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Analogs of zanamivir with modified C4-substituents as the inhibitors against the group-1 neuraminidases of influenza viruses

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ABSTRACT

Unlike the group-2 neuraminidase, the group-1 neuraminidase of influenza virus possesses a flexible loop (the 150-loop) and a cavity (the 150-cavity) adjacent to the active site, and renders a conformational change from the 'open' form to the 'closed' form on binding with substrate (sialo-glycoprotein) or inhibitor (e.g., zanamivir). Zanamivir derivative **8a** having an extended (piperazinocarbonyl)propyl substituent at the internal N-position of the guanidino group is designed as a possible inhibitor on the basis of computer docking to the open form of N1 subtype neuraminidase. Indeed, compound **8a** exhibits strong neuraminidase inhibition and good anti-influenza activity against H1N1 virus with IC_{50} = 2.15 μM and EC_{50} = 0.77 μM, respectively. This study may provide a clue to future design of better group-1 neuraminidase inhibitors.

1. Introduction

Influenza is a long-standing health problem causing significant mortality in annual epidemics by infection of the human respiratory system. The worldwide spread of H5N1 avian flu and the recent outbreak of the new type H1N1 human flu have raised public concerns of the global influenza pandemics. Neuraminidase (NA), a glycoprotein expressed on the influenza virus surface, is essential for virus replication and infectivity by breaking the linkage between the progeny virus from the surface sialo-receptor of host cells. Thus, inhibition of NA by the structure-based strategy has been applied in discovery of anti-influenza drugs.[1] Though little success has been achieved in development of the substrate mimetics, for example, the thioglycoside,[2a] 3-deoxy-3-fluoro,[2b] and phosphonate[2c] of sialic acid (Neu5Ac), as the influenza virus NA inhibitors, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en, DANA, **1**)[3] is identified as the first effective NA inhibitor ($K_i = 4 \times 10^{-6}$ M) to mimic the transition state in the enzymatic cleavage of the sialo-moiety. The X-ray crystal structures[4] of the influenza virus NA (N2 subtype) and its complex with the inhibitor DANA reveal that the C4-hydroxy group is positioned in the negatively charged binding pocket S2 containing two glutamate residues (Glu119 and Glu227). This structural analysis has thus led to a finding of more potent NA inhibitors,[5] such as 4-amino-4-deoxy-Neu5Ac2en (amino-DANA, **2**, $K_i = 4 \times 10^{-8}$ M) and 4-deoxy-4-guanidino-Neu5Ac2en (zanamivir, **3**, $K_i = 2 \times 10^{-10}$ M), by replacing the C4-hydroxy group in DANA with a basic group. The enhanced affinity is attributable to the strong electrostatic interactions of the aminium or guanidinium substituents with the two glutamate residues in physiological conditions. Zanamivir, in the trade name Relenza, is the first NA inhibitor approved to be used as anti-influenza drug.[1c,6]

Generally speaking, the flu genes encoding NA are more conserved than hemagglutinin (HA), another essential glycoprotein on the surface of influenza virus for recognition and attachment to the host cell. There are two genetically distinct groups in neuraminidases of influenza A viruses: group-1 includes N1, N4, N5, and N8 subtypes, and group-2 includes N2, N3, N6, N7, and N9 subtypes. The N1 and N2 are prominent subtypes in human influenza viruses, in particular, the current pandemic H1N1 and H5N1 viruses belong to the N1 subtype. A recent X-ray crystallographic study[7] reveals that group-1 and group-2 NAs are not only genetically distinct but also structurally distinct. Group-1 NA possesses a flexible loop (known as the 150-loop) and a cavity (the 150-cavity) adjacent to the active site. Group-1 NA would render the conformational change from the 'open' form to the 'closed' form on binding with substrate (or inhibitor), whereas group-2 NA always exists in the closed form. The NA inhibitors such as zanamivir and oseltamivir carboxylic acid[8] are originally designed based on the crystal structures of group-2 NAs (N2 and N9) in the closed form. Because group-1 NA would change the conformation from the 'open' form to the 'closed' form on binding, an insight into such conformational change can provide a novel approach to design effective inhibitors specifically against

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the group-1 NAs, such as those exist in the avian H5N1 and the 2009 human H1N1 viruses, by exploiting additional interactions with the 150-cavity of the open form.

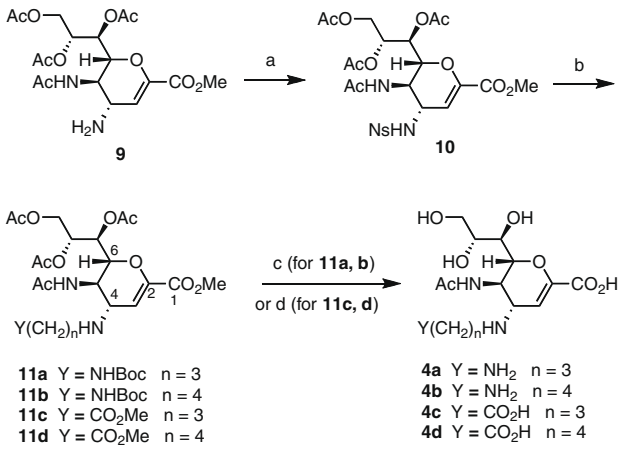
2. Molecular modeling

Accordingly, a substituent extended from the amino or guanidino groups at the C-4 position of zanamivir may exert an additional binding to the 150-cavity. Docking zanamivir to the crystal structure of the N1 neuraminidase in the open form (Fig. 1A, PDB code 2HU0) reveals room for possible side chain extension from the C-4 position of zanamivir to fit in with the 150-cavity. Further examination of the docking structure suggests that the prominent guanidinium side chain of Arg156 and the carbonyl group of the backbone of Gly147 around the 150-cavity are prospective binding partners by a salt-bridge or hydrogen bond for drug design. We thus propose compound **8a** bearing an amide group and an amino group on its side chain as a target molecule. The docking simulation of compound **8a** with the open form N1 neuraminidase shows that the carbonyl group of the **8a** side chain has a hydrogen bond (3.64 Å) with residue Arg156 in the cavity, and the terminal amino group at the tip of the side chain also displays a strong hydrogen bond (3.29 Å) with residue Gly147 in the same cavity (Fig. 1B). As a consequence, the designed side chain of **8a** may provide additional interactions with the 150-cavity in the open form of N1 neuraminidase.

In this paper, we describe the synthesis and bioactivities of a zanamivir derivative **8a** having an extended substituent at the internal N-position of the guanidino group. We also prepared the analogous compounds **4a–d**, **5**, **6a–b**, **7a–c**, and **8b–d** (Schemes 1–3) for comparison of their NA inhibitory and anti-influenza activities.

3. Chemistry

Amine **9** was prepared from sialic acid according to the previously reported method.[9] Although alkylamination of **9** has been realized,[10] attempts in direct alkylation of **9** with various halides in basic conditions resulted in complicated mixture. Therefore, amine **9** was first treated with *o*-nitrobenzenesulfonyl chloride (NsCl)[11] to give sulfonamide **10** as a key intermediate (Scheme 1). After Mitsunobu reaction of **10** with appropriate alcohols, the Ns group was subsequently removed by thiophenol in the presence of cesium carbonate to afford **11a–d** in high yields (80–86%). Saponification of **11a–d**, followed by removal of the Boc group, produced the desired compounds **4a–d**.



Scheme 1. Synthesis of compound **4a–d** bearing modified amino substituents at C-4 position. Reagents and conditions: (a) *o*-$O_2NC_6H_4SO_2Cl$, CH_2Cl_2, rt, 83%; (b) (i) $Y(CH_2)_nOH$, DIAD, PPh_3, THF, rt; (ii) PhSH, Cs_2CO_3, DMF, rt, 80–86%; (c) (i) NaOH, MeOH, H_2O, rt; (ii) CF_3CO_2H, CH_2Cl_2, rt, 92–95%; (d) NaOH, MeOH, H_2O, rt, 67–85%.

To prepare guanidine derivatives, amine **9** was treated with *N*-Boc-2-methylthio-2-imidazoline, followed by saponification and removal of the Boc group, to afford a cyclic guanidine compound **5** (Scheme 2A). Alternatively, amine **9** was reacted with 9-fluorenylmethoxycarbonyl isocyanate (FmocNCS) to give a thiourea **13** (Scheme 2B). After the Fmoc group was switched to a Boc group, the thiourea **14** was reacted with appropriate alkylidenediamine mono-carbamates in the presence of $HgCl_2$ to give guanidines **15a** and **15b**. By saponification and a subsequent treatment with TFA, the derivatives **6a** and **6b** bearing substituents at a terminal N-position of the guanidine moiety were obtained in high yields.

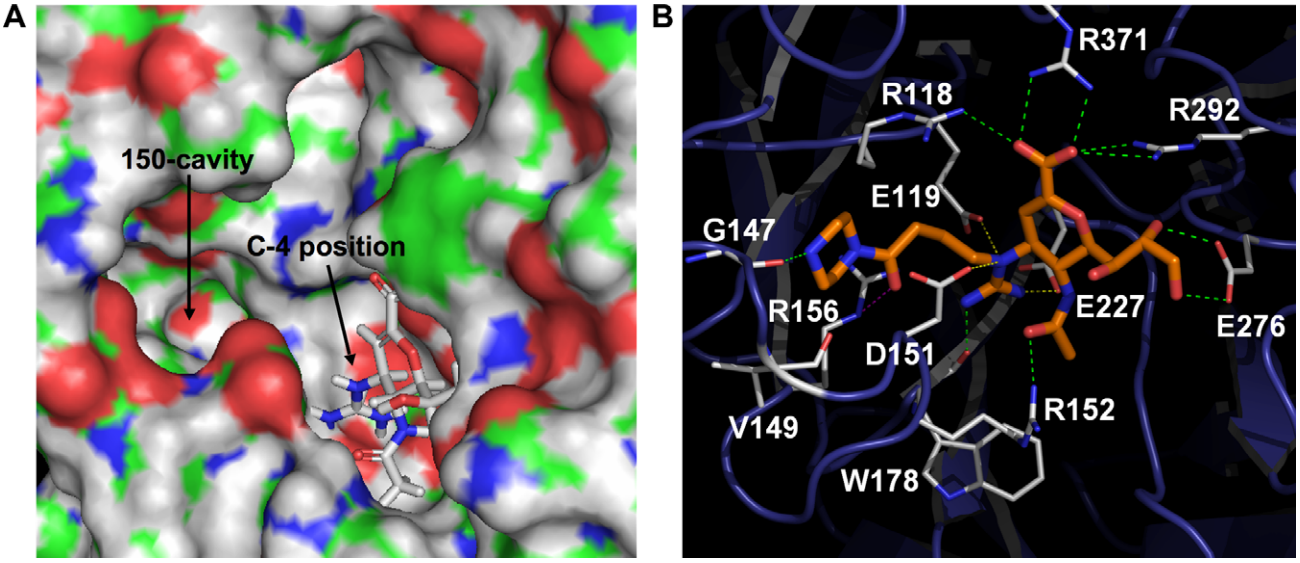
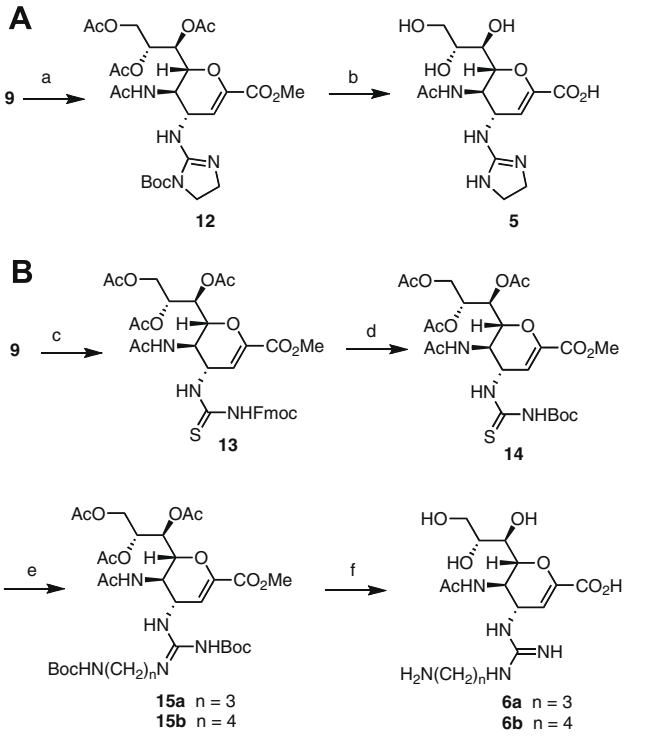


Figure 1. Zanamivir (**3**) and compound **8a** binding to the active sites of group-1 neuraminidase (N1) in the 'open' form. (A) Molecular surface of N1 neuraminidase with bound zanamivir; (B) The docking result of compound **8a** with neuraminidase in the open form. Zanamivir and compound **8a** are highlighted in atom-colored stick model. Individual atoms are displayed in gray (carbon), blue (nitrogen), and red (oxygen). The neuraminidases are shown in cyan, whereas R118, E119, G147, V149, D151, R152, R156, W178, E227, E276, R292, and R371 are also highlighted in atom-colored stick model. Hydrogen bonds are represented by dotted lines.



Scheme 2. Synthesis of zanamivir derivatives **5**, **6a**, and **6b** with modification at terminal positions of the guanidino group. Reagents and conditions: (a) *N*-Boc-2-methylthio-2-imidazoline, AcOH, EtOH, 50 °C, 62%; (b) (i) CF_3CO_2H, CH_2Cl_2, rt; (ii) NaOH, MeOH, H_2O, rt, 94%; (c) FmocNCS, CH_2Cl_2, rt, 90%; (d) (i) piperidine, CH_2Cl_2, rt; (ii) Boc_2O, CH_2Cl_2, rt, 87%; (e) $BocHN(CH_2)_nNH_2$, $HgCl_2$, Et_3N, DMF, rt, 66−74%; (f) (i) NaOH, MeOH, H_2O, rt; (ii) CF_3CO_2H, CH_2Cl_2, rt, 90−94%.

In another approach, the alkylamino groups in **11a–c** were effectively transformed into di-Boc-alkylguanidino groups by treatment with *N*,*N*′-di-Boc-*S*-methylisothiourea in the presence of $HgCl_2$ (Scheme 3A). After saponification, the Boc groups in **16a–c** were removed by TFA to afford the desired products **7a–c** bearing substituents at the internal N-position of the guanidine group. By a similar procedure, **16d** was prepared and its allyl group was subsequently removed by the catalysis of $Pd(PPh_3)_4$ to give acid **18** (Scheme 3B). The acid was converted to amides **20a–d** by coupling with *N*-Boc-piperazine, *N*-methylpiperazine, morpholine, and piperidine, respectively, using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as the promoter. Saponification, followed by removal of Boc groups, culminated in the synthesis of the target zanamivir derivatives **8a–d**.

4. Bioassay

All the target compounds were evaluated for their abilities to inhibit the hydrolysis of a fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) by the N1 neuraminidase of influenza A/WSN/1933 (H1N1) virus. The active inhibitors were further evaluated by their anti-influenza activities using cytopathic prevention assays. The results are listed in Table 1 including the values of DANA (**1**), compound **2** and zanamivir (**3**) for comparison. Compounds **4a–b**, **6a–b**, and **7a–b** with the amino or guanidino group at C-4 bearing a substituent annexed with amino group showed no significant activities (IC_{50} >200 μM), whereas compounds **4c–d** and **7c** having the terminal amino group replaced by a carboxylic group improved the NA inhibitory activities considerably (e.g., the IC_{50} of **7c** was 7.2 μM). The zanamivir analog **5** having the guanidino group confined by an ethylene unit exhibited no NA inhibitory activity. Elaboration of the terminal carboxylic group in **7c** to amides **8a** and **8b**, further improved the NA inhibitory activity. Among them, the zanamivir analog **8a** bearing a (piperazinocarbonyl)propyl substituent at the internal position of guanidino group was the most potent NA inhibitor with IC_{50} = 2.15 μM and EC_{50} = 0.77 μM. In comparison, compound **8a** showed about 88-fold potency than DANA (**1**) in NA inhibition and twofold potency than compound **2** in anti-influenza activity. However, compound **8a** was still inferior to zanamivir with regard to the enzyme inhibition and anti-influenza activity.

5. Discussion

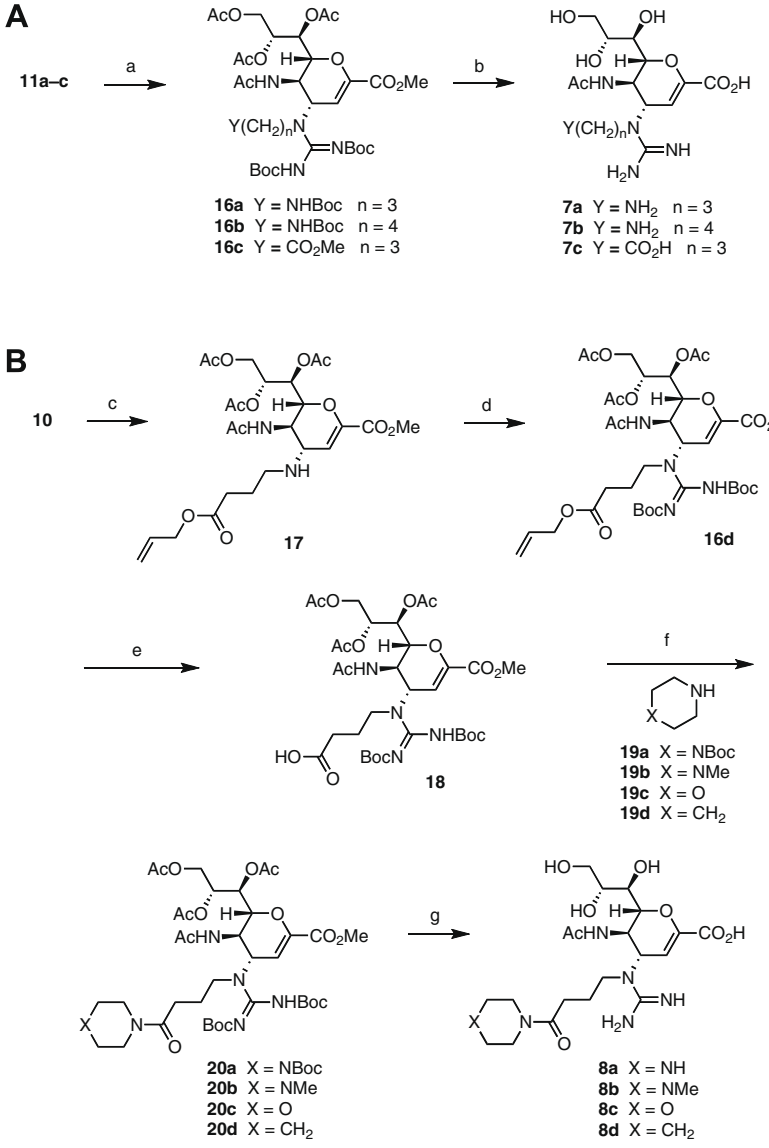
From the binding model (Fig. 1), we can find that there is a spatially restricted region in the S2 pocket defined by Glu119, Trp178 and Glu227 of the active site in open form. Thus the N-substituted guanidino group of **5**, **6a**, and **6b** may be too large to be accommodated into the S2 pocket. In the case of **7c**, the carboxylic acid of the side chain could make a salt-bridge interaction with Arg156, whereas the amino group of **7a**,**b** could result in repulsive interaction. Compound **8a** is the most potent NA inhibitor among the test samples, presumably because the piperazino group of the side chain in **8a** can exert a strong hydrogen bond interaction with Gly147. The analogs **8b–d** having *N*-methylpiperazino, morpholino, and piperidino groups lack such hydrogen bonding with Gly147, and their inhibitory activities were reduced.

In order to understand the activity discrepancy between compound **8a** and zanamivir, we also examined the 3D model of zanamivir binding to neuraminidase (N1) in closed form[6] (Fig. 2) to compare with that of compound **8a** binding to neuraminidase in open form (Fig. 1B). In the closed form of neuraminidase, the guanidinium group of zanamivir makes tight salt-bridge interactions with negatively charged S2 pocket created by Glu119, Asp151 and Glu227 in the interaction distances of 3.33, 2.92 and 3.04 Å, respectively. On the other hand, when compound **8a** binds to the open form of neuraminidase, the interaction between the guanidinium group and the S2 pocket will weaken to 4.21, 4.24 and 3.24 Å, respectively. This change may explain why compound **8a** shows less potency than zanamivir in NA inhibition, even though compound **8a** possesses additional interaction with the 150-cavity adjacent to the active site of NA.

From a different perspective, Stanislav and co-workers[12] have recently carried out the molecular dynamics simulation for the complexes of group-1 NA with zanamivir and its phosphonate congener that contains a phosphonyl group to replace the carboxylate group in zanamivir. Their study indicates that the neuraminidase N1 quickly interchanges between the closed and open conformations on binding with inhibitors. Zanamivir prefers the closed NA form, whereas its phosphonate congener having a negative total charge can repel the Asp151 residue to induce opening of the150-loop of NA. In combination of Stanislav's and our studies, one may consider design of better group-1 NA inhibitors such as the phosphonate congener of **8a**, in which the strong electrostatic interactions of phosphonyl group with arginine residues[13] can compensate the reduced salt-bridge interaction due to the extended side chain.

6. Conclusion

On the basis of computer docking to the open form of N1 subtype neuraminidase, we have designed and prepared compound **8a** with an extended substituent at the internal N-position of the guanidino group, in addition to other analogous compounds **4a–d**, **5**, **6a–b**, **7a–c**, and **8b–d**, as a possible inhibitor specifically against the group-1 neuraminidases. Though direct alkylation of amine **9** did not give clean product, introduction of an extended substituent at the N-position is achieved by Mitsunobu reaction of the *o*-nitrobenzenesulfonamide **10** in good yields. Compound



Scheme 3. Synthesis of zanamivir derivatives **7a**–**c** and **8a**–**c** with modification at the internal N-position of guanidino group. Reagents and conditions: (a) *N*,*N*′-di-Boc-*S*-methylisothiourea, $HgCl_2$, Et_3N, DMF, rt, 86−91%; (b) (i) NaOH, MeOH, H_2O, rt; (ii) CF_3CO_2H, CH_2Cl_2, rt, 92−95%; (c) (i) allyl 4-hydroxybutanoate, DIAD, PPh_3, THF, rt; (ii) PhSH, Cs_2CO_3, DMF, rt, 81%; (d) *N*,*N*′-di-Boc-*S*-methylisothiourea, $HgCl_2$, Et_3N, DMF, rt, 89%; (e) $Pd(PPh_3)_4$, morpholine, THF, rt, 85%; (f) **19a**–**d**, PyBOP, Et_3N, DMF, rt, 73−89%; (g) (i) NaOH, MeOH, H_2O, rt, (ii) CF_3CO_2H, CH_2Cl_2, rt, 88−96%.

8a exhibited strong NA inhibition and good anti-influenza activity with IC_{50} = 2.15 μM and EC_{50} = 0.77 μM, respectively. According to the molecular modeling, the extended side chain of **8a** can provide additional interactions with the 150-cavity in the open form of N1 neuraminidase. However, this disposition also weakens the salt-bridge or hydrogen bond interaction between the guanidino group of **8a** and the S2 pocket in the active site. This study may provide a clue to future design of better group-1 neuraminidase inhibitors against influenza virus.

7. Experimental

7.1. Chemistry

All the reagents and solvents were reagent grade and were used without further purification unless otherwise specified. All solvents were anhydrous grade unless indicated otherwise. CH_2Cl_2 was distilled from CaH_2. All non-aqueous reactions were carried out in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel using aqueous *p*-anisaldehyde as visualizing agent. Silica gel (0.040–0.063 mm particle sizes) was used for column chromatography. Flash chromatography was performed on silica gel of 60–200 μm particle size. Molecular sieves were activated under high vacuum at 220 °C over 6 h.

Melting points were recorded on a Yanaco or Electrothermal MEL-TEMP 1101D apparatus in open capillaries and are not corrected. Optical rotations were measured on digital polarimeter of Japan JASCO Co. DIP-1000. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Infrared (IR) spectra were recorded on Nicolet Magna 550-II or Thermo Nicolet 380 FT-IR spectrometers. Nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Plus-400 (400 MHz) or Bruker Avance-400 (400 MHz) spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to δ_H 7.24/δ_C 77.0 (central line of t) for $CHCl_3/CDCl_3$, δ_H 4.80 for H_2O/D_2O, δ_H 3.31/δ_C 48.2 for CD_3OD-d_4, or δ_H 2.49/δ_C 39.5 for DMSO-d_6. The splitting patterns are reported as s (singlet),

Table 1
Neuraminidase inhibitory and anti-influenza activities of compounds **1**–**8**

Inhibitor	IC_{50}[a] (μM)	EC_{50}[b] (μM)
1 (DANA)	190[c]	–
2 (amino-DANA)	–	1.5[d]
3 (zanamivir)	0.004	0.013
4a	>200	ND[e]
4b	>200	ND[e]
4c	~70	ND[e]
4d	~80	ND[e]
5	>200	ND[e]
6a	>200	ND[e]
6b	>200	ND[e]
7a	>200	ND[e]
7b	>200	ND[e]
7c	7.12	52.0
8a	2.15	0.77
8b	6.50	10.7
8c	33.3	37.6
8d	23.1	45.0

[a] Neuraminidase inhibition against influenza virus A/WSN/1933 (H1N1).
[b] Concentration of NA inhibitors for 50% protection of the cytopathic effects due to influenza (A/WSN/1933) infection.
[c] Neuraminidase inhibition against influenza virus A/Vienna/83/58 (H2N2) which adapted from Ref. [3]
[d] Concentration required to reduce 50% plaque formation in MDCK cells due to flu (A/Singapore/1/57, H2N2) which adapted from Ref. [5a]
[e] Not determined.

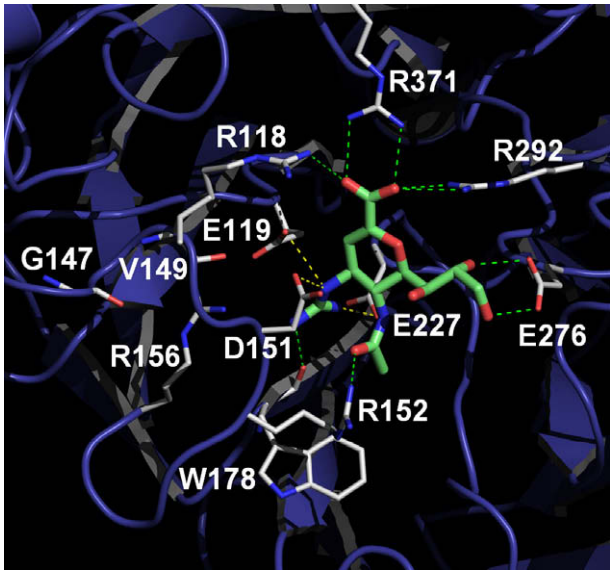


Figure 2. A crystal complex of zanamivir with N1 neuraminidase in 'closed' form. Hydrogen bonds are represented by dotted lines. The neuraminidase and zanamivir are displayed in the colors as that described in Figure 1.

d (doublet), t (triplet), q (quartet), m (multiplet), dd (double of doublets) and br (broad). Coupling constants (*J*) are given in Hz. Distortionless enhancement polarization transfer (DEPT) spectra were taken to determine the types of carbon signals. The ESI-MS experiments were conducted on a Bruker Daltonics BioTOF III high-resolution mass spectrometer.

7.1.1. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(2-nitrobenzenesulfonamido)-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (10)

A solution of amine **9** (2.38 g, 5.34 mmol) and 2-nitrobenzenesulfonyl chloride (NsCl, 1.18 g, 5.34 mmol) in CH_2Cl_2 (50 mL) was stirred at room temperature for 6 h. The mixture was washed with saturated $NaHCO_3$ and brine. After concentration under reduced pressure, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 25:1) to give the sulfonamide product **10** (3.0 g, 92%). $C_{24}H_{29}N_3O_{14}S$; colorless solid, mp 105–107 °C; TLC (CH_2Cl_2/MeOH = 9:1) R_f = 0.23; $[\alpha]_D^{26}$ +28.9 (*c* 0.89, EtOAc); IR ν_{max} (neat) 1739, 1660, 1542, 1370, 1222 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.07–8.05 (1H, m), 7.83–7.80 (1H, m), 7.74–7.69 (2H, m), 6.20 (1H, d, *J* = 9.2 Hz), 6.05 (1H, br s), 5.71 (1H, d, *J* = 2.0 Hz), 5.44 (1H, dd, *J* = 4.8, 1.2 Hz), 5.32–5.29 (1H, m), 4.63 (1H, dd, *J* = 12.0, 2.0 Hz), 4.54–4.49 (2H, m), 4.15 (1H, dd, *J* = 12.0, 6.8 Hz), 4.05–3.98 (1H, m), 3.74 (3H, s), 2.08 (3H, s), 2.05 (3H, s), 2.04 (3H, s), 1.82 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 169.9 (2×), 169.2, 161.1, 146.8, 144.0, 134.2, 133.1, 132.6, 129.8, 124.6, 110.2, 77.1, 71.8, 68.6, 62.3, 53.6, 52.5, 47.1, 23.1, 21.1 (2×), 21.0; ESI-HRMS calcd for $C_{24}H_{29}N_3O_{14}NaS$: 638.1268, found: *m/z* 638.1250 $[M+Na]^+$.

7.1.2. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(3-*tert*-butoxycarbonylamino)propylamino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (11a)

To a solution of sulfonamide **10** (245 mg, 0.4 mmol) in anhydrous THF (5 mL) were added PPh_3 (157 mg, 0.6 mmol), 4-(*tert*-butoxycarbonylamino)propanol (84 mg, 0.6 mmol) and diisopropyl azodicarboxylate (DIAD, 0.169 mL, 0.6 mmol). The mixture was stirred at room temperature for 21 h, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 40:1) to afford *N*-alkyl product with contamination of triphenylphosphine oxide.

To the above-prepared *N*-alkyl compound in DMF (4 mL) were added Cs_2CO_3 (362 mg, 1.11 mmol) and PhSH (0.23 mL, 2.22 mmol). The reaction mixture was stirred at room temperature for 2.5 h, concentrated, and then dissolved in CH_2Cl_2. The mixture was filtered; the filtrate was concentrated and purified by silica gel column chromatography (gradients CH_2Cl_2/MeOH = 30:1 to CH_2Cl_2/MeOH/Et_3N = 15:1:0.5) to give amine **11a** (202 mg, 86% for two steps). $C_{26}H_{41}N_3O_{12}$; pale yellow solid, mp 139–140 °C; $[\alpha]_D^{22}$ +49.5 (*c* 2.0 EtOAc); IR ν_{max} (neat) 1743, 1677, 1530, 1368, 1250, 1223 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.22 (1H, d, *J* = 8.8 Hz), 6.02 (1H, d, *J* = 1.2 Hz), 5.47 (1H, dd, *J* = 3.6, 3.2 Hz), 5.31–5.27 (1H, m), 5.01 (1H, br), 4.63 (1H, d, *J* = 12.0 Hz), 4.22 (1H, d, *J* = 7.2 Hz), 4.13 (1H, dd, *J* = 12.0, 7.2 Hz), 4.04–4.00 (1H, m), 3.74 (3H, s), 3.40 (1H, d, *J* = 6.8 Hz), 3.27–3.22 (1H, m), 3.05–3.00 (1H, m), 2.76–2.72 (1H, m), 2.60–2.55 (1H, m), 2.07 (3H, s), 2.01 (6H, s), 1.95 (1H, br s), 1.93 (3H, s), 1.56–1.53 (2H, m), 1.39 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 170.6, 170.4, 170.0, 169.9, 162.0, 156.0, 143.4, 111.8, 79.0, 76.9, 71.0, 68.1, 62.2, 55.6, 52.3, 46.8, 42.6, 38.0, 30.0, 28.5 (3×), 23.3, 21.0, 20.9 (2×); ESI-HRMS calcd for $C_{26}H_{42}N_3O_{12}$: 588.2763, found: *m/z* 588.2768 $[M+H]^+$.

7.1.3. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(4-*tert*-butoxycarbonylamino)butylamino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (11b)

By a procedure similar to that for **11a**, Mitsunobu reaction of sulfonamide **10** (430 mg, 0.7 mmol) with 4-(*tert*-butoxycarbonylamino)butanol (159 mg, 0.84 mmol) gave amine **11b** (345 mg, 82%) after removal of the sulfonamide group by PhSH/Cs_2CO_3. $C_{27}H_{43}N_3O_{12}$; pale yellow solid, mp 135–136 °C; $[\alpha]_D^{24}$ +12.2 (*c* 2.0, EtOAc); IR ν_{max} (neat) 1746, 1684, 1532, 1368, 1251, 1220 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.70 (1H, d, *J* = 9.2 Hz), 5.98 (1H, s), 5.43 (1H, dd, *J* = 4.4, 3.2 Hz), 5.25–5.22 (1H, m), 5.01 (1H, br s), 4.59 (1H, d, *J* = 11.6 Hz), 4.22 (1H, d, *J* = 8.0 Hz), 4.08 (1H, dd, *J* = 11.6, 7.2 Hz), 4.04–4.00 (1H, m), 3.70 (3H, s), 3.47 (1H, d, *J* = 6.4 Hz), 3.35 (1H, br s), 3.00 (2H, br s), 2.72–2.68 (1H, m), 2.52–2.47 (1H, m), 2.01 (3H, s), 1.97 (6H, s), 1.86 (3H, s), 1.41 (4H, br), 1.35 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 171.0, 170.6, 170.2, 170.1, 162.1, 156.1, 143.8, 111.2, 78.9, 76.7, 71.1, 68.0, 62.0, 55.4, 52.2, 46.1, 44.6, 40.0, 28.3 (3×), 27.1, 26.6, 22.9, 20.7,

20.6 (2×); ESI-HRMS calcd for $C_{27}H_{43}N_3O_{12}$: 602.2920, found: *m/z* 602.2919 $[M+H]^+$.

7.1.4. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(3-methxy carbonyl)propylamino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (11c)

By a procedure similar to that for **11a**, Mitsunobu reaction of sulfonamide **10** (332 mg, 0.54 mmol) with methyl 4-hydroxybutanoate (77 mg, 0.65 mmol) gave amine **11c** (170 mg, 80%) after removal of the sulfonamide group by $PhSH/Cs_2CO_3$. $C_{23}H_{34}N_2O_{12}$; pale yellow foam; $[\alpha]_D^{23}$ +60.0 (*c* 1.0, CH_2Cl_2); IR ν_{max} (neat) 1738, 1660, 1544, 1439, 1371, 1251, 1222 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.98 (1H, br), 5.98 (1H, d, *J* = 2.8 Hz), 5.46 (1H, dd *J* = 4.0, 3.6 Hz), 5.26–5.23 (1H, m), 4.62 (1H, dd, *J* = 12.0, 2.8 Hz), 4.20 (1H, dd, *J* = 8.8, 3.6 Hz), 4.10 (1H, dd, *J* = 12.0, 7.6 Hz), 4.00 (1H, dd, *J* = 8.8, 8.0 Hz), 3.71 (3H, s), 3.59 (3H, s), 3.33 (1H, *J* = 8.0, 2.8 Hz), 2.72–2.69 (1H, m), 2.52–2.49 (1H, m), 2.32–2.28 (2H, m), 2.04 (3H, s), 1.98 (6H, s), 1.89 (3H, s), 1.69–1.65 (2H, m)1.60 (1H, br); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 170.3, 170.1, 169.9 (2×), 161.9, 143.2, 111.9, 76.7, 71.0, 68.0, 62.0, 55.7, 52.3, 51.5, 46.7, 44.9, 31.6, 25.3, 23.3, 20.9, 20.8 (2×); ESI-HRMS calcd for $C_{23}H_{35}N_2O_{12}$: 531.2185, found: *m/z* 531.2189 $[M+H]^+$.

7.1.5. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(4-methox ycarbonyl)butylamino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (11d)

By a procedure similar to that for **11a**, Mitsunobu reaction of sulfonamide **10** (430 mg, 0.7 mmol) with methyl methyl 5-hydroxypentanoate (110 mg, 0.84 mmol) gave amine **11d** (310 mg, 82%) after removal of the sulfonamide group by $PhSH/Cs_2CO_3$. $C_{24}H_{36}N_2O_{12}$; colorless solid, mp 70–72 °C; $[\alpha]_D^{23}$ +26.6 (*c* 1.0, CH_2Cl_2); IR ν_{max} (neat) 1746, 1621, 1371, 1217 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.02 (1H, d, *J* = 2.8 Hz), 5.96 (1H, d, *J* = 9.2 Hz), 5.49 (1H, dd *J* = 4.4, 3.6 Hz), 5.30–5.27 (1H, m), 4.62 (1H, dd, *J* = 12.4, 2.8 Hz), 4.21 (1H, dd, *J* = 8.8, 3.6 Hz), 4.12 (1H, dd, *J* = 12.4, 7.2 Hz), 4.06–4.00 (1H, m), 3.73 (3H, s), 3.61 (3H, s), 3.35 (1H, *J* = 8.0, 2.8 Hz), 2.72–2.67 (1H, m), 2.56–2.51 (1H, m), 2.30–2.26 (2H, m), 2.06 (3H, s), 2.00 (6H, s), 1.97 (1H, br s), 1.91 (3H, s), 1.65–1.57 (2H, m), 1.44–1.38 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 170.3, 170.2, 170.0, 169.9, 162.0, 143.1, 111.7, 76.7, 71.0, 68.0, 62.1, 55.5, 52.3, 51.5, 46.8, 45.3, 33.7, 29.3, 23.3, 22.3, 20.9, 20.8 (2×); ESI-HRMS calcd for $C_{24}H_{37}N_2O_{12}$: 545.2341, found: *m/z* 545.2333 $[M+H]^+$.

7.1.6. 5-Acetamido-2,6-anhydro-4-(3-aminopropyl)amino-3,4,5 -trideoxy-D-glycero-D-galacto-non-2-enonic acid (4a)

A solution of ester **11a** (60 mg, 0.10 mmol) in MeOH (1 mL) and aqueous NaOH (1 M, 1 mL) was stirred at room temperature for 30 min. The mixture was neutralized by Dowex 50 W × 8 (H^+), filtered, and rinsed with ammonia water. The combined aqueous solution was concentrated under reduced pressure, and the residue of acid product was dissolved in CH_2Cl_2 (1 mL) and TFA (1 mL). The mixture was stirred at room temperature for 5 h, and concentrated under reduced pressure. The residue was triturated with Et_2O, and centrifuged to give the desired product **4a** (42 mg, 74%). $C_{14}H_{25}N_3O_7{\cdot}2(CF_3CO_2H)$; pale yellow foam; ^1H NMR (400 MHz, D_2O) δ 5.99 (1H, d, *J* = 1.2 Hz), 4.50 (1H, dd, *J* = 10.0, 9.2 Hz), 4.41 (1H, d, *J* = 10.0 Hz), 4.36 (1H, dd, *J* = 9.2, 1.2 Hz), 3.95–3.91 (1H, m), 3.86 (1H, dd, *J* = 12.0, 2.4 Hz), 3.70 (1H, d, *J* = 9.6 Hz), 3.65 (1H, dd, *J* = 12.0, 6.0 Hz), 3.39–3.32 (1H, m), 3.20–3.13 (1H, m), 3.08 (2H, t, *J* = 8.0 Hz), 2.15–2.02 (5H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.9, 164.9, 162.9 (CO_2 of TFA, q, *J* = 35.7 Hz), 148.6, 116.4 (CF_3 of TFA, q, *J* = 288.8 Hz), 101.4, 76.0, 69.9, 67.8, 63.1, 56.3, 43.9, 40.9, 36.8, 24.2, 22.4; ESI-HRMS calcd for $C_{14}H_{26}N_3O_7$: 348.1765, found: *m/z* 348.1763 $[M+H]^+$.

7.1.7. 5-Acetamido-2,6-anhydro-4-(4-aminobutyl)amino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (4b)

By a procedure similar to that for **4a**, saponification of ester **11b** (153 mg, 0.25 mmol), followed by treatment with TFA, gave **4b** (114 mg, 67%). $C_{15}H_{27}N_3O_7{\cdot}2(CF_3CO_2H)$; pale yellow solid, mp 140–142 °C; ^1H NMR (400 MHz, D_2O) δ 5.93 (1H, s), 4.49 (1H, dd, *J* = 10.0, 9.2 Hz), 4.40 (1H, d, *J* = 10.0 Hz), 4.32 (1H, d, *J* = 9.2 Hz), 3.95–3.91 (1H, m), 3.87 (1H, dd, *J* = 12.0, 2.4 Hz), 3.69 (1H, d, *J* = 9.2 Hz), 3.64 (1H, dd, *J* = 12.0, 6.0 Hz), 3.27 (1H, m), 3.11 (1H, m), 3.02 (2H, m), 2.06 (3H, s), 1.75 (4H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.9, 165.7, 162.9 (CO_2 of TFA, q, *J* = 34.9 Hz), 149.2, 117.9 (CF_3 of TFA, q, *J* = 289.6 Hz), 100.9, 75.9, 69.9, 67.9, 63.2, 56.2, 44.0, 43.2, 39.0, 24.2, 23.3, 22.4; ESI-HRMS calcd for $C_{15}H_{28}N_3O_7$: 362.1927, found: *m/z* 362.1924 $[M+H]^+$.

7.1.8. 5-Acetamido-2,6-anhydro-4-(3-carboxypropyl)amino-3,4, 5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (4c)

A solution of **11c** (80 mg, 0.15 mmol) in MeOH (1 mL) and aqueous NaOH (1 M, 1 mL) was stirred at room temperature for 1 h. The mixture was chromatographed on a Dowex 50 W × 8 (H^+) ion exchange column. The column was first eluted with H_2O to discard impurities, and then with ammonia water to afford the desired product **4c** (45 mg, 79%). $C_{15}H_{24}N_2O_9$; colorless solid, mp 90–91 °C; ^1H NMR (400 MHz, D_2O) δ 5.77 (1H, d, *J* = 2.4 Hz), 4.47 (1H, dd, *J* = 10.0, 9.6 Hz), 4.37 (1H, d, *J* = 10.0 Hz), 4.30 (1H, dd, *J* = 8.8, 1.6 Hz), 3.99–3.94 (1H, m), 3.90 (1H, dd, *J* = 11.6, 2.4 Hz), 3.72–3.64 (2H, m), 3.33–3.26 (1H, m), 3.18–3.13 (1H, m), 2.53 (2H, t, *J* = 7.2 Hz), 2.09 (3H, s), 2.06–1.96 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 177.0, 174.5, 167.5, 162.6 (CO_2 of TFA, q, *J* = 34.9 Hz), 151.3, 116.4 (CF_3 of TFA, q, *J* = 288.3 Hz), 98.6, 75.8, 70.1, 68.2, 63.5, 56.5, 44.7, 43.8, 31.9, 23.0, 21.9; ESI-HRMS calcd for $C_{15}H_{25}N_2O_9$: 377.1555, found: *m/z* 377.1567 $[M+H]^+$.

7.1.9. 5-Acetamido-2,6-anhydro-4-(4-carboxybutyl)amino-3,4,5 -trideoxy-D-glycero-D-galacto-non-2-enonic acid (4d)

By a procedure similar to that for **4c**, saponification of ester **11d** (90 mg, 0.16 mmol) gave **4d** (68 mg, 84%). $C_{16}H_{26}N_2O_9$; colorless solid, mp 100–101 °C; ^1H NMR (400 MHz, D_2O) δ 5.72 (1H, d, *J* = 2.0 Hz), 4.15–4.06 (2H, m), 3.93–3.85 (2H, m), 3.66–3.56 (3H, m), 2.70–2.64 (1H, m), 2.60–2.55 (1H, m), 2.16 (2H, t, *J* = 7.2 Hz), 2.03 (3H, s), 1.57–1.50 (2H, m), 1.49–1.42 (2H, m); ESI-HRMS calcd for $C_{16}H_{27}N_2O_9$: 391.1711, found: *m/z* 391.1704 $[M+H]^+$.

7.1.10. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-(1-*tert*-bu toxycarbonyl-4,5-dihydroimidazol-2-yl)amino-3,4,5-trideoxy-D -glycero-D-galacto-non-2-enonic acid methyl ester (12)

A solution of amine **9** (210 mg, 0.47 mmol) and *N*-Boc-2-methylthio-2-imidazoline (66 mg, 0.38 mmol) in EtOH/AcOH (v/v = 9:1, 3 mL) was stirred at 50 °C for 24 h. The mixture was concentrated, and then partitioned between CH_2Cl_2 and saturated $NaHCO_3$ aqueous solution. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 15:1) to give **12** (175 mg, 62%). $C_{26}H_{38}N_4O_{12}$; colorless solid, mp 145–147 °C; ^1H NMR (400 MHz, D_2O) δ 7.09 (1H, br s), 6.27 (1H, br s), 5.95 (1H, d, *J* = 2.0 Hz), 5.39 (1H, dd, *J* = 4.8, 1.6 Hz), 5.30–5.26 (1H, m), 4.66 (1H, br s), 4.64 (1H, dd, *J* = 12.4, 2.8 Hz), 4.27–4.17 (2H, m), 4.13 (1H, dd, *J* = 12.4, 7.2 Hz), 3.75 (3H, s), 3.77–3.66 (2H, m), 3.59–3.54 (2H, m), 2.10 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 1.87 (3H, s), 1.46 (9H, s); ESI-HRMS calcd for $C_{26}H_{39}N_4O_{12}$: 599.2559, found: *m/z* 599.2626 $[M+H]^+$.

7.1.11. 5-Acetamido-2,6-anhydro-4-(4,5-dihydroimidazol-2-yl) amino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (5)

A solution of **12** (107 mg, 0.18 mmol) was treated with trifluoroacetic acid (1 mL) in CH_2Cl_2 (1 mL) at room temperature for 5 h to remove the *t*-Boc group. The mixture was concentrated under reduced pressure. The residue was triturated with Et_2O, and the precipitated product was collected by centrifuge. The precipitate was subjected to saponification in aqueous NaOH (1 M, 1 mL) and MeOH (1 mL). After stirring at room temperature for 5 min, the mixture was neutralized by Dowex 50 W × 8 (H^+), filtered, and rinsed with ammonia water. The combined aqueous solution was concentrated to give compound **5** (79 mg, 94%). $C_{14}H_{22}N_4O_7{\cdot}2(CF_3CO_2H)$; colorless solid, mp 167–168 °C; ^{1}H NMR (400 MHz, D_2O) δ 5.72 (1H, s), 4.41–4.36 (2H, m), 4.25 (1H, dd, J = 10.0, 9.6 Hz), 3.97–3.88 (2H, m), 3.72–3.64 (6H, m), 2.05 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 173.6, 167.2, 162.2 (CO_2 of TFA, q, J = 34.9 Hz), 158.9, 147.5, 116.0 (CF_3 of TFA, q, J = 287.3 Hz), 104.8, 75.5, 69.8, 68.0, 63.1, 52.5, 49.0, 47.5, 42.9, 22.3; ESI-HRMS calcd for $C_{14}H_{23}N_4O_7$: 359.1561, found: *m/z* 359.1642 $[M+H]^+$.

7.1.12. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′-(9-fluorenylmethoxycarbonyl)thiourea]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (13)

To a solution of **9** (366 mg, 0.82 mmol) in anhydrous CH_2Cl_2 (5 mL) was added a solution of 9-fluorenylmethxoxycarbonyl isocyanate (FmocNCS, 253 mg, 0.9 mmol) in anhydrous CH_2Cl_2 (5 mL) dropwise. The mixture was stirred at room temperature for 15 min, concentrated, and purified by silica gel column chromatography (EtOAc/hexane = 1:1) to give thiourea **13** (524 mg, 90%). $C_{34}H_{37}N_3O_{12}S$; yellow solid, mp 103–105 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (1H, d, J = 8.8 Hz), 8.53, (1H, s), 7.72 (2H, d, J = 7.2 Hz), 7.51 (2H, dd, J = 7.6, 0.8 Hz), 7.37 (2H, dd, J = 7.6, 7.2 Hz), 7.28 (2H, ddd, J = 7.6, 7.2, 0.8 Hz), 6.14 (1H, d, J = 10.0 Hz), 5.97 (1H, d, J = 2.4 Hz), 5.49 (1H, dd, J = 4.8, 2.0 Hz), 5.45 (1H, ddd, J = 10.8, 8.8, 2.4 Hz), 5.31 (1H, m), 4.69 (1H, dd, J = 12.4, 2.8 Hz), 4.50–4.33 (4H, m), 4.18–4.14 (2H, m), 3.73 (3H, s), 2.09 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 1.87 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.0, 170.5, 170.4, 170.1, 169.8, 161.3, 151.9, 144.7, 142.6, 142.5, 141.0 (2×), 127.8 (2×), 127.8 (2×), 127.0 (2×), 124.6 (2×), 120.0, 108.8, 71.3, 68.2, 67.6, 62.1, 54.3, 52.5, 46.6, 46.4, 23.2, 21.0, 20.8 (2×); ESI-HRMS calcd for $C_{34}H_{38}N_3O_{12}S$: 712.2176, found: *m/z* 712.2180 $[M+H]^+$.

7.1.13. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′-(*tert*-butoxycarbonyl)thiourea]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (14)

To a solution of **13** (4.5 g, 6.3 mmol) in CH_2Cl_2 (50 mL) was added piperidine (12 mL). The mixture was stirred at room temperature for 15 min, concentrated, and washed with hexane to remove 9-methylidenefluorene. The crude product was dissolved in CH_2Cl_2, and di-*tert*-butyl dicarbonate (Boc_2O, 2.06 g, 9.45 mmol) and 4-dimethylaminopyridine (DMAP, 1.15 g, 9.45 mmol) were added. The mixture was stirred at room temperature for 7 h, concentrated, and purified by silica gel column chromatography (EtOAc/hexane = 3:2) to give thiourea **14** (3.23 g, 87%). $C_{24}H_{35}N_3O_{12}S$; colorless solid, mp 100–102 °C; TLC (EtOAc/hexane = 4:1) R_f = 0.55; $[\alpha]_D^{20}$ +58.2 (*c* 2.0, EtOAc); IR ν_{max} (neat) 2980, 1743, 1525, 1370, 1249, 1223, 1143 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.78 (1H, d, J = 8.4 Hz), 7.90 (1H, s), 5.94 (1H, d, J = 2.4 Hz), 5.86 (1H, d, J = 5.2 Hz), 5.56 (1H, m), 5.45 (1H, dd, J = 4.8, 2.0 Hz), 5.29 (1H, m), 4.65 (1H, dd, J = 12.4, 2.4 Hz), 4.42–4.31 (2H, m), 4.13 (1H, dd, J = 12.4, 7.2 Hz), 3.77 (3H, s), 2.10 (3H, s), 2.06 (3H, s), 2.04 (3H, s), 1.90 (3H, s), 1.46 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 180.3, 170.0, 169.9, 169.6, 169.4, 160.9, 150.6, 144.6, 108.7, 84.3, 77.6, 71.3, 67.6, 62.2, 54.1, 52.6, 47.5, 28.2 (3×), 23.6, 21.3, 21.1 (2×); ESI-HRMS calcd for $C_{24}H_{34}N_3O_{12}S$: 588.1863, found: *m/z* 588.1856 $[M-H]^-$.

7.1.14. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′-(*tert*-butoxycarbonyl)-*N*″-(3-*tert*-butoxycarbonylaminopropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (15a)

To a solution of thiourea **14** (150 mg, 0.254 mmol) in DMF (3 mL) were added *tert*-butyl *N*-(3-aminopropyl) carbamate (66 mg, 0.38 mmol), $HgCl_2$ (83 mg, 0.305 mmol) and Et_3N (0.054 mL, 0.38 mmol). The mixture was stirred at room temperature for 2 h, and then concentrated under reduced pressure. The residue was dissolved in EtOAc, and filtered through a pad of Celite. The filtrate was concentrated, and the crude product was purified by silica gel column chromatography (EtOAc/hexane = 2:1) to give **15a** (122 mg, 66%). $C_{32}H_{51}N_5O_{14}$; colorless solid, mp 108–110 °C; TLC (EtOAc/hexane = 4:1) R_f = 0.39; $[\alpha]_D^{25}$ +56.6 (*c* 1.43, EtOAc); IR ν_{max} (neat) 2976, 1746, 1638, 1603, 1368, 1250, 1218, 1147 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.05 (1H, br), 6.53 (1H, d, J = 9.2 Hz), 5.91 (1H, s), 5.44 (1H, dd, J = 5.2, 2.0 Hz), 5.40 (2H, br), 5.28 (1H, m), 4.65 (1H, d, J = 12.4 Hz), 4.27–4.17 (2H, m), 4.12 (1 H, dd, J = 12.4, 7.2 Hz), 3.75 (3H, s), 3.21–3.02 (4 H, m), 2.06 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 1.85 (3H, s), 1.77 (1H, m), 1.75 (1H, m), 1.43 (9H, s), 1.39 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.1, 169.8, 169.3, 169.0, 162.6, 161.0, 159.4, 155.8, 143.4, 110.9, 79.1, 78.2, 77.2, 70.9, 67.8, 62.1, 52.4, 49.7, 47.8, 38.6, 37.6, 30.0, 28.6 (3×), 28.5 (3×), 23.2, 21.1, 21.0, 20.9; ESI-HRMS calcd for $C_{32}H_{52}N_5O_{14}$: 730.3511, found: *m/z* 730.3512 $[M+H]^+$.

7.1.15. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′-(*tert*-butoxycarbonyl)-*N*″-(4-*tert*-butoxycarbonylaminobutyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (15b)

The reaction of thiourea **14** (190 mg, 0.32 mmol) with *N*-(4-aminobutyl) carbamic *tert*-butyl ester (73 mg, 0.39 mmol) was carried out by a procedure similar to that for **15a** to give **15b** (176 mg, 74%). $C_{33}H_{53}N_5O_{14}$; colorless solid, mp 87–89 °C; TLC (EtOAc/hexane = 4:1) R_f = 0.36; $[\alpha]_D^{25}$ +50.4 (*c* 1.0, EtOAc); IR ν_{max} (neat) 2976, 1745, 1639, 1604, 1368, 1250, 1219, 1147 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.02 (1H, br), 6.71 (1H, d, J = 9.2 Hz), 5.91 (1H, d, J = 2.0 Hz), 5.42 (1H, dd, J = 5.2, 2.0 Hz), 5.25 (1H, m), 5.11 (1H, br), 4.99 (2H, m), 4.60 (1H, d, J = 11.6 Hz), 4.25–4.12 (2H, m), 4.07 (1H, dd, J = 11.6, 7.2 Hz), 3.74 (3H, s), 3.06 (4 H, m), 2.03 (3H, s), 2.01 (3H, s), 2.00 (3H, s), 1.84 (3H, s), 1.54 (2H, m), 1.48 (2H, m), 1.41 (9H, s), 1.37 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 170.4, 169.9, 169.7, 163.4, 161.6, 159.8, 156.0, 144.0, 111.1, 79.1, 78.2, 77.2, 71.2, 67.9, 62.2, 52.4, 49.7, 40.8, 39.7, 29.7, 28.5 (3×), 28.4 (3×), 27.4, 26.2, 23.0, 20.9, 20.8, 20.7; ESI-HRMS calcd for $C_{33}H_{52}N_5O_{14}$: 742.3511, found: *m/z* 742.3507 $[M-H]^-$.

7.1.16. 5-Acetamido-2,6-anhydro-4-[*N*′-(3-aminopropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (6a)

By a procedure similar to that for **4a**, saponification of **15a** (120 mg, 0.16 mmol), followed by removal of the Boc group with TFA, gave **6a** (92 mg, 94%). $C_{15}H_{27}N_5O_7{\cdot}2(CF_3CO_2H)$; pale yellow foam; IR ν_{max} (neat) 3411, 1682, 1638, 1400, 1205, 1138 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 5.83 (1H, s), 4.50 (1H, d, J = 8.4 Hz), 4.41 (1H, d, J = 10.4 Hz), 4.29 (1H, dd, J = 10.4, 8.4 Hz), 3.94–3.86 (2H, m), 3.71–3.62 (2 H, m), 3.32 (2 H, m), 3.05 (2 H, t, J = 8.0 Hz), 2.03 (3H, s), 1.98 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.2, 166.7, 163.0 (CO_2 of TFA, q, J = 35 Hz), 156.0, 146.8, 116.4 (CF_3 of TFA, q, J = 289.6 Hz), 106.9, 75.9, 70.0, 68.1, 63.2, 51.5, 47.7, 38.7, 37.0, 26.4, 22.2; ESI-HRMS calcd for $C_{15}H_{28}N_5O_7$: 390.1989, found: m/*z* 390.2004 $[M+H]^+$.

7.1.17. 5-Acetamido-2,6-anhydro-4-[*N*′-(4-aminobutyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (6b)

By a procedure similar to that for **4a**, saponification of **15b** (176 mg, 0.24 mmol), followed by removal of the Boc group with TFA, gave **6b** (136 mg, 90%). $C_{16}H_{29}N_5O_7{\cdot}2(CF_3CO_2H)$; pale yellow foam; $[\alpha]_D^{20}$ +21.6 (*c* 3.39, H_2O); IR ν_{max} (neat) 3457, 1694, 1400, 1203, 1136 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 5.89 (1H, d, *J* = 2.0 Hz), 4.47 (1H, dd, *J* = 9.2, 2.0 Hz), 4.31 (1H, d, *J* = 10.8 Hz), 4.19 (1H, dd, *J* = 10.8, 9.2 Hz), 3.85–3.81 (1H, m), 3.79 (1H, dd, *J* = 12.0, 2.4 Hz), 3.64 (1H, d, *J* = 9.6 Hz), 3.58 (1H, dd, *J* = 12.0, 6.0 Hz), 3.15 (2 H, t, *J* = 6.4 Hz), 2.93 (2 H, t, *J* = 6.8 Hz), 1.94 (3H, s), 1.62–1.56 (4H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.4, 164.8, 162.5 (CO_2 of TFA, q, *J* = 35.6 Hz), 155.9, 144.6, 116.2 (CF_3 of TFA, q, *J* = 289.7 Hz), 109.0, 76.1, 69.9, 67.9, 63.0, 50.7, 47.5, 40.7, 39.0, 25.0, 23.9, 21.9; ESI-HRMS calcd for $C_{16}H_{30}N_5O_7$: 404.2145, found: m/*z* 404.2238 $[M+H]^+$.

7.1.18. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-(3-*tert*-butoxycarbonylaminopropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (16a)

To a solution of **11a** (104 mg, 0.177 mmol) in DMF (2 mL) were added 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (61.5 mg, 0.21 mmol), $HgCl_2$ (57.5 mg, 0.21 mmol) and Et_3N (0.05 mL, 0.35 mmol). The mixture was stirred at room temperature for 3 h, and then concentrated in vacuo. The residue was dissolved in EtOAc and filtered through a pad of Celite. The organic solution was washed with 1 M HCl, saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/hexane = 2:1) to give **16a** (134 mg, 91%). $C_{37}H_{59}N_5O_{16}$; colorless solid, mp 85–86 °C; TLC (EtOAc) R_f = 0.48; $[\alpha]_D^{24}$ +43.5 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$, 50 °C) δ 9.51 (1H, br s), 6.20 (1H, br s), 5.94 (1H, s), 5.39 (1H, d, *J* = 6.8 Hz), 5.30–5.26 (1H, m), 5.21 (1H, br s), 5.06 (1H, br s), 4.63 (1H, dd, *J* = 12.4, 2.8 Hz), 4.35 (1H, *J* = 8.4 Hz), 4.17–4.12 (2H, m), 3.76 (3H, s), 3.30–3.24 (2H, m), 3.15–3.10 (1H, m), 2.99–2.91 (1H, m), 2.10 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.84 (3H, s), 1.79–1.73 (1H, m), 1.69–1.62 (1H, m), 1.45 (18H, s), 1.40 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 170.3, 170.1, 170.0, 161.3, 161.2, 156.1, 146.0, 109.9, 82.8, 80.1, 79.1, 78.0, 77.2, 71.4, 67.9, 62.2, 57.3, 52.5, 45.7, 37.7, 29.1, 28.5 (6×), 28.2 (3×), 23.2, 21.1, 21.0, 20.9; ESI-HRMS calcd for $C_{37}H_{60}N_5O_{16}$: 830.4030, found: *m/z* 830.4033 $[M+H]^+$.

7.1.19. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-(4-*tert*-butoxycarbonylaminobutyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (16b)

By a procedure similar to that for **16a**, the reaction of **11b** (120 mg, 0.2 mmol) with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of $HgCl_2$ and Et_3N gave **16b** (148 mg, 88%). $C_{38}H_{61}N_5O_{16}$; colorless solid, mp 69–71 °C; TLC (EtOAc) R_f = 0.49; ^1H NMR (400 MHz, $CDCl_3$, 50 °C) δ 6.33 (1H, br s), 5.92 (1H, s), 5.38 (1H, dd, *J* = 4.8, 2.0 Hz), 5.26–5.23 (1H, m), 5.25 (1H, br s), 5.05 (1H, br s), 4.63 (1H, dd, *J* = 12.4, 2.8 Hz), 4.30 (1H, *J* = 10.0 Hz), 4.18 (1H, br s), 4.11 (1H, dd, *J* = 12.4, 7.2 Hz), 3.75 (3H, s), 3.15 (2H, m), 3.05–3.04 (2H, m), 2.08 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.82 (3H, s), 1.58–1.48 (2H, m), 1.43 (18H, s), 1.38 (9H, s), 1.38–1.30 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 170.2, 169.9, 169.8, 161.1, 155.7, 155.4, 150.2, 145.7, 110.1, 82.1, 80.1, 78.7, 77.9, 77.2, 71.3, 67.8, 62.1, 57.0, 52.4, 45.4, 44.8, 39.5, 29.6, 28.4 (6×), 28.0 (3×), 27.0, 23.0, 20.9 (2×), 20.7; ESI-HRMS calcd for $C_{38}H_{62}N_5O_{16}$: 844.4186, found: *m/z* 844.4184 [M+H]+.

7.1.20. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-(3-methoxycarbonylpropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (16c)

By a procedure similar to that for **16a**, the reaction of **11c** (90 mg, 0.17 mmol) with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of $HgCl_2$ and Et_3N gave **16c** (120 mg, 91%). $C_{34}H_{52}N_4O_{16}$; colorless solid, mp 75–77 °C; TLC (EtOAc/hexane) R_f = 0.29; $[\alpha]_D^{23}$ +11.4 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 9.17 (1H, br s), 6.3 (1H, br s), 5.9 (1H, s), 5.45 (1H, br s), 5.41 (1H, 5.2 Hz), 5.30–5.26 (1H, m), 4.63 (1H, d, *J* = 12.0 Hz), 4.33 (1H, d, *J* = 9.6 Hz), 4.24 (1H, br s), 4.13 (1H, dd, *J* = 12.4, 7.2 Hz), 3.78 (3H, s), 3.65 (3H, s), 3.20 (2H, br), 2.34–2.28 (2H, m), 2.10 (3H, s), 2.07 (6H, s), 1.85 (3H, s), 1.85–1.82 (2H, m), 1.46 (18H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 170.4, 169.6, 169.3, 160.7, 159.8, 153.9, 149.5, 145.3, 109.9, 84.7, 81.8, 79.4, 77.7, 77.2, 71.0, 67.8, 62.2, 56.4, 52.4, 51.7, 44.7, 44.2, 30.9, 28.2 (6×), 23.2, 21.1, 21.0, 20.9; ESI-HRMS calcd for $C_{34}H_{53}N_4O_{16}$: 773.3451, found: *m/z* 773.3450 $[M+H]^+$.

7.1.21. 5-Acetamido-2,6-anhydro-4-[*N*-(3-aminopropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (7a)

By a procedure similar to that for **4a**, saponification of **16a** (110 mg, 0.13 mmol), followed by removal of the Boc group with TFA, gave **7a** (72 mg, 92%). $C_{15}H_{27}N_5O_7{\cdot}2(CF_3CO_2H)$; colorless solid, mp >200 °C (decomposed); ^1H NMR (400 MHz, D_2O) δ 5.70 (1H, s), 4.89 (1H, m), 4.42–4.35 (2H, m), 3.98–3.85 (2H, m), 3.69–3.63 (2H, m), 3.44–3.36 (1H, m), 3.30 (1H, br), 3.04 (2H, t, *J* = 7.2 Hz), 2.15–2.09 (1H, m), 2.07–1.97 (1H, m), 2.02 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.1, 168.0, 163.0 (CO_2 of TFA, q, *J* = 35.0 Hz), 157.7, 151.5, 117.9 (CF_3 of TFA, q, *J* = 288.1 Hz), 103.4, 75.8, 70.0, 68.2, 63.3, 58.4, 45.4, 41.2, 37.1, 25.2, 22.2; ESI-HRMS calcd for $C_{15}H_{28}N_5O_7$: 390.1983, found: *m/z* 390.1977 $[M+H]^+$.

7.1.22. 5-Acetamido-2,6-anhydro-4-[*N*-(4-aminobutyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (7b)

By a procedure similar to that for **4a**, saponification of **16b** (114 mg, 0.13 mmol), followed by removal of the Boc group with TFA, gave **7b** (77 mg, 94%). $C_{16}H_{29}N_3O_7{\cdot}2(CF_3CO_2H)$; colorless solid, mp 167–168 °C; ^1H NMR (400 MHz, D_2O) δ 5.73 (1H, s), 4.89 (1H, m), 4.43–4.34 (2H, m), 4.00–3.95 (1H, m), 3.93–3.86 (1H, m), 3.70–3.64 (2H, m), 3.36–3.33 (1H, m), 3.20 (1H, br), 3.02 (2H, t, *J* = 7.2 Hz), 2.03 (3H, s), 1.84 (1H, br), 1.71–1.70 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 174.0, 168.1, 163.0 (CO_2 of TFA, q, *J* = 35.0 Hz), 157.6, 150.9, 116.5 (CF_3 of TFA, q, *J* = 289.6 Hz), 103.8, 75.8, 70.0, 68.2, 63.3, 58.2, 45.5, 43.5, 39.3, 24.4, 23.9, 22.2; ESI-HRMS calcd for $C_{16}H_{30}N_5O_7$: 404.2140, found: *m/z* 404.2135 $[M+H]^+$.

7.1.23. 5-Acetamido-2,6-anhydro-4-[*N*-(3-carboxypropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (7c)

By a procedure similar to that for **4a**, saponification of **16c** (50 mg, 0.06 mmol), followed by removal of the Boc group with TFA, gave **7c** (30 mg, 95%). $C_{16}H_{26}N_4O_9{\cdot}CF_3CO_2H$; colorless solid, mp 152–153 °C; ^1H NMR (400 MHz, D_2O) δ 5.74 (1H, s), 4.90 (1H, m), 4.42 (1H, d, *J* = 10.4 Hz), 4.35–4.34 (1H, br), 3.99–3.95 (1H, m), 3.92–3.89 (1H, m), 3.71–3.64 (2H, m), 3.32–3.28 (2H, m), 2.45 (2H, t, *J* = 6.8 Hz), 2.03 (3H, s), 2.00–1.93 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 177.6, 173.8, 167.7, 162.6 (CO_2 of TFA, q, *J* = 35.0 Hz), 157.4, 151.6, 116.5 (CF_3 of TFA, q, *J* = 288.3 Hz), 104.0, 75.9, 70.2, 68.5, 63.6, 58.0, 45.9, 43.6, 31.2, 23.1, 22.7; ESI-HRMS calcd for $C_{16}H_{25}N_4O_9$: 417.1622, found: *m/z* 417.1624 $[M-H]^-$.

7.1.24. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*-(3-allyloxycarbonylpropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (17)

By a procedure similar to that for **11a**, Mitsunobu reaction of sulfonamide **10** (1.2 g, 1.95 mmol) with allyl 3-hydroxypropanoate (420 mg, 2.9 mmol) gave amine **17** (878 mg, 81%) after removal of the sulfonamide group by $PhSH/Cs_2CO_3$. $C_{25}H_{36}N_2O_{12}$; colorless foam; TLC (CH_2Cl_2/MeOH = 3:1) R_f = 0.12; $[\alpha]_D^{23}$ +58.2 (*c* 1.0, CH_2Cl_2); IR ν_{max} (neat) 1740, 1659, 1544, 1440, 1371, 1248, 1221 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.02 (1H, d, *J* = 2.8 Hz), 5.96–5.81 (1H, m), 5.78 (1H, br s), 5.49 (1H, dd, *J* = 4.0, 3.6 Hz), 5.30–5.17 (3H, m), 4.62 (1H, dd, *J* = 12.0, 2.8 Hz), 4.53–4,51 (2H, m), 4.24 (1H, dd, *J* = 8.8, 3.6 Hz), 4.14 (1H, dd, *J* = 12.0, 7.2 Hz), 4.0 (1H, m), 3.74 (3H, s), 3.36 (1H, dd, *J* = 8.0, 2.8 Hz), 2.77–2.71 (1H, m), 2.59–2.52 (1H, m), 2.35 (2H, t, *J* = 8.4 Hz), 2.07 (3H, s), 2.01 (6H, s), 1.92 (3H, s), 1.76–1.68 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) 172.8, 170.2, 170.1, 169.7, 161.7, 143.0, 131.7, 117.7, 112.0, 76.6, 71.0, 67.8, 64.7 (2×), 61.9, 55.8, 52.0, 46.3, 44.5, 31.5, 25.2, 23.0, 20.6 (3×); ESI-HRMS calcd for $C_{25}H_{37}N_2O_{12}$: 557.2341, found: *m/z* 557.2340 $[M+H]^+$.

7.1.25. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-(3-allyloxycarbonylpropyl)guanidino]-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (16d)

By a procedure similar to that for **16a**, the reaction of **17** (683 mg, 1.21 mmol) with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of $HgCl_2$ and Et_3N gave **16d** (800 mg, 83%). $C_{36}H_{54}N_4O_{16}$; colorless solid, mp 59–60 °C; TLC (EtOAc/hexane = 3:1) R_f = 0.39; $[\alpha]_D^{23}$ +8.9 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 9.00 (1H, br s), 6.34 (1H, br s), 5.88–5.77 (2H, m), 5.36 (1H, d, *J* = 5.2 Hz), 5.35 (1H, br s), 5.28–5.14 (3H, m), 4.58 (1H, d, *J* = 12.0 Hz), 4.49 (2H, dd, *J* = 5.6, 0.8 Hz), 4.30 (1H, d, *J* = 9.6 Hz), 4.21 (1H, br s), 4.08 (1H , dd, *J* = 12.0, 6.8 Hz), 3.72 (3H, s), 3.16 (2H, br s), 2.27–2.23 (2H, m), 2.05 (3H, s), 1.99 (3H, s), 1.98 (3H, s), 1.80 (5H, s), 1.4 (18H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 171.7, 170.4, 169.6, 169.3, 160.7, 160.0, 153.9, 149.7, 145.3, 131.4, 117.9, 109.9, 81.9, 79.3, 77.8, 77.2, 71.1, 67.8, 65.1, 62.2, 56.2, 52.4, 45.3, 44.2, 31.0, 28.2 (6×), 24.2, 23.2, 21.1 (2×), 20.9; ESI-HRMS calcd for $C_{36}H_{55}N_4O_{16}$: 799.3608, found: *m/z* 799.3603 $[M+H]^+$.

7.1.26. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-{*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-[3-(4-*tert*-butoxycarbonylpiperazino)carbonylpropyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (20a)

To a solution of **16d** (800 mg, 1.0 mmol) in THF (10 mL) were added $Pd(PPh_3)_4$ (230 mg, 0.2 mmol) and morpholine (1.74 mL, 20 mmol). The mixture was stirred at room temperature for 3.5 h, and then filtered through a pad of Celite. The filtrate was concentrated, and partitioned between 1 M HCl and CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **18** (645 mg, 85%). ESI-HRMS calcd for $C_{13}H_{49}N_4O_{16}$: 757.3149, found: *m/z* 757.3143 $[M-H]^-$.

To a solution of piperazine (4.3 g, 50 mmol) in CH_2Cl_2 (125 ml) was added a solution of Boc_2O (5.45 g, 25 mmol) in CH_2Cl_2 (50 mL) dropwise at ice-bath temperature. The mixture was warmed to room temperature, stirred for 1 h, and filtered. The filtrate was concentrated in vacuo. The residue was added H_2O, and filtered. The filtrate was saturated with K_2CO_3 and extracted with Et_2O (3×). The combined organic phase was dried over $MgSO_4$, filtered and concentrated to afford *N*-Boc-piperazine **19a** (2.95 g, 63%). $C_9H_{18}N_2O_2$; colorless oil; ^1H NMR (400 MHz, $CDCl_3$) δ 3.34 (4H, t, *J* = 4.8 Hz), 2.76 (4H, t, *J* = 4.8 Hz), 1.42 (9H, s); ESI-HRMS calcd for $C_9H_{19}N_2O_2$: 187.1447, found: *m/z* 187.1446 $[M+H]^+$.

To a solution of **18** (200 mg, 0.26 mmol) in DMF (3 mL) were added the above-prepared *N*-Boc-piperazine (**19a**, 58 mg, 0.31 mmol), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 160 mg, 0.31 mmol) and Et_3N (0.054 mL, 0.39 mmol). The mixture was stirred for 3 h, and then concentration under reduced pressure. The residue was dissolved in EtOAc, and washed sequentially with 1 M HCl, saturated $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give the amide product **20a** (198 mg, 82%). $C_{42}H_{66}N_6O_{17}$; colorless solid, mp 115–117 °C; TLC (EtOAc) R_f = 0.32; $[\alpha]_D^{23}$ −3.9 (*c* 0.5, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 9.45 (1H, br s), 6.53 (1H, br s), 5.87 (1H, s), 5.64 (1H, br s), 5.40 (1H, d, *J* = 5.2 Hz), 5.29–5.26 (1H, m), 4.59 (1H, dd, *J* = 12.4, 2.0 Hz), 4.33–4.26 (2H, br s), 4.10 (1H, dd, *J* = 12.4, 7.2 Hz), 3.75 (3H, s), 3.55–3.53 (2H, m), 3.41–3.37 (6H, m), 3.09 (2H, br s), 2.33–2.20 (2H, m), 2.07 (3H, s), 2.04 (3H, s), 2.02 (3H, s), 1.86 (3H, s), 1.44 (27H, s); ^{13}C NMR (100 MHz, $CDCl_3$) 171.6, 170.4, 169.9, 169.8, 161.4, 160.0, 154.2, 153.6, 150.6, 145.7, 110.3, 81.5, 80.3, 79.1, 78.0, 77.2, 76.7, 71.0, 67.9, 62.2, 54.5, 52.5, 45.3, 44.9, 43.6, 41.5, 29.7, 29.4, 28.4 (6×), 28.2 (3×), 24.9, 23.2, 21.0, 20.9 (2×); ESI-HRMS calcd for $C_{42}H_{67}N_6O_{17}$: 927.4557, found: *m/z* 927.4562 $[M+H]^+$.

7.1.27. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-{*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-[3-(4-methylpiperazino)carbonylpropyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (20b)

By a procedure similar to that for **20a**, the coupling reaction of acid **18** (237 mg, 0.31 mmol) with 1-methylpiperazine (**19b**, 37 mg, 0.37 mmol) in the presence of PyBOP and Et_3N gave amide **20b** (190 mg, 73%). $C_{38}H_{60}N_6O_{15}$; colorless solid, mp 121–122 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 9.48 (1H, br s), 6.82 (1H, br s), 5.81 (1H, s), 5.61 (1H, br), 5.35 (1H, d, *J* = 5.2 Hz), 5.21–5.15 (1H, m), 4.53 (1H, d, *J* = 12.4 Hz), 4.28 (2H, br s), 4.04 (1H, dd, *J* = 12.4, 7.2 Hz), 3.69 (3H, s), 3.57–3.48 (2H, m), 3.38–3.29 (2H, m), 3.06–2.95 (2H, m), 2.82 (2H, br s), 2.32–2.28 (4H, m), 2.26–2.21 (5H, m), 2.01 (3H, s), 1.96 (6H, s), 1.79 (3H, s), 1.37 (18H, s); ESI-HRMS calcd for $C_{38}H_{61}N_6O_{15}$: 841.4195, found: *m/z* 841.4196 $[M+H]^+$.

7.1.28. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-{*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-[3-(morpholinocarbonyl)propyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (20c)

By a procedure similar to that for **20a**, the coupling reaction of acid **18** (185 mg, 0.24 mmol) with morpholine (**19c**, 25 mg, 0.29 mmol) in the presence of PyBOP and Et_3N gave amide **20c** (170 mg, 85%). $C_{37}H_{57}N_5O_{16}$; colorless solid, mp 133–134 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 9.45 (1H, br s), 6.51 (1H, br s), 5.87 (1H, s), 5.64 (1H, br s), 5.40 (1H, d, *J* = 5.2 Hz), 5.29–5.25 (1H, m), 4.60 (1H, dd, *J* = 12.4, 2.8 Hz), 4.33 (2H, br s), 4.10 (1H, dd, *J* = 12.4, 7.2 Hz), 3.75 (3H, s), 3.67–3.61 (4H, m), 3.58–3.56 (2H, m), 3.39–3.38 (2H, m), 3.31–3.07 (2H, m), 2.37–2.17 (4H, m), 2.07 (3H, s), 2.04 (3H, s), 2.02 (3H, s), 1.86 (3H, s), 1.44 (18H, s); ESI-HRMS calcd for $C_{37}H_{58}N_5O_{16}$: 828.3873, found: *m/z* 828.3870 $[M+H]^+$.

7.1.29. 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-{*N*′,*N*″-di(*tert*-butoxycarbonyl)-*N*-[3-(piperidinocarbonyl)propyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (20d)

By a procedure similar to that for **20a**, the coupling reaction of acid **18** (163 mg, 0.21 mmol) with piperidine (**19d**, 22 mg, 0.25 mmol) in the presence of PyBOP and Et_3N gave amide **20d** (154 mg, 89%). $C_{38}H_{59}N_5O_{15}$; TLC (EtOAc) R_f = 0.42; colorless solid, mp 116–117 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 9.65 (1H, br s), 6.57 (1H, br s), 5.87 (1H, s), 5.68 (1H, br s), 5.41 (1H, d, *J* = 5.2 Hz),

5.30–5.26 (1H, m), 4.59 (1H, dd, *J* = 12.4, 2.8 Hz), 4.33 (2H, br s), 4.11 (1H, dd, *J* = 12.4, 7.2 Hz), 3.75 (3H, s), 3.57–3.46 (2H, m), 3.32–3.30 (2H, m), 3.12–3.01 (2H, m), 2.25–2.16 (2H, m), 2.08 (3H, s), 2.04 (3H, s), 2.02 (3H, s), 1.87 (3H, s), 1.87–1.78 (2H, m), 1.64–1.60 (2H, m), 1.54–1.48 (4H, m), 1.44 (18H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 170.4, 169.8, 169.7, 161.4, 159.5, 153.4, 150.7, 145.5, 110.5, 81.2, 78.9, 78.2, 77.2, 71.0, 67.9, 62.2, 54.3, 52.5, 46.1, 45.3, 43.6, 42.8, 29.1, 28.3 (3×), 28.2 (3×), 26.3, 25.4, 24.5, 23.2 (2×), 21.0 (2×), 20.9; ESI-HRMS calcd for $C_{38}H_{60}N_5O_{15}$: 826.4080, found: *m/z* 826.4083 $[M+H]^+$.

7.1.30. 5-Acetamido-2,6-anhydro-4-[*N*-(3-piperazinocarbonyl)propyl]guanidino-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (8a)

By a procedure similar to that for **4a**, saponification of ester **20a** (76 mg, 0.082 mmol), followed by removal of the Boc groups with TFA, gave **8a** (51 mg, 88%). $C_{20}H_{34}N_6O_8{\cdot}2(CF_3CO_2H)$, colorless solid, mp 160–161 °C; $[\alpha]_D^{22}$ +26.6 (*c* 1.0 H_2O); ^{1}H NMR(400 MHz, D_2O) δ 5.74 (1H, s), 4.86 (1H, m), 4.41 (1H, d, *J* = 10.8 Hz), 4.36–4.32 (1H, m), 3.99–3.95 (1H, m), 3.92–3.79 (5H, m), 3.70–3.63 (2H, m), 3.35–3.24 (5H, m), 3.20–3.18 (1H, m), 2.54 (2H, m), 2.02 (4H, m), 1.92–1.89 (1H, m), ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1, 173.4, 167.5, 162.9 (CO_2 of TFA, q, *J* = 35.0 Hz), 157.6, 150.3, 116.5 (CF_3 of TFA, q, *J* = 289.6 Hz), 104.4, 75.9, 70.0, 68.2, 63.3, 57.5, 45.5, 43.5, 43.2 (2×), 42.4, 38.8, 29.2, 22.6, 22.3; ESI-HRMS calcd for $C_{20}H_{35}N_6O_8$: 487.2511, found: *m/z* 487.2510 $[M+H]^+$.

7.1.31. 5-Acetamido-2,6-anhydro-4-{*N*-[3-(4-methylpiperazinocarbonyl)propyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (8b)

By a procedure similar to that for **4a**, saponification of ester **20b** (63 mg, 0.075 mmol), followed by removal of the Boc groups with TFA, gave **8b** (49 mg, 91%). $C_{21}H_{36}N_6O_8{\cdot}2(CF_3CO_2H)$; colorless solid, mp 155–156 °C; $[\alpha]_D^{22}$ +43.3. (*c* 1.0 H_2O); ^{1}H NMR (400 MHz, D_2O) δ 5.96 (1H, s), 4.59 (1H, d, *J* = 11.6 Hz), 4.47 (1H, d, *J* = 10.8 Hz), 4.37 (1H, br s), 4.18 (1H, d, *J* = 14.4 Hz), 3.98–3.86 (2H, m), 3.73–3.51 (6H, m), 3.29–3.05 (5H, m), 2.96 (3H, s), 2.56–2.54 (2H, m), 2.06 (3H, s), 2.03 (1H, br s), 1.91 (1H, br s); ESI-HRMS calcd for $C_{21}H_{37}N_6O_8$: 501.2673, found: *m/z* 501.2671 $[M+H]^+$.

7.1.32. 5-Acetamido-2,6-anhydro-4-{*N*-[3-(morpholinocarbonyl)propyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (8c)

By a procedure similar to that for **4a**, saponification of ester **20c** (47 mg, 0.057 mmol), followed by removal of the Boc groups with TFA, gave **8c** (33 mg, 96%). $C_{20}H_{33}N_5O_9{\cdot}CF_3CO_2H$; colorless solid; mp 170–171 °C; $[\alpha]_D^{22}$ +11.9 (*c* 0.5 H_2O); ^{1}H NMR (400 MHz, D_2O) δ 5.85 (1H, s), 4.75–4.70 (1H, m), 4.32 (1H, d, *J* = 10.8 Hz), 4,23 (1H, br s), 3.84–3.80 (1H, m), 3.76 (1H, d, *J* = 12.4 Hz), 3.61–3.49 (6H, m), 3.45–3.41 (4H, m), 3.18–3.13 (1H, m), 3.05 (1H, br s), 2.41–2.36 (2H, m), 1.92–1.00 (1H, m), 1.89 (3H, s), 1.80–1.68 (1H, m); ESI-HRMS calcd for $C_{20}H_{34}N_5O_9$: 488.2351, found: *m/z* 488.2342 $[M+H]^+$.

7.1.33. 5-Acetamido-2,6-anhydro-4-{*N*-[3-(peperidinocarbonyl)propyl]guanidino}-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (8d)

By a procedure similar to that for **4a**, saponification of ester **20d** (73 mg, 0.088 mmol), followed by removal of the Boc groups with TFA, gave **8d** (49 mg, 93%). $C_{21}H_{35}N_5O_8{\cdot}CF_3CO_2H$; colorless solid; mp 152–153 °C; $[\alpha]_D^{22}$ +17.0 (*c* 1.0 H_2O); ^{1}H NMR (400 MHz, D_2O) δ 5.96 (1H, s), 4.78–4.75 (1H, m), 4.48 (1H, d, *J* = 10.8 Hz), 4.39–4.37 (1H, m), 4.00–3.95 (1H, m), 3.91 (1H, dd, *J* = 12.0, 2.4 Hz), 3.73–3.66 (2H, m), 3.56–3.49 (4H, m), 3.30–3.27 (1H, m), 3.20 (1H, br s), 2.51 (2H, br s), 2.04 (4H, s), 1.88 (1H, br s), 1.67–1.55 (6H, m); ESI-HRMS calcd for $C_{21}H_{36}N_5O_8$: 486.2558, found: *m/z* 486.2557 $[M+H]^+$.

7.2. Biology

7.2.1. Materials

Influenza A/WSN/1933 (H1N1) (from Dr. Shin-Ru Shih, Chang Gung University, Taiwan) was 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.

7.2.2. Determination of NA activity by a fluorescent assay

The neuraminidase activity was measured using diluted allantoic fluid harvest from influenza A/WSN/1933 (H1N1) 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) using excitation and emission wavelengths 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.

7.2.3. Determination of IC_{50} of NA inhibitor

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

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

The $TCID_{50}$ (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock 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 wells with 100 μL per well of CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega). After incubation at 37 °C for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus $TCID_{50}$ was determined using Reed–Muench method.[14]

7.2.5. Determination of EC_{50} of NA inhibitor

The anti-flu activities of neuraminidase inhibitors were measured by the EC_{50} values that were the concentrations of NA inhibitor for 50% protection of the H1N1 CPE activities. Fifty microliter diluted H1N1 at 100 $TCID_{50}$ were mixed with equal volumes of NA inhibitors at varied concentrations. The mixtures were used to infect 100 μL of previously seeded MDCK cells at 1×10^5 cells/mL in 96-wells. After 48 h incubation at 37 °C under 5% CO_2, the cytopathic effects (CPE) were determined with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent as described above. Inhibitor EC_{50} values were determined by fitting the curves of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

7.3. Computer modeling

The model of compound **8a** in complex with the NA was constructed through docking compound **8a** to the crystallographic

structure of N1 neuraminidase (PDB code 2HU0). The 3D structure of compound **8a** was build by modifying the 3D structure of zanamivir (PDB code 1NNC) with SYBYL 8.0 program (Tripos Associates). GOLD 4.0.1[15,16] was used to dock compound **8a** onto the protein with flexible docking option turned on. Kollmann-all atom charges[17] were assigned to the protein atoms, and Gasteiger–Hückel charges[18–20] were assigned to ligand atoms using the SYBYL 8.0 program. During the following docking procedure, the side chain structure of the Asp151 and Arg156 amino acid residues remained flexible, modeled with the built-in rotamer libraries of the GOLD 4.0.1 package. Initial 1000 independent genetic algorithm cycles of computation were carried out with ligand torsion angles varying between −180° and 180°. The search efficiency was set at 200% to ensure the most exhaustive search for the docking conformational space. All other parameters were kept the same as the default settings. The docking processes were distributed to a 40-processor Linux cluster with Intel(R) Xeon(TM) CPU 3.00 GHz CPUs. The resultant ligand–protein complex structures were ranked with the GOLDSCORE scoring function to determine the top 1000 hits.

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Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra of new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.010.

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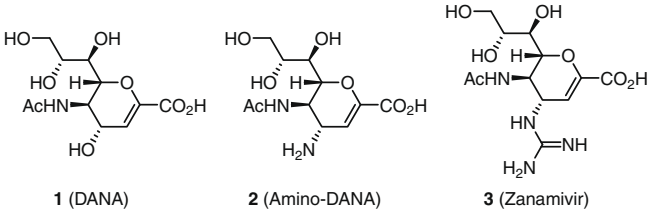
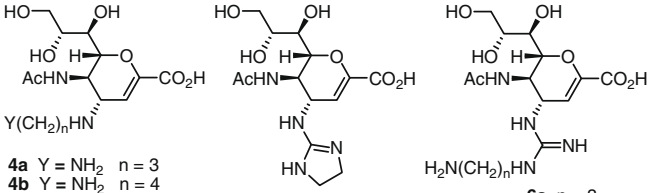


1 (DANA) **2** (Amino-DANA) **3** (Zanamivir)



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4c Y = CO_2H n = 3
4d Y = CO_2H n = 4

5

6a n = 3
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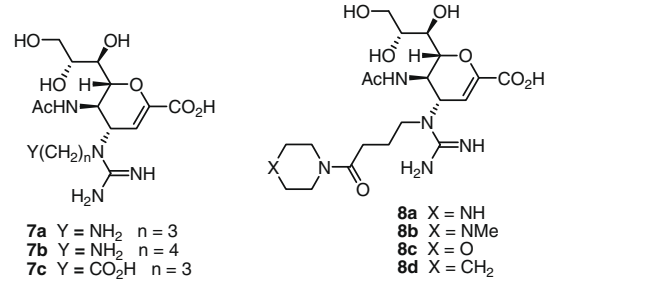

7a Y = NH_2 n = 3
7b Y = NH_2 n = 4
7c Y = CO_2H n = 3

8a X = NH
8b X = NMe
8c X = O
8d X = CH_2