Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 1383–1393
Bioorganic & Medicinal Chemistry

Chemoenzymatic synthesis of artificial glycopolypeptides containing multivalent sialyloligosaccharides with a γ-polyglutamic acid backbone and their effect on inhibition of infection by influenza viruses

Makoto Ogata,[a] Takeomi Murata,[b] Kouki Murakami,[c] Takashi Suzuki,[c] Kazuya I. P. J. Hidari,[c] Yasuo Suzuki[d] and Taichi Usui[a,b,*]

[a]*Science of Biological Resource, The United Graduate School of Agricultural Science, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan*
[b]*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Ohya 836, Suruga ward, Shizuoka 422-8529, Japan*
[c]*Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences and COE Program in the 21st century, Shizuoka, Japan*
[d]*College of Life and Science, Chubu University, Japan*

Received 6 September 2006; revised 3 November 2006; accepted 6 November 2006
Available online 9 November 2006

Abstract—Highly water-soluble, artificial glycopolypeptides with a γ-polyglutamic acid (γ-PGA) backbone derived from *Bacillus subtilis* sp. and multivalent sialyloligosaccharide units have been chemoenzymatically synthesized as potential polymeric inhibitors of infection by bird and human influenza viruses. 5-Trifluoroacetamidopentyl β-*N*-acetyllactosaminide and 5-trifluoroacetamidopentyl β-lactoside were enzymatically synthesized from LacNAc and lactose, respectively, by cellulase-mediated condensation with 5-trifluoroacetamido-1-pentanol. After deacetylation, the resulting 5-aminopentyl β-LacNAc and β-lactoside glycosides were coupled to the α-carboxyl groups of the γ-PGA side chains. The artificial glycopolypeptides carrying LacNAc and lactose were further converted to Neu5Acα2-(3/6)Galβ1–4Glcβ and Neu5Acα2-(3/6)Galβ1–4GlcNAcβ sialyloligosaccharide units by α2,3- and α2,6-sialyltransferase, respectively. The interaction of these glycopolypeptides with various influenza virus strains has been investigated by three different methods. Glycopolypeptides carrying Neu5Acα2,6LacNAc inhibited hemagglutination mediated by influenza A and B viruses, and their relative binding affinities for hemagglutinin were 10^2- to 10^4-fold higher than that of the naturally occurring fetuin control. A glycopolypeptide carrying Neu5Acα2,6LacNAc inhibited infection by A/Memphis/1/71 (H3N2) 93 times more strongly than fetuin, as assessed by cytopathic effects on virus-infected MDCK cells. The avian virus [A/duck/Hong kong/4/78 (H5N3)] bound strongly to Neu5Acα2,3LacNAc/Lac-carrying glycopolypeptides, whereas the human virus [A/Memphis/1/71 (H3N2)] bound to Neu5Acα2,6LacNAc in preference to Neu5Acα2,6Lac. Taken together, these results indicate that the binding of viruses to terminal sialic acids is markedly affected by the structure of the asialo portion, in this case either LacNAc or lactose, in the sugar chain of glycopolypeptides.

1. Introduction

Influenza A and B viruses infect host cells through the binding of viral hemagglutinins (HAs) to sialoglycoproteins or sialoglycolipids on receptors on the host cell surface. The viruses recognize not only sialic acids (Sia) on the receptors but also particular sugar chain structures, such as sialyllacto-series type I (Siaα2-(3/6)Galβ1–3GlcNAcβ1) and type II (Siaα2-(3/6)Galβ1–4GlcNAcβ1) structures.[1] Influenza viruses vary in their recognition of different types (Neu5Ac or Neu5Gc) and linkages (α2–3 or α2–6) of Sia residues.[2] Human influenza A viruses, which have been isolated from humans over the past 30 years, preferentially recognize α2,6-linked Neu5Ac residues.[1] The host cell specificity of viruses is dependent on the linkage between Sia and the

Keywords: Glycopolypeptides; Influenza virus; Inhibition; γ-Polyglutamic acid; Sialyloligosaccharides.
* Corresponding author. Tel./fax: +81 54 238 4873; e-mail: actusui@agr.shizuoka.ac.jp

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.11.006

penultimate galactose, as well as on the number of Sia residues and the core structure. Multivalent interactions must occur between viral HAs and cell-surface Sia residues, which must then be amplified by so-called 'glycoside cluster effects.'[3]

Various synthetic glycopolymers carrying multivalent Sia residues that target viral HAs have been prepared as influenza virus inhibitors by using polyacrylamide (PA),[3–9] poly(acrylacid) (PAA),[10,11] polystyrene,[12] chitosan,[13] and cyclic peptide[14] as the polymer backbone. However, synthetic glycopolymers potentially pose several problems for in vivo use, such as low solubility, significant cytotoxicity,[15] and immunogenicity.[16] From this viewpoint, we have previously reported that artificial glycopolymers containing multivalent sialooligosaccharides with a poly(α-L-glutamic acid)s (α-PGA) backbone are useful for inhibiting infection by influenza viruses, as assessed by measuring cytopathic inhibitory effects in virus-infected cells.[17] Our strategy of molecular design is to construct an amphiphilic structure, taking into account immunogenicity, by arranging hydrophobic main chains and hydrophilic pendant oligosaccharides via convenient synthetic routes.

In this study, we propose a simple synthesis of a new type of asialo-type polymer starting from *N*-acetyllactosamine or lactose. Scheme 1 represents the pathway from *N*-acetyllactosamine or lactose via the following three steps: (1) enzymatic synthesis of a spacer-linked disaccharide glycoside; (2) coupling of the resulting glycoside to γ-polyglutamic acid (γ-PGA); and (3) sialylation of the resulting polymer to produce highly water-soluble glycopolymers carrying clustered identical sialyldisaccharide segments. The inhibitory effects of these glycopolypeptides on infection by bird and human influenza viruses have been subsequently investigated by measuring the cytopathic outcome in virus-infected Madine–Darby canine kidney (MDCK) cells.

2. Results

2.1. Convenient synthetic route to a new type of artificial glycoconjugate polymer

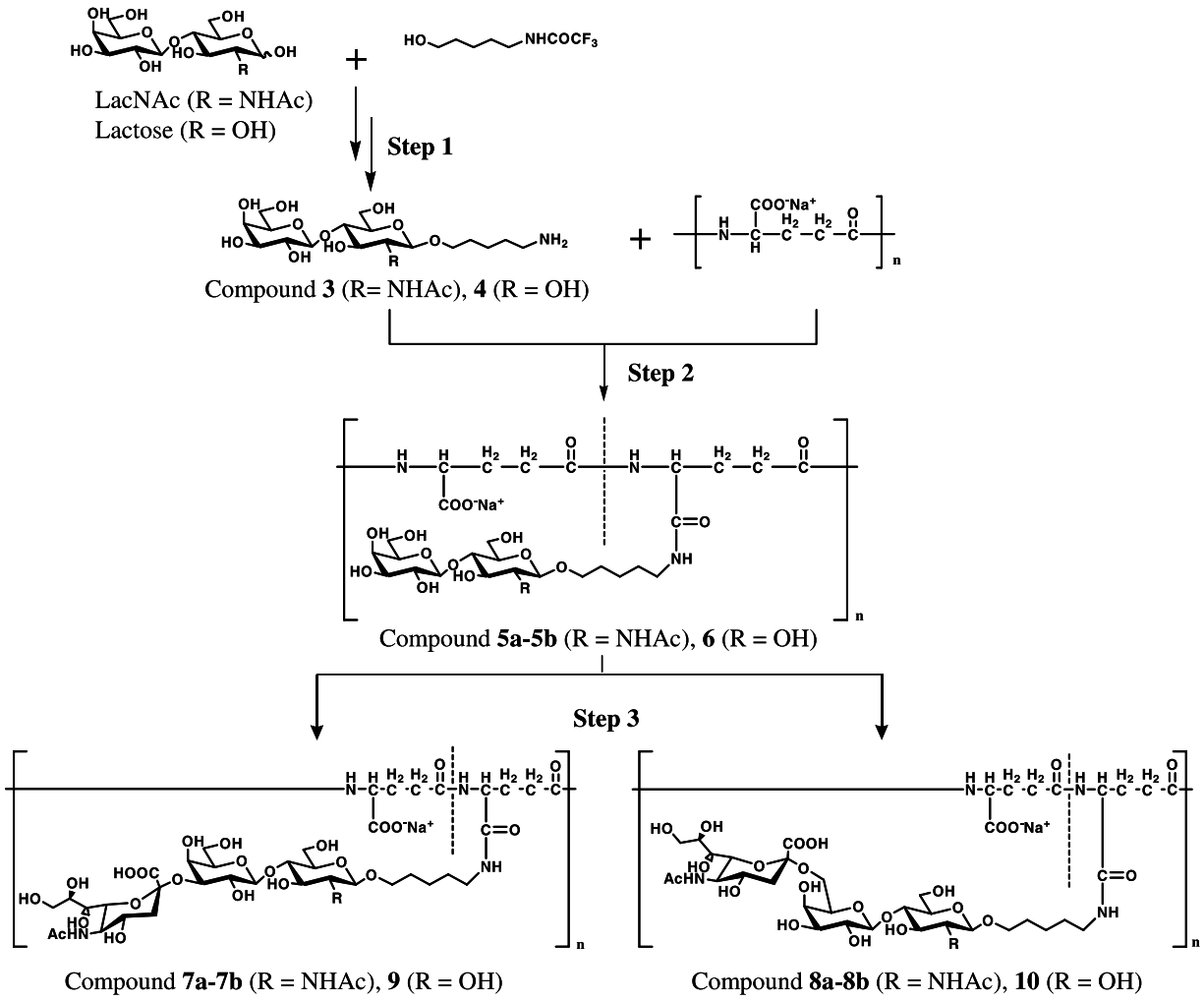
2.1.1. Synthesis of aminoalkyl disaccharide glycoside. A condensation reaction between LacNAc and 5-trifluoroacetamido-1-pentanol was performed using a partially purified enzyme preparation from *Trichoderma reesei*.[18] The molar ratio of LacNAc to 5-trifluoroacetamido-1-pentanol was 1:1.5, and the total substrate concentration in the reaction mixture was 70% (w/v). The reaction mixture was easily fractionated with a charcoal–Celite column. The target 5-trifluoroacetamidopentyl β-*N*-acetyllactosaminide (**1**) was obtained in a low yield of 1.1% based on the initial amount of LacNAc. In a similar way, 5-trifluoroacetamidopentyl β-lactoside (**2**) was synthesized by a condensation reaction between lactose and 5-trifluoroacetamido-1-pentanol. The target product was obtained by a Silica gel column in 1.0% yield based on the initial amount of lactose.

The structures of the aminoalkyl spacer-linked disaccharide glycosides **1** and **2** were confirmed by ^{1}H- and ^{13}C NMR analyses. The O-linked β-anomeric bond between the glycosyl and aglycon moieties was confirmed by the following ^{1}H- and ^{13}C NMR assignments. The protons of the O-linked β-anomeric bond between the glycosyl and aglycon moieties showed lower chemical shifts with larger coupling constants (compound **1**, δ 4.53 ppm ($J_{1,2}$ = 7.0 Hz); compound **2**, δ 4.48 ppm ($J_{1,2}$ = 7.9 Hz)). In the ^{13}C NMR spectra, the O-β-linked C-1β signal was characterized by higher chemical shifts (compound **1**, δ 103.8 ppm; compound **2**, δ 104.8 ppm). The resulting disaccharide glycosides were easily converted to 5-aminopentyl β-*N*-acetyllactosaminide (**3**) and 5-aminopentyl β-lactoside (**4**) by hydrolysis in an alkaline solution with yields in the range of 96–99%.

2.1.2. Synthesis of spacer-linked asialoglycopolypeptides carrying LacNAc or lactose. The amino function of the resulting disaccharide glycosides reacted with the carboxyl group of γ-PGA in the presence of the condensation reagents BOP and HOBt, as previously described.[19] In the synthesis of glycopolypeptides carrying LacNAc, two γ-PGAs of different molecular weight (77,000 and 990,000) were used. The reaction solution was applied to a column of Sephadex G-25M PD-10 to separate the glycosylated γ-PGA from the low-molecular-weight reactants. The degree of substitution (DS) in the mole fraction of the substituted residues in the asialoglycopolypeptides **5a** and **5b** was 61% and 58%, respectively, as calculated from the relative intensities of ^{1}H NMR signals due to peptide methylene protons and those due to spacer-linked methylene protons in aglycon. The structures of synthesized glycopolymers were confirmed by ^{1}H- and ^{13}C NMR analyses. In the ^{1}H NMR spectrum of **5a**, characteristic signals at δ 3.19 ppm were assigned to aglycon ε-methylene protons and those at δ 2.41 ppm were assigned to peptide γ-methylene protons. The analogue asialoglycopolypeptide **6** carrying a lactose residue was synthesized in a similar way, as summarized in Table 1.

2.1.3. Synthesis of sialoglycopolypeptides carrying α-Neu5Ac-(2,3/6)-β-LacNAc or α-Neu5Ac-(2,3/6)-β-lactoside. Asialoglycopolypeptides **5a**,**5b**, and **6** were sialylated, respectively, to **7a**, **7b**, and **9**, and **8a**, **8b**, and **10** by α2,3- and α2,6-sialyltransferase (Scheme 1). After separation through a column of Sephadex G-25M PD-10, the target glycopolypeptides were obtained. The structures of the synthesized sialoglycopolypeptides were confirmed by ^{1}H- and ^{13}C NMR analyses.

In the ^{1}H NMR spectrum of **7a**, characteristic signals at δ 2.76 (dd, 1H, $J_{3ax,3eq}$ 11.9 $J_{3eq,4}$ 3.0 Hz, H-3″eq) and δ 1.81 (t, 1H, $J_{3ax,3eq}$ 11.9 $J_{3ax,4}$ 11.9 Hz, H-3″ax) were assigned to the H-3″ proton. In **8a**, δ 2.67 (dd, 1H, $J_{3ax,3eq}$ 12.2 $J_{3eq,4}$ 4.2 Hz, H-3″eq) and δ 1.74 (t, 1H, $J_{3ax,3eq}$ 12.2 $J_{3ax,4}$ 12.2 Hz, H-3″ax) were assigned to the H-3″ proton. The ^{1}H NMR spectrum also showed that the degree of sialylation was 96–100% from the integration data of the proton signals (Table 2). The degree of substitution of neutral sugar derivatives (NS) and sialyl sugar derivatives (Sia) based on DP of γ-PGA as 100% (Table 2).



Scheme 1. (1) Enzymatic synthesis of spacer-linked disaccharide glycoside, (2) coupling of the resulting glycoside with γ-polyglutamic acid (γ-PGA), and (3) sialylation to highly water-soluble glycopolymers carrying clustered identical sialyldisaccharide segments.

Table 1. Synthesis of asialoglycopolypeptides with different degrees of polymerization of glutamic acid residues

Products	Compound	γ-PGA			BOP[b] (mg)	HOBt[c] (mg)	Amino-sugar[d] (mg)	Yield (mg)	DS[e] (%)
		M_W	DP[a]	mg					
Poly (5-aminopentyl β-*N*-acetyllactosaminide/γ-PGA)	**5a**	77,000	510	15.1	119	15	140	17.0	61
	5b	9,90,000	6557	15.0	118	15	139	24.0	58
Poly (5-aminopentyl β-lactoside/γ-PGA)	**6**	77,000	510	16.5	130	16	140	29.6	69

[a] Degree of polymerization of glutamic acid residues.
[b] Benzotriazol-1-yloxvtris-(dimethylamino)phosphonium hexafluorophosphate.
[c] l-Hydroxybenzotriazole hydrate.
[d] 5-Aminopentyl β-*N*-acetyllactosaminide for **5a**–**5b** and 5-aminopentyl β-lactoside **6**.
[e] Degree of substitution of sugar derivatives based on DP of γ-PGA as 100%. Calculated from ^{1}H NMR data at 25 °C.

In the ^{13}C NMR spectra of **7a** and **8a**, the respective C-3′ and C-6′ signals were distinguished by a downfield position with chemical shifts at δ 77.9 and δ 66.1. These data indicate that the galactosyl residues of the sugar chains are regiospecifically sialylated in the α2–3 and α2–6 linkages.

The structures of the sialoglycopolypeptides**7b**, **8b**, **9** and **10** were similarly confirmed by ^{1}H and ^{13}C NMR analyses. It was possible to evaluate the extent of sialylation from the integration data of the resulting proton signals (Table 2). The properties of the glycopolypeptides synthesized are summarized in Table 2.

2.2. Interaction of synthetic glycopolypeptides with influenza viruses

2.2.1. Hemagglutination inhibition (HI) assay of the glycopolypeptides using influenza viruses. Various substances carrying sialyloligosaccharides inhibit

Table 2. Glycopolypeptides for inhibiting influenza virus infection

Back bone	Sugar moiety	Compound	DP[a]	DS[e] (%)		kDa[d]
				NS[b]	Sia[c]	
γ-PGA	Galβ1–4GlcNAcβ–	**5a**	510	61	—	210
		5b	6557	58	—	2600
	Galβ1–4Glcβ–	**6**	510	69	—	210
	Neu5Acα2–3Galβ1–4GlcNAcβ–	**7a**	510	3	65	330
	Neu5Acα2–6Galβl–4GlcNAcβ–	**7b**	6557	0	58	3800
		8a	510	0	68	330
		8b	6557	0	58	3800
	Neu5Acα2–3Galβ1–4Glcβ–	**9**	510	21	48	290
	Neu5Acα2–6Galβl–4Glcβ–	**10**	510	30	39	280

[a] Degree of polymerization of glutamic acid residues.
[b] Neutral sugar derivatives substituted.
[c] Sialyl sugar derivatives substituted (=Sia contents).
[d] Calculated kDa.
[e] Degree of substitution of sugar derivatives based on DP of γ-PGA as 100%. Calculated from ^{1}H NMR data at 25 °C.

hemagglutination mediated by viral hemagglutinin. We therefore tested the inhibitory effect of the glycopolypeptides on hemagglutination by influenza viruses [A/Memphis/1/71 (H3N2), A/PR/8/34 (H1N1), and B/Lee/40], as summarized in Table 3. The Neu5Acα2,6LacNAc-carrying glycopolymers **8a** and **8b** predominantly inhibited the hemagglutinin of influenza viruses A/Memphis/1/71 (H3N2) and B/Lee/40. These glycopolypeptides showed 10^2- to 10^4-fold higher affinities for the viral hemagglutinins relative to the control fetuin. The Neu5Acα2,6-Lac-carrying glycopolymer **10** was observed to bind A/Memphis/1/71 (H3N2) and B/Lee/40 more weakly than compound **8a**.

By contrast, the Neu5Acα2,3LacNAc and Neu5Acα2,3-Lac-carrying glycopolymers **7a** and **9** inhibited hemagglutination by A/PR/8/34 (H1N1). These glycopolypeptides showed 30- to 34-fold higher affinities relative to fetuin. Compounds **8a** and **10** were observed to bind A/PR/8/34 (H1N1) very weakly. The asialoglycopolypeptides **5a**, **5b**, and **6** did not affect influenza virus-mediated hemagglutination.

2.2.2. Inhibition of influenza virus infection by glycopolypeptides. Next, we tested whether our series of synthetic glycopolypeptides inhibited infection by the influenza virus A/Memphis/1/71 (H3N2) in focus-forming assays. The virus was incubated with the glycopolypeptides at the indicated concentrations, and inoculated onto MDCK cells. The results are summarized in Table 4. Infection of A/Memphis/1/71 (H3N2) was inhibited by **8a** carrying Neu5Acα2,6LacNAc (IC_{50} 69 μg/ml), but not **7a** carrying Neu5Acα2,3LacNAc. Similar results were also observed for **10** carrying Neu5Acα2,6Lac (IC_{50} 317 μg/ml), but its activity was weaker than **8a**. The inhibition activity of **8a** in comparison with **10** indicates that an internal core carbohydrate residue, such as LacNAc, in the sugar chain is important for A/Memphis/1/71 (H3N2) to bind to terminal Neu5Ac residues. The inhibitory activity of glycopolypeptide **8a** was 93 times higher than that of fetuin. The desialylated glycopolymers (**5a** and **6**) and γ-PGAs showed no inhibitory effects, even at concentrations as high as 1000 μg/ml.

2.2.3. Binding specificity of influenza viruses to glycopeptides. The binding specificity of avian [A/duck/Hong kong/4/78 (H5N3)] and human [A/Memphis/1/71 (H3N2)] influenza viruses to glycopolypeptides was determined by a solid-phase binding assay.[20,21] Avian influenza viruses are known to preferentially bind to oligosaccharides that terminate in an α2,3-linked Neu5Ac, whereas human viruses preferentially bind to those that end in an α2,6-linked Neu5Ac. The viruses demonstrated the anticipated binding specificity to the synthesized glycopeptides (Fig. 1). The avian virus predominantly bound to **7a**, **7b**, and **9** carrying Neu5Acα2,3LacNAc/Lac (Fig. 1a), whereas the human virus preferentially bound to **8a** and **8b** carrying Neu5Acα2,6LacNAc and bound to**10** carrying Neu5Acα2,6Lac to a lesser extent (Fig. 1b). The effect of the molecular weight of the artificial glycopolypeptides on the binding activity was also assessed using **7a** and **7b**, and **8a** and **8b**, which had different molecular weights (**7a** and **8a**, 330 kDa; **7b** and **8b**, 3800 kDa) but roughly equivalent Sia contents (around 60%). Binding of **8a** and **8b** by A/Memphis/1/71 (H3N2) increased in a molecular-weight-dependent manner, but that of **7a** and **7b** by A/duck/Hong kong/4/78 (H5N3) did not. This observation indicates that binding by the human virus may be influenced not only by the carbohydrate structure of the asialo moiety in the sugar unit, but also by molecular weight. Neither virus strain showed binding to asialoglycopolypeptides.

3. Discussion

Artificial glycopolymers generated to possess a single type of Neu5Ac linkage (either α2,3 or α2,6) could be an ideal tool to study the molecular interactions between receptors and viruses. We therefore designed a series of glycopolypeptides consisting of three parts: glycan, spacer, and polypeptide backbone. The *N*-acetyllactosamine and lactose residues were at first enzymatically

Table 3. Inhibition of influenza virus hemagglutination by sialoglycopolypeptides

Glycopolypeptides	Compound	HI activity (nM)		
		A/Memphis/1/71 (H3N2)	A/PR/8/34 (H1N1)	B/Lee/40
γ-PGA[a]		ND[b]	ND	ND
Poly (5-aminopentyl β-LacNAc/γ-PGA)	**5a**	ND	ND	ND
	5b	ND	—[e]	—
Poly (5-aminopentyl β-Lac/γ-PGA)	**6**	ND	ND	ND
Poly (Neu5Acα2–3LacNAc β-5-aminopentyl/γ-PGA)	**7a**	ND	95 (34)	189(17)
	7b	16[c](102)[d]	—	—
Poly (Neu5Acα2–6LacNAc β-5-aminopentyl/γ-PGA)	**8a**	12(136)	1515(2.1)	24(136)
	8b	0.13 (12,500)	—	—
Poly (Neu5Acα2–3Lac β-5-aminopentyl/γ-PGA)	**9**	862(1.9)	108(30)	431 (7.6)
Poly (Neu5Acα2–6Lac β-5-aminopentyl/γ-PGA)	**10**	112(15)	1785(1.8)	54 (60)
Fetuin		1628(1)	3255(1)	3255(1)

[a] γ-PGA: M_W = 77,000.
[b] ND: not detected (no activity).
[c] Minimum concentrations required for complete inhibition of hemagglutination.
[d] Relative inhibitory potency. (All data normalized to those of fetuin. Higher values indicate greater inhibitory potency).
[e] '—' not determined.

Table 4. IC_{50} of glycopolypeptides in focus-forming assays of influenza A virus [A/Memphis/1/71(H3N2)]

Glycopolypeptides	Compound	A/Memphis/1/71 (H3N2)	
		IC_{50} (μg/ml)	IC_{50}[b] (Sia μM)
γ-PGA[a]		>1000	>1000
Poly (5-aminopentylβ-LacNAc/γ-PGA)	**5a**	>1000	>1000
Poly (5-aminopentylβ-Lac/γ-PGA)	**6**	>1000	>1000
Poly (Neu5Acα2–3LacNAcβ-5-aminopentyl/γ-PGA)	**7a**	>1000	>1000
Poly (Neu5Acα2–6LacNAcβ-5-aminopentyl/γ-PGA)	**8a**	69	72
Poly (Neu5Acα2–3Lacβ-5-aminopentyl/γ-PGA)	**9**	>1000	>1000
Poly (Neu5Acα2–6Lacβ-5-aminopentyl/γ-PGA)	**10**	317	225
Fetuin		6448	—

[a] γ-PGA: M_W = 77,000.
[b] Concentration of Sia units.

connected to 5-trifluoroacetamido-1-pentanol. Recently, we found that endo-β-glycosidase, a kind of cellulase from *T. reesei*, catalyzes two types of reaction, transglycosylation and condensation.[18] Thus, this enzymatic catalysis was harnessed for the present synthesis of spacer-linked trifluoroacetamidoalkyl *N*-acetyllactosaminyl and lactosyl glycosides. The efficiency of the reaction is not always high, but this method has two advantages: first, the excess of unreacted LacNAc substrate, which is a valuable substrate, is recovered by straightforward column chromatography and can be reutilized for synthesis; and second, the O-glycosidation process stereospecifically gives only the β-glycoside without the need for any protection or deprotection steps.

After deacylation of the resulting disaccharide glycosides, the amino function was easily coupled to the carboxyl group of γ-PGA as the backbone of each glycopolymer. The DS (%) of 5-aminopentyl disaccharide glycosides in the resulting asialoglycopolypeptides reached a high proportion of 58–69% by controlling the coupling reaction (Table 1). The asialoglycopolypeptides were finally sialylated to obtain sialoglycopolypeptides carrying α-Neu5Ac-(2,3/6)-β-LacNAc or α-Neu5Ac-(2,3/6)-β-Lac by α2,3- and α2,6-sialyltransferase. We have previously reported that artificial glycopolypeptides with a poly(α-L-glutamic acid) backbone can be used as acceptors of a CMP-Neu5Ac donor by utilizing α2,3- or α2,6-sialyltransferase.[17] This method was applicable to the elongation of sugar chains in the present glycopolypeptides with a γ-PGA backbone. Sialylation of the LacNAc unit was almost quantitative, but sialylation of the lactose unit occurred at low efficiency, around 60–70% (Table 2). This indicates that the sialylation in such glycopolymers occurs preferentially on a LacNAc structure than on a lactose structure in the asialo-portion in the sugar chain.

Fetuin, a glycoprotein that contains three Neu5Acα2,6 and Neu5Acα2,3 residues, is used as a control against which to measure the interaction between influenza viruses and host cell-surface receptors. This molecule binds both human and animal types of influenza virus, because it contains two different Neu5Ac linkages, Neu5Acα2,6 and Neu5Acα2,3.[22,23] Among the glycopolypeptides tested in the hemagglutination inhibition assay, **8b** carrying multivalent Neu5Acα2,6LacNAc units inhibited the hemagglutination of A/Memphis/1/

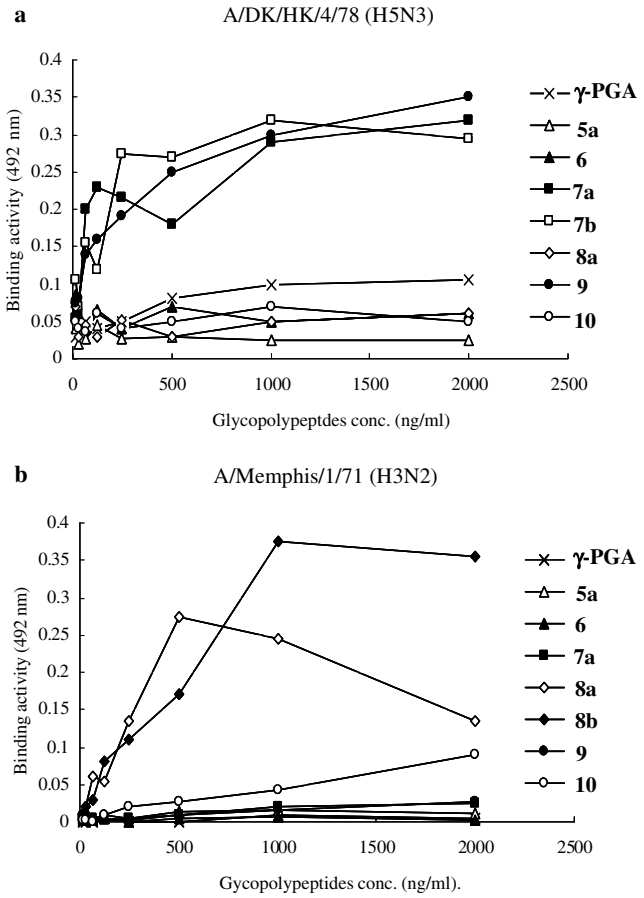


Figure 1. The direct binding activity of avian and human influenza viruses to glycopolypeptides was determined by a previously published method.[20] (a) The avian [A/DK/HK/4/78 (H5N3)] virus predominantly bound to **7a**, **7b** and **9** carrying α2,3-sialoglycopolypeptides. γ-PGA, asialoglycopolypeptides (**5a** and **6**), and α2,6-sialoglycopolypeptides (**8a** and **10**) were not bound to A/DK/HK/4/78 (H5N3) virus. (b) The human [A/Memphis/1/71 (H3N2)] virus preferentially bound to **8a** and **8b** carrying Neu5Acα2,6LacNAc and bound to**10** carrying Neu5Acα2,6Lac to a lesser extent. γ-PGA, asialoglycopolypeptides (**5a** and **6**) and α2,3-sialoglycopolypeptides (**7a** and **9**) were not bound to A/Memphis/1/71 (H3N2) virus.

71 (H3N2) most potently at a concentration of 0.13 nM (or 5×10^{-4} mg/ml), and its relative binding affinity was 12,500-fold higher than that of fetuin itself (Table 3). Compound **8b** had a 92-fold higher affinity relative to **8a**. This indicates that a α2,6-sialoglycopolymer with a high-molecular-weight PGA backbone shows enhanced binding to hemagglutinin proteins. In contrast to this result, glycopolypeptides **7a** and **9** with multivalent Neu5Acα2,3LacNAc units bound to A/PR/8/34 (H1N1) in preference to A/Memphis/1/71 (H3N2) or B/Lee/40. From these results, it can be seen that the present α2,6- and α2,3-sialylglycopolymers are useful for analyzing the preferred receptor species for three kinds of viruses based on the relative affinity of viral HA. In the focus-forming assay, **8a** and **10** inhibited infection by A/Memphis/1/71 (H3N2) at an IC_{50} of 69 and 317 μg/ml, respectively (Table 4). A clear difference due to the structure of the inner disaccharide unit (LacNAc or lactose) was observed. To date, infections by influenza viruses have been successfully inhibited by Sia-conjugated PA,[3–9,24] PAA,[10,11] dendrimers,[15] sialylphosphatidylethanolamine derivatives,[25] sialyllactose-conjugated polystyrene,[12] lyso-GM3-conjugated PGA,[26] GM3 lactose[27] and sialylLacNAc-conjugated PA.[8] The IC_{50} of glycopolymer **8a** used in the present study is roughly equal to that of polymers synthesized by Tuchida et al.[12]

The relationship between viral binding activity and the core determinant of the sugar chain in glycopolymers is not clear. In our study, the contribution of asialo-portions in sugar chains was examined in a solid-phase binding assay. The avian influenza viruses bound almost equally to both **7a** and **7b** carrying Neu5Acα2,3LacNAc, and to **9** carrying Neu5Acα2,3Lac, suggesting that the asialo-portion of the disaccharide unit does not contribute to the binding activity of the glycopolymers (Fig. 1a). In contrast to this result, the fact that human virus binds much more strongly to **8a** and **8b** than to **10** indicates that the asialo-portion (LacNAc) in the sugar chains has an important role in binding of the virus to the terminal Neu5Ac residues (Fig. 1b). The effect of molecular weight (length) of the backbone was also shown to be critical in our glycopolymers because binding by the human influenza virus was enhanced in a molecular-weight-dependent manner, whereas that of the avian influenza virus was not. Paulson and colleagues have also demonstrated that binding of influenza virus to Sia residues is influenced by the asialo-portion of the carbohydrate structure in experiments based on inhibiting the adsorption of A/Memphis/102/72(H) to erythrocytes using natural and synthetic monovalent sialosides.[28–30]

We have previously reported that glycopolymers with a poly(α-L-glutamic acid) backbone carrying multivalent sialyl oligosaccharide units have relatively low immunogenicity.[17] However, the supply of poly(α-L-glutamic acid) as a backbone, which is chemically synthesized, is limited. The objective of our work is to develop a convenient chemo-enzymatic procedure suitable for large-scale preparation of glycopolymers. Sufficient amounts of the backbone material are needed for practical synthesis. PGA, a natural substance produced from *B. subtilis sp.*, is commercially available in large amounts. The physiological merits of PGA as the backbone of glycopolymers in terms of cytotoxicity and immunogenicity are expected. Generally, conventional glycopolymers have a problem of toxicity caused by a backbone such as PA.[15,31] In our previous study, acrylamide monomer, which is known to be a potent neutrotoxin,[32] and PAA-Na were found to be cytotoxic to MDCK cells under the conditions used for the tests.[17] It has also been shown that sugar-bovine serum albumin (BSA) and PA conjugates act as antigens or immunogens.

4. Conclusion

We developed a simple synthesis of artificial glycopolypeptides suitable for large-scale preparation. It should be emphasized that our PGA-based glycopolymers have an extremely high solubility in water (>10% w/v), as

compared with PA-based glycopolymers (<1%), and that they are remarkably heat-stable with no aggregation, even in boiling water. Therefore, PGA-based glycopolymers have ideal characteristics as potential *in vivo* polymeric inhibitors.

5. Experimental

5.1. General methods

5.1.1. Materials. Cellulase (cellulose XL-522, crude enzyme) from *Trichoderma reesei* was purchased from Nagase ChemteX Co. *p*-Nitrophenyl β-lactoside (Lac β-*p*NP) and *N*-acetyllactosamine (LacNAc) were prepared by our previously described methods.[33] γ-PGA-Na (M_W 77,000, 9,90,000) from *B. subtilis sp.* was a kind gift from Meiji Food Materia Co. CMP-β-D-*N*-acetylneuraminic acid disodium salt (CMP-β-Neu5Ac, 2Na) was a kind gift from Yamasa Corporation (Chiba, Japan). α2,3-(*N*)-sialyltransferase (rat recombinant, *Spodoptera frugiperda*) and α2,6-(*N*)-sialyltransferase (rat recombinant, *S. frugiperda*) were purchased from Calbiochem–Novabiochem (San Diego, CA). Rabbit anti-influenza A virus antiserum (anti-P-50) was raised against an influenza A virus carrying the HA gene from strains A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2).[34] Human influenza A and B virus strains, A/PR/8/34 (H1N1), A/Memphis/1/71 (H3N2), B/Lee/40, and A/DK/HK/4/78 (H5N3), were grown in the allantoic sacs of 10-day-old embryonated eggs and purified by sucrose density gradient centrifugation. Viral hemagglutination units (HAUs) of purified viruses were determined as described previously.[35] MDCK cells were propagated in EMEM supplemented with 10% FBS. All other reagents were of the highest quality commercially available and were used without further purification.

5.2. Enzyme assay

To measure Gal β-*p*NP and Lac β-*p*NP hydrolytic activities, a mixture containing 25 μl of a 10 mM substrate solution (Gal β-*p*NP and Lac β-*p*NP) in 50 mM sodium acetate buffer (pH 5.0, 70 μl) and an appropriate amount of enzyme (5 μl) was incubated for 20 min at 40 °C. One-tenth of the reaction mixture was removed at 5-min intervals throughout the time course of the experiment and the reaction was stopped by adding it to 1.0 M Na_2CO_3 (190 μl) in a microplate. The amount of *p*-nitrophenol liberated was determined by measuring the absorbance at 405 nm using a microplate reader (Biolumin 960, Amersham Pharmacia, Sweden). One unit of enzyme was defined as the amount releasing 1 μmol of *p*-nitrophenol per minute.

5.3. Analytical methods

HPLC analysis was carried out using a Mightysil Si60 column (4.6 × 250 mm, KANTO CHEMICAL Co.) with a JASCO Intelligent system liquid chromatograph and detection at 210 nm. The column was eluted with 90% CH_3CN at a flow rate of 1.0 ml/min at 40 °C. FAB-mass analysis was carried out in positive ion mode using a JEOL JMS DX-303HF mass spectrometer coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 were employed. A sample in distilled water (1 μl) was loaded onto a probe tip and mixed with glycerol (1 μl) as a matrix. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer at 25 °C. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl) propionate as an external standard. The amount of protein was determined using a Bio-Rad protein assay kit.

5.4. Synthesis of 5-trifluoroacetamido-1-pentanol

5-Amino-1-pentanol (10 g, 97 mmol) was dissolved in dry pyridine (20 ml) at 0 °C. Trifluoroacetic anhydride (25 ml, 180 mmol) was added and the resulting mixture was stirred magnetically for 1 h at room temperature. The reaction was terminated by adding crushed ice, neutralized with saturated $NaHCO_3$aq (200 ml), and then concentrated to a syrup. It was dissolved in 20 ml of $CHCl_3/CH_3COCH_3$ = 8/2 and then loaded onto a Silica Gel 60 N column (∅ 4.5 × 35 cm). The column was developed with the same solvent at a flow rate of 10 ml/min and a fraction size of 25 ml/tube. Fractions 33–120 were pooled and concentrated. 5-Trifluoroacetamido-1-pentanol was obtained in a total yield of 94% (18 g).

$[\alpha]_D^{30}$ +2.7° (*c* 0.1, water); FAB-mass: *m*/*z* 200 $[M+H]^+$ (matrix: glycerol); ^1H NMR (D_2O, 500 MHz): δ 3.60 (t, 2H, H-α), 3.33 (t, 2H, H-ε), 1.61 (m, 2H, H-ω), 1.57 (m, 2H, H-β), 1.37 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 161.8 (CF_3CONH–), 119.9 (CF_3CONH–), 64.3 (C-α), 42.5 (C-ε), 33.6 (C-β), 30.2 (C-ω), 25.1 (C-γ).

5.5. Synthesis of trifluoroacetamidopentyl disaccharide glycosides

5.5.1. 5-Trifluoroacetamidopentyl β-*N*-acetyllactosaminide (1). The crude cellulase from *T. reesei* was partially purified by removing unwanted β-D-galactosidase by our previously described method.[18] A mixture containing 52.2 mmol of LacNAc, 78.4 mmol of 5-trifluoroacetamido-1-pentanol, and partially purified enzyme (6200 U of Lac β-*p*NP hydrolytic activity) in 52.2 ml of 50 mM sodium acetate buffer (pH 5.0) was incubated for 144 h at 40 °C. The reaction was terminated by heating at 100 °C for 10 min, and the supernatant obtained from centrifugation (17,000 rpm, 10 min) was loaded onto a charcoal–Celite column (∅ 4.5 × 100 cm) equilibrated with distilled water. Subsequently, the absorbed portion was eluted with a linear gradient of 0–25% ethanol in a total volume of 10 L, followed by 80% ethanol, at a flow rate of 5.0 ml/min, and a fraction size of 60 ml/tube. The neutral sugar content of the eluted fractions was measured at 485 nm by the phenol–sulfuric acid method. An aliquot from fractions 53–101 was concentrated and lyophilized: LacNAc was recovered in a yield of 86% (17.2 g). An aliquot from fractions 133–181 was then concentrated and dissolved in 5 ml of $CHCl_3/CH_3OH/H_2O$ = 7/3/0.5 and then loaded onto a Silica Gel 60 N column (∅ 4.5 × 50 cm). The column was

developed with the same solvent at a flow rate of 10 ml/min and a fraction size of 25 ml/tube. An aliquot from fractions 70–100 was then concentrated and lyophilized: compound **1** was obtained in a total yield of 1.1% (322 mg) based on the initial amount of LacNAc.

$[\alpha]_D^{30}$ −14.4° (*c* 0.1, water); FAB-mass: *m*/*z* 565 $[M+H]^+$ (matrix: glycerol); ^{1}H NMR (D_2O, 500 MHz): δ 4.53 (d, 1H, $J_{1,2}$ 7.0 Hz, H-1), 4.47 (d, 1H, $J_{1'2'}$ 7.7 Hz, H-1′), 3.99 (dd, 1H, $J_{5,6b}$ 1.5, $J_{6a,6b}$ 12.5 Hz, H-6b), 3.93 (1H, H-4′), 3.90 (m, 1H, H-αb), 3.83 (dd, 1H, $J_{5,6a}$ 5.0, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.78–3.75 (2H, H-6′a, H-6′b), 3.73–3.70 (4H, H-5′, H-4, H-3, H-2), 3.67 (dd, 1H, $J_{2'3'}$ 10, $J_{3'4'}$ 3.4 Hz, H-3′), 3.59 (m, 1H, H-αa), 3.58 (1H, H-5), 3.54 (dd, 1H, $J_{1'2'}$ 7.7, $J_{2'3'}$ 10 Hz, H-2′), 3.33 (t, 2H, H-ε), 2.03 (s, 3H, C*H*$_3$CONH–), 1.61 (m, 2H, H-ω), 1.58 (m, 2H, H-β), 1.36 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 177.2 (CH_3*C*ONH–), 161.8 (CF_3*C*ONH–), 119.9 (*C*F_3CONH–), 105.7 (C-1′), 103.8 (C-1), 81.3 (C-4), 78.2 (C-5′), 77.6 (C-5), 75.3 (C-3), 75.3 (C-3′), 73.8 (C-2′), 73.0 (C-α), 71.4 (C-4′), 63.8 (C-6′), 62.9 (C-6), 57.9 (C-2), 42.5 (C-ε), 31.0 (C-β), 30.2 (C-ω), 25.2 (C-γ), 24.9 (*C*H_3CONH–).

5.5.2. 5-Trifluoroacetamidopentyl β-lactoside 2. A mixture containing 151 mmol of lactose, 151 mmol of 5-trifluoroacetamido-1-pentanol, and partially purified enzyme (4500 U of Lac β-*p*NP hydrolytic activity) in 151 ml of 50 mM sodium acetate buffer (pH 5.0) was incubated for 144 h at 40 °C. The reaction was terminated by heating at 100 °C for 10 min, and the supernatant obtained from centrifugation (17,000 rpm, 10 min) was concentrated to a syrup. It was dissolved in 25 ml of $CHCl_3/CH_3OH/H_2O$ = 7:3:0.5 and then loaded onto a Silica Gel 60 N column (∅ 4.5 × 50 cm). The column was developed with the same solvent at a flow rate of 10 ml/min and fraction size of 25 ml/tube. An aliquot from fractions 65–103 was concentrated and lyophilized. Compound **2** was obtained in a total yield of 1.0% (849 mg) based on the initial amount of lactose.

$[\alpha]_D^{30}$ −2.5 ° (*c* 0.1, water); FAB-mass: *m*/*z* 524 $[M+H]^+$ (matrix: glycerol); ^{1}H NMR (D_2O, 500 MHz): δ 4.48 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 4.45 (d, 1H, $J_{1'2'}$ 7.7 Hz, H-1′), 3.98 (dd, 1H, $J_{5,6b}$ 1.8, $J_{6a,6b}$ 12.5 Hz, H-6b), 3.93 (1H, H-4′), 3.92 (m, 1H, H-αb), 3.80 (dd, 1H, $J_{5,6a}$ 5.2, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.81–3.76 (2H, H-6′a, H-6′b), 3.73 (1H, H-5′), 3.72 (m, 1H, H-αa), 3.69–3.62 (3H, H-3′, H-4, H-3), 3.57 (1H, H-5), 3.54 (dd, 1H, $J_{1'2'}$ 7.7, $J_{2'3'}$ 9.8 Hz, H-2′), 3.34 (t, 2H, H-ε), 3.30 (t, 1H, $J_{1,2}$ 7.9, $J_{2,3}$ 7.9 Hz, H-2), 1.66 (m, 2H, H-β), 1.61 (m, 2H, H-ω), 1.40 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 161.8 (CF_3*C*ONH–), 119.9 (*C*F_3CONH–), 105.7 (C-1′), 104.8 (C-1), 81.2 (C-4), 78.2 (C-5′), 77.6 (C-5), 77.3 (C-3), 75.7 (C-2), 75.3 (C-3′), 73.8 (C-2′), 73.1 (C-α), 71.4 (C-4′), 63.8 (C-6′), 62.9 (C-6), 42.5 (C-ε), 31.1 (C-β), 30.2 (C-ω), 25.2 (C-γ).

5.6. Synthesis of aminopentyl disaccharide glycosides

5-Trifluoroacetamidopentyl β-*N*-acetyllactosaminide (100 mg, 0.18 mmol) was dissolved in 1.0 M NaOH (1.2 ml). After the mixture was incubated for 60 min at room temperature, it was loaded onto a Sephadex G-25 column (∅ 2.5 × 55 cm) equilibrated with H_2O at a flow rate of 1.0 ml/min and fraction size of 2.0 ml/tube. An aliquot from fractions 60–71 was concentrated and lyophilized. 5-Aminopentyl β-*N*-acetyllactosaminide **3** was obtained in a total yield of 99% (82 mg).

$[\alpha]_D^{30}$ −15.5° (*c* 0.1, water); FAB-mass: *m*/*z* 469 $[M+H]^+$ (matrix: glycerol); ^{1}H NMR (D_2O, 500 MHz): δ 4.52 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.47 (d, 1H, $J_{1'2'}$ 7.7 Hz, H-1′), 3.98 (dd, 1H, $J_{5,6b}$ 2.2, $J_{6a,6b}$ 12.2 Hz, H-6b), 3.92 (1H, H-4′), 3.90 (m, 1H, H-αb), 3.82 (dd, 1H, $J_{5,6a}$ 5.2, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.78–3.74 (2H, H-6′a, H-6′b), 3.73–3.69 (4H, H-5′, H-4, H-3, H-2), 3.66 (dd, 1H, $J_{2'3'}$ 10, $J_{3'4'}$ 3.4 Hz, H-3′), 3.60 (m, 1H, H-αa), 3.59 (1H, H-5), 3.53 (dd, 1H, $J_{1'2'}$ 7.7, $J_{2'3'}$ 10 Hz, H-2′), 2.88 (t, 2H, H-ε), 2.03 (s, 3H, C*H*$_3$CONH–), 1.60 (m, 2H, H-ω), 1.59 (m, 2H, H-β), 1.37 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz): δ 177.2 (CH_3*C*ONH–), 105.7 (C-1′), 103.8 (C-1), 81.3 (C-4), 78.2 (C-5′), 77.6 (C-5), 75.3 (C-3), 75.2 (C-3′), 73.8 (C-2′), 73.0 (C-α), 71.3 (C-4′), 63.8 (C-6′), 62.9 (C-6), 57.9 (C-2), 42.4 (C-ε), 31.0 (C-β), 30.5 (C-ω), 25.0 (C-γ), 25.0 (*C*H_3CONH–).

5-Aminopentyl β-lactoside**4** was obtained by alkaline hydrolysis in a manner similar to the preparation of 5-Aminopentyl β-*N*-acetyllactosaminide described above.

$[\alpha]_D^{30}$ −7.0° (*c* 0.1, water); FAB-mass: *m*/*z* 428 $[M+H]^+$ (matrix: glycerol); ^{1}H NMR (D_2O, 500 MHz): δ 4.49 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.45 (d, 1H, $J_{1'2'}$ 7.6 Hz, H-1′), 3.98 (dd, 1H, $J_{5,6b}$ 1.3, $J_{6a,6b}$ 12.2 Hz, H-6b), 3.93 (1H, H-4′), 3.92 (m, 1H, H-αb), 3.80 (dd, 1H, $J_{5,6a}$ 4.0, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.77–3.76 (2H, H-6′a, H-6′b), 3.73 (1H, H-5′), 3.72–3.62 (4H, H-αa, H-3′, H-4, H-3), 3.60 (1H, H-5), 3.54 (dd, 1H, $J_{1'2'}$ 7.6, $J_{2'3'}$ 9.5 Hz, H-2′), 3.30 (t, 1H, $J_{1,2}$ 8.0, $J_{2,3}$ 8.0 Hz, H-2), 2.97 (t, 2H, H-ε), 1.69–1.64 (4H, H-β, H-ω), 1.46 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 105.7 (C-1′), 104.8 (C-1), 81.2 (C-4), 78.2 (C-5′), 77.6 (C-5), 77.3 (C-3), 75.7 (C-2), 75.3 (C-3′), 73.8 (C-2′), 72.9 (C-α), 71.4 (C-4′), 63.8 (C-6′), 62.9 (C-6), 42.3 (C-ε), 31.0 (C-β), 29.7 (C-ω), 24.9 (C-γ).

5.7. Synthesis of asialoglycopolypeptides with γ-PGA backbones

Poly(5-aminopentyl β-*N*-acetyllactosaminide/γ-PGA) **5a** was synthesized as follows. The compositions of aminopentyl disaccharides, γ-PGAs, and reagents used for the coupling reaction are summarized in Table 1. The DS in the mole fraction of substituted residues in the glycopolypeptides was calculated as a percentage from the relative intensities of the ^{1}H NMR signal areas of peptide γ-methylene protons and aglycon ε-methylene protons. γ-PGA (M_W 77,000, 15.1 mg) was dissolved in 1.3 ml of 100 mM Na_2CO_3/Na_2HCO_3 buffer (pH 10.0). BOP (119 mg) and HOBt (15 mg) in Me_2SO (3.5 ml) were then added and the resulting mixture was stirred magnetically for 15 min at room temperature. 5-Aminopentyl β-*N*-acetyllactosaminide (140 mg) in 100 mM Na_2CO_3/Na_2HCO_3 buffer (pH 10.0, 0.9 ml) was added with continuous stirring for 24 h under the

same conditions. The reaction mixture was loaded onto a Sephadex G-25M PD-10 column (Amersham Biosciences Corp., NJ, USA) equilibrated with 100 mM PBS (pH 7.4). The high-molecular-weight fraction collected was dialyzed against distilled water for 3 days and lyophilized to afford compound **5a** (17.0 mg).

^{1}H NMR (D_2O, 500 MHz): δ 4.52 (d, 1H, $J_{1,2}$ 6.8 Hz, H-1), 4.47 (d, 1H, $J_{1'2'}$ 8.0 Hz, H-1′), 4.31–4.21 (1H, α-methine, γ-PGA), 3.98 (dd, 1H, $J_{5,6b}$ 1.5, $J_{6a,6b}$ 12.2 Hz, H-6b), 3.93 (1H, H-4′), 3.87 (m, 1H, H-αb), 3.83 (dd, 1H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.80–3.71 (7H, H-6′a, H-6′b, H-5′, H-αa, H-4, H-3, H-2), 3.67 (dd, 1H, $J_{2'3'}$ 10.0, $J_{3'4'}$ 3.1 Hz, H-3′), 3.58 (1H, H-5), 3.55 (dd, 1H, $J_{1'2'}$ 8.0, $J_{2'3'}$ 10.0 Hz, H-2′), 3.19 (t, 2H, H-ε), 2.41 (2H, γ-methylene, γ-PGA), 2.18–1.96 (2H, β-methylene, γ-PGA), 2.03 (s, 3H, CH_3CONH–), 1.55 (2H, H-ω), 1.51 (2H, H-β), 1.31 (m, 2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 177.1 (HOO*C*-, γ-PGA), 177.1 (CH$_3$*C*ONH–), 105.7 (C-1′), 103.8 (C-1), 81.3 (C-4), 78.2 (C-5′), 77.6 (C-5), 75.3 (C-3), 57.9 (C-2), 75.2 (C-3′), 73.8 (C-2′), 73.1 (C-α), 71.4 (C-4′), 63.8 (C-6′), 62.9 (C-6), 55.8 (α-methine, γ-PGA), 42.1 (C-ε), 34.4 (γ-methylene, γ-PGA), 31.1 (C-β), 30.7 (C-ω), 29.8 (β-methylene, γ-PGA), 25.2 (C-γ), 25.1 (*C*H$_3$CONH–). Compound **5b** gave similar NMR data.

Poly(5-aminopentyl β-lactoside/γ-PGA) **6** was obtained in a manner similar to that used to prepare compound **5a** (see Table 1).

^{1}H NMR data of compound **6** (D_2O, 500 MHz): δ 4.47 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.45 (d, 1H, $J_{1'2'}$ 8.3 Hz, H-1′), 4.31–4.20 (1H, α-methine, γ-PGA), 3.97 (dd, 1H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 11.6 Hz, H-6b), 3.93 (1H, H-4′), 3.90 (m, 1H, H-αb), 3.80 (dd, 1H, $J_{5,6a}$ 5.8, $J_{6a,6b}$ 11.6 Hz, H-6a), 3.78–3.76 (2H, H-6′a, H-6′b), 3.74 (1H, H-5′), 3.72 (m, 1H, H-αa), 3.68–3.62 (3H, H-3′, H-4, H-3), 3.59 (1H, H-5), 3.55 (t, 1H, $J_{1'2'}$ 8.3, $J_{2'3'}$ 8.3 Hz, H-2′), 3.31 (t, 1H, $J_{1,2}$ 8.0, $J_{2,3}$ 8.0 Hz, H-2), 3.20 (t, 2H, H-ε), 2.41 (2H, γ-methylene, γ-PGA), 2.19–1.85 (2H, β-methylene, γ-PGA), 1.63 (2H, H-ω), 1.53 (2H, H-β), 1.36 (m, 2H, H-γ); ^{13}C NMR data of compound **6** (D_2O, 500 MHz):δ 177.8 (HOO*C*-, γ-PGA), 105.7 (C-1′), 104.9 (C-1), 81.2 (C-4), 78.2 (C-5′), 77.6 (C-5), 77.3 (C-3), 75.7 (C-2), 75.3 (C-3′), 73.8 (C-2′), 73.2 (C-α), 71.4 (C-4′), 63.8 (C-6′), 62.9 (C-6), 55.4 (α-methine, γ-PGA), 42.1 (C-ε), 34.5 (γ-methylene, γ-PGA), 31.2 (C-β), 30.9 (C-ω), 29.5 (β-methylene, γ-PGA), 25.2 (C-γ).

5.8. Synthesis of sialoglycopolypeptides with γ-PGA backbones

Poly(Neu5Acα2–3LacNAc β-5-aminopentyl/γ-PGA) **7a** was enzymatically synthesized from compound **5a** as follows. A mixture containing 5.0 mg of compound **5a**, 16.0 mM CMP-β-Neu5Ac, 40 mU/ml of rat recombinant α2,3-(*N*)-sialyltransferase, 2.5 mM $MnCl_2$, 0.1% BSA, and 10 U/ml of calf intestine alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany) in 50 mM MOPS buffer (pH 7.4) was incubated at 37 °C for 48 h in a total volume of 1.0 ml. After heating at 100 °C and centrifugation, the supernatant from the reaction mixture was loaded onto a Sephadex G-25 M PD-10 column equilibrated with 100 mM PBS (pH 7.4). The high-molecular-weight fraction collected was dialyzed against distilled water for 3 days and lyophilized to afford compound **7a** (6.4 mg).

^{1}H NMR (D_2O, 500 MHz): δ 4.55 (d, 1H, $J_{1'2'}$ 7.7 Hz, H-1′), 4.51 (d, 1H, $J_{1,2}$ 6.5 Hz, H-1), 4.32–4.21 (1H, α-methine, γ-PGA), 4.13–3.57 (21H, from sugar, H-α), 3.18 (2H, H-ε), 2.76 (dd, 1H, $J_{3''ax,3''eq}$ 11.9, $J_{3''eq,4''}$ 3.0 Hz, H-3″eq), 2.41 (2H, γ-methylene, γ-PGA), 2.18–1.85 (2H, β-methylene, γ-PGA), 2.03 (s, 6H, CH_3CONH″–, CH_3CONH–), 1.81 (t, 1H, $J_{3''ax,3''eq}$ 11.9, $J_{3''ax,4''}$ 11.9 Hz, H-3″ax), 1.55 (2H, H-ω), 1.50 (2H, H-β), 1.30 (2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 177.8 (CH$_3$*C*ONH–), 177.6 (CH$_3$*C*ONH″–), 177.6 (HOO*C*-, γ-PGA), 175.9 (HOO*C*-″), 105.4 (C-1′), 103.8 (C-1), 102.6 (C-2″), 81.1 (C-4), 78.3 (C-5′), 77.9 (C-3′), 77.6 (C-5), 75.7 (C-6″), 75.2 (C-3), 74.5 (C-8″), 73.0 (C-α), 72.2 (C-2′), 71.1 (C-4″), 70.9 (C-7″), 70.3 (C-4′), 65.4 (C-9″), 63.8 (C-6′), 62.9 (C-6), 57.9 (C-2), 56.4 (α-methine, γ-PGA), 54.5 (C-5″), 42.4 (C-3″), 42.1 (C-ε), 34.3 (γ-methylene, γ-PGA), 31.0 (C-β), 30.8 (C-ω), 29.8 (β-methylene, γ-PGA), 25.3 (C-γ), 25.1 (*C*H$_3$CONH–), 24.9 (*C*H$_3$CONH″–).

Poly(Neu5Acα2–3LacNAc β-5-aminopentyl/γ-PGA) **7b** was enzymatically synthesized from compound **5b** in a manner similar to that used to prepare compound **7a**. Compound **7b** gave similar NMR data.

Poly(Neu5Acα2–6LacNAc β-5-aminopentyl/γ-PGA) **8a** was similarly synthesized from compound **5a** using rat recombinant α2,6-(*N*)-sialyltransferase (40 mU/ml) to afford compound **8a** (6.1 mg).

^{1}H NMR (D_2O, 500 MHz): δ 4.55 (d, 1H, $J_{1,2}$ 6.7 Hz, H-1), 4.45 (d, 1H, $J_{1'2'}$ 7.6 Hz, H-1′), 4.33–4.21 (1H, α-methine, γ-PGA), 4.01–3.52 (21H, from sugar, H-α), 3.19 (2H, H-ε), 2.67 (dd, 1H, $J_{3''ax,3''eq}$ 12.2, $J_{3''eq,4''}$ 4.2 Hz, H-3″eq), 2.40 (2H, γ-methylene, γ-PGA), 2.18–1.85 (2H, β-methylene, γ-PGA), 2.06 (s, 3H, CH_3CONH″–), 2.03 (s, 3H, CH_3CONH–), 1.74 (t, 1H, $J_{3''ax,3''eq}$ 12.2, $J_{3''ax,4''}$ 12.2 Hz, H-3″ax), 1.56 (2H, H-ω), 1.51 (2H, H-β), 1.31 (2H, H-γ); ^{13}C NMR (D_2O, 500 MHz):δ 177.7 (CH$_3$*C*ONH–), 177.6 (CH$_3$*C*ONH″–), 177.2 (HOO*C*-, γ-PGA), 175.9 (HOO*C*-″), 106.3 (C-1′), 103.7 (C-1), 102.8 (C-2″), 83.5 (C-4), 77.3 (C-5′), 76.4 (C-5), 75.4 (C-6″), 75.3 (C-3), 75.2 (C-3′), 74.4 (C-8″), 73.6 (C-2′), 73.0 (C-α), 71.2 (C-4′), 71.2 (C-4″), 70.9 (C-7″), 66.1 (C-6′), 65.5 (C-9″), 63.2 (C-6), 57.7 (C-2), 56.4 (α-methine, γ-PGA), 54.7 (C-5″), 42.7 (C-3″), 42.1 (C-ε), 34.3 (γ-methylene, γ-PGA), 31.0 (C-β), 30.8 (C-ω), 29.8 (β-methylene, γ-PGA), 25.2 (C-γ), 25.2 (*C*H$_3$CONH–), 24.9 (*C*H$_3$CONH″–).

Poly(Neu5Acα2–6LacNAc β-5-aminopentyl/γ-PGA) **8b** was enzymatically synthesized from compound **5b** in a manner similar to that used to prepare compound **8a**. Compound **8b** gave similar NMR data.

Poly(Neu5Acα2–3Lac β-5-aminopentyl/γ-PGA) **9** was enzymatically synthesized from compound **6** as follows.

A mixture containing 5.5 mg of compound **6**, 16.0 mM CMP-β-Neu5Ac, 40 mU/ml of rat recombinant α2,3-(*N*)-sialyltransferase, 2.5 mM $MnCl_2$, 0.1% BSA, and 10 U/ml calf intestine alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany) in 50 mM MOPS buffer (pH 7.4) was incubated at 37 °C for 48 h in a total volume of 1.1 ml. After heating at 100 °C and centrifugation, the supernatant from the reaction mixture was loaded onto a Sephadex G-25M PD-10 column equilibrated with 100 mM PBS (pH 7.4). The high-molecular-weight fraction collected was dialyzed against distilled water for 3 days and lyophilized to afford compound **9** (6.7 mg).

^{1}H NMR (D_2O, 500 MHz): δ 4.53 (d, 1H, $J_{1'2'}$ 7.6 Hz, H-1′), 4.47 (d, 1H, $J_{1,2}$ 7.3 Hz, H-1), 4.34–4.19 (1H, α-methine, γ-PGA), 4.13–3.58 (20H, from sugar, H-α), 3.30 (t, 1H, $J_{1,2}$ 7.3 Hz, $J_{2,3}$ 7.3 Hz, H-2), 3.20 (2H, H-ε), 2.76 (dd, 1H, $J_{3''ax,3''eq}$ 11.3, $J_{3''eq,4''}$ 3.0 Hz, H-3″eq), 2.42 (2H, γ-methylene, γ-PGA), 2.18–1.90 (2H, β-methylene, γ-PGA), 2.03 (s, 3H, CH_3CONH″–), 1.82 (t, 1H, $J_{3''ax,3''eq}$ 11.3, $J_{3''ax,4''}$ 11.3 Hz, H-3″ax), 1.63 (2H, H-ω), 1.53 (2H, H-β), 1.36 (2H, H-γ); ^{13}C NMR (D_2O, 500 MHz): δ 177.7 (CH_3*C*ONH″–), 177.7 (HOO*C*–, γ-PGA), 175.9 (HOOC″–), 105.5 (C-1′), 104.9 (C-1), 102.5 (C-2″), 81.1 (C-4), 78.3 (C-5′), 77.9 (C-3′), 77.6 (C-5), 77.3 (C-3), 75.7 (C-2), 75.7 (C-6″), 74.5 (C-8″), 73.2 (C-α), 72.2 (C-2′), 71.1 (C-4″), 70.9 (C-7″), 70.3 (C-4′), 65.4 (C-9″), 63.8 (C-6′), 62.9 (C-6), 56.4 (α-methine, γ-PGA), 54.5 (C-5″), 42.3 (C-3″), 42.1 (C-ε), 34.3 (γ-methylene, γ-PGA), 31.2 (C-β), 30.9 (C-ω), 29.8 (β-methylene, γ-PGA), 25.3 (C-γ), 24.9 (*C*H_3CONH″–).

Poly(Neu5Acα2–6Lac β-5-aminopentyl/γ-PGA) **10** was also synthesized from compound **6** using rat recombinant α2,6-(*N*)-sialyltransferase (40 mU/ml) in a similar manner to afford compound **10** (6.8 mg).

^{1}H NMR (D_2O, 500 MHz): δ 4.47 (d, 1H, $J_{1,2}$ 8.6 Hz, H-1), 4.43 (d, 1H, $J_{1'2'}$ 7.6 Hz, H-1′), 4.32–4.20 (1H, α-methine, γ-PGA), 3.99–3.52 (20H, from sugar, H-α), 3.33 (t, 1H, $J_{1,2}$ 8.6 Hz, $J_{2,3}$ 8.6 Hz, H-2), 3.21 (2H, H-ε), 2.72 (dd, 1H, $J_{3''ax,3''eq}$ 11.9, $J_{3''eq,4''}$ 4.0 Hz, H-3″eq), 2.42 (2H, γ-methylene, γ-PGA), 2.23–1.90 (2H, β-methylene, γ-PGA), 2.03 (s, 3H, CH_3CONH″–), 1.75 (t, 1H, $J_{3''ax,3''eq}$ 11.9, $J_{3''ax,4''}$ 11.9 Hz, H-3″ax), 1.63 (2H, H-ω), 1.52 (2H, H-β), 1.36 (2H, H-γ); ^{13}C NMR (D_2O, 500 MHz): δ 177.7 (CH_3*C*ONH″–), 177.7 (HOO*C*–, γ-PGA), 175.9 (HOOC″–), 106.1 (C-1′), 105.8 (C-1), 104.9 (C-2″), 82.5 (C-4), 77.4 (C-5′), 77.3 (C-3), 76.4 (C-5), 75.7 (C-2), 75.6 (C-6″), 75.3 (C-3′), 74.5 (C-8″), 73.8 (C-2′), 73.2 (C-α), 71.3 (C-4′), 71.2 (C-4″), 71.1 (C-7″), 66.3 (C-6′), 65.5 (C-9″), 63.0 (C-6), 56.4 (α-methine, γ-PGA), 54.6 (C-5″), 42.8 (C-3″), 42.1 (C-ε), 34.4 (γ-methylene, γ-PGA), 31.2 (C-β), 30.9 (C-ω), 29.8 (β-methylene, γ-PGA), 25.2 (C-γ), 24.9 (*C*H_3CONH″–).

5.9. Hemagglutination inhibition assay

The hemagglutination inhibition (HI) assay was carried out using 96-well microtiter plates as described previously.[8] Phosphate-buffered saline (PBS, pH 6.5) was used as a dilution buffer. Human erythrocytes were used as indicator cells. Virus suspension (4 HA units in 0.025 ml of PBS) was added to each well containing the artificial glycopolypeptides (1000 to 0.244 μg/ml) in a twofold serial dilution in dilution buffer. After incubation for 1 h at 4 °C, 0.05 ml of 0.5% (v/v) guinea-pig suspension erythrocytes was added to the plates, and allowed to settle for 2 h at 4 °C. The maximum dilution of the samples showing complete inhibition of hemagglutination was defined as the HI titer. As a control, the HA inhibitory activity of fetuin (10 mg/ml) was assayed at the same time.

5.10. Solid-phase binding assay

Direct binding of the viruses and the glycopeptides was determined as described previously.[20] In brief, each glycopeptide (2 μg/ml) in PBS was serially diluted twofold with PBS. Fifty microliters of each glycopeptide dilution was added to the wells of microtiter plates (Corning Polystyrene Universal-BIND Microplate, USA) and incubated at 4 °C for 2 h. The plates were irradiated under UV light at 254 nm for 10 min. The glycopolymer solution was then removed and the plates were blocked with 0.3 ml of PBS containing 0.25% lipid-free BSA at room temperature for 12 h, as described previously.[21] After being washed with PBS five times, the plates were incubated with a solution containing influenza virus (128 HAU in PBS) on ice for 5 h. The plates were washed with PBS containing 0.01% Tween 20 (T-PBS) three times, and antibodies to the virus were added. After incubation for 2 h on ice, the plates were washed three times with ice-cold T-PBS and then incubated with horseradish peroxidase (HRP)-conjugated protein A (Organon Teknika N.V. Cappel Products, Turnhout, Belgium, diluted 2000-fold in PBS containing 0.25% lipid-free BSA) on ice for 2 h. The plates were washed with ice-cold T-PBS four times and then incubated with OPD (o-phenylenediamine, Sigma, USA) in PBS containing 0.01% H_2O_2 for 10 min at room temperature. The reaction was stopped with 0.05 ml of 1 N HCl and the absorbance was determined at 492 nm.

5.11. Inhibition of influenza virus infection by glycopeptides

Each glycopeptide (1 mg/ml) in serum-free medium (SFM) (hybridoma-SFM complete DPM, Invitrogen Corp. NY, USA) was serially diluted twofold with SFM. Seventy-five microliters of each glycopeptide dilution was mixed with 75 μl of influenza virus suspension (100 FFU) in SFM and then incubated at 4 °C for 1 h. Confluent monolayers of Madine–Darby canine kidney (MDCK) cells in 96-well microplates (Corning Costar Corporation, Cambridge, MA) were inoculated with 100 μl of the mixture at room temperature. After 1 h at 34 °C, the inoculum was removed from each plate, and the monolayers were washed three times with PBS and incubated for 16 h at 34 °C in 100 μl of SFM. The monolayers in each well were washed three times with PBS, fixed with 50 μl of methanol at room temperature for 5 min, and washed three more times with PBS. Infection foci of cells were detected by a focus-forming assay,

as described previously,[36,37] using Anti-NP monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G plus M (IgG+M) antibody. Infected cells were defined as the mean of three counts of blue-strained cells within one well. Virus infection was determined as focus-forming units (FFU). The concentration causing 50% inhibition of FFU was determined by plotting the percentage inhibition against the concentration of each glycopeptide.

Acknowledgments

We are grateful to the Yamasa Corporation for the generous gift of the CMP-Neu5Ac. This work was supported by a Grant-in-Aid for scientific research (Nos. 16380077 and 16580072) from the Ministry of Education, Science, Sports, and Culture of Japan, and a research grant from Cooperation of Innovative Technology and Advanced Research in Evolutional Area (CITY AREA) –in the Central Shizuoka Area-, Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

References and notes

1. Suzuki, Y. *Prog. Lipid Res.* **1994**, *33*, 429.
2. Suzuki, Y.; Ito, T.; Suzuki, T.; Holland, R. E., Jr.; Chambers, T. M.; Kiso, M.; Ishida, H.; Kawaoka, Y. *J. Virol.* **2000**, *74*, 11825.
3. Sigal, G. B.; Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Am. Chem. Soc.* **1996**, *118*, 3789.
4. Gamian, A.; Chomik, M.; Laferriere, C. A.; Roy, R. *Can. J. Microbiol.* **1991**, *37*, 233.
5. Spaltenstein, A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 686.
6. Lees, W. J.; Spaltenstein, A.; Kingery-Wood, J. E.; Whitesides, G. M. *J. Med. Chem.* **1994**, *37*, 3419.
7. Itoh, M.; Hetterich, P.; Isecke, R.; Brossmer, R.; Klenk, H. D. *Virology* **1995**, *212*, 340.
8. Furuike, T.; Aiba, S.; Suzuki, T.; Takahashi, T.; Suzuki, Y.; Yamada, K. *J. Chem. Soc., Perkin Trans. 1* **2000**, 3000.
9. Wu, W. Y.; Jin, B.; Krippner, G. Y.; Watson, K. G. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 341.
10. Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 4179.
11. Choi, S. K.; Mammen, M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1997**, *119*, 4103.
12. Tsuchida, A.; Kobayashi, K.; Matsubara, N.; Muramatu, T.; Suzuki, T.; Suzuki, Y. *Glycoconjugate J.* **1998**, *15*, 1047.
13. Makimura, Y.; Watanabe, S.; Suzuki, T.; Suzuki, Y.; Ishida, H.; Kiso, M.; Katayama, T.; Kumagai, H.; Yamamoto, K. *Carbohydr. Res.* **2006**, *341*, 1803.
14. Ohta, T.; Miura, N.; Fujitani, N.; Nakajima, F.; Niikura, K.; Sadamoto, R.; Guo, C. T.; Suzuki, T.; Suzuki, Y.; Monde, K.; Nishimura, S. I. *Angew. Chem., Int. Ed.* **2003**, *42*, 5186.
15. Reuter, J. D.; Myc, A.; Hayes, M. M.; Gan, Z.; Roy, R.; Qin, D.; Yin, R.; Piehler, L. T.; Esfand, R.; Tomalia, D. A., ; Baker, J. R., Jr. *Bioconjugate Chem.* **1999**, *10*, 271.
16. Iurovskii, V. V.; Bovin, N. V.; Safonova, N. G.; Vasilov, R. G.; Khorlin, A. *Bioorg. Khim.* **1986**, *12*, 100.
17. Totani, K.; Kubota, T.; Kuroda, T.; Murata, T.; Hidari, I.-P. J. K.; Suzuki, T.; Suzuki, Y.; Kobayashi, K.; Ashida, H.; Yamamoto, K.; Usui, T. *Glycobiology* **2003**, *13*, 315.
18. Yasutake, N.; Totani, K.; Harada, Y.; Haraguchi, S.; Murata, T.; Usui, T. *Biochim. Biophys. Acta* **2003**, *1620*, 252.
19. Zeng, X.; Nakaaki, Y.; Murata, T.; Usui, T. *Arch. Biochem. Biophys.* **2000**, *383*, 28.
20. Le, Q. M.; Kiso, M.; Someya, K.; Sakai, T. Y.; Nguyen, T. H.; Nguyen, H. L. K.; Pham, H. DE.; Ngyen, H. H.; Yamada, S.; Muramoto, Y.; Horimoto, T.; Takada, A.; Goto, H.; Suzuki, T.; Suzuki, Y.; Kawaoka, Y. *Nature* **2005**, *437*, 1108.
21. Suzuki, T.; Portner, A.; Scroggs, R. A.; Uchikawa, M.; Koyama, N.; Matsuo, K.; Suzuki, Y.; Takimoto, T. *J. Virol.* **2001**, *75*, 4604.
22. Baenziger, J. U.; Fiete, D. *J. Biol. Chem.* **1979**, *254*, 789.
23. Edge, A. S.; Spiro, R. G. *J. Biol. Chem.* **1987**, *262*, 16135.
24. Sparks, M. A.; Williams, K. W.; Whitesides, G. M. *J. Med. Chem.* **1993**, *36*, 778.
25. Guo, C. T.; Wong, C. H.; Kajimoto, T.; Miura, T.; Ida, Y.; Juneja, L. R.; Kim, M. J.; Masuda, H.; Suzuki, T.; Suzuki, Y. *Glycoconjugate J.* **1998**, *15*, 1099.
26. Kamitakahara, H.; Suzuki, T.; Nishigori, N.; Suzuki, Y.; Kanie, O.; Wong, C. H. *Angew. Chem., Int. Ed.* **1998**, *37*, 1524.
27. Sato, T.; Ishii, M.; Ohtake, F.; Nagata, K.; Terabayashi, T.; Kawanishi, Y.; Okahata, Y. *Glycoconjugate J.* **1999**, *16*, 223.
28. Rogers, G. N.; Paulson, J. C. *Virology* **1983**, *127*, 361.
29. Pritchett, T. J.; Brossmer, R.; Rose, U.; Paulson, J. C. *Virology* **1987**, *160*, 502.
30. Matrosovich, M. N.; Gambaryan, A. S.; Tuzikov, A. B.; Byramova, N. E.; Mochalova, L. V.; Golbraikh, A. A.; Shenderovich, M. D.; Finne, J.; Bovin, N. V. *Virology* **1993**, *196*, 111.
31. Ikeda, S.; Neyts, J.; Verma, S.; Wikramasinghe, A.; Mohan, P.; De Clercq, E. *Antimicrob. Agents Chemother.* **1994**, *38*, 256.
32. Spencer, P. S.; Schaumburg, H. H. *Environ. Health Perspect.* **1975**, *11*, 129.
33. Usui, T.; Morimoto, S.; Hayakawa, Y.; Kawaguchi, M.; Murata, T.; Matahira, Y.; Nishida, Y. *Carbohydr. Res.* **1996**, *285*, 29.
34. Suzuki, T.; Sometani, A.; Yamazaki, Y.; Horiike, G.; Mizutani, Y.; Masuda, H.; Yamada, M.; Tahara, H.; Xu, G.; Miyamoto, D. *Biochem. J.* **1996**, *318*, 389, and others.
35. Suzuki, Y.; Suzuki, T.; Matsumoto, M. *J. Biochem. (Tokyo)* **1983**, *93*, 1621.
36. Suzuki, T.; Takahashi, T.; Guo, C.-T.; Hidari, I. P.; Jwa, K.; Miyamoto, D.; Goto, H.; Kawaoka, Y.; Suzuki, Y. *J. Virol.* **2005**, *79*, 11705.
37. Aoki, C.; Hidari, I. P. J. K.; Itonori, S.; Yamada, A.; Takahashi, N.; Kasama, T.; Hasebe, F.; Islam, M. A.; Hatano, K.; Matsuoka, K.; Taki, T.; Guo, C. T.; Takahashi, T.; Sakano, Y.; Suzuki, T.; Miyamoto, D.; Sugita, M.; Terunuma, D.; Morita, K.; Suzuki, Y. *J. Biochem.* **2006**, *139*, 607.