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S-Linked Sialyloligosaccharides Bearing Liposomes and Micelles as Influenza Virus Inhibitors

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Abstract
An efficient, homogeneous synthesis of phospholipid conjugation of S-Neu5Acα2-6Galβ1-4GluNAcβ1-3 (3) and its 6-sulphate analogue 4 has been developed. The self-assembled micelles and liposomes of these trisaccharides formed in solution were found to be inhibitors to interfere with the entry of the H1N1 influenza virus into MDCK cells. Compound 3 bearing liposome and micelle displayed superior inhibitory activity than its 6-sulfate congener 4 in both the virus neutralization assay and the hemagglutination inhibition assay.

Introduction

Human influenza, a highly contagious viral disease that infect the epithelial cells of the respiratory tract, is responsible for over half million deaths worldwide every year. The initiation and sustenance of the infection process between a viral particle and the host cell depends on two membrane-bounded glycoproteins: hemagglutinin (HA) and neuraminidase (NA). To fight influenza, much effort has been devoted to the design of neuraminidase inhibitors, such as oseltamivir and zanamivir, which have seen clinical use for a decade. Oseltamivir resistance, however, is a growing problem, and new drugs to combat influenza are required. Although the targeting of NA has been proven as a viable strategy for anti-influenza agents, glycan microarray studies revealed that HAs recognize more specific structural features of the glycan on host cells. For example, HAs of human-adapted influenza viruses have binding preferences for a host glycan that contain Neu5Acα2-6Galβ1-4GluNAcβ1-3 (Figure 1, structures 1 and 2); whereas avian influenza prefers a terminal α2-3 sialic acid linkage. Array results also indicated that the extended chain (Galβ1-4GluNAcβ1-3) was critically important for viral recognition. However, although HA inhibition is an option for the treatment of influenza, very few studies regarding HA inhibitors have been reported, perhaps because of the complexity that synthesizing HA binding glycans entails (design of HA inhibitors is challenging because NA cleaves O-sialoside rapidly in vivo). In addition, the binding between multiple HA ligands of an influenza virus and sialic acid surface receptors of an erythrocyte during viral infection is estimated to occur with an affinity of 10^{13} M^{-1}, while the association constant of a single sialic acid-HA interaction is 10^{3} M^{-1}, and therefore high multivalency is required for strong binding affinity between HA and HA-bound glycans.

Several multivalent vectors functionalized with sialic acid, such as dendrimers, polymers, liposomes, and nanoparticles have been reported and their ability to inhibit activities of the corresponding lectins proven, but all are either heterogeneous mixtures, unsuitable to be used in biochemical issues, and/or contained only a monosaccharide (sialic acid) for protein recognition which may be not sufficient for HA inhibition. Also, natural O-linked sialic acid is NA labile, and
thus targeting of HA without considering the effect of NA would be problematic. However, the $S$-linkage is impervious to endogenous glycosidase.$^{12}$ In addition, sulfur is electronically and sterically similar to the oxygen and therefore $S$-linked oligosaccharides are frequently used when a stable $O$-linked oligosaccharide mimic is required. Several $S$-linked sialic oligosaccharides have been demonstrated to inhibit glycosidases.$^{21,22}$ In addition, the $S$-linkage is highly flexible between glycosyl units, and so $S$-linked oligosaccharide generally possess more low energy conformers in solution than natural oligosaccharide, presenting more opportunities for advance binding with the natural protein.$^{23,24}$ Recently an $\alpha2$-$6$-thio-linked sialic acid functionalized nanoparticle was reported to bind with human influenza virus with high specificity.$^{25}$ Therefore, inspired by the interaction of HA ligands– Neu5Aca$^{2}$-6Gal$^{1}$-4GluNAc$^{1}$-3 derivatives (1-2) with human influenza virus detected by glycan array,$^{10}$ we synthesized compound 3 and 4 as virus inhibitor mimics of 1 and 2, wherein the stability of the Neu5Aca$^{2}$-6Gal glycosidic bond was enhanced using a thio-linkage (Figure 1). These sialyloligosaccharides were then attached onto a biocompatible phospholipid- DLPE (1,2-dilauroyl-sn-glycero-3-phosphoethanolamine), in order to obtain a multivalent ligand. To the best of our knowledge, there is no previous report of an $S$-linked trisaccharide able to directly interact with intact virus in solution.

![Figure 1](image-url). Structure of $O$-linked Neu5Aca$^{2}$-6Gal$^{1}$-4GluNAc$^{1}$-3 glycans 1, 2 and $S$-linked glycosides 3, and 4 conjugated with phospholipid (DLPE).

**Results and Discussion**

The method for the synthesis of $\alpha2$-$6$ thioglycosides 3 and 4 is outlined in Scheme 1. Compound 5$^{26}$ was first coupled with methyl glycolate, which served as a linker for further conjugation with phospholipid, using NIS/TfOH to afford 6 in 85% yield and excellent selectivity. Hydrogenation of the benzylidene acetal of 6 with H$_2$ and 10% Pd/C afforded the diol intermediate, and the resulting primary alcohol was then selectively protected using TBDMSCl and imidazole$^{27}$ to give 7. Glycosylation of 7 with thioglycoside donor 8$^{28}$ was performed using BSP/Tf$_2$O activation in the presence of two equivalences of TTBP to give 9 in 90% yield.$^{29}$ Removal of the
$N$-Troc group of 9 was carried out using the Zn/Ac$_2$O reduction method, which allowed a one-pot conversion of a $N$-Troc group into an $N$-acetyl group of 10.

Before the construction of $\alpha$2-6 thio-linkage between sialic acid and galactose could take place, the 4, 6-benzyldiene of 10 had to be removed. Selective deprotection of the benzylidene acetal without affecting the TBDMS group proved impossible, however. Accordingly, we decided to change the TBDMS group to the more stable protecting group-TBDPS. Thus, TBAF/AcOH was used to remove the TBDMS, and the resulting hydroxyl group protected as a silyl ether by TBDPSCl in the presence of Et$_3$N to afford compound 11. It is noteworthy that using TBDPS in place the TBDMS group of compound 7 was impractical as the subsequent glycosylation step (with compound 8) did not work, perhaps due to the higher steric hindrance of TBDPS group compared with TBDMS group. After this transformation, the benzylidene acetal of 11 was successfully hydrogenolyzed using H$_2$ and 10% Pd/C to afford the diol 12, in which the TBDPS group remained intact. With compound 12 in hand, sulfonylation of the primary alcohol using Tf$_2$O and pyridine in dichloromethane yielded the triflate intermediate. Nucleophilic substitution of the triflate with Neu5Ac thiolate yielded the $S$-linked trisaccharide 14 in 85% yield. Notably, none of the beta anomer was obtained. After the formation of thio-linkage, the remaining 4'-OH of 14 was acetylated using Ac$_2$O, and then the 6-$O$-TBDPS was deprotected using HF-pyridine to give trisaccharide 15 (Scheme 1). Lead compound 16 was obtained by treating with catalytic NaOMe in methanol for 12 h to remove the acetyl and benzyl ester groups and then using sodium hydroxide to remove the methyl ester of compound 15. The 6-sulfate group of compound 17 was constructed by treating compound 15 with SO$_3$·TMA in DMF at 60 °C, then followed by a similar sequence of deprotections as for compound 16.

Both $S$-linked trisaccharides 16 and 17 were subjected to conjugation with DLPE via amide bond formation. DLPE is a kind of phospholipid found in biological membranes, which contains an amino group on the hydrophilic side. Coupling of 16 with DLPE to give compound 3 using various amide bond formation reagents (HBTU or EDC+HOBt or EDC+sulfo:NHS) at first was failed. Attempting the reaction in various buffer systems (MES buffer or PBS) or with two-step pH adjustments (maintaining the pH at around 5-6 during acid activation, and then increasing to over 7 for the amide bond formation step) were also unsuccessful (very little or no product). Finally, using strong acid cation exchanging resin to remove sodium cations of $S$-linked trisaccharides 16, the resulting glycolic acid was able to conjugate with DLPE via EDC/HOBt/NMM mediated amide bond formation to afford compound 3. Compound 16 has two carboxylic groups, but only the glycolic acid moiety was able to conjugate to DLPE; the carboxylic group at the 2 position of the sialic acid being too unreactive for conjugation with DLPE, due to the steric hindrance. Coupling of
17 with DLPE was more challenging, because the sulfate group is moderately sensitive to acidic environments. To avoid cleavage of the sulfate group in the presence of the strongly acidic cation exchanging resin, a pH of 3.5-4.5 was maintained. After amide bond formations, crudes 3 and 4 were purified by Sephadex LH-20. OTBDMS

Scheme 1. Synthesis of DLPE conjugated 3 and 4. (a) NIS, TfOH, methyl glycolate, DCM, 4Å MS, -40 °C, 1 h, 85%; (b) 1) H$_2$, 10% Pd/C, rt, 12 h; 2) TBDMSI, imidazole, THF, rt, 8 h, 58% two steps; (c) 8, BSP, TTBP, Tf$_2$O / DCM, -60 °C, 1.5 h, 90%; (d) Zn, Ac$_2$O, Et$_3$N, DCM, rt, 6 h, 51%; (e) 1) TBAF, AcOH, THF, rt, 18 h, 79%; 2) TBDPSI, Et$_3$N, DMAP, THF, rt, 12 h, 95%; (f) H$_2$, 10% Pd/C, rt, 12 h, 79%; (g) 1) Tf$_2$O, pyridine, DCM, -25°C, 1.5 h; 2) Et$_3$NH, DMF, -20°C, 3 h, 85% two steps; (h) 1) Ac$_2$O, pyridine, DMAP, 0 °C to rt, 5 h; 2) HF-pyridine, THF, rt, 12 h, 68% (two steps); (i) 1) NaOMe/MeOH, rt, 16 h; 2) 0.2 N NaOH, rt, 10 h, for 16 (96% for 2 steps); (j) 1) SO$_3$•TMA, DMF, 60 °C, 12 h, 86%; 2) NaOMe/MeOH, rt, 12 h; 3) 0.2 N NaOH, rt, 10 h, 3 steps.
for 17 (82 % for 3 steps) (k) 1) Amberlite® IR120 H+; 2) DLPE, EDC, HOBt, NMM / DMF, 35 °C, 9 h, two steps 39% for 3 and 11% for 4.

Products 3 and 4 were mixed with dipalmitoylphosphatidyl-choline (DPPC) and cholesterol (CH) in specific ratios (DPPC: CH: 3 or 4 = 4: 1: 1, molar ratio) by sonication to form liposomes (Lipo-3 and Lipo-4). The size distribution profiles were monitored using dynamic light scattering (DLS, see Supporting Information). The synthetic S-linked glycosides 3 and 4 and their liposomal forms Lipo-3 and Lipo-4 were subjected to the A/WSN/33 H1N1 influenza entry inhibition assay wherein neutralization of the virus is defined as the loss of infectivity through reaction of the virus with specific blockers, such as antibodies and inhibitors. In this neutralization assay, the amount of viral nucleoprotein (NP) in Madin-Darby canine kidney (MDCK) cells was detected after infection. Since the virus entered the cell through HA:sialic acid associated endocytosis, the ability of the test compound to inhibit virus entry can be evaluated by quantifying the amount of NP.33 As shown in Figure 2, the lipo-control exhibited weak entry inhibition (about 15% inhibition in 1mM), perhaps due to colloidal dispersions (steric stabilization), and/or limited mobility of the virus due to the high concentration of liposome increasing the viscosity of the environment. Lipo-3, however, markedly inhibited virus entry (IC\textsubscript{50} of 71 ± 5.5 µM, Table 1) compared with Lipo-4 (IC\textsubscript{50} of 280 ± 30 µM, Table 1), a result consistent with a previous glycan array study of HA receptor specificity,34 indicating that human influenza virus recognized the Neu5Acα2:6Galβ1:4GluNAcβ1:3 (1) moiety better than its sulfated congener (2). In addition, the relatively larger particle size of Lipo-4 (356 ± 34 nm, see Supporting Information) compared to Lipo-3 (196 ± 16 nm) might cause some sugar incorporation inside the liposome or between lipid bilayers. Thus, the density of sugar on Lipo-4 might be lower than Lipo-3, leading to decrease of activity and avidity.

To our surprise, compound 3 (EC\textsubscript{50} 99 ± 6.9 µM, Table 1) alone was as potent as Lipo-3 in this assay, and inhibited 100% virus entry at 500 µM (Figure 2). It was suggested compound 3 may self-assembled into micelles in aqueous solutions, because it bears both hydrophobic (DLPE) and hydrophilic (sugar) moieties.35 To investigate this, a solution of compound 3 in PBS (5 mM) was subjected to analysis by transmission electron microscope (TEM). Spherical micelles with the diameter of around 50 nm were observed (Figure 3a), confirming that 3 can form micelles spontaneously in aqueous solution. TEM image of Lipo-3 in solution showed particles of 100-200 nm (Figure 3b), which correlated well with the results from particles size analysis by DLS. Since spontaneously formation of micelles (compound 3 and 4 in PBS solution) have smaller particle size than liposomal Lipo-3 and Lipo-4, they are anticipated to express a higher surface density of glycans, explaining why compound 3 alone was potent and exhibited full inhibition of influenza
entry. The superior potency of compound 4 (EC$_{50}$ 140 ± 12 µM) compared to its liposomal form Lipo-4 (EC$_{50}$ 280 ± 30 µM) can be similarly explained.

The influenza virus HA protein also binds sialic acids on red blood cells (RBCs) resulting in hemagglutination. The ability of influenza-specific molecules that bind to HA protein to prevent hemagglutination of RBCs is the basis for the hemagglutination inhibition (HAI) assay, a quantitative and inexpensive method for diagnosing influenza infection serologically.$^{36,37}$ The lowest concentration of inhibitor that prevented hemagglutination is defined as $K_i$ (Table 1).$^{38}$ Results from HAI assay are consistent to the virus entry assay, i.e. compound 3 and Lipo-3 displayed better inhibitory activity than compound 4 and Lipo-4, respectively. In addition, sialic acid, compound 16, and 17 all failed to display any inhibitory activity (data not shown). This evidence indicates that sialic oligosaccharide conjugation with DLPE for multivalent carbohydrate display either by liposome or micelle is important in the development of HA inhibitors. Sialyneolacto-N-tetraose-bearing liposomes reported recently performed well in virus-entry inhibition,$^{39}$ the inconsistent nature of our results to these might be due to the different assay conditions used or that a longer sialic containing oligosaccharide was used for HA binding. This is currently being investigated, and results will be reported in due course.

**Figure 2.** A/WSN/33 H1N1 neutralization assay of Lipo-control, 3, 4, Lipo-3, and Lipo-4. The presence of NPs were detected by ELISA with a monoclonal antibody to the influenza A NP followed by incubation with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG, and then O-phenylenediamine dihydrochloride (OPD) was used as substrate with absorbance read at 492 nm.
Table 1. Assessment of different compounds and their liposome formulations by A/WSN/33 H1N1 neutralization assay (EC_{50}) and hemagglutination inhibition assay (K_i).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC_{50} (µM)</th>
<th>K_i (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipo-control</td>
<td>15 %^a</td>
<td>NI^b</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td><strong>Lipo-3</strong></td>
<td>71 ± 5.5^c</td>
<td>125</td>
</tr>
<tr>
<td><strong>Lipo-4</strong></td>
<td>280 ± 30</td>
<td>500</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>99 ± 6.9</td>
<td>125</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>140 ± 12</td>
<td>250</td>
</tr>
</tbody>
</table>

^a inhibition at 1 mM; ^b NI: no inhibition; ^c Data represents as mean ±SD from at least three determinations.

(a) ![Image](image1.png) (b) ![Image](image2.png)

**Figure 3.** TEM images of (a) 3 in PBS as micelle morphology; (b) **Lipo-3** in PBS as liposome morphology.

**Conclusions**

High-yielding syntheses of thio-linked sialic containing trisaccharides 16 (20 steps, 8% overall yield) and its sulfated congener 17 (21 steps, 7% overall yield) have been developed. Upon conjugation of 16 and 17 with DLPE, the resulting trisaccharides 3 and 4 were found to moderately inhibit the A/WSN/33 H1N1 influenza virus by interfering with the initial virus entry process, whether used directly (when it was found using TEM that they self-assembled into micelles), or first made into liposomes. Compound 3 when displayed on liposome and micelle surface was found to have superior inhibitory activity than its 6-sulfate congener in both the virus neutralization assay and hemagglutination inhibition assay. The ability of these trisaccharides as targeting ligands on the liposomal and micelle surfaces makes them extremely versatile tools for the future evaluation of new strategies against influenza, and research into the interactions of sialic acid with lectins, such as
Sambucus nigra agglutinin (SNA),\textsuperscript{40} Limax flavus agglutinin (LFA),\textsuperscript{41} human polyoma virus,\textsuperscript{42} and CD22 related studies.\textsuperscript{43}

**Experimental Section**

**General methods**

All reagents and solvents were reagent grades and were used without further purification unless otherwise stated. The progress of the reaction was monitored by analytical TLC on 0.25 mm E. Merck silica gel 60 F\textsubscript{254} using p-anisaldehyde, ninhydrine, and cerium as visualizing agents. Bruker-AV-400 (400 MHz) and Bruker-AV-600 (600 MHz) with TCI cryo probe and a BACS 60 sample changer. Chemical shift (\(\delta\)) are given in parts per million relative to \(^1\text{H}: 7.24\) ppm, \(^{13}\text{C}: 77.0\) ppm for CDCl\(_3\), \(^1\text{H}: 8.74\) ppm, \(^{13}\text{C}: 150.35\) ppm for pyridine-D5, \(^1\text{H}: 4.80\) ppm for D\(_2\)O, and \(^1\text{H}: 4.78\) ppm, \(^{13}\text{C}: 49.2\) ppm for methanol-D4. The splitting patterns are reported as s (singlet), bs (broad singlet), d (doublet), bd (broad doublet), t (triplet), q (quartet), dd (double doublet), m (multiplet). Coupling constant (\(J\)) was given in Hz. All peak assignments were confirmed using 2DNMR (COSY, HSQC) techniques. Exact mass measurements were performed on VG platform electrospray ESI/MS or BioTOF II (Taiwan). DLS experiment used equipment - BIC 90 PLUS, Brookhaven Instrument Co. Holtsville, NY, USA. Tecnai G\textsuperscript{2} Transmission Electron Microscope, FEI Co. was used for TEM images.

**3-O-Benzoyl-6-O-tert-butyldimethylsilyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-\(\beta\)-\(\delta\)-glucopyranoside (7).** To a stirred solution of thioglycoside 5 (20.0 g, 30.6 mmol) and methyl glycolate (4.66 mL, 61.26 mmol) in dry DCM (200 mL) was added 4A molecule sieves, NIS (8.27 g, 36.75 mmol) and TfOH (0.54 mL, 6.13 mmol) at -40 °C and the mixture was stirred for 1 h. After the reaction completed, it was quenched by sat. aqueous sodium thiosulfate, and then the mixture was diluted with DCM, washed with sat. NaHCO\(_3\), and brine. The organic layers were collected, dried over MgSO\(_4\), filtered and concentrated under reduced pressure to give residue as colorless oil. The crude was subjected to a short silica packing column elution with ethyl acetate : hexane = 1 : 1, and then concentrated under reduced pressure to give compound 6 (16.1 g, 85\%). To a stirred solution of this residue 6 (6.77 g, 10.95 mmol) in chloroform (50 mL) and methanol (50 mL) was added 10% Pd/C (677 mg) and stirred under H\(_2\) (1 atm) for 12 h. The mixture was filtered through a celite pad, and the filter cake was washed with chloroform. The filtrate was concentrated under reduced pressure to obtain the crude residue. To a stirred solution of the crude residue in dry THF (70 mL) containing imidazole (1.84 g, 27.39 mmol), and TBDMSCl (2.48 mL, 16.4 mmol) were added in four portions. After being stirred for 8 h, the reaction mixture was quenched by methanol and evaporated under reduced pressure. The residue was diluted with DCM and washed with sat. NaHCO\(_3\), water and brine. The organic layers were collected and
concentrated under reduced pressure. The residue was purified by chromatography (silica gel; ethyl acetate : hexane = 1 : 3) to give compound 7 (4.94 g, 2 steps yield 70 %) as colorless solid; mp 62 °C; \(^{1}\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 8.02 (dd, \(J = 8.0, 1.2\) Hz, 2H, Ar-H), 7.52 (dd, \(J = 7.2, 1.2\) Hz, 1H, Ar-H), 7.38 (dd, \(J = 8.0, 7.2\) Hz, 2H, Ar-H), 5.82 (bd, \(J = 9.2\) Hz, 1H, N-H), 5.35 (t, \(J = 9.6\) Hz, 1H, H-3), 4.73 (d, \(J = 9.6\) Hz, 1H, Troc-CH\(_2\)), 4.72 (d, \(J = 12.0\) Hz, 1H, Troc-CH\(_2\)), 4.47 (d, \(J = 12.0\) Hz, 1H, Troc-CH\(_2\)), 4.33 (d, \(J = 16.8\) Hz, 1H, -OCH\(_2\)C=O), 3.95–3.87 (m, 3H), 3.85 (t, \(J = 9.6\) Hz, 1H), 3.70 (s, 3H, :OMe), 3.51–3.46 (m, 2H), 0.87 (s, 9H, TBDMS-tert-butyl), 0.07 (s, 3H, TBDMS:CH\(_3\)), 0.06 (s, 3H, TBDMS:CH\(_3\)) ppm; \(^{13}\)C-NMR (CDCl\(_3\), 100 MHz) \(\delta\) 170.3, 167.1, 154.8, 133.4, 130.0, 129.2, 128.3, 100.0 (C:1), 95.4 (CH\(_2\)CCl\(_3\)), 76.0 (C-3), 75.0 (C-4), 74.2 (C-5), 71.1 (CH\(_2\)CCl\(_3\)), 64.4 (C-6), 63.9 (C-2), 63.5 (C:2), 18.2 (Si:C(CH\(_3\))\(_3\)), -6.3 (Si:CH\(_3\)) ppm; HRMS (ESI) calcd. for C\(_{25}\)H\(_{36}\)Cl\(_3\)NO\(_{10}\)NaSi [M+Na\(^+\)]: 666.1066, found: m/z 666.1097.

4-O-[2,3-Di-O-benzoyl-4,6-di-O-benzylidene-\(\beta\)-D-galactopyranosyl]-1→4)-3-benzoyl-6-O-tert-butylidimethylsilyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-\(\beta\)-D-g lucopyranoside (9). To a stirred solution containing the thioglycoside 8 (4.52 g, 7.76 mmol), glycosyl acceptor 7 (3.5 g, 5.43 mmol), BSP (1.62 g, 7.76 mmol), TTBP (3.85 g, 15.52 mmol), and activated 4Å powdered molecular sieves in DCM (80 mL) at -60 °C under N\(_2\), was added Tf\(_2\)O (1.44 mL, 8.54 mmol) dropwise. After being stirred at -60 °C for 1.5 h, the reaction mixture was warmed to rt, filtered, washed with sat. NaHCO\(_3\), followed by brine, dried over MgSO\(_4\), and concentrated under reduced pressure. The residue was purified by chromatography (silica gel; toluene : acetone = 20 : 1) to give compound 8 (5.4 g, 90 % yield) as oil; \(^{1}\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 8.10 (d, \(J = 7.2\) Hz, 2H, Ar:H), 7.88–7.83 (m, 4H, Ar :H), 7.47–7.42 (m, 4H, Ar :H), 7.32–7.18 (m, 10H, Ar :H), 5.60 (dd, \(J = 10.2, 7.8\) Hz, 1H, H-2′), 5.54 (dd, \(J = 10.2, 2.8\) Hz, 1H, H-3), 5.25 (s, 1H, CHPh), 5.09 (dd, \(J = 10.2, 3.6\) Hz, 1H, H-3′), 4.92 (d, \(J = 7.8\) Hz, 1H, H-1′), 4.72 (d, \(J = 12.0\) Hz, 1H, Troc-CH\(_2\)), 4.67 (d, \(J = 9.0\) Hz, 1H, H-1), 4.50 (d, \(J = 12.0\) Hz, 1H, Troc-CH\(_2\)), 4.36 (d, \(J = 3.6\) Hz, 1H, H-4′), 4.36–4.23 (m, 2H, H-6a, H-6b), 4.06 (t, \(J = 10.2\) Hz, 1H, H-4), 4.01 (dd, \(J = 10.2, 9.0\) Hz, 1H, H-2), 4.06–3.99 (m, 2H, H-6a′, H-6b′), 3.82 (d, \(J = 12.6\) Hz, 1H, -OCH\(_2\)C=O), 3.75 (d, \(J = 12.6\) Hz, 1H, -OCH\(_2\)C=O), 3.51 (s, 3H, -OMe), 3.22–3.21 (m, 1H, H:5), 3.20–3.19 (m, 1H, H-5′), 0.90 (s, 9H, TBDMS-tert-butyl), 0.05 (s, 3H, TBDMS-CH\(_3\)), 0.00 (s, 3H, TBDMS-CH\(_3\)) ppm; \(^{13}\)C-NMR (CDCl\(_3\), 100 MHz) \(\delta\) 170.1, 166.3, 166.1, 164.6, 155.0, 137.3, 133.2, 133.0, 130.2, 129.9, 129.6, 129.4, 129.1, 128.9, 128.7, 128.3, 128.2, 128.1, 128.0, 127.8, 126.3, 100.9 (CHPh), 100.7 (C-1), 100.1 (C-1′), 95.4 (CH\(_2\)CCl\(_3\)), 75.7 (C-3), 75.4 (C-3′), 74.3 (C-2′), 73.5 (C-5), 73.1(CH\(_2\)CCl\(_3\)), 69.5 (C-4), 68.1 (C-5′), 68.1 (C-4′), 66.4 (C-6), 64.5 ((-OCH\(_2\)C=O), 61.2 (C-6′), 56.2 (C-2) 51.8 (OCH\(_3\)), 25.9 (Si-C(CH\(_3\)))3, 18.2 (Si-C(CH\(_3\)))3, -5.0
(Si-CH₃), -5.4 (Si-CH₃) ppm; HRMS (ESI) calcd. for C₅₂H₅₈Cl₂NO₁₇NaSi [M+Na]⁺: 1124.2432, found: m/z 1124.2454.

4-O-[2,3-Di-O-benzoyl-4,6-di-O-benzylidene-β-D-galactopyranosyl]-(1→4)-2-acetamido-3-benzoyl-6-O-tert-butyldimethylysilyl-1-O-methylglycolate-2-deoxy-β-D-glucopyranoside (10). Freshly activated Zn dust (10.0 g) was added to a solution of the N-Troc equipped disaccharide 9 (5.4 g, 4.9 mmol) in DCM (50 mL) containing Ac₂O (0.692 mL, 7.34 mmol) and Et₃N (2.04 mL, 14.7 mmol). The reaction bottle was then sonicated in a classic ultrasonic cleaning bath below room temperature until the disappearance of starting material, which was determined by TLC (approximately 6 h). Then the mixture was filtered through celite pad, and the filtrate was washed with sat. NaHCO₃, followed by brine. The organic layers were collected and evaporated under reduced pressure. The crude residue was purified by column chromatography (silica gel; ethyl acetate : hexane = 3 : 2) to give NH-Ac product 10 (2.75 g, 51% yield) as foam; 

1H-NMR (CDCl₃, 400 MHz) δ 8.05 (d, J = 7.2 Hz, 2H, Ar:H), 7.89−7.87 (m, 4H, Ar:H), 7.48−7.42 (m, 4H, Ar:H), 7.40−7.26 (m, 10H, Ar:H), 6.38 (d, J = 9.2 Hz, 1H, N:H), 5.66 (dd, J = 10.0, 8.0 Hz, 1H, H-2′), 5.42 (t, J = 8.2 Hz, 1H, H-3), 5.29 (s, 1H, CHPh), 5.12 (dd, J = 10.0, 3.6 Hz, 1H, H-3′), 4.90 (d, J = 8.0 Hz, 1H, H-1′), 4.59 (d, J = 8.2 Hz, 1H, H-1), 4.36 (d, J = 3.6 Hz, 1H, H-4′), 4.35−4.21 (m, 3H, H:6a′, H:6a, H:6b), 4.04 (t, J = 8.2 Hz, 1H, H-4), 3.85 (d, J = 12.4 Hz, 1H, -OCH₂C=O), 3.68 (t, J = 8.2 Hz, 1H, H-2), 3.70−3.68 (m, 1H, H-6b), 3.67 (s, 3H, -OMe), 3.22 (d, J = 8.2 Hz, 1H, H-5), 3.21-3.19 (m, 1H, H-5′), 1.87 (s, 3H, N-Ac), 0.84 (s, 9H, TBDMS-tert-butyl), -0.04 (s, 3H, TBDMS-CH₃), -0.07 (s, 3H, TBDMS-CH₃) ppm; 13C-NMR (CDCl₃, 100 MHz) δ 170.6, 170.3, 166.4, 166.0, 164.8, 137.4, 133.3, 133.2, 133.0, 129.9, 129.8, 129.6, 129.5, 129.1, 128.9, 128.7, 128.3, 128.2, 128.0, 127.8, 126.3, 101.0 (C-1), 100.6 (CHPh), 100.0 (C-1′), 75.5 (C-3), 75.4 (C-3′), 73.7 (C-5), 72.9 (C-2′), 69.5 (C-4), 68.1 (C-5′), 65.8 (C-4′), 66.4 (C-6), 64.1 (-OCH₂C=O), 61.3 (C-6′), 53.2 (C-2), 51.8 (OCH₃), 25.7 (Si-C(CH₃)₃), 23.2 (Ac), 18.2 (Si-C(CH₃)₃), -5.2 (Si-CH₃), -5.5 (Si-CH₃) ppm; HRMS (ESI) calcd. for C₅₂H₅₈Cl₂NO₁₇NaSi [M+Na]⁺: 1124.2432, found: m/z 1124.2454.

4-O-[2,3-Di-O-benzoyl-4,6-di-O-benzylidene-β-D-galactopyranosyl]-(1→4)-2-acetamido-3-benzoyl-6-O-tert-butyldiphenylsilyl-1-O-methylglycolate-2-deoxy-β-D-glucopyranoside (11). To a stirred solution of compound 10 (2.41 g, 2.18 mmol) in THF (30mL) was added the mixture of TBAF/THF (1 M, 7.45 mL) and AcOH (0.22 mL) dropwise. After being stirred for 18 h, the solvent was evaporated under reduced pressure and then the residue was diluted with EtOAc (50 mL), washed with sat. NaHCO₃, water and brine and then the organic layers were collected and evaporated. The residue was purified by chromatography (silica gel; ethyl acetate : hexane = 3 : 1) to give the intermediate (1.47 g, 79% yield) as foam. To this intermediate (1.68 g, 1.96 mmol) in
dry THF was added DMAP (10 mg, 0.098 mmol), triethylamine (816 µL, 5.88 mmol) and TBDPSCI (1.07 mL, 3.92 mmol). After being stirred for 12 h at rt, the reaction mixture was quenched by methanol and evaporated under reduced pressure. The residue was diluted with DCM (30 mL) and washed with sat. NaHCO₃, water, and brine. The organic layers were concentrated under reduced pressure. The residue was purified by chromatography (silica gel; ethyl acetate : hexane = 1 : 1) to give product 11 (2.04 g, 95% yield) as white foam; ¹H-NMR (CDCl₃, 400 MHz) δ 8.09 (d, J = 7.2 Hz, 2H, Ar-H), 7.90 (d, J = 7.2 Hz, 2H, Ar-H), 7.88–7.70 (m, 5H, Ar-H), 7.54 (d, J = 7.2 Hz, 1H, Ar-H), 7.45–7.25 (m, 17H, Ar-H), 7.03 (t, 2H, J = 7.2 Hz, Ar-H), 6.11 (bd, J = 9.2 Hz, 1H, N:H), 5.65 (dd, J = 10.4, 8.0 Hz, 1H, H:2′), 5.34 (s, 1H, CHPh), 5.31 (t, J = 9.2 Hz, 1H, H-1), 4.39 (d, J = 3.6 Hz, 1H, H-4′), 4.34 (t, J = 9.2 Hz, 1H, H-4), 4.31 (t, J = 12.4 Hz, 1H, H-6a), 4.28 (d, J = 16.4 Hz, 1H, -OCH₂C=O), 4.20 (d, J = 16.4 Hz, 1H, -OCH₂C=O), 4.13 (d, J = 12.4 Hz, 1H, H-6b), 3.86 (d, J = 11.2 Hz, 1H, H-6a'), 3.82–3.80 (m, 2H, H:5, H:6a'), 3.64 (s, 3H, -OMe), 3.27–3.26 (m, 1H, H:5′), 1.90 (s, 3H, N:Ac), 1.06 (s, 9H, TBDPS-tert-butyl) ppm; ¹³C-NMR (CDCl₃, 100 MHz) δ 170.6, 170.3, 170.0, 166.0, 164.7, 137.4, 136.0, 135.9, 135.4, 133.5, 133.3, 132.9, 132.8, 132.4, 130.0, 130.0, 129.9, 129.6, 129.6, 129.0, 128.9, 128.7, 128.5, 128.2, 128.0, 127.8, 127.7, 126.4, 126.0, 100.8 (C-1), 100.2 (CHPh), 100.2 (C-1'), 75.6 (C-3), 73.5 (C-5), 73.2 (C-3′), 73.1 (C-4′), 72.8 (C-2'), 69.3 (C-4), 68.3 (C-5'), 66.5 (C-6), 63.9 (-OCH₂C=O), 61.4 (C-6'), 53.2 (C-2), 51.8 (OCH₃), 26.9 (Si-C(CH₃)₃), 23.3 (Ac), 19.4 ppm; HRMS (ESI) calcd. for C₆₁H₆₄NO₁₆Si [M+H]+: 1094.3989, found: m/z 1094.4008.

4-O-[2,3-Di-O-benzoyl-β-D-galactopyranosyl]-1-O-methylglycolate-2-deoxy-β-D-glucopyranoside (12). To a solution of compound 11 (2.04 g, 1.86 mmol) in chloroform (20 mL) and methanol (20 mL) was added 10% Pd/C (200 mg) and stirred under H₂ (1 atm) for 12 h. The mixture was filtered through a celite pad, washed with chloroform. The filtrates were concentrated under reduced pressure. The residue was purified by column chromatography (silica gel; ethyl acetate : hexane = 1 : 2) to give product 12 (1.47 g, 79% yield) as white foam; ¹H-NMR (CDCl₃, 600 MHz) δ 8.16 (d, J = 7.2 Hz, 2H, Ar-H), 7.89 (d, J = 7.2 Hz, 2H, Ar-H), 7.48 (t, J = 7.2 Hz, 1H, Ar-H), 7.45–7.27 (m, 14H, Ar-H), 6.96 (t, 2H, J = 7.2 Hz, Ar-H), 6.46 (bd, J = 9.2 Hz, 1H, N-H), 5.57 (dd, J = 10.8, 7.8 Hz, 1H, H-2′), 5.42 (t, J = 9.0 Hz, 1H, H-3), 5.03 (dd, J = 10.8, 3.0 Hz, 1H, H-3′), 4.93 (d, J = 7.8 Hz, 1H, H-1'), 4.69 (d, J = 7.8 Hz, 1H, H-1), 4.40 (t, J = 9.0 Hz, 1H, H-4), 4.28 (dd, J = 9.0, 7.8 Hz, 1H, H-2), 4.44 (d, J = 16.8 Hz, 1H, -OCH₂C=O), 4.33 (d, J = 16.8 Hz, 1H, -OCH₂C=O), 4.17 (d, J = 3.0 Hz, 1H, H-4'), 3.72 (bd, J = 12.0 Hz, 1H, H-6a), 3.67 (s, 3H, -OMe), 3.66 (bd, J = 12.0 Hz, 1H, H-6b), 3.46-3.44 (m, 2H, H-5', H-6a'), 3.35–3.28 (m, 1H, H-6b'), 3.17 (d, J = 9.0 Hz, 1H, H-5),
1.92 (s, 3H, N-Ac), 1.06 (s, 9H, TBDPS-tert-butyl) ppm; $^{13}$C-NMR (CDCl$_3$, 150 MHz) δ 170.8, 170.8, 166.7, 165.7, 164.9, 135.9, 135.4, 133.5, 133.4, 133.3, 133.0, 132.5, 130.3, 130.1, 129.9, 129.8, 129.7, 129.4, 129.1, 128.7, 128.6, 128.3, 128.0, 127.7, 100.3 (C-1'), 100.0 (C-1), 75.4 (C-3'), 74.4 (C-3), 74.3 (C-4), 74.3 (C-5), 73.5 (C-5'), 69.7 (C-2'), 68.3 (C-4'), 63.9 (OCH$_2$C=O), 62.4 (C-6), 61.1 (C-6'), 52.9 (C-2), 51.9 (OCH$_3$), 26.9 (Si-C(CH$_3$)$_3$), 23.4 (Ac), 19.4 ppm; HRMS (ESI) calcd. for C$_{54}$H$_{60}$NO$_{16}$Si [M+H]$^+$: 1006.3681, found: m/z 1006.3698.

$S$-(Methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-$\alpha$-d-glycero-$\alpha$-d-galacto-non-2-ulpopyranosylonate)-(2→6)-(2,3-di-O-benzoyl-6-thio-6-deoxy-$\beta$-d-galactopyranosyl)-(1→4)-2-acetamido-3-benzoyl-6-O-tert-butyldiphenylsilyl-1-$O$-methylglycolate-2-deoxy-$\beta$-d-glucopyranoside (14). Compound 12 (1.46 g, 1.45 mmol) was dissolved in dry DCM (15 mL) and pyridine (580 µL, 7.25 mmol, 5 equiv.) with stirring under N$_2$. After cooling the solution to –25°C, trifluoromethanesulfonic anhydride (283 µL, 1.68 mmol, 1.2 equiv.) was added dropwise over one minute. After stirring at –25°C for 1.5 h, the reaction mixture was diluted with DCM (15 mL), washed with 1.0 N HCl, saturated NaHCO$_3$ solution, ice water, and brine. The organic layers were dried over MgSO$_4$, filtered, and concentrated. The resting crude residue was combined with methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-2-$S$-acetyl-3,5-dideoxy-2-thio-$D$-glycero-$\alpha$-$D$-galacto-non-2-ulopyranosylate 13 (0.8 g, 1.45 mmol, 1 equiv.), and the mixture was dissolved in DMF (3.0 mL) under N$_2$. After cooled to -20°C, diethylamine (1.5 mL, 14.5 mmol, 10 equiv.) was added and the solution was stirred for 3 h. The mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica gel; toluene : acetone = 95 : 5) to afford product 14 (1.84 g, 85% yield for 2 steps) as white foam; $^1$H-NMR (CDCl$_3$, 600 MHz) δ 8.20 (d, $J = 7.2$ Hz, 2H, Ar-H), 7.92 (d, $J = 7.2$ Hz, 2H, Ar-H), 7.73–7.71 (m, 4H, Ar-H), 7.57–7.55 (m, 3H, Ar-H), 7.52–7.40 (m, 7H, Ar-H), 7.36 (t, $J = 7.8$ Hz, Ar-H), 7.34 (t, 2H, $J = 7.8$ Hz, Ar-H), 7.28 (t, 1H, $J = 7.8$ Hz, Ar-H), 7.00 (t, 2H, $J = 6.0$ Hz, Ar-H), 6.23 (bd, $J = 7.2$ Hz, 1H, N-H), 5.52 (dd, $J = 10.8$, 7.8 Hz, 1H, H-2'), 5.34–5.29 (m, 3H, H-3, H-7″, H-9b″), 5.18 (bd, $J = 9.6$ Hz, 1H, N-H), 5.06 (dd, $J = 10.8$, 3.0 Hz, 1H, H-3′), 4.94 (d, $J = 7.8$ Hz, 1H, H-1′), 4.92 (dd, $J = 10.8$, 4.8 Hz, 1H, H-4″), 4.59 (d, $J = 8.4$ Hz, 1H, H-1), 4.35 (t, $J = 8.4$ Hz, 1H, H-2), 4.33–4.28 (m, 3H, H-9a″, H-4, H-8″), 4.27 (d, $J = 16.8$ Hz, 1H, -OCH$_3$C=O), 4.40 (bt, $J = 3.0$ Hz, 1H, H-4″), 4.05 (dd, $J = 12.0$, 4.8 Hz, 1H, H-5″), 4.02 (d, $J = 16.8$ Hz, 1H, -OCH$_3$C=O), 3.75 (d, $J = 12.0$ Hz, 1H, H-6‴″), 3.74 (s, 3H, -OMe), 3.71–3.69 (m, 1H, H-5), 3.68 (s, 3H, -OMe), 3.51 (dd, $J = 7.8$, 4.8 Hz, 1H, H-6a″), 3.20 (bd, $J = 7.8$ Hz, 1H, H-6b), 2.75 (dd, $J = 9.6$, 4.8 Hz, 1H, H-3_eq‴), 2.75–2.74 (m, 1H, H-5′), 2.64–2.63 (m, 2H, H-6a″, H-6b″), 2.18 (s, 3H, -C=OCH$_3$), 2.08 (t, $J = 9.6$ Hz, 1H, H-3_ax‴), 2.07 (s, 3H, -C=OCH$_3$), 2.03 (s, 3H, -C=OCH$_3$), 1.93 (s, 3H, -C=OCH$_3$), 1.92 (s, 3H, -C=OCH$_3$), 1.87 (s, 3H, -C=OCH$_3$), 1.05 (s, 9H, TBDPS-tert-butyl) ppm; $^{13}$C-NMR (CDCl$_3$, 150 MHz) δ 171.0, 170.9,
170.7, 170.6, 170.5, 170.1, 166.8, 165.8, 165.9, 165.1, 164.8, 135.9, 135.4, 133.7, 133.3, 133.0, 132.9, 132.4, 130.1, 129.8, 129.9, 129.1, 129.0, 128.4, 128.3, 127.7, 100.4 (C:1), 99.7 (C:1′), 82.7 (C-2′′), 75.6 (C:5′), 75.6 (C:7′′), 74.1 (C:3′), 74.0 (C:6′′), 72.9 (C-5), 72.8 (C-6), 69.6 (C-2′), 69.5 (C-3′), 68.5 (C-4′), 67.2 (C-5′′), 63.8 (OCH$_2$C=O), 62.2 (C-4), 61.2 (C-8″), 53.2 (C-2), 53.1 (OCH$_3$), 51.9 (OCH$_3$), 49.5 (C-4′′), 37.9 (C:4), 29.4 (C:6′), 27.1 (Si-C(CH$_3$)$_3$), 23.4 (Ac), 23.2 (Ac), 21.2 (Ac), 20.9 (Ac), 20.7 (Ac), 19.4 ppm; HRMS (ESI) calcd. for C$_{74}$H$_{87}$N$_2$O$_{27}$SSi [M+H]+: 1495.4981, found: m/z 1495.4989.

$S$-(Methyl-5-acetamido-4,7,8,9-tetra-$O$-acetyl-3,5-dideoxy-$\alpha$-$D$-galacto-non-2-ulopyranosylonate)-(2→6)-(4-$O$-acetyl-2,3-di-$O$-benzoyl-6-thio-6-deoxy-$\beta$-$D$-galactopyranosyl)-(1→4)-2-acetamido-3-benzoyl-$1$-$O$-methylglycolate-2-deoxy-$\beta$-$D$-glucopyranoside (15). Compound 14 (1.84 g, 1.23 mmol) in pyridine (10 mL) was added DMAP (12 mg, 0.12 mmol) and Ac$_2$O (189 µL, 1.85 mmol) at 0°C, and then the mixture was stirred and warmed to rt for 5 h. The mixture was concentrated under reduced pressure. The residue was diluted with DCM (15 mL), and washed with 1.0 N HCl, sat. NaHCO$_3$, water, and brine. The organic layers were collected, dried over MgSO$_4$, filtered and then concentrated to obtain the crude residue which was then dissolved THF (30 mL) and pyridine (30 mL) and was subjected to add HF:pyridine (1.87 mL) dropwise. After being stirred for 12 h, the reaction was quenched by sat. NaHCO$_3$. Evaporated under reduced pressure to afford the residue, which was diluted with DCM, washed with sat. NaHCO$_3$, water and brine and then the organic layers were collected and evaporated. The residue was purified by column chromatography (silica gel; DCM: methanol = 97 : 3) to afford product 15 (1.08 g, 2 steps yield 68%) as brown solid; mp 173 °C; $^1$H-NMR (CDCl$_3$, 600 MHz) δ 8.11 (d, $J = 7.8$ Hz, 2H, Ar:H), 7.92 (d, $J = 7.8$ Hz, 2H, Ar-H), 7.78 (d, $J = 7.8$ Hz, 2H, Ar-H), 7.50 (t, $J = 7.2$ Hz, 1H, Ar-H), 7.47 (t, $J = 7.2$ Hz, 1H, Ar-H), 7.43 (t, $J = 7.2$ Hz, 1H, Ar-H), 7.38 (t, 2H, $J = 7.8$ Hz, Ar-H), 7.29 (t, 2H, $J = 7.8$ Hz, Ar-H), 5.96 (bd, $J = 9.0$ Hz, 1H, N:H), 5.63 (d, $J = 3.0$ Hz, 1H, H-4′), 5.43 (dd, $J = 10.2$, 7.8 Hz, 1H, H-2′), 5.36 (dd, $J = 10.2$, 3.0 Hz, 1H, H-3′), 5.32 (t, $J = 9.0$ Hz, 1H, H-3), 5.28 (bd, $J = 9.0$ Hz, 1H, H-6″), 5.28–5.25 (m, 1H, H-7″), 5.20 (bd, $J = 9.0$ Hz, 1H, N-H), 4.99 (d, $J = 7.8$ Hz, 1H, H-1′), 4.91 (dd, $J = 12.6$, 7.8, 4.2 Hz, 1H, H-4″), 4.56 (d, $J = 9.0$ Hz, 1H, H-1), 4.42 (d, $J = 12.6$, 3.0 Hz, 1H, -OCH$_3$C=O), 4.32 (t, $J = 9.0$ Hz, 1H, H-2), 4.31 (t, $J = 9.0$ Hz, 1H, H-4), 4.26–4.22 (m, 3H, OCH$_3$C=O, H-9a″, H-9b″), 3.92 (dd, $J = 9.0$, 7.8 Hz, 1H, H-5″), 3.92–3.87 (m, 2H, H-5, H-5′), 3.89 (s, 3H, -OMe), 3.85–3.82 (m, 1H, H-6b), 3.76–3.72 (m, 1H, H-6a), 3.69 (s, 3H, -OMe), 3.32 (d, $J = 9.6$ Hz, 1H, H-8″), 2.95 (bt, $J = 6.0$ Hz 1H, OH), 2.69 (dd, $J = 12.6$, 4.2 Hz, 1H, H-3″), 2.35 (d, $J = 15.0$, 6.6 Hz, 1H, H-6a′), 2.25 (s, 3H, -C=OCH$_3$), 2.19 (s, 3H, -C=OCH$_2$), 2.03 (s, 3H, -C=OCH$_3$), 1.96 (t, $J = 12.6$ Hz, 1H, H-3ax″), 1.93 (dd, $J = 15.0$, 7.8 Hz, 1H, H-6b′), 1.89 (s, 3H, -C=OCH$_3$), 1.88 (s, 3H, -C=OCH$_3$).
1.87 (s, 3H, -C=OCH₃) ppm; ¹³C-NMR (CDCl₃, 150 MHz) δ 171.4, 170.9, 170.9, 170.6, 170.6, 170.1, 169.7, 168.1, 166.3, 165.2, 165.0, 133.2, 133.1, 132.8, 130.4, 130.2, 129.6, 129.3, 129.1, 128.4, 128.3, 128.0, 127.7, 100.7 (C-1), 99.5 (C-1’), 84.2 (C-2′′), 75.8 (C-5′), 73.8 (C-7′′), 73.1 (C-3), 73.1 (C-4), 73.0 (C-6′′), 71.9 (C-5), 70.1 (C-6), 69.1 (C-2′), 68.1 (C-3′), 68.0 (C-4′), 66.8 (C-5′′), 64.7 (OCH₂C=O), 62.1 (C-9′′), 60.0 (C-8′′), 53.3 (C-2′′), 53.2 (OCH₃), 51.9 (OCH₃), 49.6 (C-4′′), 38.3 (C-3′′), 29.1 (C-6′), 23.3 (Ac), 23.2 (Ac), 21.2 (Ac), 20.8 (Ac), 20.7 (Ac), 20.4 (Ac) ppm; HRMS (ESI) calcd. for C₆₀H₇₁N₂O₂₈S [M+H]^+: 1299.3909, found: m/z 1299.3951.

S-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic)-(2→6)-(6-thio-6-dideoxy-β-D-galactopyranosyl)-(1→4)-2-acetamido-1-O-glycolic-2-deoxy-β-D-glucopyranoside disodium (16). To a solution of the compound 15 (100 mg, 0.077 mmol) in dry MeOH (2 mL) was added sodium methoxide (1 mg). After being stirred at rt for 16 h, the solution was neutralized by Amberlite® IR 120 H⁺ resin, and the solution was concentrated. The residue was dissolved in 0.2 N NaOH (2 mL). After stirred at rt for 10 h, the solution was purified by Sephadex G-10 column chromatography and lyophilized to afford compound 16 (59 mg, 96% yield), as amorphous white foam; ¹H-NMR (D₂O, 600 MHz) δ 4.63 (d, J = 8.4 Hz, 1H, H:1′), 4.49 (d, J = 7.8 Hz, 1H, H-1), 4.19 (d, J = 15.6 Hz, 1H, OCH₂C=O), 4.15 (d, J = 15.6 Hz, 1H, OCH₂C=O), 4.04 (bd, J = 3.0 Hz, 1H, H:8′′), 4.01 (bd, J = 12.0 Hz, 1H, H:6a), 3.91 (bd, J = 12.0 Hz, 1H, H:6b), 3.89 (bd, J = 8.4 Hz, 1H, H:9a′′), 3.86 (dd, J = 9.6, 8.4 Hz, 1H, H:2′), 3.86−3.82 (m, 2H, H:4, H:6′′), 3.80 (m, 1H, H:5′), 3.77−3.69 (m, 3H, H:4′′, H:7′′, H:5), 3.69 (t, J = 7.8 Hz, 1H, H-3), 3.67 (bd, J = 8.4 Hz, 1H, H:9b′′), 3.66−3.61 (m, 1H, H:5′′), 3.64 (bs, 1H, H-4′), 3.63 (bd, J = 9.6 Hz, 1H, H-3′), 3.54 (t, J = 7.8 Hz, 1H, H-2), 3.00 (dd, J = 13.8 Hz, 9.6 Hz, 1H, H-6a′), 2.96 (dd, J = 13.8 Hz, 7.8 Hz, 1H, H-6b′), 2.84 (dd, J = 12.6 Hz, 4.8 Hz, 1H, H-3_α′′), 2.11 (s, 3H, -C=OCH₃), 2.07 (s, 3H, -C=OCH₃), 1.81 (t, J = 12.6 Hz, 1H, H-3_α′′) ppm; ¹³C-NMR (D₂O, 150 MHz) δ 177.2, 175.0, 174.9, 174.1, 103.0 (C-1), 100.3 (C-1′), 85.7 (C-2′′), 79.2 (C-4′′), 74.9 (C-3′), 74.9 (C-5′), 74.1 (C-4), 72.5 (C-5′′), 72.5 (C-2), 72.2 (C-3), 70.7 (C-2′), 69.1 (C-6′′), 68.6 (C-4′), 68.1 (C-5′), 67.9 (OCH₂C=O), 62.6 (C-9′′), 60.2 (C-6), 55.1 (C-7′′), 51.7 (C-8′′), 40.8 (C-3′′), 29.5 (C-6′), 22.4 (Ac), 22.0 (Ac), ppm; HRMS (ESI) calcd. for C₂⁷H₄₂N₂O₂⁸S [M+Na]^+: 769.1949, found: m/z 769.1961.

S-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic)-(2→6)-(6-thio-6-dideoxy-β-D-galactopyranosyl)-(1→4)-2-acetamido-1-O-glycolic-6-O-sulfo-2-deoxy-β-D-glucopyranoside trisodium (17). Compound 15 (350 mg, 0.27 mmol) was dissolved in dry DMF (3 mL) and then SO₃ • TMA (375 mg) was added in one portion. The reaction mixture was stirred at 60 °C for 12 h. The reaction was quenched with excess NaHCO₃ powder and solvent was evaporated under reduced pressure. The residue was purified by column chromatography (silica gel; DCM: methanol = 8 : 1) to give 6-O-sulfo product (320 mg, 86% yield) as foam. To a solution of this product (270
mg, 0.193 mmol) in dry MeOH (3 mL) was added sodium methoxide (2.0 mg). After being stirred at rt for 12 h, the solution was concentrated and then the residue was dissolved in 0.2 N NaOH (3 mL). After 10 h stirring at rt, the solution was purified by Sephadex G-10 column chromatography and lyophilized to afford compound 17 (164 mg, 95% yield) as amorphous white foam; $^1$H-NMR (D$_2$O, 600 MHz) δ 4.67 (d, J = 8.4 Hz, 1H, H-1'), 4.54 (d, J = 7.8 Hz, 1H, H-1), 4.01-4.00 (m, J = 11.4 Hz, 1H, H-6a), 3.91 (dd, J = 11.4, 5.4 Hz, 1H, H-6b), 4.22 (d, J = 15.6 Hz, 1H, OCH$_2$C=O), 4.14 (d, J = 15.6 Hz, 1H, OCH$_2$C=O), 4.01 (d, J = 3.0 Hz, 1H, H-8''), 3.92 (d, J = 11.4 Hz, 1H, H-9a''), 3.89 −3.83 (m, 3H, H:4, H:5'′, H:6′′), 3.82 −3.73 (m, 3H, H:4′′, H:7′′, H:5), 3.71 (t, J = 7.8 Hz, 1H, H:3), 3.67 (bd, J = 11.4 Hz, 1H, H:9b''), 3.74 −3.69 (m, 1H, H:5′′), 3.63:3.62 (m, 1H, H:4′), 3.62 (d, J = 9.6 Hz, 1H, H:3′), 3.54 (t, J = 7.8 Hz, 1H, H-2), 3.03 (dd, J = 13.8, 8.4 Hz, 1H, H-6a'), 2.97 (dd, J = 13.8, 8.4 Hz, 1H, H-6b'), 2.84 (dd, J = 12.6, 4.2 Hz, 1H, H-3′′), 2.11 (s, 3H, :C=OCH$_3$), 2.07 (s, 3H, :C=OCH$_3$), 1.82 (t, J = 12.6 Hz, 1H, H-3ax'').

$^{13}$C-NMR (D$_2$O, 150 MHz) δ 177.0, 175.0, 174.9, 174.2, 102.9 (C:1), 100.2 (C:1′), 85.4 (C-2''), 79.1 (C-4''), 74.8 (C-3''), 73.9 (C-4), 72.7 (C-5''), 72.5 (C-2), 72.4 (C-5), 72.1 (C-3), 70.8 (C-2'), 69.3 (C-6''), 68.6 (C-4''), 68.2 (C-5''), 67.9 (OCH$_2$C=O), 66.7 (C-6), 62.6 (C-9''), 55.0 (C-7''), 51.7 (C-8''), 40.7 (C-3''), 29.4 (C-6'), 22.4 (Ac), 22.0 (Ac) ppm; HRMS (ESI) calcd. For C$_{27}$H$_{41}$N$_2$Na$_2$O$_2$S$_2$ [M:Na]: 871.1337, found: m/z 871.1347.

S-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl-(2→6)-(6-thio-6-deoxy-β-D-galactopyranosyl)-(1→4)-2-acetamido-1-O-(glycolic,1,2-didodecanoyl-sn-glycero-3-p hospheethanolamido)-2-deoxy-β-D-glucopyranoside (3). To a stirred solution of compound 16 (28 mg, 0.031 mmol) in methanol was added Amberlite® IR 120 H$^+$ in one portion. After stirred for 30 min, the solution was filtered and evaporated under reduced pressure. The dry residue was dissolved in DMF (2 mL), followed by addition of EDC-HCl (14 mg, 0.07 mmol, 2 equiv.), HOBt (24 mg, 0.18 mmol, 5 equiv.), DLPE (19 mg, 0.035 mmol, 1 equiv.), and NMM (19 µL, 0.18 mmol, 5 equiv.) gradually. After the desired product was detected, the solution was evaporated and purified by column chromatography three times (Sephadex LH-20, chloroform : methanol = 1 : 9) to afford compound 3 (16 mg, 39% yield) as brown oil. The purity of compound 3 was 100% as shown by HPLC on an syncronis C18 column (4.6 mm × 250 mm, 5 µm; Thermo), t$_R$ = 7.18 min (isocratic 55% MeCN in water at flow rate of 1.0 mL/min, UV 214 nm). $^1$H-NMR (pyridine-D$_5$, 600 MHz) δ 5.74 −5.73 (m, 1H , CH$_2$:C(O)H:CH), 5.33 (d, J = 7.2 Hz, 1H, H-1'), 5.09 (d, J = 8.4 Hz, 1H, H-1), 4.87-4.85 (m, 1H, H-9a''), 4.80 (dd, J = 8.4, 3.6 Hz, 1H, CH$_2$C(O)H-CH$_2$), 4.62−4.51 (m, 4H, H-4, H-6a, CH$_2$C(O)H-CH$_2$), 4.50−4.38 (m, 6H, H-2, H-6b, H-3'′, H-9b''), OCH$_2$C=O), 4.37−4.31 (m, 4H, H-2′, H-5′, H-8''), CH$_2$C(O)H-CH$_2$), 4.27−4.22 (m, 2H, H-3, H-4'), 4.19 (dd, J = 10.2, 5.4 Hz, 1H, H-7'′), 3.93−3.87 (m, 1H, C-6''), 3.67−3.52 (m, 5H, H-5, H-4''', H-5''',NH-CH$_2$-CH$_2$O), 3.17-3.1.10 (m, 2H, NH-CH$_2$), 2.46 (t, J = 7.2 Hz, 2H, C(=O)-CH$_2$), 2.40 (t, J
= 7.2 Hz, 2H, C(=O)-CH₂), 2.34-2.27 (m, 4H, -C(=O)CH₂ H-3₃'), 2.08-2.01 (m, 4H, -C(=O)CH₂), 1.69-1.66 (m, 4H, C(=O)CH₂CH₂), 1.25-1.18 (m, 32H), 0.87 (t, J = 7.2 Hz, 6H, -CH₂-CH₃) ppm; ¹³C-NMR (pyridine-D₅, 150 MHz) δ 174.0, 173.8, 173.8, 173.6, 172.1, 170.2, 105.5 (C-1'), 102.5 (C-1), 89.0 (C-2''), 82.2, 78.1 (C-5'), 75.7 (C-2'), 74.4 (C-3'), 73.2 (CH₂-C(O)H-CH), 73.0 (NH-CH₂-CH₂-O), 71.7 (C-4), 70.5 (C-3), 69.6 (C-8''), 69.1 (OCH₂C=O), 68.9 (C-4'), 65.1 (C-4''), 64.7 (C-6), 63.5 (C-7''), 61.8 (C-9''), 57.0 (C-2), 56.2 (NH-CH₂-CH₂-O), 54.6 (C-5), 53.9 (C-6'), 44.1, 43.4 (C-5''), 41.8 (C-3''), 37.9, 35.7, 34.8 (C(=O)CH₂), 34.6 (C(=O)CH₂), 32.5, 30.25, 30.15, 30.0, 29.95, 29.8, 26.9, 25.7 (C(=O)CH₂CH₂), 25.6 (Ac), 23.3 (Ac), 16.4, 14.6 (CH₃) ppm; HRMS (ESI) calcd. For C₅₆H₉₉N₃O₂₇PS [M+H]: 1308.5924, found: m/z 1308.5943.

**S-(5-Acetamido-3,5-dideoxy-δ-glycero-α-d-galacto-non-2-ulopyranosyl)-(2→6)-(6-thio-δ-dideoxy-β-d-galactopyranosyl)-(1→4)-2-acetamido-1-O-(glycolic-1,2-didodecanoylsn-glycero-3-phosphoethanolamido)-2-deoxy-6-sulfo-β-d-glucopyranoside (4).** To a stirred solution of compound 17 (36 mg, 0.04 mmol) in methanol was added Amberlite® IR 120 H⁺ carefully by controlling pH value at 3.5-4.5. After stirred for 10 minutes, the solution was filtered and evaporated under reduced pressure. The dry residue was then dissolved in DMF (3 mL), followed by addition of EDC-HCl (16 mg, 0.08 mmol, 2 equiv.), HOBt (28 mg, 0.21 mmol, 5 equiv.), DLPE (23 mg, 0.04 mmol, 1 equiv.), and NMM (23 µL, 0.21 mmol, 5 equiv.) gradually. After the desired product was detected, the solution was evaporated and purified by column chromatography three times (Sephadex LH-20, chloroform : methanol = 1 : 9) to afford compound 4 (6 mg, 11 % yield) as yellow oil. The purity of compound 4 was 100% as shown by HPLC on an syncronis C18 column (4.6 mm × 250 mm, 5µm; Thermo), tᵣ = 7.18 min (isocratic 55% MeCN in water at flow rate of 1.0 mL/min, UV 214 nm).

¹H-NMR (Methanol-D₄ and CDCl₃, ratio = 4 : 1, 600 MHz) δ 5.25 −5.21 (m, 1H, CH₂:CH(O):CH₂), 4.55-4.51 (m, 2H, H:1, H:1'), 4.43 (dd, J = 12.0, 3.0 Hz, 1H), 4.35 (dd, J = 11.4, 4.2 Hz, 1H, H:6a), 4.32 (dd, J = 11.4, 3.6 Hz, 1H, H:6b), 4.19 (d, J = 15.6 Hz, 1H, OCH₂C=O), 4.18 (dd, J = 12.0, 6.6 Hz, 1H, CH₂(CH₂-CH(O)-CH₂), 4.05 (d, J = 15.6 Hz, 1H, OCH₂C=O), 4.05-4.02 (m, 4H, H-3, CH₂-CH(O)-CH₂), 4.01 (t, J = 5.4 Hz, 2H, NH-CH₂-CH₂-O), 3.92 (dd, J = 11.4, 2.4 Hz, 1H, H-9b''), 3.89 (dd, J = 9.0, 5.4 Hz, 1H, H-5'), 3.82 (t, J = 9.6 Hz, 1H, H-2'), 3.80-3.61 (m, 7H, H-4, H-4', H-5'', H-6'', H-7'', H-8'', H-9b''), 3.54 (dd, J = 9.6, 3.6 Hz, 1H, H-3''), 3.50-3.46 (m, 3H, H-2, H-5, H-4''), 3.14-3.16 (m, 2H, NH-CH₂), 2.98 (dd, J = 13.8, 8.4 Hz, 1H, H-6a'), 2.90 (dd, J = 13.8, 5.4 Hz, 1H, H-6b'), 2.84 (dd, J = 12.6, 4.4 Hz, 1H, H-3''), 2.34 (t, J = 7.8 Hz, 2H, C(=O)-CH₃), 2.31 (t, J = 7.8 Hz, 2H, C(=O)-CH₃), 2.03 (s, 3H, -C=OCH₃), 2.01 (s, 3H, -C=OCH₃), 1.66 (t, J = 12.6 Hz, 1H, H-3''), 1.62-1.59 (m, 4H, C(=O)CH₂CH₂), 1.31-1.29 (m, 32H), 0.89 (t, J = 7.2 Hz, 6H, -CH₂-CH₂) ppm; ¹³C-NMR (Methanol-D₄ and CDCl₃, ratio = 4 : 1, 150 MHz) δ 176.9, 175.5, 175.1, 174.9, 174.8, 173.1, 104.7 (C-1'), 102.1 (C-1), 83.3 (C-2''), 81.0
(C-6\textsuperscript{''}), 76.1 (C-2\textsuperscript{'}), 74.8 (C-5), 74.7 (C-5\textsuperscript{'}), 73.5 (C-4\textsuperscript{''}), 72.6 (H-7\textsuperscript{'}), 71.9 (C-3\textsuperscript{'}), 71.83 (CH\textsubscript{2}:CH(O):CH\textsubscript{2}), 71.81 (C-4), 70.3 (C-2), 69.8 (C-4\textsuperscript{''}), 69.6 (CH\textsubscript{2}:CH(O):CH\textsubscript{2}), 69.5 (CH\textsubscript{2}:CH(O):CH\textsubscript{2}), 67.4 (C-6), 65.0 (C-3), 64.5 (C-9\textsuperscript{'}), 63.7(NH-CH\textsubscript{2}-CH\textsubscript{2}-O), 63.1(C-8\textsuperscript{''}), 61.7, 56.4(C-5\textsuperscript{'}), 53.9, 43.1(C-3\textsuperscript{'}), 41.8 (NH-CH\textsubscript{2}-CH\textsubscript{2}-O), 35.2 (C(=O)CH\textsubscript{2}), 35.1(C(=O)CH\textsubscript{2}), 33.1, 30.8, 30.7, 30.3, 30.3, 26.1 (C(=O)CH\textsubscript{2}CH\textsubscript{2}), 26.1 (C(=O)CH\textsubscript{2}CH\textsubscript{2}), 23.8, 23.3, 22.8 (Ac), 21.1 (Ac), 14.6 (CH\textsubscript{3}) ppm; HRMS (ESI) calcd. For C\textsubscript{56}H\textsubscript{99}N\textsubscript{3}O\textsubscript{3}PS\textsubscript{2} [M+H]\textsuperscript{+}: 1388.5492, found: m/z 1388.5508.

**Liposome preparation**

DLPE, or compound 3, or 4 and cholesterol (CH) were dissolved with dipalmitoylphosphatidyl-choline (DPPC) in CH\textsubscript{3}Cl (DPPC : CH : DLPE, or 3, or 4 = 4 : 1 : 1, mole ratio). The lipid sample mixtures were then concentrated under high vacuum for 30 minutes. Film hydration were then reconstituted by gentle mixing in PBS buffer to reach final lipid concentration of 20 mM. The contents of the vial were sonicated in a tip sonicator to give Lipo-control, Lipo-3, Lipo-4, which was monitored by Dynamic Light Scattering (DLS, BIC 90 PLUS, Brookhaven Instrument. Co. Holtsville, NY, USA.) The parameters of these liposomes are listed in Supporting Information.

**Hemagglutination inhibition assay**

WSN H1N1 virus (2\textsuperscript{3} HA units) and inhibitors were mixed and allowed to preincubate for 30 min at 4 °C. Erythrocytes (0.5% v/v) were then added and mixed, and the system was allowed to incubate for 30 min at 35 °C before being observed. If the agglutination was inhibited, the RBCs sedimented to the bottom of the well and formed a pellet. Otherwise, they were aggregated by the virus particles and formed a lattice.

**Neutralization Assay**

MDCK cells at 1 × 10\textsuperscript{4}/ml was added to each well. The 96 wells plates were incubated for 24 h at 35 °C. WSN virus (H1N1) and inhibitors were mixed and preincubated for 1 hour at 4 °C, and then added to each well. After infection at 35 °C for 1 h, cells were washed with PBS twice and incubated for 40 hours. Cells were fixed in methanol and lysed by 0.5% Triton X-100. After treatment with 5% Skim Milk at rt for 1 h, the presence of viral nucleoprotein (NP) was detected by ELISA with a monoclonal antibody to influenza A NP followed by incubation with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG. O-Phenylenediamine dihydrochloride (OPD) was used as a substrate with absorbance read at 492 nm (Perkin Elmer Envision Microplate Reader, CA, USA) after stopping the reaction by addition of 1N H\textsubscript{2}SO\textsubscript{4}.

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Supplementary data

Electronic supplementary information (ESI) available: NMR spectra for new compounds, see DOI:

References

19 H. Driguez, Chembiochem 2001, 2, 311-318;


