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Syntheses and biological evaluations of carbosilane dendrimers uniformly functionalized with sialyl α(2→3) lactose moieties as inhibitors for human influenza viruses ☆

Hiroyuki Oka [a], Tomotsune Onaga [a], Tetsuo Koyama [a], Chao-Tan Guo [b,c,d], Yasuo Suzuki [b,c], Yasuaki Esumi [e], Ken Hatano [a], Daiyo Terunuma [a], Koji Matsuoka [a,*]

[a] Division of Material Science, Graduate School of Science and Engineering, Saitama University, Sakura, Saitama 338-8570, Japan
[b] Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi 487-8501, Japan
[c] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
[d] Zhejiang Academy of Medical Science, 182 Tianmushan Road, Hangzhou 310016, China
[e] The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan

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ABSTRACT

A series of carbosilane dendrimers uniformly functionalized with sialyl lactose moieties (Neu5Acα2→3-Galβ1→4Glc) was systematically synthesized, and biological evaluations for anti-influenza virus activity using the glycodendrimers were performed. The results suggested that the glycodendrimers had unique biological activities depending on the form of their core frame, and Dumbbell(1)6-amide type glycodendrimer **7** showed particularly strong inhibitory activities against human influenza viruses [A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2)]. The results suggested that the structure–activity relationship (SAR) on the glycolibrary against various influenza viruses was observed, and dumbbell-shaped dendrimers as supporting carbohydrate moieties were found to be the most suitable core scaffolds in this study.

1. Introduction

Influenza virus is a highly pathogenic virus, and an average of about 226,000 patients with influenza virus have been hospitalized every year in the USA.[1] Influenza A viruses have two unique glycoproteins, hemagglutinin (HA) and neuraminidase (NA), on the surfaces of viral particles for infection and replication.[2] Neuraminidase inhibitors, including zanamivir[3] and oseltamivir,[4] have been synthesized and widely used worldwide for clinical treatment of influenza A virus. Although these agents have strong inhibitory activities against influenza virus, several strains of resistant viruses were emerged and their crystal structures of neuraminidase mutants have been studied.[5] In view of the outbreak of neuraminidase-resistant viruses as well as the possibility of appearance of further mutants related to influenza virus, alternative types of therapeutic agents are needed. One of the approaches to prevent first contact of the virus and a host cell has focused on HAs, which participate in infection of influenza virus to host cells.

Sialyl lactose (Neu5Acα2→3Galβ1→4Glc), a sugar chain of ganglioside GM3 on the cell surface, is known to a specific receptor of HAs of influenza A viruses.[6] HA has an important role on the basis of carbohydrate–protein interaction in order to adhere the surface glycosyl receptor on the host cell. Three HAs aggregate to form a trimeric structure by hydrophobic interaction on the viral particle, and HAs therefore have three sugar-binding sites on each unit.[6] These three sugar-binding sites locate on a each end of ca. 45 Å triangle.[6] Cluster-type carbohydrate derivatives[7] are expected to be effective artificial receptors to the binding sites. Synthetic assembly of sialyl lactose against the trimeric binding sites of HAs was valuable approach for the prevention from infection of influenza A viruses. Similar strategies to inhibit receptor-ligand interaction have recently been reported.[8] For example, linear-type polystyrene bearing sialyl lactose moieties as a polymeric HA receptor has been synthesized.[9] Linear-type polyacylamide as backbone has been also synthesized.[10] Both of them were showed high biological activities against viral HAs. In order to construct clustered sialyl lactose moieties, carbosilane dendrimers were conveniently selected as core scaffolds. The advantages of using carbosilane dendrimers are easy control of the number of functional groups at terminal ends, easy accessibility to a macromolecule having suitable

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* Corresponding author. Tel./fax: +81 48 858 3099.
E-mail address: koji@fms.saitama-u.ac.jp (K. Matsuoka).

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molecular size, and low toxicity for living organisms.[11] In our previous studies, some functionalized carbosilane dendrimers uniformly functionalized with cyclodextrins for host–guest interactions,[12] with globotriaoses for Shiga toxin–carbohydrate interactions,[13] and with lacto-*N*-neotetraoses for Dengue virus–carbohydrate interactions,[14] were efficiently synthesized and showed attractive biological activities.

In this paper, we report the systematic synthesis of various carbosilane dendrimers having sialyl lactose moieties at each terminal end on the surface of dendritic structures, as shown in Figure 1, and the results of biological evaluations of the synthesized glycodendrimers for human influenza viruses.

2. Results and discussion

Sialyl lactose is chemically labile under strong basic conditions resulting to form intra-molecular lactam.[15] In our previous study, introduction of sugar moieties into carbosilane dendrimers in liquid ammonia in the presence of Na metal was reported.[12,13] This method could not be used for introduction of sialyl acid-containing sugar moieties because of the strong basic conditions. Thus, we have established an alternative route for the introduction of sialyl lactose moieties into carbosilane dendrimers using lactose as a model sugar.[16] Application of the methodology for assembly of sialooligosaccharide using carbosilane scaffolds was tested to provide a library of carbosilane dendrimers carrying sialyl lactose moieties as the epitope for influenza HAs.

2.1. Synthesis of carbosilane dendrimers having lactose moieties as model introduction for unstable sialyl lactose moieties

Synthetic introduction of a thioacetate moiety into unsaturated aglycons of lactosides is demonstrated in Scheme 1. A pentenyl lactoside **12** was readily synthesized from β-lactose per acetate and 4-penten-1-ol in the presence of BF_3–OEt_2 as Lewis acid.[13a] Radical addition of alkyl thiol to a C=C double bond at the terminal of sugar aglycon, such as a butenyl glycoside, was progressed in good yield.[13] Although the acetyl-thio functional group at the end of sugar aglycon is readily de-S-acetylated to give the corresponding thiolate anion, which is a superior nucleophile for S_N2 reaction with alkyl halide, a radical addition of thioacetic acid to the terminal double bond, especially sugar aglycon, had not been reported. Thus, radical additions of thioacetic acid into allyl glycosides, either acetate **8** or benzylate **10**, were successfully performed to give corresponding thioacetate **9** (67%) or **11** (56%) and recovered starting materials **8** (31%) or **10** (23%). In the addition reaction, 0.5 equiv molar use of AIBN for the double bond was required to achieve the above yields. When a small amount of AIBN was use for the same reaction, prolonged reaction time was needed. Addition of more AIBN (1 equiv molar or more) to the reaction mixture did not improve the reaction yields. However, when pentenyl glycosides, either acetate **12** or benzylate **14**, were used for the reaction, addition of 0.5 equiv molar of AIBN gave the corresponding thioacetate **13** or **15** in quantitative yield. The results suggest that it is important to choose appropriate chain length for a radical addition of thioacetic acid to the terminal C=C double bond of sugar aglycon.[16]

Scheme 2 summarizes synthetic assembly of lactose moieties using dendritic core scaffolds as shown in Figure 2. A one-pot procedure involving de-S-acetylation of **13** with NaOMe and subsequent coupling reaction to a known carbosilane dendrimer **16**[13a] in MeOH–DMF was carried out, and the reaction proceeded smoothly to afford **22** in 71% yield. Introduction of lactosyl derivatives **13** into a dumbbell-type carbosilane dendrimer **18**[13b] was then carried out in the same manner as that described for fan-type carbosilane dendrimers to provide carbosilane dendrimer **24** having six lactose moieties in 62% yield. A disulfide-linked lactoside dimer **23** was also obtained as by-product in the reaction.

Deprotection of fan-type dendrimer **22** was performed with a combination of Zemplén's transesterification and subsequent saponification reaction to yield the corresponding water-soluble carbosilane dendrimer **25** having three lactose moieties quantitatively. In the course of this reaction, partially deprotected products

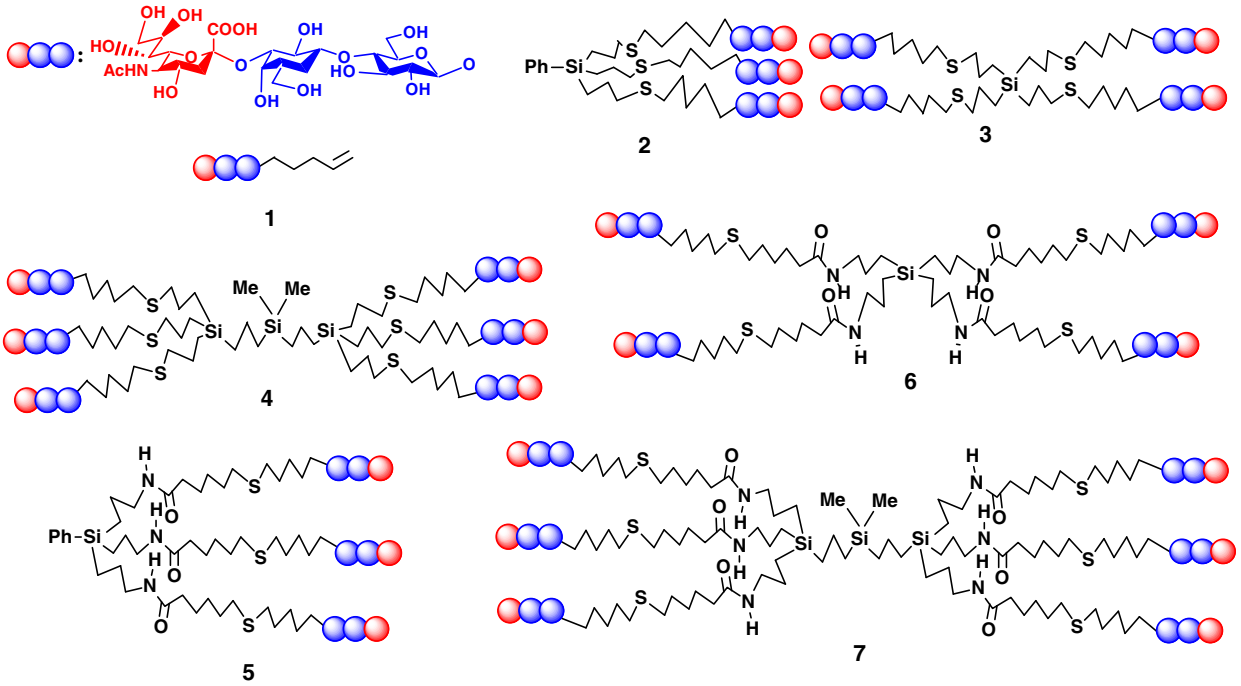
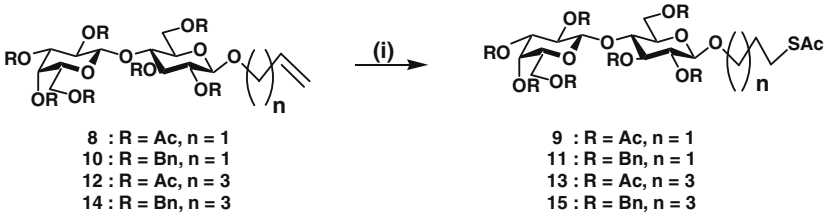
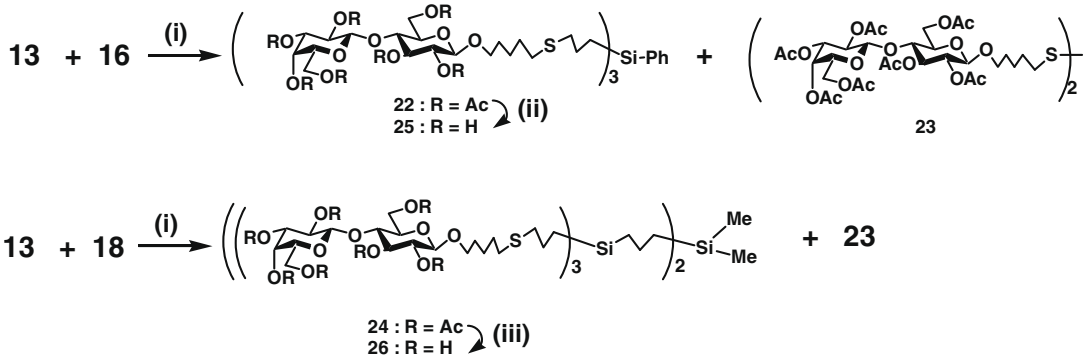


Figure 1. A series of carbosilane dendrimers having sialyl α(2→3) lactose.



Scheme 1. Reagents and conditions: (i) AIBN, HSAc, 1,4-dioxane, 50 °C→80 °C, 3 h, for **9** (67%), for **11** (56%), for **13** (quant), for **15** (quant).



Scheme 2. Reagents and conditions: (i) NaOMe, MeOH–DMF, rt, overnight, then Ac_2O–Pyr, rt, overnight, for **22** (71%), for **24** (62%); (ii) NaOMe, MeOH, rt, overnight, then 0.1 M aq NaOH, rt, overnight, quant.; (iii) NaOMe, MeOH, rt, overnight, then 0.07 M aq NaOH, rt, overnight, quant.

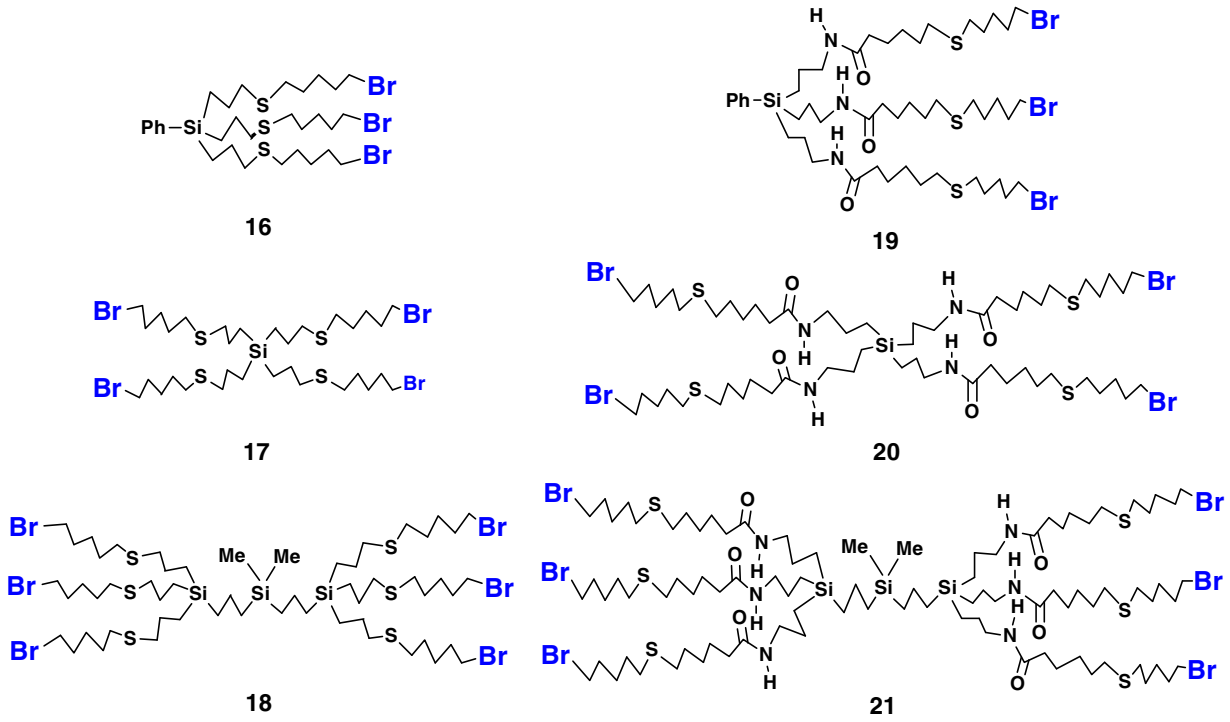
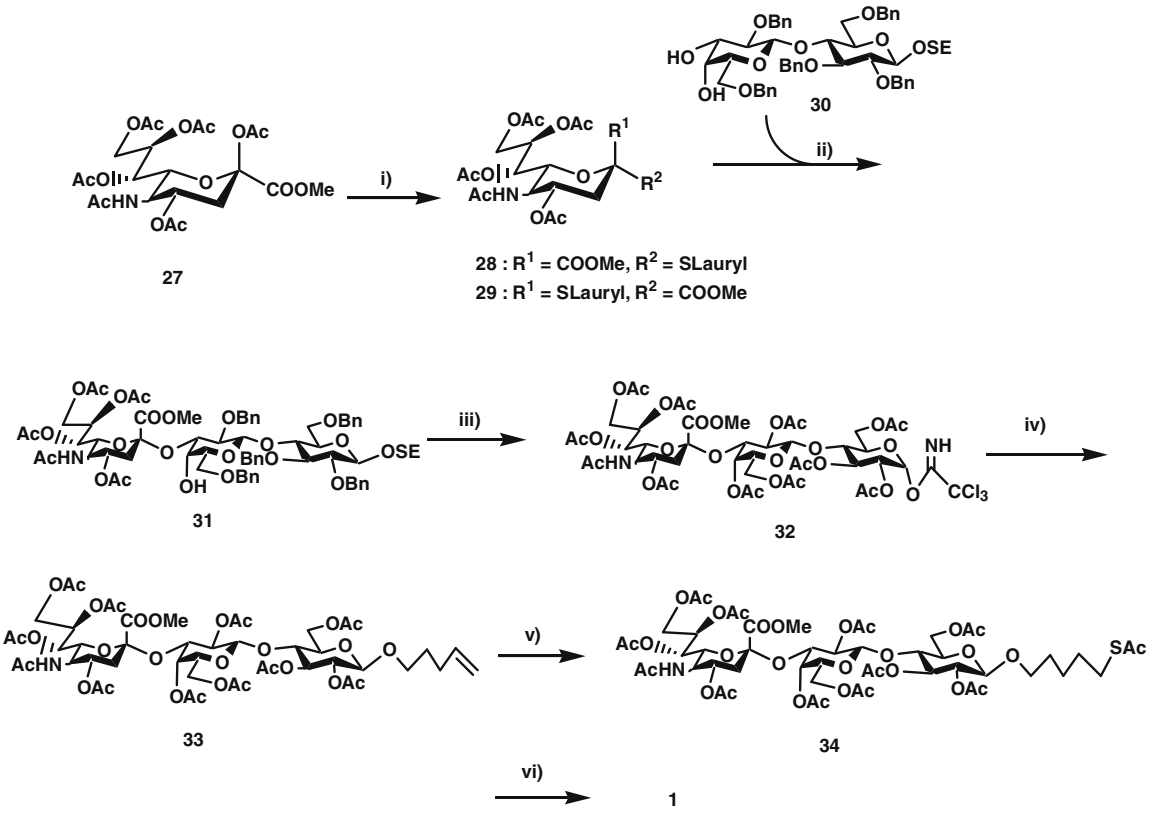


Figure 2. A series of carbosilane dendrimers uniformly functionalized with a bromo atom at each terminal end.

were observed on TLC and the reaction mixture became a suspension due to precipitation of the partially deprotected compounds. Therefore, two-step reaction, such as ester exchange reaction and subsequent saponification, was needed for complete deacetylation. In the same manner, deprotection of dumbbell-type dendrimer **24** gave the corresponding water-soluble carbosilane dendrimer **26** having six sialyl lactose moieties in quantitative yield.

2.2. Synthesis of sialyl lactoside having a thioacetylated pentyl group as the aglycon

Construction and chemical modification of a sialyl lactose derivative are shown in Scheme 3. Sialyl lactose moiety of **31** was prepared by coupling reaction between lauryl thioglycoside of Neu5Ac **28**[17] and a known lactosyl diol **30**[18] with good α-selectivity. An



Scheme 3. Reagents and conditions: (i) 1-dodecanethiol, BF_3–OEt_2, CH_2Cl_2, 0 °C→rt, 3 h, α-laurylate **28** (26%) and β-laurylate **29** (59%); ii) **28** (2 equiv molar), **30** (1 equiv. molar), NIS, TfOH, 3 Å MS, CH_3CN, 55%; (iii) Ref. 14; (iv) 4-penten-1-ol, BF_3–OEt_2, 4 Å MS, CH_2Cl_2, −25 °C→-5 °C, 4 h, 84%; (v) AIBN, HSAc, 1,4-dioxane, 50 °C→80 °C, 3 h, 99%; (vi) NaOMe, MeOH, rt, overnight, then 0.05 M aq NaOH, rt, 2 h, 84%.

anomeric mixture of lauryl thiosialosides **28** and **29** was found to be an efficient glycosyl donor for sialylation, and the thioglycoside was easily prepared from a known acetate **27**.[19] Trichloroacetoimidate **32** was derived from **31** by applying the method reported by Tietze et al. in four steps in good total yield.[20]

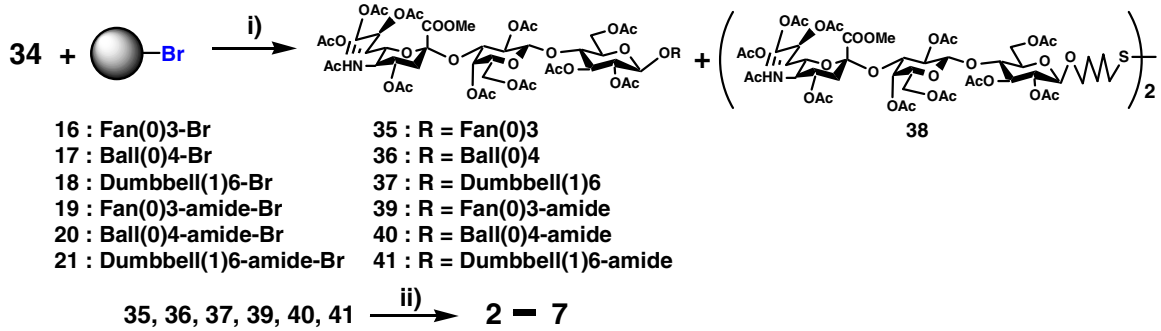
Glycosidation reaction between imidate **32** and 4-penten-1-ol (*n*-pentenyl alcohol) was carried out in the presence of a Lewis acid as a promoter to give β-pentenyl sialyl lactoside **33** with high anomeric β-selectivity in high yield. Radical addition of thioacetic acid into the terminal C=C double bond of **33** required one more equivalent molar of AIBN per double bond for completion of the reaction. When 0.5 equiv molar of AIBN was used for the reaction, unreacted **33** remained. NMR spectra of **34** suggested that the radical addition reaction of thioacetic acid into the alkenyl moiety was accomplished with *anti*Markovnikov's orientation.

2.3. Synthesis of carbosilane dendrimers uniformly functionalized with sialyl lactosyl moieties as multivalent-type epitopes

We have established a model reaction for introduction of lactose derivatives **13** into carbosilane dendrimers **16** and **18** by making sulfide linkage in DMF–MeOH as a solvent system in the presence of NaOMe. This reaction involves removal of the *S*-acetyl group to generate thiolate anion and subsequent replacement of the bromine atom in alkyl halide-type dendrimers as shown in Figure 2. The one-pot reaction, de-S-acetylation, followed by coupling reaction, was applied for sialyl lactosyl derivatives **34**. Under these conditions, partial de-O-acetylation and demethylation of the methyl ester occurred, and the purification of the reaction mixture was impossible. Thus, re-O-acetylation and methyl esterification of the carboxyl group of sialic acid were carried out for temporal purification of the desired dendrimers. A combined purification by silica gel column chromatography and gel permeation chromatography gave pure **35** in 80.2% yield including 1″,2′-lactone form, which was formed by intramolecular ester exchange reaction. In the same manner, coupling reactions into ball-type dendrimer **17**,[13b] dumbbell-type dendrimer **18** and amide-type dendrimers **19**, **20**, **21**[12] were performed and gave corresponding sugar-modified dendrimers **36**, **37**, **39**, **40**, and **41** in adequate yields. A disulfide-linked sialyl lactoside dimer **38** was also obtained as by-product in the reaction. Ball-type dendrimers **36** and **40** showed unfortunately lower yields than those of other dendrimers.

As a model reaction for complete deprotection of sialyl lactose moieties in Scheme 3, removal of all protection of **33** was achieved with two-step reaction, such as ester exchange reaction and subsequent saponification reaction, to provide corresponding sialyl lactoside **1** having a pentenyl moiety as the aglycon in 84.4% yield. Saponification of sialoside **33** was carried out using 0.05 M aq NaOH in order to avoid lactam formation under strong basic conditions. The analogues of GM_3 were known to show an equilibrium between normal sialic acid formation and it is lactone formation in aqueous solution.[21] Pentenyl sialyl lactoside **1** also formed an equilibrium between free sialic acid and it's lactone in aq solution in a few days.

Deprotection of a series of dendrimers was demonstrated as shown in Scheme 4. Thus, deprotection of fan-type dendrimer **35** was carried out with ester exchange reaction and subsequent saponification reaction to yield the corresponding water-soluble carbosilane dendrimer having three sialyl lactose moieties **2** quantitatively. In the same manner, removal of all protections of **36**, **37**,



Scheme 4. Reagents and conditions: (i) NaOMe, MeOH–DMF, rt, then Ac_2O–Pyr, rt, then CH_2N_2, Et_2O, rt, for **35** (80%), for **36** (33%), for **37** (77%), for **39** (66%), for **40** (16%), for **41** (35%); (ii) NaOMe, MeOH, rt, then 0.05 M aq NaOH, rt, for **2** (quant), for **3** (94%), for **4** (quant), for **5** (quant), for **6** (64%), for **7** (77%).

39, **40**, and **41** provided corresponding water-soluble dendrimers **3**, **4**, **5**, **6**, **7**, respectively, in good yields. Structural elucidation was performed by a combination of ^{1}H NMR spectroscopic analysis and mass spectroscopy. The results of the NMR showed near-doublet peaks at around 2.6–2.7 ppm, which were assignable to H-3″*eq* of sialyl acid residue in dendrimers, while H-3″*eq* of lactone showed doublet–doublet peaks at around 2.2–2.3 ppm.

2.4. Biological evaluations of the activities of carbosilane dendrimers having sialyl lactose moieties against human influenza virus

Since systematic synthesis of a glycolibrary of sialyl lactose using carbosilane dendrimers was successfully accomplished, our attention was directed toward the biological activity of the glycolibrary against influenza viruses. The inhibitory activities of these dendrimers against three types of human influenza A virus, (A/PR/8/34, H1N1), (A/Aichi/2/68, H3N2) and (A/Memphis/1/71, H3N2), were initially evaluated by means of hemagglutination inhibition (HAI) assays. The hemagglutinin on viral particles of the viral strain A/PR/8/34 recognizes Neu5Acα(2→3)Gal residues in glycoconjugates, and the virus adheres to erythrocytes through protein—carbohydrate interactions. The hemagglutinin of A/Aichi/2/68 strain recognizes both Neu5Acα(2→3)Gal and Neu5Acα(2→6)-Gal sequences, and the hemagglutinin of A/Memphis/1/71 strain recognizes Neu5Acα(2→6)Gal saccharidic unit. Inhibitory activity of the series of glycodendrimers on hemagglutination was clearly observed when A/PR/8/34 and A/Aichi/2/68 were used as viruses.[22] A/Memphis/1/71 was used as the viral strain for HAI assay using the glycolibrary, and the inhibitory potencies of all of the glycodendrimers were extremely weak. The results of HAI assays using the glycodendrimers are summarized in Table 1, where the inhibitor concentrations were calculated on the basis of a sialyl lactose unit. The results suggested that the hemagglutinin of each virus certainly recognizes the Neu5Acα(2→3)Gal disaccharidic unit in dendritic structures and the results agree well with those in a previous report.[23] A substrate Dumbbell(1)6-amide-SLac **7** showed the highest inhibitory potency in the HAI assay, and the effective concentration of **7** was 16-times lower than that of Pent-SLac **1** against A/PR/8/34 and 63-times lower than that of **1** against A/Aichi/2/68. Furthermore, inhibitory activity of glycodendrimers in HAI assays was significantly related to the shape of core dendrimers and numbers of sugar epitopes.

In order to elucidate further the structure–activity relationships (SARs) of the glycodendrimer against influenza virus, hemolysis inhibition assay and infection inhibition assay were carried out using human influenza virus (A/PR/8/34) strain. The strain A/PR/8/34 was chosen for both hemolysis inhibition assay and infection inhibition assay because the virus strain of A/PR/8/34 strongly recognizes the Neu5Acα(2→3)Gal structure, which was appropriate for our synthesized glycodendrimers. The inhibitory activities of glycodendrimers in the hemolysis inhibition assay of influenza virus to erythrocytes are shown in Figure 3, where concentrations of glycodendrimers are represented by [SLac], which was corrected on the basis of a Neu5Acα(2→3)Galβ(1→4)Glc unit. According to Figure 3, both Dumbbell(1)6-SLac **4** and Dumbbell(1)6-amide-SLac **7** showed strong inhibitory activity. The results suggested that the shape of the core dendrimer has a remarkable influence on the inhibitory activities in the hemolysis inhibition assay. The half maximal inhibitory concentration (IC_{50}) values of the inhibitors from Figure 3 were estimated and are shown in Table 2. Other glycodendrimers also have stronger inhibitory activity than Pent-SLac **1**, and the order of the inhibitory effects of the glycolibrary in the hemolysis inhibition assay was **4** [Dumbbell(1)6-SLac] = **7** [Dumbbell(1)6-amide-SLac] > **5** [Fan(0)3-amide-SLac] = **6** [Ball(0)4-amide-SLac] > **3** [Ball(0)4-SLac] > **2** [Fan(0)3-SLac] > **1** [Pent-SLac].

Table 1
Inhibitory activities of a series of glycodendrimers against hemagglutination of human influenza viruses (type-A) to erythrocytes

Compounds	Inhibition activities (μM)		
	A/PR/8/34 (H1N1)	A/Aichi/2/68 (H3N2)	A/Memphis/1/71 (H3N2)
1 [Pent-SLac]	125	250	500
2 [Fan(0)3-SLac]	64	125	250
3 [Ball(0)4-SLac]	64	32	250
4 [Dumbbell(1)6-SLac]	32	32	250
5 [Fan(0)3-amide-SLac]	16	8	250
6 [Ball(0)4-amide-SLac]	16	4	250
7 [Dummbell(1)6-amide-Slac]	8	4	250

Values are IC_{50} values based on a sialyl lactose unit.

Neutralization assays of glycodendrimers to the infection of influenza virus (A/PR/8/34) using Madin-Darby canine kidney (MDCK) cells were also performed and the results are shown in Figure 4. As was found in hemolysis inhibition assays, both Dumbbell(1)6-SLac **4** and Dumbbell(1)6-amide-SLac **7** showed strong inhibitory activity compared to the activities of others. IC_{50} of the glycolibrary in the neutralization assay was estimated from the results shown in Figure 4 and the values are summarized in Table 2. All glycodendrimers having multivalent sialyl lactose moieties showed higher levels of inhibitory activities against human influenza virus than that of monomeric-type Pent-SLac **1**. Especially, Dumbbell(1)6-amide-SLac **7** showed 22-times stronger inhibitory activity than that of **1**. The order of the inhibitory effects of the glycolibrary in the infection inhibition assay was **7** [Dumbbell(1)6-amide-SLac] > **4** [Dumbbell(1)6-SLac] > **6** [Ball(0)4-amide-SLac] > **3**

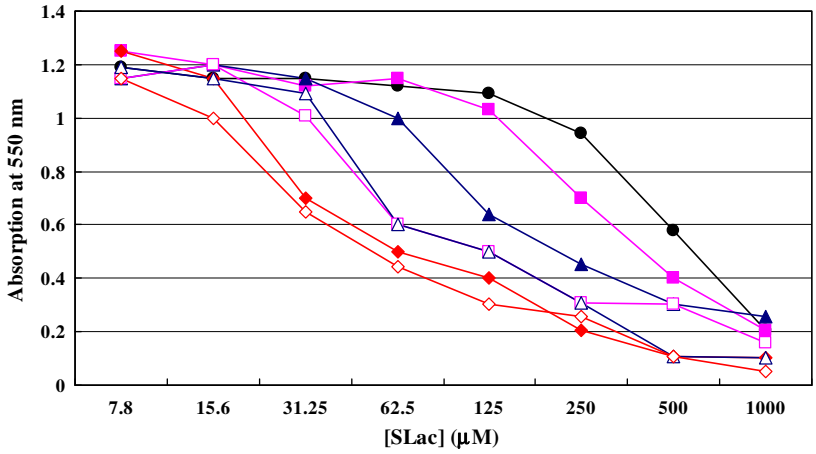


Figure 3. Inhibitory activities of glycodendrimers against hemolysis of human influenza virus (A/PR/8/34 (H1N1)) to erythrocytes [-●-: **1** (Pent-SLac), -■-: **2** (Fan(0)3-SLac), -▲-: **3** (Ball(0)4-SLac), -◆-: **4** (Dumbbell(1)6-SLac), -□-: **5** (Fan(0)3-amide-SLac), -△-: **6** (Ball(0)4-amide-SLac), -◇-: **7** (Dumbbell(1)6-amide-SLac)]. Concentrations of glycodendrimers are represented by [SLac], which was calculated on the basis of a Neu5Acα2→3Galβ1→4Glc unit.

Table 2
Inhibitory activities of a series of glycodendrimers against human influenza virus (A/PR/8/34 (H1N1) strain)

Compounds	Inhibition activities (μM)		
	Hemagglutination[a]	Hemolysis (IC_{50})	Infection (IC_{50})
1 [Pent-SLac]	125	500	125
2 [Fan(0)3-SLac]	64	250	64
3 [Ball(0)4-SLac]	64	125	32
4 [Dumbbell(1)6-SLac]	32	32	7.8
5 [Fan(0)3-amide-SLac]	16	62.5	25
6 [Ball(0)4-amide-SLac]	16	62.5	16
7 [Dummbell(1)6-amide-Slac]	8	32	5.6

Concentrations of glycodendrimers were calculated on the basis of a sialyl lactose unit.
[a] The data are also shown in Table 1.

[Ball(0)4-SLac] > **5** [Fan(0)3-amide-SLac] > **2** [Fan(0)3-SLac] > **1** [Pent-SLac]. Furthermore, these glycodendrimers having a Neu5Acα(2→3)Gal residue at each terminal end are promising agents for prevention of infection with avian influenza virus (H5N1), since avian influenza virus recognized Neu5Acα(2→3)Gal residues on host cells.[24]

3. Conclusion

We have systematically succeeded in the synthesis of a series of carbosilane dendrimers uniformly functionalized with sialyl lactose moieties (Neu5Acα2→3Galβ1→4Glc). Biological evaluations of the glycolibrary against human influenza viruses were also carried out. Inhibitory activities of the glycodendrimers were stronger than that of monomeric sialyl lactoside. The strongest inhibitor against human influenza virus in this study was Dumbbell(1)6-amide-SLac **7**. SAR studies of the glycolibrary also suggested that dumbbell-shaped dendrimers as supporting carbohydrate epitopes were found to be the most suitable core scaffolds. Furthermore, we have reported a series of thiosialoside clusters as NA inhibitors[25] and both HA inhibitors in this study and NA inhibitors are promising therapeutic agents for influenza disease.

4. Experimental

4.1. Materials and methods

Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Pyridine

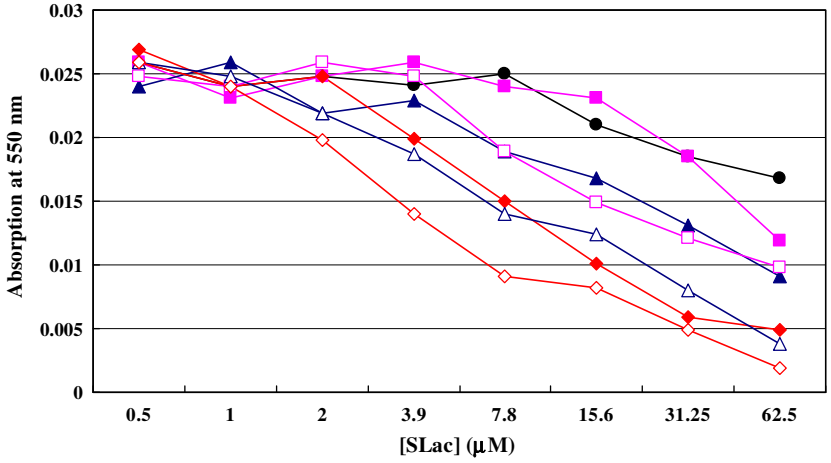


Figure 4. Inhibitory activities of glycodendrimers against infection of human influenza virus (A/PR/8/34 (H1N1)) to MDCK cells [-●-: **1** (Pent-SLac), -■-: **2** (Fan(0)3-SLac), -▲-: **3** (Ball(0)4-SLac), -◆-: **4** (Dumbbell(1)6-SLac), -□-: **5** (Fan(0)3-amide-SLac), -△-: **6** (Ball(0)4-amide-SLac), -◇-: **7** (Dumbbell(1)6-amide-SLac)]. Concentrations of glycodendrimers are represented by [SLac], which was calculated on the basis of a Neu5Acα2→3Galβ1→4Glc unit.

(Pyr), *N,N*-dimethylformamide (DMF), and 1,4-dioxane were stored over molecular sieves (4 Å MS), and methanol (MeOH) was stored over 3 Å MS before use. Tetrahydrofuran (THF) was distilled in the presence of sodium benzophenone ketyl just before use. Melting points were measured with a Laboratory Devices MELTEMP II apparatus and were uncorrected. The optical rotations were determined with a JASCO DIP-1000 digital polarimeter. IR spectra were obtained using a JASCO FT/IR-300E spectrophotometer. The ^{1}H NMR spectra were recorded at 400 MHz with a Bruker AM-400 spectrometer or at 300 MHz with a Bruker AC-300P spectrometer in chloroform-*d*, deuterium oxide or methyl-d_3 alcohol-*d*. The ^{13}C NMR spectra were recorded at 100.6 MHz using the same instruments. Tetramethylsilane (TMS), $CHCl_3$ (7.26 ppm for ^{1}H or 77.0 ppm for ^{13}C), and MeOD (3.3 ppm for ^{1}H or 49.0 ppm for ^{13}C) were used as internal standards. Ring-proton assignments in NMR were made by first-order analysis of the spectra and were supported by the results of homonuclear decoupling experiments. Elemental analyses were performed with a Fisons EA1108 on samples extensively dried at 50–60 °C over phosphorus pentoxide for 4–5 h. Fast atom bombardment mass (FAB-MS) spectra were recorded with a JEOL JMS-HX110 spectrometer. Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF-MS) were obtained using a Perseptive Biosystems Voyager Elite spectrometer. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of Silica Gel 60F_{254} (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). For detection of the intermediates, TLC sheets were dipped in (a) a solution of 85:10:5 (v/v/v) MeOH-*p*-anisaldehyde-concd H_2SO_4 and heated for a few minutes (for carbohydrate), (b) an aq solution of 5 wt % $KMnO_4$ and heated similarly (for C=C double bond), or (c) an ethanolic solution of 7% phosphomolybdic acid and heated similarly (for organic compound). Column chromatography was performed on silica gel (Silica Gel 60; 63–200 μm, E. Merck). Flush column chromatography was performed on silica gel (Silica Gel 60, spherical neutral; 40–100 μm, E. Merck). All extractions were concentrated below 45 °C under diminished pressure.

4.1.1. ω-Acetylthiopentyl 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (13)

To a solution of **12** (5.00 g, 7.10 mmol) in 1,4-dioxane (25 mL) was added thioacetic acid (7.45 mL, 106 mmol), and the solution was heated to 50 °C. To the solution was added AIBN (583 mg, 3.55 mmol) and the solution was heated to 80 °C and stirred for 3 h. The solution was cooled to room temperature. Then, to the solution was added cyclohexene (10.8 mL, 106 mmol) and the solution was stirred for a few minutes. The solution was co-evaporated with toluene. Purification of the residue by silica gel column chromatography with 8:7 (v/v) hexane–EtOAc gave **13** (5.52 g, 99.6%).

R_f 0.43 [2:1 (v/v) EtOAc–hexane], 0.69 [5:1 (v/v) EtOAc–hexane]; $[\alpha]_D^{21}$ −15.1 (*c* 1.77, $CHCl_3$); IR (KBr) 2945 (ν_{C-H}), 1751 ($\nu_{C=O}$), 1690 ($\nu_{C=O}$, SAc), 1238 (ν_{C-O-C}), 1057 (ν_{C-O-C}) cm^{-1}; ^{1}H NMR δ ($CDCl_3$) 5.35 (dd, 1H, $J_{3',4'}$ = 3.2 Hz, $J_{4',5'}$ = 1.1 Hz, H-4′), 5.19 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.1 Hz, H-3), 5.11 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 10.4 Hz, H-2′), 4.95 (dd, 1H, H-3′), 4.88 (dd, 1H, $J_{1,2}$ = 7.8 Hz, H-2), 4.48 (d, 1H, H-1′), 4.48 (m, 1H, H-6b), 4.45 (d, 1H, H-1), 4.16–4.06 (m, 3H, H-6a, H-6′a, H-6′b), 3.89–3.77 (m, 3H, H-4, H-5′, one of OCH_2–), 3.59 (ddd, 1H, *J* = 2.2 Hz, *J* = 5.2 Hz, *J* = 9.9 Hz, H-5), 3.48 (dt, 1H, J_{gem} = 9.6 Hz, J_{vic} = 6.4 Hz, one of OCH_2–), 2.32 (s, 3H, S*Ac*), 2.15, 2.12, 2.06, 2.04, and 1.97 (each s, 21H, O*Ac*), 1.56 (m, 4H, OCH$_2$*CH*$_2$CH$_2$*CH*$_2$CH$_2$SAc), 1.64 (m, 2H, –*CH*$_2$CH$_2$CH$_2$SAc); ^{13}C NMR δ ($CDCl_3$) 195.34(S*C*=O), 170.12, 170.07, 169.90, 169.78, 169.52, 169.39, 168.83, 100.80, 100.29, 76.05, 72.57, 72.39, 71.41, 70.75, 70.44, 68.90, 67.95, 66.45, 61.81, 60.64, 30.36, 29.12, 25.35, 25.10, 20.61, 20.55, 20.45, 20.38, 20.26. FAB-MS Calcd for $C_{33}H_{48}O_{19}S_1$ $[M+H]^+$: 781, $[M+Na]^+$: 803. Found: *m/z*: 781, *m/z*: 803. Anal. Calcd for $C_{33}H_{48}O_{19}S_1$: C, 50.76; H, 6.20; N, 0.00. Found: C, 50.67; H, 6.21; N, 0.00.

4.1.2. Fan(0)3-Lac-Ac (22)

To a suspension of **16** (50.0 mg, 0.107 mmol) in DMF (0.6 mL) was added compound **13** (500 mg, 0.64 mmol), and the solution was stirred until it became clear. Then, to the solution was added MeOH (0.6 mL), and the solution was stirred at room temperature for 2 h. NaOMe (38.0 mg) was added to the solution, and the solution was stirred overnight. After addition of AcOH (0.3 mL) to the solution, the solution was concentrated. The residue was acetylated with Ac_2O and pyridine and then concentrated. The residue was extracted with $CHCl_3$, and the organic layer was washed successively with cold 1 M aq HCl, cold satd aq $NaHCO_3$ and brine, dried over anhyd $MgSO_4$, filtered, and evaporated in vacuo. Purification of the residue by silica gel column chromatography with 2:1 (v/v) hexane–EtOAc gave pure **22** (184 mg, 70.8%) and a disulfide-linked lactoside dimer **23** (181 mg), respectively.

4.1.2.1. Data for Fan(0)3-Lac-Ac 22. R_f 0.50 [5:1 (v/v) EtOAc–hexane]; $[\alpha]_D^{21}$ −14.2 (*c* 1.40, $CHCl_3$); IR (KBr) 2941 (ν_{C-H}), 1753 ($\nu_{C=O}$), 1234 (ν_{CO-C}), 1057 (ν_{C-OC}) cm^{-1}; ^{1}H NMR δ ($CDCl_3$) 7.41 (m, 5H, Si*Ph*), 5.35 (dd, 3H, $J_{3',4'}$ = 3.5 Hz, $J_{4',5'}$ = 0.9 Hz, H-4′), 5.19 (t, 3H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.11 (dd, 3H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 10.3 Hz, H-2′), 4.95 (dd, 3H, H-3′), 4.88 (dd, 3H, $J_{1,2}$ = 8.1 Hz, H-2), 4.49 (d, 3H, H-1′), 4.47 (m, 3H, H-6b), 4.45 (d, 3H, H-1), 4.11 (m, 9H, H-6a, H-6′a, H-6′b), 3.83 (m, 9H, H-4, H-5′, one of OCH_2–), 3.59 (ddd, 3H, *J* = 2.2 Hz, *J* = 5.2 Hz, *J* = 9.9 Hz, H-5), 3.44 (dt, 3H, J_{gem} = 9.6 Hz, J_{vic} = 6.6 Hz, one of OCH_2–), 2.49 (t, 6H, SCH_2), 2.43 (t, 6H, SCH_2), 2.15, 2.11, 2.06, 2.04, 2.04, 2.03, and 1.97 (each s, 63H, O*Ac*), 1.54 (m, 18H, –CH$_2$), 1.40 (m, 6H, –CH$_2$), 0.90 (m, 6H, –CH$_2$); ^{13}C NMR δ ($CDCl_3$) 170.19, 170.16, 169.98, 169.87, 169.63, 169.41, 168.91, 136.13, 133.85, 128.99, 127.74, 100.89, 100.40, 76.14, 72.68, 72.44, 71.54, 70.83, 70.49, 69.74, 68.96, 66.48, 61.90, 60.67, 35.73, 31.83, 29.19, 28.88, 24.97, 23.89, 20.71, 20.65, 20.56, 20.47, 20.34, 11.73. FAB-MS Calcd for $C_{108}H_{158}O_{54}S_{13}Si$ $[M+H]^+$: 2444.9. Found: *m/z* 2444.6. Anal. Calcd for $C_{108}H_{158}O_{54}S_3Si_1$: C, 53.06; H, 6.51; N, 0.00. Found: C, 52.94; H, 6.54; N, 0.00.

4.1.2.2. Data for dimer 23. R_f 0.53 [5:1 (v/v) EtOAc–hexane]; ^{1}H NMR δ ($CDCl_3$) 5.35 (dd, 2H, H-4′), 5.19 (t, 2H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.10 (dd, 2H, $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 10.2 Hz, H-2′), 4.96 (dd, 2H, $J_{3',4'}$ = 3.5 Hz H-3′), 4.88 (dd, 2H, $J_{1,2}$ = 8.0 Hz, H-2), 4.49 (d, 2H, H-1′), 4.49 (m, 2H, H-6b), 4.46 (d, 2H, H-1), 4.11 (m, 6H, H-6a, H-6′a, H-6′b), 3.84 (m, 6H, H-4, H-5′, one of OCH_2–), 3.60 (ddd, 2H, H-5), 3.46 (dt, 2H, J_{gem} = 9.6 Hz, J_{vic} = 6.7 Hz, one of OCH_2–), 2.65 (t, 4H, *J* = 7.2 Hz SCH_2), 2.06 (each s, 42H, O*Ac*), 1.61 (m, 8H, OCH$_2$*CH*$_2$CH$_2$*CH*$_2$CH$_2$S), 1.41 (m, 4H, OCH$_2$CH$_2$*CH*$_2$–). FAB-MS Calcd for $C_{62}H_{90}O_{36}S_2$ $[M+H]^+$: 1475. Found: *m/z* 1475.

4.1.3. Dumbbell(1)6-Lac-Ac (24)

Compound **18** (49.7 mg, 0.053 mmol) in DMF (0.6 mL) was coupled with **13**[16] (500 mg, 0.64 mmol) by the method described for the preparation of **22**. Purification of the reaction mixture by silica gel column chromatography with 1:5 (v/v) hexane–EtOAc gave pure **24** (159 mg, 61.5%) and a disulfide-linked lactoside dimer **23**, respectively.

vR_f 0.17 [5:1 (v/v) EtOAc–hexane], 0.39 [10:1 (v/v) $CHCl_3$–MeOH]; $[\alpha]_D^{21}$ −15.3 (*c* 0.80, $CHCl_3$); IR (KBr) 2941 (ν_{C-H}), 1753 ($\nu_{C=O}$), 1234 (ν_{C-O-C}), 1053 (ν_{C-O-C}) cm^{-1}; ^{1}H NMR δ ($CDCl_3$, 7.26 ($CHCl_3$)) 5.33 (d, 6H, *J* = 2.9 Hz, H-4′), 5.18 (t, 6H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.09 (dd, 6H, $J_{1',2'}$ = 7.9 Hz, $J'_{2,3'}$ = 10.5 Hz, H-2′), 4.94 (dd, 6H, $J_{3''4}$ = 3.3 Hz, H-3′), 4.86 (dd, 6H, $J_{1,2}$ = 8.1 Hz, H-2), 4.48 (d, 6H, H-1′), 4.46 (m, 6H, H-6b), 4.44 (d, 6H, H-1), 4.09 (m, 18H, H-6a, H-6′a, H-6′b), 3.82 (m, 18H, H-4, H-5′, one of OCH_2–), 3.58 (ddd, 6H,

J = 1.9 Hz, J = 4.9 Hz, J = 9.8 Hz, H-5), 3.44 (dt, 6H, J_{gem} = 9.5 Hz, J_{vic} = 6.7 Hz, one of OCH_2–), 2.48 (t, 12H, SCH_2), 2.46 (t, 12H, SCH_2), 2.14–1.95 (each s, 126H, OA*c*), 1.58–1.39 (m, 52H, OCH$_2$CH_2CH_2CH_2CH$_2$SCH$_2$CH_2–, SiCH$_2$CH_2CH$_2$Si), 0.55 (m, 20H, –CH_2SiCH_2CH$_2$CH_2SiMe$_2$), −0.08 (s, 6H, Si(CH_3)$_2$); ^{13}C NMR δ (CDCl$_3$) 170.20, 170.19, 169.99, 169.89, 169.64, 169.43, 168.93, 100.92, 100.44, 77.20, 76.18, 72.73, 72.48, 71.59, 70.86, 70.52, 69.77, 69.00, 66.51, 61.93, 60.67, 35.98, 31.98, 29.52, 29.27, 28.96, 25.03, 24.19, 20.74, 20.67, 20.59, 20.49, 20.36, 20.28, 18.22, 16.98, 11.99, −3.32. FAB-MS Calcd for $C_{212}H_{324}O_{108}S_6Si_3$ [M+H]$^+$: 4877.8. Found: *m/z* 4877.6. Anal. Calcd for $C_{212}H_{324}O_{108}S_6Si_3$: C, 52.21; H, 6.70; N, 0.00. Found: C, 51.99; H, 6.65; N, 0.00.

4.1.4. Fan(0)3-Lac (25)

To a solution of dendrimer **22** (32.8 mg, 0.0134 mmol) in MeOH (2 mL) was added NaOMe, and the mixture was stirred overnight at room temperature. IR-120B (H$^+$) resin was added to the mixture to remove Na$^+$, and the suspension was filtered, and the filtrate was concentrated. 0.1 M aq NaOH (3 mL) was added to the residue and the solution was stirred overnight at room temperature. IR-120B (H$^+$) resin was added to the solution, and the mixture was filtered and concentrated. Gel filtration of the residue using Sephadex G-25 with 5% aq AcOH gave **25** quantitatively.

$[\alpha]_D^{19}$ −4.98 (*c* 0.16, H$_2$O); IR (KBr) 3400 (ν_{O-H}), 2922 (ν_{C-H}), 1066 (ν_{C-O}); ^{1}H NMR δ (D$_2$O) 7.30 (m, 5H, Si*Ph*), 4.39 (d, 3H, $J_{1''2}$ = 7.0 Hz, H-1′), 4.33 (d, 3H, $J_{1,2}$ = 7.0 Hz, H-1), 3.85–3.47 (m), 3.27 (br, 3H, H-2), 2.39 (br, 12H, –CH_2SCH_2–), 1.46 (br), 0.80 (br, 6H, CH_2Si); ^{13}C NMR δ (D$_2$O, 49.00 (MeOD)) 136.70, 134.17, 129.45, 128.17, 103.16, 102.54, 78.65, 75.48, 74.83, 74.71, 72.97, 72.77, 71.11, 70.37, 68.69, 61.17, 60.47, 35.72, 31.89, 29.56, 29.10, 25.17, 24.14, 11.81. FAB-MS Calcd for $C_{66}H_{116}O_{33}S_3Si$ [M+H]$^+$: 1561.6. Found: *m/z* 1561.9. Anal. Calcd for $C_{66}H_{116}O_{33}S_3Si_1{\cdot}4H_2O$: C, 48.51; H, 7.65; N, 0.00. Found: C, 48.52; H, 7.34; N, 0.00.

4.1.5. Dumbbell(1)6-Lac (26)

An acetate **24** (159 mg, 0.0326 mmol) was deprotected in a manner similar to that described for **25** to give **26** quantitatively.

$[\alpha]_D^{20}$ −4.44 (*c* 0.76, H$_2$O); IR (KBr) 3400 (ν_{O-H}), 2918 (ν_{C-H}), 1068 (ν_{C-O}); ^{1}H NMR δ (D$_2$O) 4.39 (near t, 12H, $J_{1,2}$ = $J'_{1,2'}$ = 9.6 Hz, H-1, H-1′), 3.84–3.46 (m), 3.26 (br, 6H, H-2), 2.49 (br, 24H, –CH_2SCH_2–), 1.49 (br), 0.63 (br), −0.12 (br, 6H, Si*Me*$_2$); ^{13}C NMR δ (D$_2$O) 103.00, 102.40, 78.51, 75.32, 74.69, 74.54, 72.81, 72.61, 70.94, 70.19, 68.54, 61.01, 60.32, 35.78, 31.84, 29.47, 28.98, 25.07, 24.25, 20.60, 20.24, 18.58, 17.50, 11.89, −2.40. MALDI-TOF-MS Calcd for $C_{128}H_{240}O_{66}S_6Si_3$ [M+Na]$^+$: 3134.30. Found: *m/z* 3138.09. Anal. Calcd for $C_{128}H_{240}O_{66}S_6Si_3{\cdot}6H_2O$: C, 47.74; H, 7.89; N, 0.00. Found: C, 47.78; H, 7.77; N, 0.00.

4.1.6. Methyl (lauryl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-glycero-D-galacto-2-nonulopyranosid)onate (28[26] and 29)

To a solution of known acetate **27** (5.6 g, 10.5 mmol) in dichloromethane (60 mL) was added 1-dodecanethiol (10 mL, 42.0 mmol), and the solution was cooled to 0 °C. Then, to the solution was added BF$_3$–OEt$_2$ (215 μL, 1.71 mmol), and the stirring was continued at 0 °C for 30 min and at room temperature for 3 h. The solution was diluted with CHCl$_3$ and washed successively with cold water, cold satd aq NaHCO$_3$ and brine, dried over anhyd MgSO$_4$, filtered, and evaporated in vacuo. Complete separation of an anomeric mixture of the residue was performed by silica gel column chromatography with 1:2 (v/v) toluene–EtOAc to afford pure α-laurylate **28**[26] (1.9 g, 26.2%) and β-laurylate **29** (4.3 g, 59.2%) as foam, respectively.

4.1.6.1. Data for α-laurylate 28. R_f 0.58 [5:1 (v/v) CHCl$_3$–MeOH]; $[\alpha]_D^{28}$ +26.0 (*c* 1.09, CHCl$_3$) [lit.[26] $[\alpha]_D^{25}$ +21.4 (*c* 0.69, CHCl$_3$)]; IR (KBr) 1751 ($\nu_{C=O}$), 1658 ($\nu_{C=O}$), 1550 (ν_{N-H}), 1222 (ν_{C-O-C}), 1037 (ν_{C-O-C}) cm^{-1}; ^{1}H NMR δ (CDCl$_3$) 5.36 (ddd, 1H, H-8), 5.33 (dd, 1H, H-7), 5.21 (d, 1H, $J_{NH,5}$ = 10.0 Hz, *NH*), 4.86 (ddd, 1H, $J_{3eq,4}$ = 4.7 Hz, $J_{3ax,4}$ = $J_{4,5}$ = 10.7 Hz, H-4), 4.32 (dd, 1H, $J_{8,9a}$ = 2.2 Hz, H-9a), 4.12 (dd, 1H, $J_{9a,9b}$ = 12.4 Hz, $J_{8,9b}$ = 4.7 Hz, H-9b), 4.05 (m, 1H, H-5), 3.83 (dd, 1H, H-6), 3.80 (s, 3H, COOCH_3), 2.53 (m, 2H, SCH_2), 2.16, 2.14, 2.04, 2.02, and 1.88 (each s, 15H, NH*Ac*, 4O*Ac*), 1.49 (m, 2H, SCH$_2$CH_2), 1.25 (m, 18H, 9CH_2), 0.88 (t, 3H, CH$_2$CH_3). Anal. Calcd for $C_{32}H_{53}N_1O_{12}S$: C, 56.87; H, 7.90; N, 2.07. Found: C, 56.72; H, 7.95; N, 2.01.

4.1.6.2. Data for β-laurylate 29. R_f 0.60 [5:1 (v/v) CHCl$_3$–MeOH]; $[\alpha]_D^{28}$ −67.0 (*c* 0.91, CHCl$_3$); ^{1}H NMR δ (CDCl$_3$) 5.44 (m, 1H, H-7), 5.37 (d, 1H, $J_{NH,5}$ = 10.2 Hz, *NH*), 5.26 (ddd, 1H, $J_{3eq,4}$ = 4.8 Hz, $J_{3ax,4}$ = $J_{4,5}$ = 10.7 Hz, H-4), 5.11 (ddd, 1H, $J_{7,8}$ = 2.7 Hz, H-8), 4.81 (dd, 1H, $J_{8,9a}$ = 2.1 Hz, H-9a), 4.33 (dd, 1H, $J_{6,7}$ = 2.2 Hz, H-6), 4.17 (dd, 1H, $J_{9a,9b}$ = 12.3 Hz, $J_{8,9b}$ = 8.0 Hz, H-9b), 4.08 (ddd, 1H, $J_{4,5}$ = $J_{5,6}$ = 10.2 Hz, H-5), 3.80 (s, 3H, COOCH_3), 2.52 (m, 3H, SCH_2, H-3*eq*), 2.13, 2.07, 2.03, 2.02, and 1.88 (each s, 15H, NH*Ac*, 4 O*Ac*), 1.51 (m, 2H, SCH$_2$CH_2), 1.25 (m, 18H, 9CH_2), 0.88 (t, 3H, CH$_2$CH_3). Anal. Calcd for $C_{32}H_{53}N_1O_{12}S$: C, 56.87; H, 7.90; N, 2.07. Found: C, 56.68; H, 7.97; N, 2.00.

4.1.7. 2-(Trimethylsilyl)ethyl [methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-(2→3)-*O*-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (31)

To a solution of lauryl thio glycoside **28** (4.20 g, 8.1 mmol) and lactosyl diol **30** (0.432 mL, 4.27 mmol) in acetonitrile (82 mL) was added 3 Å MS powder (8.2 g) and the mixture was stirred at room temperature for a few hours. After the suspension had been cooled to −35 °C, *N*-iodosuccinimide (4.00 g, 17.8 mmol) and trifluoromethanesulfonic acid (159 μL, 1.8 mmol) were added and the stirring was continued at −35 °C for 4 h. The suspension was filtered through a pad of Celite, and the filtrate was diluted with CHCl$_3$ and washed successively with cold satd aq NaHCO$_3$, 1 M aq sodium thiosulfate and brine, dried over anhyd MgSO$_4$, filtered, and evaporated in vacuo. Chromatographic purification of the residue by silica gel with 3:2 (v/v) toluene–EtOAc as the eluent gave known **31** (3.34 g, 54.6%). Analytical data for this compound agree with those in previous report.[20]

4.1.8. [Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-(2→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetoimidate (32)

Trichloroacetoimidate **32** was efficiently synthesized from **31** by the method previously reported by Tietze et al.[20]

4.1.9. Pentenyl [Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-(2→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (33)

To a solution of an imidate **32** (1.03 g, 0.853 mmol) and 4-penten-1-ol (0.432 mL, 4.27 mmol) in dichloromethane (24.0 mL) was added 4 Å MS powder (2.0 g), and the mixture was stirred at room temperature for a few minutes. After the mixture had been cooled to −25 °C, BF$_3$–OEt$_2$ (215 μL, 1.71 mmol) was added to the mixture with stirring, and the stirring was continued at −25 °C for 20 min and at −5 °C for 4 h. The mixture was filtered through a pad of Celite, and the filtrate was diluted with CHCl$_3$ and washed successively with satd aq NaHCO$_3$ and brine, dried over anhyd MgSO$_4$, filtered, and evaporated in vacuo. The residual syrup was applied to a column of silica gel with 20:1 (v/v) CHCl$_3$–MeOH as the eluent to yield **33** (816 mg, 84.3%) as a foam.

R_f 0.50 [10:1 (v/v) CHCl$_3$–MeOH]; $[\alpha]_D^{18}$ −6.87 (*c* 2.02, CHCl$_3$); IR (KBr) 2949 (ν_{C-H}), 1745 ($\nu_{C=O}$), 1674 ($\nu_{C=O}$, SAc, NAc), 1554 (ν_{N-H}),

1230 (ν_{C-O-C}), 1045 (ν_{C-O-C}) cm^{-1}; ^1H NMR δ ($CDCl_3$) 5.78 (m, 1H, –*CH*=CH_2), 5.54 (m, 1H, H-8″), 5.39 (dd, 1H, $J_{6'',7''}$ = 2.7 Hz, $J_{7'',8''}$ = 9.3 Hz, H-7″), 5.18 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 5.08 (d, 1H, $J_{NH,5''}$ = 10.2 Hz, *NH*), 5.02–4.85 (m, H-2, H-2′, H-4′, –CH=CH_2), 4.67 (d, 1H, $J_{1',2'}$ = 10.0 Hz, H-1′), 4.52 (dd, 1H, $J_{2',3'}$ = 10.2 Hz, $J_{3',4'}$ = 3.3 Hz, H-3′), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.43 (m, 2H, H-6b, H-9″), 4.18 (dd, 1H, $J_{5,6a}$ = 5.4 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6a), 4.02 (m), 3.87 (m, 3H, H-4, one of OCH_2–, –*CH*), 3.84 (s, 3H, COOCH_3), 3.63 (dd, 1H, $J_{5'',6''}$ = 10.8 Hz, $J_{6'',7''}$ = 2.7 Hz, H-6″), 3.59 (m, 1H, H-5), 3.49 (dt, 1H, J_{gem} = 9.6 Hz, J_{vic} = 6.7 Hz, one of OCH_2–), 2.58 (dd, 1H, $J_{3''a,3''e}$ = 12.6 Hz, $J_{3''e,4''}$ = 4.5 Hz, H-3″*eq*), 2.25, 2.16, 2.09, 2.08, 2.06, 2.04, 2.03, 2.01, and 1.86 (each s, 33H, NH*Ac*, O*Ac*), 2.08 (m, $OCH_2CH_2CH_2CH$=CH_2), 1.66 (m, OCH_2CH_2–, H-3″*ax*); ^{13}C NMR δ ($CDCl_3$) 170.66, 170.51, 170.48, 170.45, 170.35, 170.25, 170.17, 170.05, 169.65, 169.54, 169.48, 169.44, 167.81, 137.68, 114.91, 100.82, 100.40, 96.63, 76.19, 73.27, 72.45, 71.86, 71.70, 71.20, 70.30, 69.77, 69.20, 69.13, 67.70, 67.14, 66.78, 62.20, 62.12, 61.37, 52.98, 48.88, 37.25, 29.67, 28.44, 22.99, 21.36, 20.78, 20.67, 20.62, 20.56, 20.52, 20.45. Anal. Calcd for $C_{49}H_{69}N_1O_{29}$: C, 51.80; H, 6.12; N, 1.23. Found: C, 51.50; H, 6.14; N, 1.19.

4.1.10. Pentenyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranoside (1)

An acetate **33** (50.0 mg, 0.044 mmol) was dissolved in MeOH (1.0 mL), and 1 M NaOMe in MeOH (44 μL) was added dropwise to the solution. The reaction mixture was stirred overnight at room temperature. IR-120B (H^+) resin was added to the solution, and the mixture was filtered and evaporated. 0.05 M aq NaOH (2 mL) was added to the residue, and the solution was stirred at room temperature for 2 h. IR-120B (H^+) resin was added to the solution, and the mixture was filtered and concentrated to afford **1** (26.0 mg, 84.4%).

$[\alpha]_D^{18}$ −3.45 (*c* 0.96, H_2O); IR (KBr) 3394 (ν_{O-H}), 2937 (ν_{C-H}), 1732 ($\nu_{C=O}$), 1639 ($\nu_{C=O}$), 1560 (ν_{N-H}), 1036 (ν_{C-O-C}), 619 (ν_{N-H}) cm^{-1}; ^1H NMR δ (D_2O) 5.78 (m, 1H, –*CH*=CH_2), 4.96 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 15.4 Hz, one of –CH=*CH*$_2$), 4.96 (d, 1H, J_{cis} = 10.3 Hz, one of –CH=*CH*$_2$), 4.40 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′), 4.35 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1), 4.03 (dd, 1H, $J_{2',3'}$ = 9.7 Hz, $J_{3',4'}$ = 2.8 Hz, H-3′), 3.88–3.42 (m), 3.18 (t, 1H, $J_{2,3}$ = 7.9 Hz, H-2), 2.63 (dd, 1H, $J_{3''a,3''e}$ = 12.5 Hz, $J_{3''e,4''}$ = 4.0 Hz, H-3″*eq*), 2.02 (q, 2H, *J* = 7.1 Hz, –*CH*$_2$CH=CH_2), 1.91 (s, 3H, ND*Ac*), 1.77 (t, 1H, $J_{3''a,4''}$ = 12.1 Hz, H-3″*ax*), 1.60 (m, 2H, $OCH_2CH_2CH_2$). Anal. Calcd for $C_{28}H_{47}N_1O_{19}\cdot1.5H_2O$: C, 46.15; H, 6.92; N, 1.92. Found: C, 46.29; H, 6.77; N, 1.82.

4.1.11. ω-Acetylthio-pentyl [methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)-onate]-(2→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (34)

To a solution of **33** (100 mg, 0.088 mmol) in 1,4-dioxane (209 μL) was added thioacetic acid (209 μL, 3.0 mmol), and the solution was heated to 50 °C. After addition of AIBN (24.6 mg, 0.15 mmol) to the solution, the temperature of the solution was raised to 80 °C, and the heated solution was stirred for 3 h at the same temperature. After cooling the solution to room temperature, cyclohexene (608 μL) was added to the solution, and the reaction mixture was further stirred for a few minutes. The solution was evaporated and co-evaporated with toluene. The residue was diluted with $CHCl_3$, and the $CHCl_3$ layer was washed successively with satd aq $NaHCO_3$ and brine, dried over anhyd $MgSO_4$, filtered, and evaporated in vacuo. Chromatographic purification of the residue by silica gel with 1:0–30:1 (v/v) $CHCl_3$–MeOH as the eluent afforded **34** (105 mg, 99.1%).

R_f 0.51 [10:1 (v/v) $CHCl_3$–MeOH]; $[\alpha]_D^{29}$ −6.28 (*c* 1.29, $CHCl_3$); IR (KBr) 2959 (ν_{C-H}), 1756 ($\nu_{C=O}$), 1689 ($\nu_{C=O}$, Sac, NAc), 1544 (ν_{N-H}), 1436 (ν_{C-N}), 1250 (ν_{C-O-C}), 1040 (ν_{C-O-C}), 631–603 (ν_{N-H}) cm^{-1}; ^1H NMR δ ($CDCl_3$) 5.49 (m, 1H, H-8″), 5.39 (dd, 1H, $J_{6'',7''}$ = 2.8 Hz, $J_{7'',8''}$ = 9.4 Hz, H-7″), 5.18 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 5.12 (d, 1H, $J_{NH,5''}$ = 9.9 Hz, *NH*), 4.93 (dd, 1H, $J_{1',2'}$ = 8.1 Hz, $J_{2',3'}$ = 10.3 Hz, H-2′), 4.88 (m, H-2, H-4″, –*CH*), 4.67 (d, 1H, H-1′), 4.52 (dd, 1H, $J_{3',4'}$ = 3.3 Hz, H-3′), 4.45 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1), 4.43 (m, H-6b, H-9″, –*CH*), 4.18 (dd, 1H, $J_{6a,6b}$ = 11.9 Hz, $J_{5,6a}$ = 5.4 Hz, H-6a), 4.09–3.82 (2 m), 3.84 (s, 3H, COOCH_3), 3.63 (dd, 1H, $J_{5'',6''}$ = 10.7 Hz, $J_{6'',7''}$ = 2.6 Hz, H-6″), 3.59 (m, 1H, H-5), 3.46 (dt, 1H, J_{gem} = 9.7 Hz, J_{vic} = 6.6 Hz, one of OCH_2–), 2.85 (t, 2 H, *J* = 7.2 Hz, SCH_2–), 2.58 (dd, 1 H, $J_{3''a,3''e}$ = 12.7 Hz, $J_{3''e,4''}$ = 4.6 Hz, H-3″*eq*), 2.32 (s, 3H, S*Ac*), 2.24 (s, 3H, NH*Ac*), 2.16, 2.094, 2.086, 2.086, 2.077, 2.061, 2.040, 2.040, 2.008, and 1.855 (each s, 30H, 10 O*Ac*), 1.61 (m, 5 H, H-3″*ax*, $OCH_2CH_2CH_2CH_2CH_2S$), 1.39 (m, 2H, –$CH_2CH_2CH_2SAc$). FAB-MS Calcd for $C_{51}H_{73}N_1O_{30}S_1$ $[M+H]^+$: 1212, $[M+Na]^+$: 1234. Found: *m/z*: 1212, *m/z*: 1234. Anal. Calcd for $C_{51}H_{73}N_1O_{30}S_1$: C, 50.53; H, 6.07; N, 1.16. Found: C, 50.03; H, 6.03; N, 1.14.

4.1.12. Fan(0)3-SLac-Ac (35)

To a solution of **16** (6.48 mg, 0.014 mmol) in DMF was added thioacetate **34** (100 mg, 0.082 mmol), and the mixture was stirred until it became clear. MeOH (0.2 mL) was added to the solution, and the solution was stirred at room temperature for 1 h. To the solution was added NaOMe (4.90 mg), and the mixture was stirred overnight. After addition of AcOH (0.1 mL) to the mixture, the mixture was concentrated. The residue was treated with Ac_2O (2 mL) and pyridine (2 mL) and then concentrated. The residue was diluted with $CHCl_3$, and the organic solution was washed successively with 1 M aq HCl and brine, dried over anhyd $MgSO_4$, filtered, and evaporated in vacuo. The residual syrup was dissolved in MeOH–Et_2O, and diazomethane in Et_2O was added dropwise to the solution at room temperature. After addition of AcOH to the solution, the solution was concentrated. A combination of chromatographic purification of the residue by silica gel with 30:1 (v/v) $CHCl_3$–MeOH as the eluent and by gel permeation chromatography using Sephadex LH-20 with MeOH as the eluent gave **35** (41.2 mg, 80.2%) including 1″,2′-lactone form.

R_f 0.3 [10:1 (v/v) $CHCl_3$–MeOH]; IR (KBr) 2941 (ν_{C-H}), 1749 ($\nu_{C=O}$), 1687 ($\nu_{C=O}$), 1543 (ν_{N-H}), 1435 (ν_{C-N}), 1230 (ν_{C-O-C}), 1039 (ν_{C-O-C}) cm^{-1}; ^1H NMR δ ($CDCl_3$) 7.40 (m, 5H, *Ph*), 5.53 (m, 3H, H-8″), 5.39 (dd, 3H, $J_{6'',7''}$ = 2.7 Hz, $J_{7'',8''}$ = 9.1 Hz, H-7″), 4.52 (dd, 1H, $J_{2',3'}$ = 10.2 Hz, $J_{3',4'}$ = 3.2 Hz, H-3′), 3.84 (s, COOCH_3), 2.58 (dd, $J_{3''a,3''e}$ = 12.6 Hz, $J_{3''e,4''}$ = 4.6 Hz, H-3″*eq*), 2.49 (t, J_{vic} = 7.0 Hz, SCH_2–), 2.43 (t, J_{vic} = 7.0 Hz, SCH_2–), 2.25–1.85 (each s, NH*Ac*, O*Ac*), 1.55 (br, $OCH_2CH_2CH_2CH_2CH_2S$), 1.39 (br, 6H, –$CH_2CH_2CH_2S$), 0.92 (br, 6H, SiCH_2). FAB-MS Calcd for $C_{162}H_{233}N_3O_{87}S_3Si$ $[M+Na]^+$: 3761.28. Found: *m/z* 3760.84.

4.1.13. Ball(0)4-SLac-Ac (36)

Coupling reaction between ball-type dendrimer **17** (47 mg, 0.06 mmol) and thioacetate **34** (430 mg, 0.35 mmol) was carried out by the method described for **35** to give Ball(0)4-Slac-Ac **36** (50 mg, 33.1%).

^1H NMR δ ($CDCl_3$) 4.68 (d, 4 H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.49 (d, 4H, $J_{1,2}$ = 8.0 Hz, H-1), 3.84 (s, COOCH_3), 2.59–2.44 (m, 20 H, H-3″*eq*, –CH_2SCH_2–), 0.56 (br, 8H, SiCH_2–).

4.1.14. Dumbbell(1)6-SLac-Ac (37)

Coupling reaction between dumbbell-type dendrimer **18** (47 mg, 0.06 mmol) and thioacetate **34** (430 mg, 0.35 mmol) was carried out by the same method as that described for **35** to give Dumbbell(1)6-Slac-Ac **37** (79 mg, 76.7%) including 1″,2′-lactone form. In addition, dimer **38** (54 mg) and starting material **34** (38.4 mg) were also obtained.

R_f 0.3 [10:1 (v/v) $CHCl_3$–MeOH]; IR (KBr) 2941 (ν_{C-H}), 1749 ($\nu_{C=O}$), 1670 ($\nu_{C=O}$), 1541 (ν_{N-H}), 1437 (ν_{C-N}), 1234 (ν_{C-C}), ****1041 (ν_{C-O-C}) cm^{-1}; ^1H NMR δ ($CDCl_3$) 5.50 (m, H-8″), 5.36 (dd, 3H, $J_{6'',7''}$ = 2.7 Hz,

$J_{7'',8''}$ = 9.1 Hz, H-7″), 5.19–4.59 (m), 4.49 (dd, 1H, $J_{2',3'}$ = 10.4 Hz, $J_{3',4'}$ = 3.0 Hz, H-3′), 4.43–3.70 (m), 3.81 (s, COOCH_3), 3.62 (m, 12H, H-5, H-6″), 3.43 (m, 6H, one of OCH_2–), 2.55 (dd, H-3″*eq*), 2.46 (t, J_{vic} = 7.0 Hz, SCH_2–), 2.45 (t, J_{vic} = 7.0 Hz, SCH_2–), 2.43–1.83 (m, NH*Ac*, O*Ac*, –*CH*), 1.53 (br), 1.38 (br), 1.23 (br, SiCH$_2$*CH*$_2$CH$_2$Si), 0.56 (br, 20H, SiCH_2–), −0.09 (s, 6H, SiMe_2). FAB-MS Calcd for $C_{320}H_{474}N_6O_{174}S_6Si_3$ [M+Na]$^+$: 7488.61. Found: *m/z* 7488.30.

4.1.15. Fan(0)3-amide-SLac-OAc (39)

Coupling reaction between Fan(0)3-amide-Br dendrimer **19** (47 mg, 0.06 mmol) and thioacetate **34** (430 mg, 0.35 mmol) was carried out by the same method as that described for **35** to give **39** (155 mg, 65.7%).

^{1}H NMR δ ($CDCl_3$) 7.39 (m, 5H, *Ph*), 4.67 (d, 3H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.52 (dd, 3H, $J_{2',3'}$ = 10.2 Hz, $J_{3',4'}$ = 3.2 Hz, H-3′), 3.84 (s, COOCH_3), 3.59 (m, 3H, one of OCH_2–), 3.21 (m, 6H, NCH_2–), 2.58 (dd, 3H, $J_{3''a,3''e}$ = 12.3 Hz, $J_{3''e,4''}$ = 4.3 Hz, H-3″*eq*), 2.47 (m, 12H, SCH_2–), 0.79 (m, 6H, SiCH_2–).

4.1.16. Ball(0)4-amide-SLac-OAc (40)

Coupling reaction between Ball(0)4-amide-Br dendrimer **20** (33 mg, 0.06 mmol) and thioacetate **34** (350 mg, 0.29 mmol) was carried out by the same method as that described for **35** to give **40** (28 mg, 15.5%).

^{1}H NMR δ ($CDCl_3$) 4.67 (d, 4 H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.44 (d, 4H, $J_{1,2}$ = 8.0 Hz, H-1), 3.84 (s, COOCH_3), 3.18 (m, 8H, NCH_2–), 2.48 (q, 16H, J = 6.8 Hz, SCH_2–), 0.51 (m, 8H, SiCH_2–).

4.1.17. Dumbbell(1)6-amide-SLac-OAc (41)

Coupling reaction between Dumbbell(1)6-amide-Br dendrimer **21** (48 mg, 0.03 mmol) and thioacetate **34** (400 mg, 0.33 mmol) was carried out by the same method as that described for **35** to give **41** (85 mg, 34.8%).

^{1}H NMR data could not be assigned due to intramolecular lactonization.

4.1.18. Fan(0)3-SLac (2)

To a solution of fully protected dendrimer **35** (26 mg, 0.0070 mmol) in MeOH (2 mL) was added NaOMe, and the mixture was stirred overnight at room temperature. IR-120B (H^+) resin was added to the mixture, and the mixture was filtered and concentrated. 0.025 M aq NaOH (2 mL) was added to the residue, and the solution was stirred overnight at room temperature. IR-120B (H^+) resin was added to the mixture, and the suspension was filtered and concentrated. The residual syrup was purified by gel permeation chromatography using Sephadex G-25 with 5% aq AcOH to afford **2** in quantitative yield.

IR (KBr) 3396 (ν_{O-H}), 2927 (ν_{C-H}), 1730 ($\nu_{C=O}$), 1633 ($\nu_{C=O}$), 1566 (ν_{N-H}), 1072 (ν_{C-O-C}), 1034 (ν_{C-O-C}), 706 (ν_{N-H}), 619 (ν_{N-H}) cm^{-1}; ^{1}H NMR δ (D_2O) 7.30 (m, 5H, Si*Ph*), 4.45 (br, 3H, H-1′), 4.32 (br, 3H, H-1), 4.07 (near d, 3H, H-3′), 3.98–3.46 (m), 3.24 (br, 3H, H-2), 2.66 (near d, 3H, H-3″*eq*), 2.38 (br, 12H, –CH_2SCH_2–), 1.95 (s, 9H, ND*Ac*), 1.84, 1.46, and 1.32 (3m), 0.81 (br, 6H, CH_2Si).

$^*\delta$ 2.33 (near dd, H-3″*eq* of lactone) included about 8%.

FAB-MS Calcd for $C_{99}H_{167}N_3O_{57}S_3Si$ [M+H]$^-$: 2433.9. Found: *m/z* 2434.1.

4.1.19. Ball(0)4-SLac (3)

Removal of all protection of Ball-type dendrimer **36** (50 mg, 0.010 mmol) was carried out by the same method as that described for **2** to give water-soluble **3** (30 mg, 93.8%).

^{1}H NMR δ (D_2O) 4.46 (d, 4H, $J_{1',2'}$ = 7.0 Hz, H-1′), 4.38 (d, 4H, $J_{1,2}$ = 7.5 Hz, H-1), 2.68 (dd, 4H, $J_{3''a,3''e}$ = 12.9 Hz, $J_{3''e,4''}$ = 4.3 Hz, H-3″*eq*), 1.82 (m, 4H, H-3″*ax*), 0.66 (br, 6H, CH_2Si). MALDI-TOF-MS Calcd for $C_{124}H_{216}N_4O_{76}S_4SiNa$ [M+Na]$^+$: 3158.4. Found: *m/z* 3159.4.

4.1.20. Dumbbell(1)6-SLac (4)

Removal of all protection of Dumbbell-type dendrimer **37** (45.4 mg, 0.0365 mmol) was carried out by the same method as that described for **2** to give water-soluble **4** in quantitative yield.

IR (KBr) 3406 (ν_{O-H}), 2931 (ν_{C-H}), 1730 ($\nu_{C=O}$), 1643 ($\nu_{C=O}$), 1566 (ν_{N-H}), 1032 ($\nu_{C-°C}$), 624 (ν_{N-H}), cm^{-1}; ^{1}H NMR δ (D_2O) 4.46 (near d, 6H, H-1′), 4.36 (near d, 6H, H-1), 4.08 (near d, 6H, H-3′), 3.99–3.46 (m), 3.26 (br, 6H, H-2), 2.67 (near d, 3H, H-3″*eq*), 2.50 (br, 24H, –CH_2SCH_2–), 1.96 (s, 18H, ND*Ac*), 1.85, 1.57, and 1.41 (3 br), 0.63 (br, 20H, Si CH_2), −0.06 (br, 6H, SiMe_2).

$^*\delta$ 2.23 (near dd, $J_{3''a,3''e}$ = 12.9 Hz, $J_{3''e,4''}$ = 4.8 Hz, H-3″*eq* of lactone) included about 3%.

FAB-MS Calcd for $C_{194}H_{342}N_6O_{114}S_6Si_3$ [M+H]$^-$: 4857.9. Found: *m/z* 4859.2.

4.1.21. Fan(0)3-amide-SLac (5)

Removal of all protection of Fan-amide-type dendrimer **39** (106 mg, 0.026 mmol) was carried out by the same method as that described for **2** to give water-soluble **5** in quantitative yield.

^{1}H NMR δ (D_2O) 7.26 (m, 5H, *Ph*), 4.40 (d, 3H, $J_{1',2'}$ = 7.5 Hz, H-1′), 4.29 (d, 3H, $J_{1,2}$ = 7.5 Hz, H-1), 2.61 (br, 3H, H-3″*eq*), 2.34 (m, 12H, –CH_2SCH_2–), 0.65 (br, 6H, CH_2Si). MALDI-TOF-MS Calcd for $C_{117}H_{200}N6O_{60}S3SiNa$ [M+Na]$^+$: 2798.1. Found: *m/z* 2799.3.

4.1.22. Ball(0)4-amide-SLac (6)

Removal of all protection of Ball-amide-type dendrimer **40** (22 mg, 0.004 mmol) was carried out by the same method as that described for **2** to give water-soluble **6** (9.0 mg, 64%).

^{1}H NMR δ (D_2O) 4.50 (d, 4H, $J_{1',2'}$ = 7.9 Hz, H-1′), 4.43 (d, 4H, $J_{1,2}$ = 7.9 Hz, H-1), 3.27 (br, 8H, NCH_2), 2.72 (dd, 4H, $J_{3''a,3''e}$ = 12.5 Hz, $J_{3''e,4''}$ = 4.2 Hz, H-3″*eq*), 2.55 (m, 16H, SCH_2), 2.22 (m, 8H, CH_2), 2.00 (s, 12H, ND*Ac*), 1.83 (t, 4H, $J_{3''a,3''e}$ = $J_{3''a,4''}$ = 12.0 Hz, H-3″*ax*), 0.53 (br, 8H, CH_2Si). MALDI-TOF-MS Calcd for $C_{148}H_{260}N_8O_{80}S_4SiNa$ [M+Na]$^+$: 3611.04. Found: *m/z* 3610.44.

4.1.23. Dumbbell(1)6-amide-SLac (7)

Removal of all protection of Dumbbell-amide-type dendrimer **41** (85 mg, 0.010 mmol) was carried out by the same method as that described for **2** to give water-soluble **7** (44 mg, 77%).

^{1}H NMR δ (D_2O) 4.51 (br, 6H, H-1′), 4.41 (br, 6H, H-1), 4.12 (br, 6H, H-3′), 2.53 (br, 24H, –CH_2SCH_2–), 0.57 (m, 20H, –CH_2Si), 0.03 (br, 6H, SiCH_3). MALDI-TOF-MS Calcd for $C_{230}H_{408}N_{12}O_{12}S_6Si_3Na$ [M+Na]$^+$: 5561.4. Found: *m/z* 5562.4.

4.2. Viruses

Influenza viruses A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/Memphis/1/71 (H3N2) were cultured in 11-day-old embryonic chicken eggs, purified, and concentrated as described previously.[27] Viral hemagglutinin (HA) units were determined in microtiter plates using 0.5% guinea pig erythrocytes.

4.3. Hemagglutination inhibition assay (HAI)

The hemagglutination inhibition (HAI) assay was carried out using 96-well microtiter plates as described previously.[28] Phosphate buffer saline (pH 6.5) containing 0.01% gelatin was used as a dilution buffer. Human erythrocytes were used as indicator cells. Virus suspension (2^2 HA units in 25 μL of PBS) was added to each well containing an inhibitor in twofold serial dilutions with the dilution buffer. The plates were incubated for 1 h at 4 °C. After 0.05 ml of 0.5% (v/v) chicken erythrocytes in PBS had been added to the plates, the plates were kept at 4 °C for 1 h. The maximum dilution of the samples showing complete inhibition of the hemagglutination was defined as the hemagglutination inhibition titer.

4.4. Hemolysis inhibition assay

The hemolysis inhibition assay was carried out as described previously.[28] Briefly, influenza viruses (2^8 HAU) were preincubated for 1 h at 4 °C with various concentrations of each agent in 50 μL of PBS. The mixtures were then reacted for 30 min at 4 °C with 0.5 mL of PBS containing 2.5% (v/v) guinea pig erythrocytes. Erythrocytes were sedimented and resuspended in 0.5 mL of 20 mM acetate-buffered saline (pH 5.0) for hemolysis mediated by influenza A virus (A/PR/8/34). The hemolysis was carried out for 30 min at 37 °C and terminated by addition of an equal volume of PBS. The mixtures were centrifuged, and the concentrations of hemoglobin in the supernatants were determined by measuring the absorbance at 550 nm. To achieve complete hemolysis, Triton X-100 solution was added to the erythrocyte suspension instead of the virus-reagent mixture with a final concentration of 0.1% (v/v). The percent hemolysis of each agent was calculated by comparing its absorbance with the absorbance of 0.1% Triton X-100, taken as 100% hemolysis.

4.5. Neutralization assay (infection inhibition)

The neutralization of influenza virus with synthetic compounds was determined as previously described.[29] Madin-Darby canine kidney (MDCK) cell monolayers were maintained in Eagle's minimum essential medium (EMEM) containing 5% fetal calf serum. One hundred microliters of TCID50 (50% tissue culture infectious dose) of influenza A virus (A/PR/8/34) in the presence of an inhibitor (0.5–4000 μM) was inoculated at 34.5 °C for 5 h. After removal of the inoculum, the monolayers were washed three times with EMEM. The cells were examined using a light microscope for the progression of viral-induced cytopathic effects (CPE) after incubation at 34.5 °C for 20 h. The lactate dehydrogenase (LDH) that was released from MDCK cells was examined for virus neutralization by a slightly modified colorimetric assay.[30] Fetuin was used as a control. The LDH activities in the medium were determined according to the manufacturer's instructions. Briefly, the medium (12.5 μL) was diluted to 1:4 with PBS and mixed with 50 μL of LDH reagent (Shinotest, Japan). The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.1 mL of 0.5 M HCl. Absorbance was measured at 550 nm (reference at 630 nm). The assays were performed in duplicate.

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