (12d)

By a procedure similar to that for **12a**, the guanidination of compound **10d** (42 mg, 0.1 mmol) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (47 mg, 0.12 mmol) was conducted in the presence of NEt_3 (0.041 mL, 0.3 mmol) in CH_2Cl_2 (1 mL). The residue was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **11d** as pale yellow oil, which was subsequently treated with trifluoroacetic acid (0.077 mL, 1.0 mmol) to give **12d** (13 mg, 28%). The purity of product **12d** was 99.7% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm porosity), t_R = 12.7 min (MeOH/H_2O = 3:7). $C_{21}H_{31}N_5O_5S$; hygroscopic solid; $[\alpha]_D^{22}$ −14.9 (*c* 0.5, MeOH); IR (film) 3564, 3220, 2920, 1659, 1437, 1407, 1318, 1016, 952, 903, 711 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.91 (2H, d, J = 6.8 Hz), 7.51–7.42 (3H, m), 6.71 (1H, s), 4.25 (1H, br d, J = 7.2 Hz), 3.85 (1H, br t, J = 11.2 Hz), 3.73–3.67 (1H, m), 3.46 (1H, quint, J = 5.6 Hz), 2.72 (1H, dd, J = 17.6, 5.4 Hz), 2.16 (1H, dd, J = 17.6, 9.8 Hz), 2.00 (3H, s), 1.59–1.43 (4H, m), 0.95–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.5, 174.0, 158.4, 145.3, 135.6, 134.9, 132.3, 129.2 (2×), 128.1 (2×), 83.8, 76.4, 56.2, 52.0, 31.6, 27.3, 26.8, 22.9, 9.8 (2×); HRMS calcd for $C_{21}H_{32}N_5O_5S$:466.2124, found: m/z 466.2119 $[M+H]^+$.

4.4. Determination of influenza virus $TCID_{50}$

The TCID50 (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock solution onto 100 μL

MDCK cells at 1×10^5 cells/mL in 96-well microplates. The infected cells were incubated at 37 °C under 5.0% CO_2 for 48 h and added to each well with 100 μL of CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega). After the incubation at 37 °C for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus TCID50 was determined using Reed–Muench method.

4.5. Determination of neuraminidase activity by a fluorescent assay

The neuraminidase activity was measured by using diluted allantoic fluid harvested from influenza virus infected embryonated eggs. A fluorometric assay was used to determine the NA activity with the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma). The fluorescence of the released 4-methylumbelliferone was measured in Envision plate reader (Perkin-Elmer, Wellesley, MA) by using excitation and emission wavelength of 365 and 460 nm, respectively. Neuraminidase activity was determined at 200 μM of MUNANA. Enzyme activity was expressed as the fluorescence increase during 15 min incubation at room temperature.

4.6. Determination of IC_{50} of neuraminidase inhibitors

Neuraminidase inhibition was determined by mixing inhibitor and neuraminidase for 10 min at room temperature followed by the addition of 200 μM of substrate. The IC_{50} values were determined from the dose–response curves by plotting the percent inhibition of NA activity versus inhibitor concentrations using Graph Pad Prism 4.

4.7. Determination of EC_{50} and CC_{50} of neuraminidase inhibitors

The anti-flu activities of neuraminidase inhibitors were measured by the EC_{50} values, which were the concentrations of NA inhibitor for 50% protection of the influenza virus infection-mediated CPE (cytopathic effects). 50 μL of diluted influenza virus at 100 TCID50 was mixed with equal volumes of NA inhibitors at varied concentrations. The mixtures were used to infect 100 μL of MDCK cells at 1×10^5 cells/mL in 96-wells. After 48 h of incubation at 37 °C under 5.0% CO_2, the cytopathic effects were determined with CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay reagent as described above. The EC_{50} values were determined by fitting the curve of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

The CC_{50} values (50% cytotoxic concentrations) of NA inhibitor to MDCK or human 293T cells were determined by the procedures similar to the EC_{50} determination but without virus infection.

Acknowledgment

We thank the Ministry of Science and Technology (Taiwan) and Academia Sinica (Taiwan) for financial support.

Supplementary data

Supplementary data (synthetic schemes, procedures and compound characterization, ^1H, ^{13}C, and ^{19}F NMR spectra, and HPLC diagrams) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.10.005. These data include MOL files and InChiKeys of the most important compounds described in this article.

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