Synthesis and Anti-influenza Evaluation of Polyvalent Sialidase Inhibitors Bearing 4-Guanidino-Neu5Ac2en Derivatives

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Polyvalent sialidase inhibitors bearing 4-guanidino-Neu5Ac2en derivatives on a poly-L-glutamine backbone are described. Aiming for a longer retention time of 4-guanidino-Neu5Ac2en (zanamivir) in bronchi and lungs, we focused on supermolecules bearing 4-guanidino-Neu5Ac2en derivatives bound at their C-7 position through noncleavable alkyl ether linkages. We first found that alkylation of the 7-hydroxyl group of sialic acid derivative 8 proceeded smoothly, and produced 7-O-alkyl-4-guanidino-Neu5Ac2en derivatives 13, which exhibited equipotent inhibitory activity against not only influenza A virus sialidase but also influenza A virus in the cell culture. Next, we synthesized poly-L-glutamine bearing 7-O-alkyl-4-guanidino-Neu5Ac2en derivatives linked by amide bonds, 26, which showed enhanced antiviral activity against influenza A virus and more potent efficacy in vivo relative to a monomeric sialidase inhibitor.

Key words anti-influenza drug; sialidase inhibitor; sialic acid; zanamivir; poly-L-glutamine

Influenza virus infection is known to cause substantial fatigue and sometimes death worldwide. Treatments were limited before the development of influenza sialidase (neuraminidase) inhibitors, which has lead to a major breakthrough in the control of influenza.1—3) The influenza sialidase is one of two viral coat glycoproteins critical for viral replication. It is an attractive target for antiviral intervention because its active site is antigenically conserved among all clinically relevant strains.4—6)

Zanamivir (4-guanidino-Neu5Ac2en)7—10) and Oseltamivir phosphate11,12) are two influenza sialidase inhibitors that have been approved for human use for the treatment of influenza infection (Fig. 1). They are transition state analogs of sialyl glycoside linkage hydrolysis. Oseltamivir phosphate, an ethyl ester prodrug of GS 4071, is administered orally, whereas Zanamivir is delivered by oral inhalation due to its poor oral bioavailability. They have been widely used as they are safe, and effective against both A and B strains, as well as emerging resistant strains.3)

In developing our sialidase inhibitor, administration by inhalation was preferred because direct delivery of the medicine to the site of viral replication in the respiratory tract assured the use of a much smaller dose and avoidance of systemic effects. However, zanamivir would require frequent administrations because it is rapidly eliminated in the unchanged form in the urine, and completely eliminated within 24 h, after a single dose.13) Therefore, a new class of sialidase inhibitors to be inhaled at lower and less frequent doses was sought. To circumvent the short retention time of zanamivir, we modified zanamivir so that it would not be released into the systemic circulation from the respiratory tract. We constructed a polymer carrying zanamivir equivalents on its sides attached by noncleavable linkages (Fig. 2).

In designing a polymer that carries zanamivir equivalents, the important point to consider was how to connect 4-guanidino-Neu5Ac2en molecules to the polymer backbone without a loss of inhibitory activity. From X-ray studies of the complex with sialidase and zanamivir reported by Varghese's...
group, the 7-hydroxyl group of zanamivir was shown to form no direct hydrogen bonds with sialidase. Moreover, as it was oriented outward of the enzyme active site, it was considered suitable for further elongation (Fig. 4). Based on this knowledge, we anticipated that the 7-hydroxyl group would help form a suitable link to the polymer.

In the course of our previous studies, we have investigated the synthesis and biological evaluation of analogues related to zanamivir modified at position-7 by using chemoenzymatic reactions. Although the replacement of the 7-OH moiety with N-amide groups showed a decrease in virus growth inhibitory activity, replacement with methoxy and ethoxy groups retained or improved the inhibitory activity against both influenza A virus sialidase and influenza A virus growth in cell culture (Fig. 3). Therefore, we were interested in polymers with 4-guanidino-7-O-alkyl-Neu5Ac2en analogues attached as multivalent influenza sialidase inhibitors.

Here, we describe the detailed synthesis, characterization, and biological evaluation of 7-O-alkyl ether analogues related to zanamivir and polymer-type sialidase inhibitors.

Chemistry

**Synthesis of 7-O-Alkyl Ether Derivatives Related to Zanamivir**

In fact, there were no reports on the direct chemical modification of the 7-hydroxyl group of sialic acid except by acylation. We first tried methylating the 7-hydroxyl group of dihydropyranyl intermediates 2, 4, and 6, which were derived from intermediate 1. Despite many attempts, including under basic and acidic conditions, we could not obtain 7-methoxy compounds 3, 5, and 7 (Chart 1). We thought the steric hindrance of the 7-hydroxyl group of the glycerol moiety might have been the cause.

Instead of using dihydropyranyl compounds as substrates, we chose tetrahydropyranyl derivative 9, which was reported to have been 7-O-acylated. We found that the alkylation reaction proceeded smoothly with sodium hydrogen and an alkyl halide in DMF solution. Instead of sodium hydrogen and alkyl halide, activation with potassium hydroxide or potassium tert-butoxide followed by addition of various dialkylsulfates also afforded 7-O-alkylated sialic acid derivatives 9. Successive methanolysis of 9 gave methyl ester 10.

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**Fig. 4.** Perspective View into the Active Site of the N9 Sialidase (Neuraminidase) Complexed with Zanamivir

Left: CPA Model, right: Tube Model.

**Chart 1.** 7-Modification of Neu5Ac2en Intermediates
Dialkylsulfate was readily prepared by Ru-oxidation of dialkylsulfite, which was obtained by reacting thionyl chloride and alcohol with triethylamine. \(^{30}\) 10 was converted to 7-O-alkylated zanamivir derivatives 13 using conventional methods via intermediates 4-azide compounds 11, and 4-guanidino compounds 12 (Chart 3). \(^{18-22}\)

To further develop compounds 13, we modified the terminal position of the alkyl groups to allow coupling with the polymer backbone (Chart 4). Treatment of 8 with potassium hydroxide followed by di(benzyloxethyl)- or di(benzyl-oxypentyl)-sulfate gave 14j and 14k, respectively. An ensuing reduction with Pd–C gave 7-O-hydroxyethyl ether 15j and 7-O-hydroxypentyl ether 15k, respectively. Treatment of 8 with sodium hydride followed by di[10-(tetrahydro-pyran-2-yloxy)-decyl]sulfate gave 16. 15j, 15k, and 16 were converted to 4-protected guanidine compounds 18 using conventional methods followed by deprotection which gave hydroxyalkyl ether 13j, 13k, and 13l, respectively (Chart 5). Methanalysis of 18 followed by acetonization gave 8,9-O-acetonide-7-O-hydroxyalkyl ether 19. The primary alcohol of 19 was tosylated and successive treatment of the resulting compound with sodium azide gave azide compounds 20. Deprotection of 20 gave azidealkyl ether derivatives 21 (Chart 6). Reaction of 20 with Lindlar catalyst gave amine compounds 22, and further deprotection of 22 gave aminoalkyl ether derivatives 23. Acetylation of the primary amine of 22 followed by deprotection gave acetylaminoalkyl ether derivatives 24 and 25 (Chart 7).

Treatment of 7-O-aminoethyl-4-guanidino-Neu5Ac2en 23j with excess 1-(acetoxy)benzotriazole \(^\text{23}\) and pyridine in water at room temperature for 2 h exclusively afforded acetylamino derivative 25j without any N-acylguanidine (Chart 8). We
Chart 5. Synthetic Scheme of 7-O-Hydroxyalkyl Ether Derivatives 13

Chart 6. Synthetic Scheme of 7-O-Azidealkyl Ether Derivatives 21

Chart 7. Synthetic Scheme of 7-Aminoalkyl Ether 23 and 7-O-Acetylaminoalkyl Ether Derivatives 26

Chart 8. Treatment of 23j with 1-(Acetoxy)benzotriazole and Pyridine in Water
found that selective acetylation of the aminoalkyl ether group was achieved. This result suggests that the primary amino group reacted readily perhaps due to its high flexibility. However, the guanidino group was slow to react probably due to intra- or intermolecular salt formation with carboxylic acid and steric hindrance. This selectivity is desirable for the condensation of 23 to form the polymer.

Synthesis of a Poly-\(\text{L}\)-glutamine Polymer Bearing Zanamivir Molecules As we were able to obtain 7-\(O\)-alkylamino-4-guanidino-Neu5Ac2en and subject it to selective acylation, we next focused on the synthesis of supermolecules bearing 4-guanidino-Neu5Ac2en derivatives.

We selected poly-\(\text{L}\)-glutamic acid as a polymer backbone precursor because poly-\(\text{L}\)-glutamic acid possesses some advantages as a drug carrier such as good biodegradability, high water solubility, the presence of multiple carboxyl groups that are easily modified chemically, low toxicity, and low immunogenicity.24)

Reaction of poly-\(\text{L}\)-glutamic acid (MW: 50000—100000, average MW: 71400, average extent of polymerization 5470) with benzotriazole ester and 0.1 or 0.3 eq of the 7-\(O\)-aminoalkyl-4-guanidino-Neu5Ac2en 23\(j\), 23\(k\), or 23\(l\) for 1 h followed by quenching with aqueous ammonia to convert unreacted activated esters to carbamoyl groups and purification by dialysis afforded poly-\(\text{L}\)-glutamine conjugate 26. The \(\text{H}\)-NMR spectrum of conjugate 26 showed the incorporation of approximately 10 or 25% ligand 23 onto the polymer backbone confirming that a complete reaction had occurred.

Biological Evaluation. Structure–Activity Relationship of 7-\(O\)-Alkylated Derivatives Related to Zanamivir The neuraminidase inhibitory activity and the 50% effective inhibitory concentration (IC\(_{50}\)) against influenza A are shown in Table 1.15) 13\(a\) (7-\(O\)-methyl ether), 13\(b\) (7-\(O\)-ethyl ether), 13\(c\) (7-\(O\)-propyl ether), 13\(d\) (7-\(O\)-n-propyl ether), 13\(e\) (7-\(O\)-n-octyl ether), and 13\(f\) (7-\(O\)-n-dodecyl ether), whose linear alkyl ether linkages was less than 12 carbons in length exhibited similar inhibitory activity to zanamivir against influenza A virus sialidase. These compounds showed pronounced improvement in inhibitory activity against influenza A virus infection of MDCK cell compared to zanamivir. However, 13\(g\) (7-\(O\)-n-tetradecyl ether) which has a linear linkage greater than 12 carbons in length showed a slight decrease in inhibitory activity against sialidase and virus infection, probably due to unfavorable steric and electrostatic interaction within the enzyme binding site. Substituting a cyclic aliphatic moiety, aromatic moiety, and hetero atom for the hydroxyl, azide, amino, and acetylamo group did not significantly affect the binding of compounds 13\(j\), 21\(j\), 23\(j\), and 25\(j\).

Structure–Activity Relationship of Poly-\(\text{L}\)-glutamine Bearing 4-Guanidino-Neu5Ac2en Derivatives The IC\(_{50}\) against influenza neuraminidase and influenza A of the synthesized polymers 26 are shown in Table 2.25) The IC\(_{50}\) values of each polymer relative to monomeric zanamivir were calcu-
strated that all the polymers were much more active than monomeric sialidase inhibitor 25j, 25k and zanamivir, regardless of their content of 4-guanidino-Neu5Ac2en molecules and length of the carbon chain. As a control for the biological evaluation, unsubstituted poly-L-glutamic acid was used and showed no inhibitory activity. This enhancement could be explained by the contribution of multivalence or cluster effects.26—28

Efficacy of Intranasally Administered Polymeric Sialidase Inhibitor 26c Tested in the Influenza Virus-Infected Mouse Model Furthermore, the efficacy of intranasally administered polymeric sialidase inhibitor 26c (average MW: 78100) was tested in the influenza virus-infected mouse model in terms of the survival rate of treated and infected mice relative to that of control mice. Compound 26c was administered intranasally once beginning 24 h prior to infection. It was found that 26c was much more effective than zanamivir as shown in Table 3 (7/7 survived in the 26c-treated infected group while there were no survivors in the case of zanamivir). This in vivo efficacy is the first such result for polymeric sialidase inhibitors. No in vitro cytotoxicity or in vivo toxicity in mouse was observed.

In conclusion, we synthesized a series of 7-O-alkyl ether derivatives related to zanamivir by direct alkylation of the C-7 alcohol of a sialic acid derivative. Alkyl ether moiety less than 12 carbons in length showed similar activities against influenza A virus sialidase. These compounds showed improved virus growth inhibitory activity compared to zanamivir. We also synthesized poly-L-glutamines carrying 4-guanidino-Neu5Ac2en bound via alkyl ether bonds at the C-7 position. All polymers displayed less potent influenza A sialidase inhibitory activity. However, a much greater efficacy against influenza A in the mice model by intranasal administration than zanamivir was observed.

Table 2. Sialidase Inhibitory and Plaque Reduction Activities of Compounds 26 (IC50 (nv))

<table>
<thead>
<tr>
<th></th>
<th>Sialidase inhibitory</th>
<th>Plaque reduction assay</th>
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<tbody>
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<td></td>
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<tr>
<td></td>
<td>assay</td>
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<tr>
<td>Zanamivir</td>
<td>11—29 (1.0)x3</td>
<td>1.3—27 (1.0)y3</td>
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</tr>
<tr>
<td>25j</td>
<td>1</td>
<td>9.8 (1.4)</td>
<td>2.4 (0.2)</td>
<td></td>
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<tr>
<td>25k</td>
<td>4</td>
<td>90 (6.0)</td>
<td>5.9 (1.3)</td>
<td></td>
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<tr>
<td>26a</td>
<td>1</td>
<td>114 (5.2)</td>
<td>0.066 (0.077)</td>
<td></td>
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<tr>
<td>26b</td>
<td>1</td>
<td>364 (22.8)</td>
<td>0.17 (0.020)</td>
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<tr>
<td>26c</td>
<td>4</td>
<td>161 (7.3)</td>
<td>0.072 (0.0085)</td>
<td></td>
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<tr>
<td>26d</td>
<td>9</td>
<td>62 (2.1)</td>
<td>0.076 (0.023)</td>
<td></td>
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</table>

a) Relative to the monomeric sialidase inhibitor content. b) The ratios were determined by 1H-NMR. c) Since IC50 values varied depending on the experiment, the relative potencies of the compounds to zanamivir are shown in parentheses based on the IC50 values. The IC50 values of zanamivir in enzyme inhibition and plaque reduction were 11—29 nm and 1.3—27 nm, respectively.

Table 3. Survival Rates of Infected Mice17 Administered Compound 26c and Zanamivir17

<table>
<thead>
<tr>
<th></th>
<th>No. of survivors/Total No. of mice</th>
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<tr>
<td>10 d after infection</td>
<td>Zanamivir 26c</td>
</tr>
<tr>
<td>20 d after infection</td>
<td></td>
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</tbody>
</table>

a) Mice were infected with influenza A/PR/8/34 (H1N1) virus. b) Compound 26c and zanamivir were administered intranasally at doses of 0.3 mmol/kg once beginning 24 h prior to infection. The concentration of 0.3 mmol of 26c was calculated based on the molar concentration of the monomeric sialidase inhibitor. c) p = 0.0009 versus zanamivir (Log-rank test).

C-7 position. All polymers displayed less potent influenza A sialidase inhibitory activity. However, a much greater efficacy against influenza A in the mice model by intranasal administration than zanamivir was observed.

Experimental

General Methods

IR spectra were recorded on a Jasco FT-IR 8300 or 8900 spectrometer. NMR spectra were recorded on a JEOL EX 270 (270 MHz) or GSX-400 (400 MHz) spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JEOL JX-100, SX-102A or AX-505H mass spectrometer. The melting point (mp) was determined using a Yanagimoto micro-melting point apparatus and was not corrected. Optical rotations were obtained with a Jasco DIP-370 polarimeter. Column chromatography was carried out on Silica gel 60 (230—400 mesh, Art. 9385, Merck). The analytical column for HPLC was an L-Column ODS (5 mm particle size, 4.6 mm i.d.×150 mm).

5-Acetylamino-3,5-dideoxy-2,7-O-dimethyl-8,9-O-isopropylidene-4-O-tetrahydrobutylmethylsilyl-α-glycero-β-galacto-2-nonulo-pyranosonic Acid Methyl Ester 9a A solution of 5-acetylamino-3,5-dideoxy-8,9-O-isopropylidene-2-O-methyl-4-O-tetrahydrobutylmethylsilyl-α-glycero-β-galacto-2-nonulo-pyranosonic acid methyl ester 8 (1.0 g, 2.03 mmol) in acetonitrile (20 ml) was cooled in an ice bath and slowly mixed with potassium hydroxide (0.67 g, 10.17 mmol). The reaction mixture was stirred at room temperature for 1 h. Then, dimethyl sulfate (0.96 ml, 10.17 mmol) was added dropwise with cooling in an ice bath. The reaction mixture was stirred at room temperature for 1 h, diluted with aqueous ammonium hydroxide and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure.

The crude product was purified by silica gel chromatography (eluent: CH2Cl2: MeOH = 5:1) to obtain 9a as an amorphous solid (510 mg, 1.01 mmol, 50%), mp 160—161 °C. 1H-NMR (CDCl3) δ: 0.05 (3H, s), 0.06 (3H, s), 0.86 (9H, s), 1.33 (3H, s), 1.42 (3H, s), 1.73 (1H, dd, J = 13.1, 10.7 Hz), 1.99 (3H, s), 2.28 (1H, dd, J = 13.1, 5.1 Hz), 3.22 (3H, s), 3.48—3.56 (1H, m), 3.57 (3H, s), 3.78 (3H, s), 3.80—4.30 (6H, m), 5.16 (1H, d, J = 8.2 Hz). FAB-MS m/z: 506 (M+H)+. HR-FAB-MS m/z: Caled for C35H40O11N1a (M+Na)+: 528.2609. Found: 528.2605. General Methods

11a 9a 10a 10b 10c 10d

<table>
<thead>
<tr>
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<th>No. of survivors/Total No. of mice</th>
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<td>10 d after infection</td>
<td>Zanamivir 26c</td>
</tr>
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<td>20 d after infection</td>
<td></td>
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a) Mice were infected with influenza A/PR/8/34 (H1N1) virus. b) Compound 26c and zanamivir were administered intranasally at doses of 0.3 mmol/kg once beginning 24 h prior to infection. The concentration of 0.3 mmol of 26c was calculated based on the molar concentration of the monomeric sialidase inhibitor. c) p = 0.0009 versus zanamivir (Log-rank test).
ride and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (elu-
tant: hexane:ethyl acetate=2: 1) to obtain 12a as a colorless amorphous solid (4.42 g, 6.86 mmol, 76%). 3-H-NMR (CDCl3): δ: 1.49 (9H, s), 1.50 (9H, s), 1.97 (3H, s), 2.05 (3H, s), 2.07 (3H, s), 3.52 (3H, s), 3.54—3.56 (1H, m), 3.76 (3H, s), 4.10 (1H, dd, J=10.5, 10.0 Hz), 4.25—4.40 (2H, m), 4.82 (1H, dd, J=12.0, 3.2 Hz), 5.12 (1H, dd, J=11.0, 11.0, 2.5 Hz), 5.29—5.31 (1H, m), 5.85 (1H, d, J=2.3 Hz), 6.35 (1H, d, J=8.8 Hz). FAB-MS m/z: 645 (M+H).

(4S,5R,6'R,1'R,2'R,2'R)-5-Acetylamino-5,6-dihydro-6-(2',3'-diacetoxy-1'-methoxy)propyl-4-guanidino-4H-pyran-2-carboxylic Acid 13a 12a (53 mg, 0.082 mmol) was treated with methylene chloride (3 ml), and trijfluoroacetic acid (30 ml) and the reaction mixture was stirred at room tempera-
ture for 1 h. The organic layer was washed with aqueous sodium hydroxide (1%, 0.3 ml, 0.3 mmol) with cooling in an ice bath. The reaction was stirred for 30 min and neutralized with Dowex 50W X8 (H+). Then, the reaction was filtered and the residue was thoroughly washed with methanol. The filtrate was combined, and the mixture was evaporated under reduced pressure. The crude product was purified by reverse phase cosmolig chromatography (elu-ntant: water:MeOH=1: 1) to obtain 13a as a colorless amorphous solid (0.0693 ml, 77%). 3-H-NMR (CDCl3): δ: 3.37 (3H, s), 3.55 (1H, d, J=8.5 Hz), 3.65 (1H, dd, J=5.0, 12.0 Hz), 3.80 (1H, dd, J=2.6, 12.0 Hz), 3.90 (1H, m), 4.20 (1H, dd, J=10.0, 10.0 Hz), 4.40—4.50 (2H, m), 5.85 (1H, d, J=1.8 Hz). FAB-MS m/z: 347 (M+H)

5-Acetylamino-3,5-dideoxy-7-0-ethyl-8,9-0-isopropylidene-2,0-
methyl-1-o-tart-butylidemethylsilylo-glycerol-o-galacto-2-nonulo-pyra-
sonic Acid Methyl Ester 10b 8 (6.0 g, 12.2 mmol) in acetonitrile (30 ml) was treated with sodium carbonate (3.50 g, 32.5 mmol) at 0°C, and stirred at room temperature for 1 h.

NMR (CDCl3) δ: 0.89—0.99 (6H, m), 1.55—1.80 (4H, m), 2.04 (3H, s), 2.05 (3H, s), 2.09 (3H, s), 3.40—3.50 (1H, m), 3.56—3.65 (1H, m), 3.70—3.77 (1H, m), 3.92—4.02 (1H, m), 4.29 (1H, dd, J=11.9, 7.3 Hz), 4.45—4.56 (2H, m), 4.63—4.71 (1H, m), 5.30—5.39 (1H, m), 5.68—5.73 (1H, m), 9.98 (1H, s, J=3.3 Hz). FAB-MS m/z: 485 (M+H).

(4S,5R,6'R,1'R,2'R,2'R)-5-Acetylamino-4-(bis-N',N'-tert-butylxyloxy carbonyl)
guanidino-6-0,2'-3',4'-diacetoxy-1'-pentoyloxy)propyl-5,6-dihydro-4H-
pyran-2-carboxylic Acid Methyl Ester 12b 12b was obtained from 10b (180 mg, 0.21 mmol) using the same procedure employed for the preparation of 11a (colorless amorphous solid, 110 g, 2.27 mmol, 71%). 3-H-NMR (CDCl3): δ: 0.89—0.99 (6H, m), 1.55—1.80 (4H, m), 2.04 (3H, s), 2.05 (3H, s), 2.09 (3H, s), 3.40—3.50 (1H, m), 3.56—3.65 (1H, m), 3.70—3.77 (1H, m), 3.92—4.02 (1H, m), 4.29 (1H, dd, J=11.9, 7.3 Hz), 4.45—4.56 (2H, m), 4.63—4.71 (1H, m), 5.30—5.39 (1H, m), 5.68—5.73 (1H, m), 9.98 (1H, s, J=3.3 Hz). FAB-MS m/z: 485 (M+H).

(4S,5R,6'R,1'R,2'R,2'R)-5-Acetylamino-4-(bis-N',N'-tert-butylxyloxy carbonyl)
guanidino-6-0,2'-3',4'-diacetoxy-1'-pentoyloxy)propyl-5,6-dihydro-4H-
pyran-2-carboxylic Acid Methyl Ester 12c Compound 11c was obtained from 10c (180 mg, 0.21 mmol) using the same procedure employed for the preparation of 11a (colorless amorphous solid, 110 g, 2.27 mmol, 71%). 3-H-NMR (CDCl3): δ: 0.90—0.94 (3H, m), 1.30—1.38 (4H, m), 1.56—1.64 (2H, m), 2.06 (3H, s), 2.07 (3H, s), 2.10 (3H, s), 3.47—3.52 (1H, m), 3.64—3.70 (1H, m), 3.76 (1H, d, J=4.3, 3.5 Hz), 3.82 (3H, s), 3.96 (1H, dd, J=8.1, 8.1 Hz), 4.23 (1H, dd, J=12.3, 7.1 Hz), 4.49 (1H, dd, J=8.1, 3.0 Hz), 4.55 (1H, dd, J=8.8, 3.5 Hz), 4.73 (1H, dd, J=12.3, 3.0 Hz), 5.36 (1H, dd, J=7.1, 4.3, 3.0 Hz), 5.69 (1H, brd, J=8.1 Hz), 6.00 (1H, dd, J=3.0 Hz). FAB-MS m/z: 485 (M+H).
Compound 9f was obtained from 11f (64 mg, 0.110 mmol) using the same procedure employed for the preparation of 11d (colorless amorphous solid, 10 mg, 16.3 mg, 54%).

1H-NMR (CDCl3): δ 0.87—0.92 (3H, m), 1.25—1.35 (18H, m), 1.50—1.64 (2H, m), 2.04 (3H, s), 2.09 (3H, s), 3.50—3.62 (2H, m), 3.70 (1H, dd, J = 10.6, 4.0 Hz), 3.78 (3H, s), 3.92—3.98 (1H, m), 4.10 (1H, dd, J = 10.4, 1.6 Hz), 4.26 (1H, dd, J = 12.5, 7.6 Hz), 4.83 (1H, dd, J = 12.5, 2.4 Hz), 5.09—5.15 (1H, m), 5.29 (1H, ddd, J = 7.6, 4.0, 1.6 Hz), 5.38 (1H, d, J = 2.4 Hz), 6.41 (1H, br, J = 9.1 Hz), 8.53 (1H, br, J = 8.7 Hz). FAB-MS m/z: 743 (M+H)+.

5-Acetamido-3,5-dideoxy-8,9-O-isopropylidene-2-O-methyl-7-0-nortetradecyloxy-3-0-tetartbutylmethylsilyl-o-glycero-o-galacto-2-nonulo-pyranosonic Acid 11g was obtained from 11f (24 mg, 30.2 mmol) using the same procedure employed for the preparation of 11d (colorless amorphous solid, 40 mg, 0.311 mmol, 27%).

1H-NMR (CDCl3): δ 0.87—0.92 (3H, m), 1.25—1.35 (18H, m), 1.50—1.64 (2H, m), 2.04 (3H, s), 2.09 (3H, s), 3.50—3.62 (2H, m), 3.70 (1H, dd, J = 10.6, 4.0 Hz), 3.78 (3H, s), 3.92—3.98 (1H, m), 4.10 (1H, dd, J = 10.4, 1.6 Hz), 4.26 (1H, dd, J = 12.5, 7.6 Hz), 4.83 (1H, dd, J = 12.5, 2.4 Hz), 5.09—5.15 (1H, m), 5.29 (1H, ddd, J = 7.6, 4.0, 1.6 Hz), 5.38 (1H, d, J = 2.4 Hz), 6.41 (1H, br, J = 9.1 Hz), 8.53 (1H, br, J = 8.7 Hz). FAB-MS m/z: 743 (M+H)+.

5-Acetamido-3,5-dideoxy-8,9-O-isopropylidene-2-O-methyl-7-0-nortetradecyloxy-3-0-tetartbutylmethylsilyl-o-glycero-o-galacto-2-nonulo-pyranosonic Acid 11h was obtained from 11f (24 mg, 30.2 mmol) using the same procedure employed for the preparation of 11d (colorless amorphous solid, 40 mg, 0.311 mmol, 27%).

1H-NMR (CDCl3): δ 0.87—0.92 (3H, m), 1.25—1.35 (18H, m), 1.50—1.64 (2H, m), 2.04 (3H, s), 2.09 (3H, s), 3.50—3.62 (2H, m), 3.70 (1H, dd, J = 10.6, 4.0 Hz), 3.78 (3H, s), 3.92—3.98 (1H, m), 4.10 (1H, dd, J = 10.4, 1.6 Hz), 4.26 (1H, dd, J = 12.5, 7.6 Hz), 4.83 (1H, dd, J = 12.5, 2.4 Hz), 5.09—5.15 (1H, m), 5.29 (1H, ddd, J = 7.6, 4.0, 1.6 Hz), 5.38 (1H, d, J = 2.4 Hz), 6.41 (1H, br, J = 9.1 Hz), 8.53 (1H, br, J = 8.7 Hz). FAB-MS m/z: 743 (M+H)+.

5-Acetamido-3,5-dideoxy-8,9-O-isopropylidene-2-O-methyl-7-0-nortetradecyloxy-3-0-tetartbutylmethylsilyl-o-glycero-o-galacto-2-nonulo-pyranosonic Acid 11i was obtained from 11f (24 mg, 30.2 mmol) using the same procedure employed for the preparation of 11d (colorless amorphous solid, 40 mg, 0.311 mmol, 27%).

1H-NMR (CDCl3): δ 0.87—0.92 (3H, m), 1.25—1.35 (18H, m), 1.50—1.64 (2H, m), 2.04 (3H, s), 2.09 (3H, s), 3.50—3.62 (2H, m), 3.70 (1H, dd, J = 10.6, 4.0 Hz), 3.78 (3H, s), 3.92—3.98 (1H, m), 4.10 (1H, dd, J = 10.4, 1.6 Hz), 4.26 (1H, dd, J = 12.5, 7.6 Hz), 4.83 (1H, dd, J = 12.5, 2.4 Hz), 5.09—5.15 (1H, m), 5.29 (1H, ddd, J = 7.6, 4.0, 1.6 Hz), 5.38 (1H, d, J = 2.4 Hz), 6.41 (1H, br, J = 9.1 Hz), 8.53 (1H, br, J = 8.7 Hz). FAB-MS m/z: 743 (M+H)+.
0.86 (9H, s), 0.90—1.04 (4H, m), 1.12—1.30 (6H, m), 1.31 (3H, s), 1.41 (3H, s), 1.58—1.80 (13H, m). 1.97 (3H, s), 2.25 (1H, dd, J = 13.1, 5.1 Hz), 3.22 (3H, s), 3.40—3.51 (2H, m), 3.59—3.62 (1H, m), 3.80—3.86 (1H, m), 3.87—3.91 (1H, m), 3.97—4.05 (3H, m), 4.08—4.15 (2H, m), 4.22—4.26 (1H, m), 5.15 (1H, d, J = 9.0 Hz). FAB-MS m/z: 670 (M + H)

pyran-2-carboxylic Acid Cyclohexylmethyl Ester 11h Compound 11h was obtained from 9h (1.0 g, 1.19 mmol) using the same procedure employed for the preparation of 11d (colorless amorphous solid, 232 mg, 0.286 mmol, 70%). 1H-NMR (CDCl3): δ: 0.90—1.03 (4H, m), 1.10—1.32 (6H, m), 1.49 (9H, s), 1.50 (9H, s), 1.56—1.76 (12H, m), 1.94 (3H, s), 2.05 (3H, s), 2.08 (3H, s), 3.35—3.42 (2H, m), 3.69 (1H, dd, J = 3.6, 1.3 Hz), 3.95 (1H, dd, J = 10.7, 6.7 Hz), 4.03 (1H, dd, J = 10.7, 6.7 Hz), 4.07 (1H, dd, J = 10.3, 1.3 Hz), 4.24—4.32 (1H, m), 4.36 (1H, dd, J = 12.4, 7.9 Hz), 4.75 (1H, dd, J = 12.4, 2.4 Hz), 5.06—5.13 (1H, m), 5.27 (1H, dd, J = 7.9, 3.6, 2.4 Hz), 5.79 (1H, d, J = 2.3 Hz), 6.80 (1H, br d, J = 7.8 Hz), 8.53 (1H, d, J = 3.0 Hz). FAB-MS m/z: 593 (M + H+)

(4S,5R,6R,1′,2′,5′,9′,10′,11′)-5-Acetylamino-4-(bis-N,N′-tert-butyl oxy carbonyl)guanidine-6(1H)-cyclohexylmethoxy-2′,3′-dideoxy-6-[[2-(5-butyldimethylsilyl)-D-
pyran-2-carboxylic Acid Cyclohexylmethyl Ester 12h Compound 12h was obtained from 11h (243 mg, 0.410 mmol) using the same procedure employed for the preparation of 12a (colorless amorphous solid, 232 mg, 0.286 mmol, 70%). 1H-NMR (CDCl3): δ: 0.90—1.03 (4H, m), 1.10—1.32 (6H, m), 1.49 (9H, s), 1.50 (9H, s), 1.56—1.76 (12H, m), 1.94 (3H, s), 2.05 (3H, s), 2.08 (3H, s), 3.35—3.42 (2H, m), 3.69 (1H, dd, J = 3.6, 1.3 Hz), 3.95 (1H, dd, J = 10.7, 6.7 Hz), 4.03 (1H, dd, J = 10.7, 6.7 Hz), 4.07 (1H, dd, J = 10.3, 1.3 Hz), 4.24—4.32 (1H, m), 4.36 (1H, dd, J = 12.4, 7.9 Hz), 4.75 (1H, dd, J = 12.4, 2.4 Hz), 5.06—5.13 (1H, m), 5.27 (1H, dd, J = 7.9, 3.6, 2.4 Hz), 5.79 (1H, d, J = 2.3 Hz), 6.80 (1H, br d, J = 7.8 Hz), 8.53 (1H, d, J = 3.0 Hz). FAB-MS m/z: 593 (M + H+)

A procedure similar to that used for the preparation of compound 9a was employed for the preparation of the amorphous solid, 132 mg, 0.139 mmol) using the procedure employed for the preparation of 13a (colorless amorphous solid, 232 mg, 0.286 mmol, 70%). 1H-NMR (CDCl3): δ: 0.88—1.13 (2H, m), 1.10—1.32 (3H, m), 1.47—1.81 (6H, m), 1.99 (3H, s), 3.24—3.38 (2H, m), 3.54 (1H, dd, J = 8.3, 1.3 Hz), 3.63 (1H, dd, J = 11.3, 4.6 Hz), 3.81 (1H, dd, J = 11.3, 3.5 Hz), 3.89—4.05 (2H, m), 4.25—4.35 (1H, m), 5.68—5.70 (1H, m). FAB-MS m/z: 429 (M + H+)

5-Acetylamino-3,5-dideoxy-8,9-O-isopropylidene-2-hexyl-1H-pyrano[3,2-c]pyrene-4-0-phosphoryl-1,2-dihydro-4H-pyran-2-carboxylic Acid Phosphonyl Ester 9i A procedure similar to that used for the preparation of compound 10a was employed for the preparation of 10i (20.2 g, 37.7 mmol) using the same procedure employed for the preparation of the amorphous solid, 18.7 g, 26.1 mmol, 88%). 1H-NMR (CDCl3): δ: 0.48 (9H, s), 1.49 (9H, s), 1.95 (3H, s), 2.06 (3H, s), 2.10 (6H, s), 3.72—3.77 (1H, m), 3.73 (8H, s), 3.80—3.87 (2H, m), 4.09 (1H, dd, J = 11.0, 2.8 Hz), 4.22—4.28 (2H, m), 4.39—4.40 (1H, m), 4.84 (1H, dd, J = 12.6, 2.4 Hz), 5.07—5.17 (1H, m), 5.27—5.33 (1H, m), 5.83 (1H, d, J = 2.4 Hz), 6.63 (1H, brd, J = 9.0 Hz), 8.55 (1H, brd, J = 8.8 Hz). IR (CDCl3): νmax 2143, 2425, 2955, 2910, 1740, 1681, 1602, 1510, 1439 cm−1. MS (FAB) m/z 717 (M + H+) HS-FAB MS (FAB) for C14H15O7N4: 717.235. FAB-MS (FAB) for C14H15O7N4: 717.3215. FAB-MS (FAB) for C14H15O7N4: 717.3202 (M + H+).
(5R,5R,6R,1’5’2’S)-6’-[1’-(10’-Acetoxy)deoxy]-2’,3’-diacetoxy-5’-propyl-5-acetylamino-4-(bis-N’-tert-butyloxycarbonyl)guanidino-5,6-dihydro-4H-pyran-2-carboxylic Acid Methyl Ester 18j

A solution of 18j (18.7 g, 26.1 mmol) in methanol (200 ml) was cooled in an ice bath and neutralized with Dowex 50WX 8+ (H+). The reaction mixture was filtered and the precipitate was washed with methanol. The filtrate was evaporated and the residue was dissolved in acetone (200 ml). To the solution, dimethoxypropane (40 ml) and p-toluenesulfonic acid monohydrate (500 mg, 2.62 mmol) were added and the mixture was stirred for 2 h. The reaction mixture was combined with sodium hydroxide (50 %) with a solution of an ice bath. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was filtered, the precipitate was washed with ethyl acetate, and the filtrate was evaporated. The residue was dissolved in ethyl acetate (200 ml) and mixed with aqueous hydrochloride (1 x, 100 ml). The reaction mixture was stirred for 5 min, and then extracted with ethyl acetate. The organic layer was washed with aqueous sodium carbonate and sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (elucent: ethyl acetate) to give compound 19j (12.1 g, 19.1 mmol, 73 %). The reaction mixture was stirred at room temperature for 2 h with ethyl acetate. The organic layer was washed with 1 x aqueous hydrochloride, aqueous sodium hydrogen carbonate and sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (elucent: ethyl acetate) to give compound 19j (12.1 g, 19.1 mmol, 73 %). The reaction mixture was stirred at room temperature for 2 h with ethyl acetate. The organic layer was washed with 1 x aqueous hydrochloride, aqueous sodium hydrogen carbonate and sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (elucent: ethyl acetate) to give compound 19j (12.1 g, 19.2 mmol) was dissolved in anhydrous pyridine (150 ml), cooled in an ice bath, and mixed with p-toluenesulfonic acid monohydrate (7.32 g, 38.4 mmol). The reaction mixture was stirred for 2 h at room temperature, then the reaction mixture was diluted with ethyl acetate and filtered through a short pad of sodium carbonate, and concentrated under reduced pressure. The residue was dissolved in N,N-dimethylformamide (100 ml) and mixed with sodium azide (2.49 g, 38.4 mmol). The reaction mixture was stirred for 5 h at 50 °C. Then, the reaction mixture was diluted with ethyl acetate, and the ethyl acetate was concentrated under reduced pressure. The residue was purified by silica gel chromatography (elucent: ethyl acetate/ethyl acetate to give compound 19j (10.7 g, 16.3 mmol, 85 %). The reaction mixture was stirred at room temperature for 2 h with ethyl acetate. The organic layer was washed with 1 x aqueous hydrochloride, aqueous sodium hydrogen carbonate and sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (elucent: ethyl acetate/ethyl acetate to give compound 19j (10.7 g, 16.3 mmol, 85 %).
\[ J = 8.6 \text{ Hz} \]. MS (FAB) m/z 656 (M+·).

(4S,SR,6R,1'R,2'R,5)-5-Acetamino-6-[[2\'-azidoethoxy]-2\'-3\'-dihydroxypropyl]-5,6-dihydro-4-quinidine-4H-pyran-2-carboxylic Acid 21j Compound 21j was obtained from 20j (110 mg, 0.168 mmol) using the same procedure employed for the preparation of 13a (colorless amorphous solid, 46 mg, 0.114 mmol, 68%). 'H-NMR (400 MHz, CDCl₃) \( \delta = 1.99 \) (3H, s), 3.33—5.92 (2H, m), 3.55—3.80 (4H, m), 3.86 (1H, dd, \( J = 11.9 \), 2.6 Hz), 3.92—4.01 (1H, m), 4.17—4.27 (1H, m), 4.33—4.42 (2H, m), 5.57 (1H, d, \( J = 2.1 \) Hz). MS (FAB) m/z 401 (M+·).

(4S,SR,6R,1'S,4'R,5)-5-Acetamino-4-(bis-N\'-tert-butylxycarbonyl)-guanidine-6-[[2\'-amino-ethoxy]-2\'-2\'-dimethyl-[1\'-3\']dioxolan-4\'-yl]methyl-5,6-dihydro-4H-pyran-2-carboxylic Acid Methyl Estor 21k Compound 20k was obtained from 19k (10.3 g, 1.53 mmol) using the same procedure as that for the preparation of 20j (colorless amorphous solid, 46 mg, 0.114 mmol, 68%). 'H-NMR (400 MHz, CDCl₃) \( \delta = 1.36 \) (3H, s), 1.43 (3H, s), 1.49 (9H, s), 1.50—1.60 (2H, m), 1.65—1.75 (4H, m), 1.92 (3H, s), 3.49—3.75 (4H, m), 3.77 (3H, s), 3.75—3.81 (1H, m), 4.00—
4.08 (1H, m), 4.10—4.32 (4H, m), 5.12—5.21 (1H, m), 5.85 (1H, d, \( J = 2.3 \) Hz), 6.63 (1H, br, \( J = 8.3 \) Hz). 8.41 (1H, br, \( J = 8.7 \) Hz). MS (FAB) m/z 673 (M+·).

HR-MS (FAB) Calcd for C₉₆H₁₃₂O₂₈N₇Na: 1398.8291; Found 1398.8290 (M+1·).

(4S,SR,6R,1'S,4'R,5)-5-Acetamino-4-(bis-N\'-tert-butylxycarbonyl)-guanidine-6-[[2\'-amino-ethoxy]-2\'-2\'-dimethyl-[1\'-3\']dioxolan-4\'-yl]methyl-5,6-dihydro-4H-pyran-2-carboxylic Acid Methyl Estor 22k Compound 22k was obtained from 20k (20.1 g, 2.88 mmol) using the same procedure employed for the preparation of 20j (colorless amorphous solid, 19.4 g, 2.88 mmol, quant.). 'H-NMR (400 MHz, CDCl₃) \( \delta = 1.33 \) (3H, s), 1.40 (3H, s), 1.46 (9H, s), 1.53 (9H, s), 1.94 (3H, s), 2.72 (2H, t, \( J = 11.9 \) Hz), 3.06—3.18 (4H, m), 3.29—3.41 (1H, m), 3.59—3.67 (1H, m), 3.88—4.00 (1H, m), 4.10—4.22 (1H, m), 4.30—4.42 (2H, m), 5.52—5.58 (1H, d, \( J = 2.5 \) Hz). 6.05 (1H, br, \( J = 8.8 \) Hz). 8.51 (1H, br, \( J = 8.7 \) Hz). MS (FAB) m/z 672 (M+·).

HR-MS (FAB) Calcd for C₉₆H₁₃₂O₂₈N₇: 1400.8273; Found 1401.8226 (M+1·).

(4S,SR,6R,1'S,4'R,5)-5-Acetamino-4-(bis-N\'-tert-butylxycarbonyl)-guanidine-6-[[2\'-amino-ethoxy]-2\'-2\'-dimethyl-[1\'-3\']dioxolan-4\'-yl]methyl-5,6-dihydro-4H-pyran-2-carboxylic Acid Methyl Estor 23k Compound 23k was obtained from 22k (1.00 g, 1.49 mmol) using the same procedure employed for the preparation of 13a (colorless amorphous solid, 430 mg, 0.890 mmol, 54%). 'H-NMR (400 MHz, CDCl₃) \( \delta = 1.33 \) (3H, s), 1.40 (3H, s), 1.46 (9H, s), 1.53 (9H, s), 1.94 (3H, s), 2.60—2.67 (1H, m), 3.40—3.50 (1H, m), 3.73—3.83 (2H, m), 3.78 (3H, s), 4.02—4.09 (2H, m), 4.13—4.35 (3H, m), 4.90—5.02 (1H, m), 5.86 (1H, d, \( J = 2.4 \) Hz), 6.14 (1H, br, \( J = 8.6 \) Hz). 8.51 (1H, br, \( J = 8.7 \) Hz). MS (FAB) m/z 672 (M+·).

HR-MS (FAB) Calcd for C₉₆H₁₃₂O₂₈N₇Na: 1402.8320; Found 1402.8320 (M+1·).

(4S,SR,6R,1'S,4'R,5)-5-Acetamino-4-(bis-N\'-tert-butylxycarbonyl)-guanidine-6-[[2\'-amino-ethoxy]-2\'-2\'-dimethyl-[1\'-3\']dioxolan-4\'-yl]methyl-5,6-dihydro-4H-pyran-2-carboxylic Acid Methyl Estor 24j 22j (76 mg, 0.121 mmol) was treated with acetic anhydride (1 ml) and pyridine (1 ml). The reaction mixture was stirred at room temperature for 2h. The reaction mixture was poured into brine and extracted with methylene chloride. The organic layer was washed with aqueous hydrogen chloride (1H), aqueous sodium hydroxide carbonate, and brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (eluent; ethyl acetate:methanol=10:1) to give compound 24j as a colorless amorphous solid (77 mg, 0.114 mmol, 95%). 'H-NMR (400 MHz, CDCl₃) \( \delta = 1.35 \) (3H,
The residue was dissolved in water, dialyzed for 2 d in water (MW 0.364 mmol) was added and the mixture was stirred at room temperature for 2 d.

To the reaction mixture was concentrated under reduced pressure. To the room temperature for 30 min. Then, the precipitate was separated by filtration.

0.364 mmol) and a solution of 1-hydroxybenzotriazole (111 mg, 0.728

Mol fraction of sugar unit = 0.09. 1H-NMR (400 MHz, D2O) δ: 1.15—1.35 (2H, m), 1.38—1.55 (4H, m), 1.85—2.18 (22H, m), 1.98 (3H, s), 2.22—2.48 (22H, m), 3.20—3.40 (2H, m), 3.40—3.70 (4H, m), 3.78—3.88 (1H, m), 3.92—4.00 (1H, m), 4.12—4.52 (14H, m), 5.58—5.62 (1H, m).

**Cells and Viruses**

Madin-Darby canine kidney (MDCK) cells obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) were grown in Earle’s Minimum Essential Medium (MEM) (Invitrogen corp.) supplemented with 10% fetal bovine serum (HyClone), and the antibiotics penicillin G (50 units/ml) and streptomycin sulfate (50 μg/ml) (Invitrogen corp.).

Cells were routinely cultured in flasks at 37 °C and 5% CO2. The influenza virus strains A/PR/8/34 (H1N1) was provided by Dr. Peter Palese, and A/Yama-
gata/32/89 (H1N1) was obtained from Chiba Serum Institute (Japan). The A/PR/8/34 (H1N1) virus used for neuraminidase inhibition assays (the source of enzyme) was propagated in the allantoic sacs of embryonated eggs and purified by sucrose density gradient centrifugation. The A/Yama-
gata/32/89 (H1N1) virus used for plaque reduction assay was in the allantoic sacs of embryonated eggs. The viral stocks were stored at −80 °C. Influenza (A/PR/8/34) was adapted to MDCK cells by passing the virus several times in mice (MAIV, Mouse Adapted Influenza Virus).

**Neuraminidase Inhibition Assay**

A standard colorimetric assay was used to measure influenza virus neuraminidase activity. The substrate, 2(4-nitrophenyl)-o-N-acetylneuraminic acid, was cleaved by neuraminidase to yield nitrophenol, which was quantified. The assay mixture contained in inhibitors at various concentrations and the substrate in 20 μl of molar solution was incubated at 37 °C for 10 min. The reaction was started by the addition of enzyme. For incubation for 20 min, the reaction was terminated by adding 0.2 μl glycine/sodium hydroxide (pH 10.2). Then, the absorbance at 450 nm was measured using the microplate reader. The substrate blanks were subtracted from the sample readings. The IC50 was calculated by plotting the percent inhibition of neuraminidase activity versus the inhibitor concentration.

**Plaque Reduction Assays in MDCK Cells**

Anti-influenza virus activity was measured as described by Hayden et al., with some modifications. Briefly, confluent growing MDCK cells on 35 mm dish were washed with phosphate buffered saline (PBS) (Invitrogen Corp.), inoculated with virus (80 to 150 PFU/well) and incubated in a 5% CO2 incubator at 37°C for 1 h.

The virus inoculum was then discarded, and the cell monolayers were overlaid with MEM supplemented with 0.22% sodium hydrogen carbonate, 10 mM HEPES (pH 7.2) (Invitrogen Corp.), 0.21% bovine serum albumin (PANASONIC Pharmaceuticals Co., Ltd.) 1 μg/ml of trypsin, 0.01% DEAD Dextran, 0.6% aegrose (Sigma Aldrich Corp.) and the test compound. After 30 to 38 h of incubation at 37 °C, the agar overlay was removed and the cell monolayers were stained with 0.1% crystal violet in 19% methanol.

The antiviral efficacy of the test compounds was assessed by counting the plaque number visually or measuring plaque size with PIXEL CATHETER & ANALYZER PCA-11 (SYSTEM SCIENCE CO., Japan) at each compound concentration.

The IC50 was calculated by plotting the percent inhibition of plaque size or cell viability against the concentration of the test compound.

**In Vivo Antiviral Experiments**

Mice (BALB/c CrSlc, female, 5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Compounds were prepared with saline. Mice were anesthetized with chloroform/diethyl ether (1:1) 24 h before infection and each group of 7 or 8 mice received 50 μl of 26c or zanamivir solution intranasally at doses of 0.3 μmol/kg, respectively. The control group (n=8) was administered saline. Then, 500 plaque-forming units (pfu)/mouse of MAIV in 50 μl of phosphate-buffered saline (pH 7.4) containing 0.4% bovine serum albumin was inoculated intranasally to mice anesthetized with chloroform/diethyl ether. The number of surviving mice was counted everyday for 20 d after infection.

The statistical analyses were performed using the S-Plus System Release 8.2 for Windows (SAS Institute Inc.). The Log-Rank test based on the ranking method was carried out to compare the prolonged effects of 26c and zanamivir.

**References and Notes**
