Bioorganic & Medicinal Chemistry xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of *S*-linked NeuAc-α(2-6)-di-LacNAc bearing liposomes for H1N1 influenza virus inhibition assays

Hou-Wen Cheng [a], Hsiao-Wen Wang [a], Tsung-Yun Wong [a], Hsien-Wei Yeh [a], Yi-Chun Chen [a], Der-Zen Liu [b,c], Pi-Hui Liang [a,d,*]

[a] School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan
[b] Graduate Institute of Biomedical Materials and Tissue Engineering, College of Oral Medicine, Taipei Medical University, Taipei 110, Taiwan
[c] Medical and Pharmaceutical Industry Technology and Development Center, Academia Sinica, Taipei 128, Taiwan
[d] The Genomics Research Center, Academia Sinica, Taipei 128, Taiwan

ARTICLE INFO

Article history:
Received 13 December 2017
Revised 7 February 2018
Accepted 10 February 2018
Available online xxxx

Keywords:
Liposome
Influenza
Sialic acid
Hemagglutinin

ABSTRACT

S-NeuAc-α(2-6)-di-LacNAc (**5**) was efficiently synthesized by a [2+2] followed by a [1+4] glycosylation, and later conjugated with 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE) to form both single-layer and multi-layer homogeneous liposomes in the presence of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol. These liposomes were found to be weak inhibitors in both the influenza virus entry assay and the hemagglutination inhibition assay. The single layer liposome was found to more efficiently interfere with the entry of the H1N1 influenza virus into MDCK cells than the multilayer liposome containing **5**.

1. Introduction

Influenza is an infectious disease caused by the influenza virus, which infects humans and animals through contact with bronchial epithelial cells. The mechanism by which the virus attacks the cell entails recognition and cleavage of the terminal alpha-linked sialic acid on the surface of the cell by the glycoprotein hemagglutinin (HA), present on the surface of the virus. There are several different types of alpha-sialic acid linkage present on the surfaces of human mucosal cells, for which different types of influenza viruses have different binding preferences. For example, avian influenza virus binds to sialic acid in a α2-3-linkage with galactose; swine influenza virus is reported to bind sialic acid in either a α2-6 or α2-3 linkage with galactose; and human-adapted influenza viruses have binding preferences for a α2-6 sialic acid linkage.[1–3]

Following invasion of the cell in this way, a second influenza surface glycoprotein, neuraminidase (NA), is implicated in the release of the virus from the host cell. As both processes are integral to the proliferation of the virus, both HA and NA are promising targets for the development of anti-influenza drugs. NA inhibitors, such as oseltamivir and zanamivir, have seen clinical use for a decade.[4] Oseltamivir resistance,[5,6] however, is a growing problem; influenza continues to threaten humans; and new drugs to combat it are urgently required. Meanwhile, anti-influenza inhibitors that target HA are underdeveloped, compared to NA inhibitors.[7,8]

Although the targeting of HA is an option for the treatment of influenza, the binding between multiple HA of an influenza virus and the sialic acid surface receptors of an erythrocyte during viral infection is estimated to occur with an affinity of 10^{13} M^{-1}, whereas the association constant of a single sialic acid-HA interaction is 10^{3} M^{-1};[9] and thus the high degree of multi-valency required for a strong binding affinity between HA and HA-bound molecules poses a challenge.[10] For example, the sialic acid moieties presented by human mucosal cells are densely packed together, and any platform presenting an array of molecules intended to interact with the HA trimer would also have to be very densely packed.

When covalently displayed on the surface of liposome, *S*-Neu5Ac-α(2-6)-LacNAc (**2**, Fig. 1) was shown to have an EC_{50} of around 70–180 μM in a virus entry inhibitory assay.[11] Since the potency was not optimized, we sought to increase the inhibitory effect of sialic conjugation. A model to describe the binding between HA and the sialoside was recently disclosed.[12] When HA binds to α2-3-sialoside, it is believed to assume a "cone-like" topology. However, when HA binds to α2-6-sialoside, it forms an "umbrella-like" topology with additional glycan conformational flexibility. Longer α2-6 sialic acid bearing oligosaccharides would

* Corresponding author at: School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan.
E-mail address: phliang@ntu.edu.tw (P.-H. Liang).

https://doi.org/10.1016/j.bmc.2018.02.012
0968-0896/

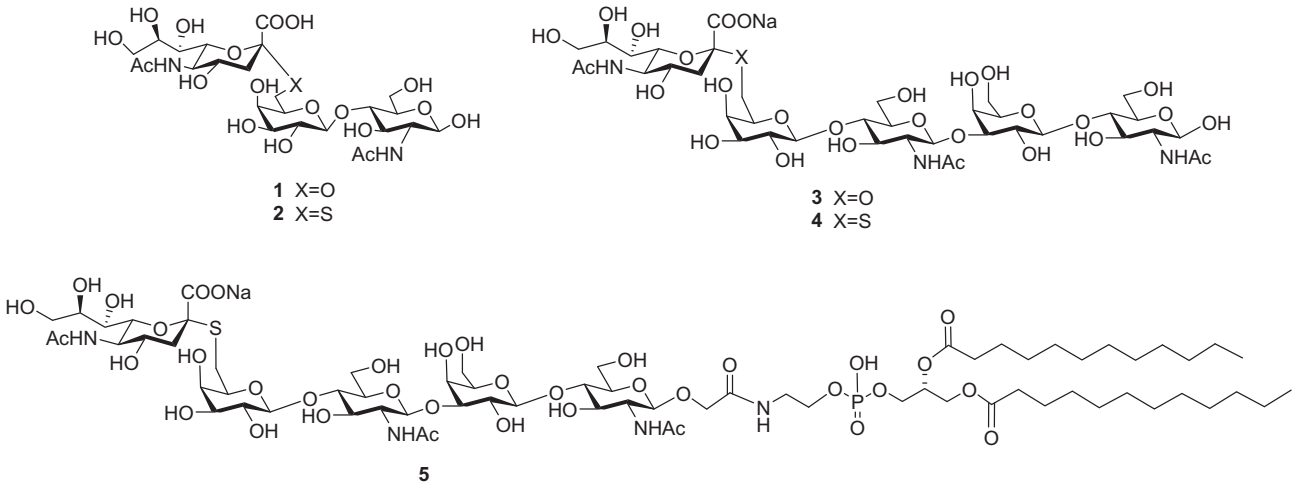


Fig. 1. Structure of sialic acid containing trisaccharides (**1**, **2**), pentasaccharides (**3**, **4**), and DLPE conjugation **5**.

favor this conformation. In mammalian respiratory epithelial cells, LacNAc is frequently displayed.[12,13] Glycan microarray studies revealed that the HAs of human influenzas bound best to *N*-glycans with the linear di-LacNAc sequence, and also to *N*-glycans with the tri-LacNAc repeat sequence.[14] Neu5Ac-α(2-6)-di-LacNAc (**3**) was indeed found to have a stronger binding preference for influenza than trisaccharide **1** (Fig. 1). Accordingly, we designed and synthesized *S*-Neu5Ac-α(2-6)-di-LacNAc (**4**), wherein the aforementioned trisaccharide **2** has been extended by LacNAc and *S*-linked Neu5Ac moieties.[15,16] This compound is expected to serve as both a novel synthetic recognition molecule of HA, and as a competitor of natural *O*-glycosides to NA.

Several multivalent vectors (dendrimers,[17] polymers,[18] liposomes,[19] and nanoparticles[20]), have been functionalized with sialic acid; and their abilities to inhibit the activities of their corresponding lectins were measured. However, in all cases, either heterogeneous mixtures of Neu5Ac were submitted for conjugation, and/or they contained only a Neu5Ac moiety for protein recognition, which may be not sufficient for HA interaction, and cannot be used to quantify the contribution of Neu5Ac in the virus interaction.[14] In order to procure a multivalent display of homogenous **4**, it was designed to conjugate with phospholipid – DLPE (1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine) to give **5** (Fig. 1), which could be formulated with DPPC (dipalmitoyl phosphatidylcholine) to form liposomes (Fig. 2) in aqueous solution. These compounds and formulations were evaluated in the A/WSN/33 H1N1 influenza entry inhibition and hemagglutination inhibition assays by red blood cells (RBC)s.

2. Results and discussion

2.1. Synthesis of DLPE bearing *5*

Our retrosynthesis of **5** is depicted in Scheme 1. The key steps are the formation of the amide bond that connects the lipid with the pentasaccharide; and two key glycosylation steps. Firstly, the *S*-glycosidic α2-6 linkage, would be formed by nucleophilic substitution of the triflate group at the galactose 6-position of compound **8** by the Neu5Ac thiolate of **7** ([1+4] glycosylation step); and secondly, the [2+2] glycosylation in step c, which would yield compound **8**. In order to control the selectivity of the glycosylations of steps c, d and e, benzoyl (Bz) and trichloroethylcarbonyl (Troc) groups would be introduced at the galactose 2-OH (compounds **11** and **14**) and glucosamine 2-NH_2 positions (compounds **12** and **13**), respectively. These are expected to confer a degree of β-selectivity on the glycosylation, by a way of neighboring-group partici-

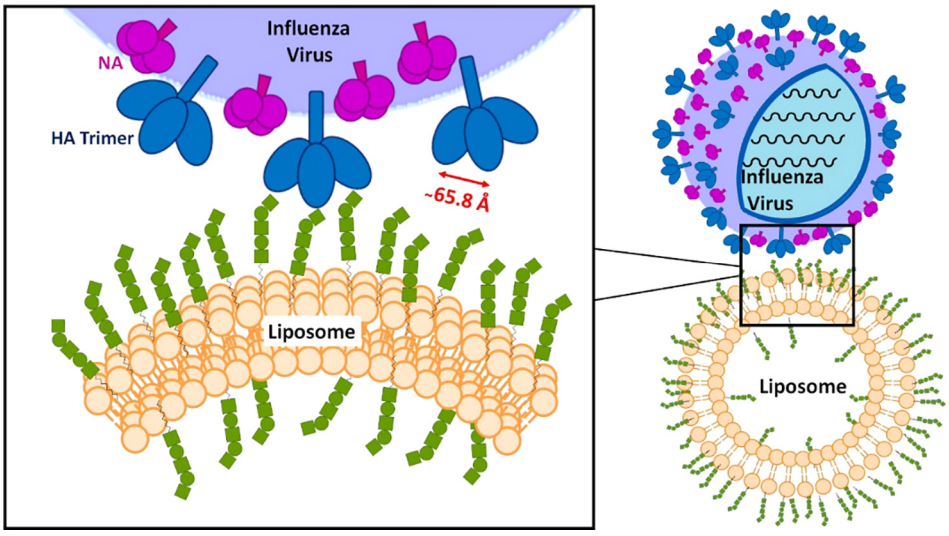
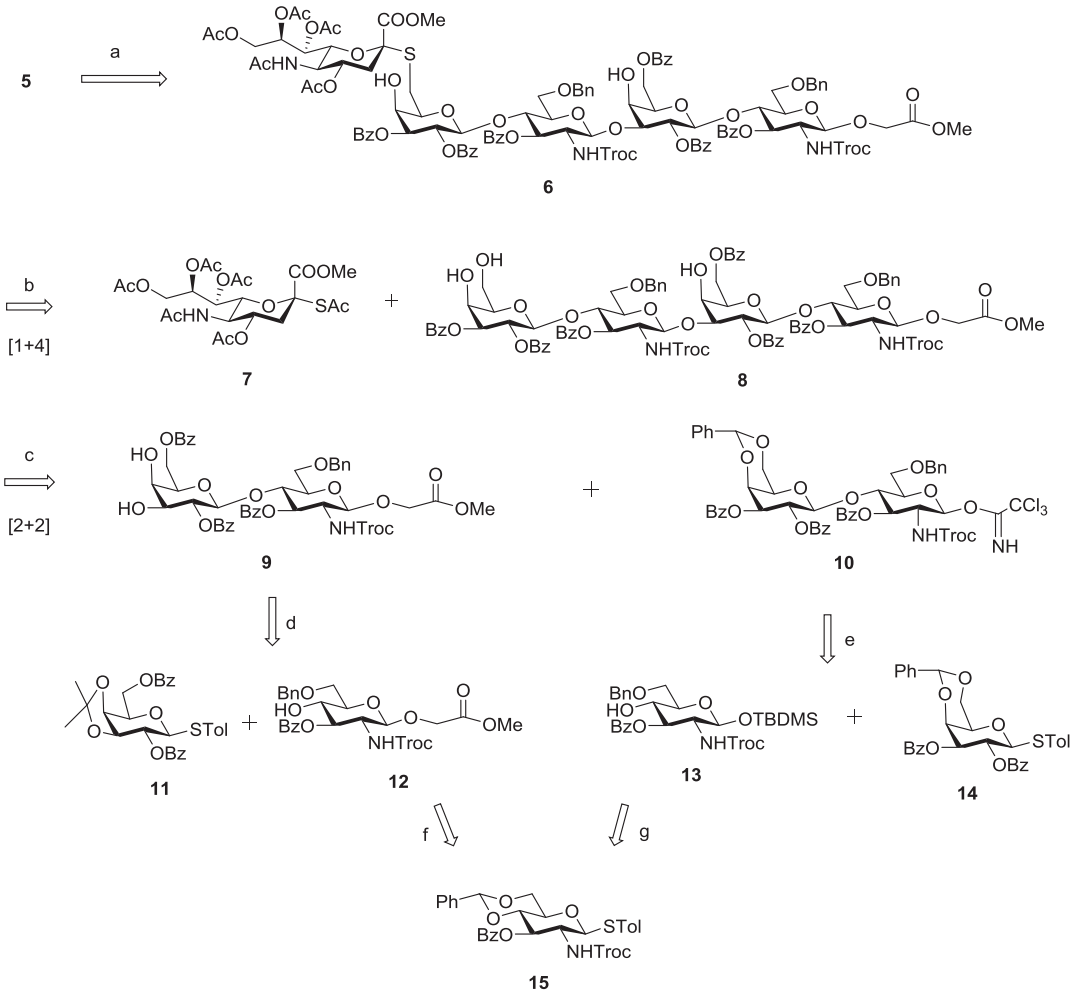


Fig. 2. Approach to present NA *S*-linked NeuAc-α(2-6)-di-LacNAc bearing liposomes to HA of influenza virus.

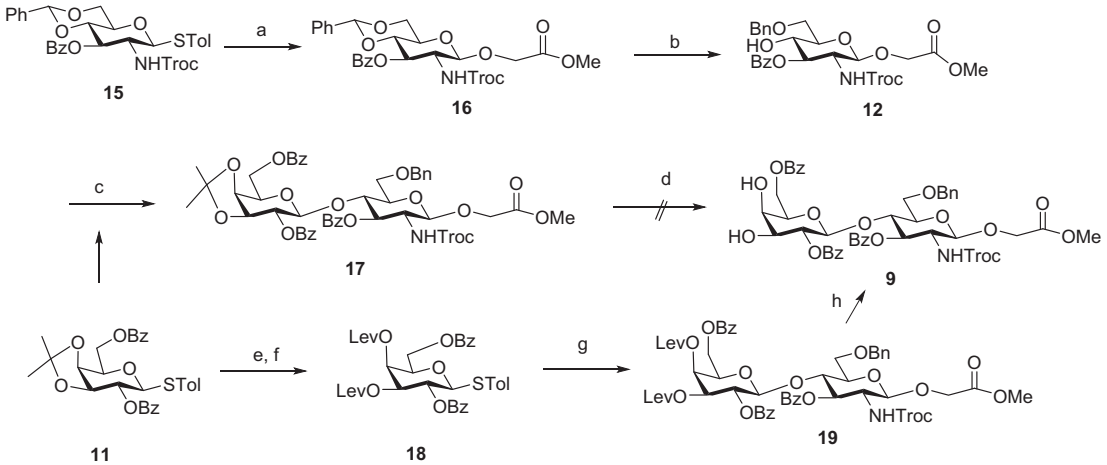
Please cite this article in press as: Cheng H.-W., et al. Bioorg. Med. Chem. (2018), https://doi.org/10.1016/j.bmc.2018.02.012



Scheme 1. Retrosynthetic analysis of *S*-Neu5Ac-α(2-6)Gal-β(1–4)GlcNAc-β(1–3)-Gal-β(1–4)GlcNAc containing **5**. (a) Deprotection of benzyl, benzoyl, Troc groups; (b) Formation of S-linkage, [1+4] glycosylation; (c) [2+2] glycosylation; (d), (e) Glycosylation; (f), (g) Glycosylation and selective ring opening reaction/or selective silyl group protection.

pation. The anomeric position of the non-reducing end of GluNAc (**15**) is modified by a methyl glycolate linker to give **12**, which can be hydrolyzed to the corresponding carboxylic acid, for conjugation with the phospholipid.

Our synthesis started with compound **15**,[11] which was coupled with methyl glycolate under NIS/TfOH conditions to give compound **16** in 85% yield (Scheme 2). The selective ring opening reaction was conducted under $NaBH_3CN$ and HCl in diethyl ether to

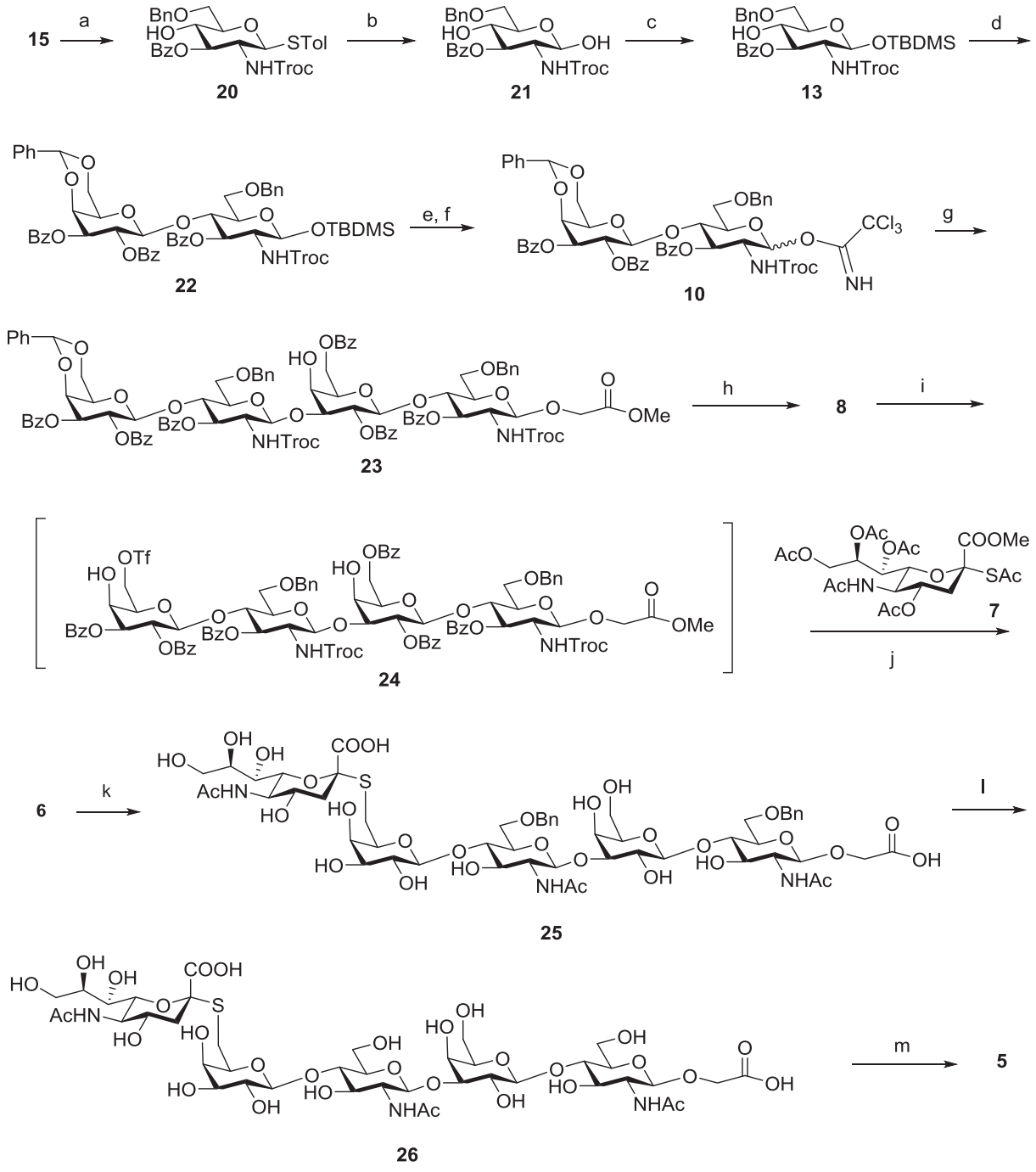


Scheme 2. Preparation of **9**. (a) NIS, TfOH, methyl glycolate, DCM, 4 Å MS, −40 °C, 1 h, 85%; (b) $NaBH_3CN$, HCl ether/THF, 0 °C, 70%; (c) BSP, TTBP, Tf_2O, DCM, 4 ÅMS, −60 °C, 1.5 h, 80%; (d) AcOH 60%, 60 °C; (e) Acetic acid 60%, 60 °C, 12 h; (f) levulinic acid, EDCI, DMAP/DCM, rt, 12 h, 80% two steps; (g) **12**, BSP, TTBP, Tf_2O/DCM, −60 °C, 1.5 h, 85%; (h) 1.0 N hydrazine acetate, toluene, rt, 10 min, 70%.

Please cite this article in press as: Cheng H.-W., et al. Bioorg. Med. Chem. (2018), https://doi.org/10.1016/j.bmc.2018.02.012

give compound **12** in 70% yield. Compounds **11** and **12** underwent glycosylation under BSP, TTBP and Tf_2O conditions to afford the disaccharide compound **17**. In order to remove the dimethyl acetonide group, compound **17** was treated with acetic acid (60%) at 60 °C. Unfortunately, these conditions resulted in hydrolysis of the glycosidic bond, and none of the desired compound **9** was isolated. Alternative reaction conditions (higher/lower temperatures) and reagents ($HClO_4$ at 0 °C or I_2 dissolved in MeOH)[21] were also unsuccessful-hydrolysis of the glycosidic bond occurred in all cases. Accordingly, we revised our protecting group strategy and replaced the acetonide group of **11** with a levulinate (Lev) group. Compound **11** was treated with 60% acetic acid at 60 °C to remove the acetonide group, which was directly coupled with levulinic acid using EDCI, and DMAP to give **18**. Glycosylation of compounds **12** and **18** took place with BSP, TTBP and Tf_2O at −60 °C to give the glycosylated intermediate **19**, whereupon the Lev groups were removed by hydrazine acetate to afford compound **9** in a yield of 60% over two steps.

Glycolipid **5** could now be pursued (Scheme 3). Compound **15** underwent selective ring opening using $NaBH_3CN$ and HCl in diethyl ether to give compound **20** in 70% yield. The thiol group of **20** was removed smoothly using NBS to provide compound **21**,



Scheme 3. Preparation of **5**. (a) $NaBH_3CN$, HCl ether/THF, 0 °C, 70%; (b)NBS/Acetone:H_2O = 5:1, −25 °C, 0.5 h; (c) TBDMSCl, imidazole/DCM, rt, 12 h, 70%; (d) **14**, NIS, TfOH/DCM, 4 Å MS, −45 °C to −25 °C, 1.5 h, 70%;(e) TBAF, acetic acid/THF, rt, 1 h; (f) CCl_3CN, DBU/DCM, rt, 4 h, two steps 70%; (g) **9**, TMSOTf/DCM, −78 °C, 36 h; (h) Acetic acid 80%, 60 °C, 24 h, 60%; (i) Tf_2O, pyridine/DCM, −25 °C, 4 h; (j) diethylamine/DMF, −20 °C, 2 h, 43% (3 steps); (k) i. Zn, Ac_2O, Et_3N, DCM, rt, 6 h; ii. NaOMe/MeOH, rt, 12 h; iii. 0.2 N NaOH, rt, 2 h, 56% for 3 steps; (l) H_2, $Pd(OH)_2$, 4 bar, rt, 12 h, 70%; (m) i. Amberlite®IR120 H^+; ii. DLPE, EDC, HOBt, NMM/DMF, 35 °C, 9 h, 50%.

Please cite this article in press as: Cheng H.-W., et al. Bioorg. Med. Chem. (2018), https://doi.org/10.1016/j.bmc.2018.02.012

the anomeric hydroxyl group of which reacted with TBDMSCl in the presence of imidazole to give compound **13**. Reaction of the 4-hydroxyl group of **21** with TBDMSCl was not observed, presumably due to its lower reactivity and more congested steric environment, compared to the anomeric hydroxyl group. Further glycosylation of **13** with thioglycoside donor **14** was performed using NIS and TfOH activation to give **22** in 70% yield. Next, the silyl protecting group of **22** was removed under TBAF and acetic acid conditions, and then reacted with trichloroacetonitrile and DBU to afford glycosyl imidate **10**. Disaccharides **9** and **10** underwent glycosylation by TMSOTf at −78 °C to give tetrasaccharide **23**. The benzylidene groups of tetrasaccharide **23** were removed by treatment with acetic acid to give a major product **8**, which was immediately reacted with Tf_2O in the presence of pyridine to yield triflate **24**. Nucleophilic substitution of the triflate and Neu5Ac thiolate, generated *in situ* by diethylamine from thioacetate **7**,[22] generated the *S*-linked pentasaccharide **6** in 70% yield. **6** was carried out using Zn/Ac_2O to remove Troc group and sequentially *N*-acetylated, and then global deprotected using cat. NaOMe in methanol followed by basic hydrolysis with 0.1 N NaOH to provide compound **25**. Hydrogenolysis of compound **25** by H_2 and Pd/C gave the desired compound **26**. Subsequently, **26** was conjugated *via* amide bond formation with DLPE, a kind of phospholipid found in biological membranes, which bears an amino group on the hydrophilic side. In order to free the carboxylic acid from its sodium salt, compound **26** was acidified with Amberlite® IR120 H^+ and the free carboxylic group activated by EDC and HOBt, prior to reaction with DLPE in the presence of NMM, to give the dipolar compound **5**. No DLPE was observed to conjugate with the 2-carboxylic group of sialyl **26**, presumably because of steric hindrance.

2.2. Preparation of liposome and zanamivir encapsulated liposomes

Single-layer (SL) and multi-layer liposomes (ML) are the most common type of liposomes. A single-layer liposome was deemed the most suitable for this study, because the surface ligands present in ML are buried within layers, which reduces the surface density of the ligand.

Fig. 2 depicts the expected interaction between HA and the liposome. In order for a trivalent interaction between the SL and the HA to occur, the surface density of the ligand presented on the surface of the liposome should be closed to the density of HA. For a liposome composed of DPPC and cholesterol in a 4:1 ratio and of an approximate diameter of 200 nm, the molar percentage of **5** on the liposome was estimated to be 0.8 mol% (see Supporting Information). Accordingly, DPPC, cholesterol (CH), and **5** were dissolved in $CHCl_3$ in a molar ratio of 4:1:0.008. The lipid sample mixtures were then concentrated for 30 min at 40 °C, then resuspended in PBS buffer (pH 7.4) to give multilayer liposomes (namely **5-ML**), which was used as a control. When the suspension was pressed through extruders of membrane pore sizes 400 nm and 100 nm, liposomes of 162.5 ± 1.3 nm diameter (monitored by Dynamic Light Scattering) were obtained, and henceforth referred to as **5-SL1**. The final concentrations were 20 mM for DPPC, and 165 μM for **5** (present as **5-SL1**). By way of comparison, SLs both without **5** and with a 0.4 mol% of **5** were also prepared by a similar method, and resulted in liposomes bearing **5** at concentrations of 0 μM (lipo-control) and 83 μM (**5-SL2**), respectively. The zanamivir encapsulated liposome **5-SL1-Za** was also prepared by adding zanamivir (2 mg) to a suspension of **5-SL1** (5 mL), and then pressing it through extruder of membrane pore size 400 nm and 100 nm. Compound **5** could also self-assemble to form micelles. The physical parameters for each of these formulations are disclosed in the Supporting Information.

2.3. Virus entry inhibition assay

To evaluate the ability of our compounds/formulations for inhibiting influenza virus entry, we adapted an effective method by co-incubating of our inhibitors with virus particles and Madin-Darby canine kidney (MDCK) cells, and then quantifying the amount of viral nucleoprotein (NP) present in the cells.[23] The presence of NP can be detected by ELISA with a primary monoclonal antibody to the influenza A NP, followed by incubation with a secondary monoclonal antibody. The fluorescence value of ex485/em535 is then detected. Here, compound **5**, **5-SL1**, **5-SL2**, and **5-ML** were subjected to an influenza entry inhibition assay with A/WSN/33 H1N1. Fluorescence values for each experiment were obtained at different concentrations, and the data is presented in Table 1. The greater the fluorescence, the lower the inhibitory ability of that compound. None of the multi-valent vectors tested displayed any inhibitory activity at 83 μM. Formulations of both **5-SL1** and **5-SL2** at 165 μM imparted weak inhibition (17%). Liposome encapsulated zanamivir (**5-SL1-Za**, zanamivir is 1.4 mM concentration herein) showed 25% virus entry inhibitory. These results indicate that zanamivir interfered with the entry of the virus into the cells,[24] and that our attempt to block influenza entry by capturing the virus using a sialic acid containing liposome failed, a result inconsistent with a recent study that demonstrated that a liposome bearing sialylneolacto-*N*-tetraose could effectively inhibit virus (Influenza A/Puerto Rico/8/34 virus) entry at 10 nM.[25] The contrasting nature of our results with this study might be due to the differences in virus strain and conditions for the influenza entry inhibition assay.

Table 1
The data of entry inhibition and hemagglutination inhibition assay.

Compd/formulation	Surface density (%)[a]	Assay conc.[b]	Entry inhibition	*Ki* (μM)[c]
Control/Single layer liposome	0 mol%	0	0%	0
5/Self assemble micelle	100 mol%	83	2%	ND[d]
		165	6%	
5-SL1/Single layer liposome	0.8 mol%	83	7%	15.6
		165	17%	
5-SL2/Single layer liposome	0.4 mol%	42	4%	15.6
		83	17%	
5-SL1-Za/Single layer liposome	0.8 mol%	83	10%	ND
		165	25%	
5-ML/Multilayer liposome	0.8 mol%	83	0%	31.2
		165	0%	

[a] Indicated the molar concentration of **4** in percentage among the formulation.
[b] Conc. of **5** in μM.
[c] Hemagglutination inhibition assay.
[d] Not determined.

Please cite this article in press as: Cheng H.-W., et al. Bioorg. Med. Chem. (2018), https://doi.org/10.1016/j.bmc.2018.02.012

2.4. Hemagglutination inhibition assay

According to the hemagglutination inhibition assay, the single layer liposomes **5-SL1** and **5-SL2**, which had 0.8 mol% and 0.4 mol% *S*-linked Neu5Ac-α(2-6)-di-LacNAc on their respective surfaces, displayed the lowest *K*i values of 15.6 μM (Table 1). Multilayer liposomes which had 0.8 mol% *S*-linked Neu5Ac-α(2-6)-di-LacNAc displayed the same K_i values of 31.2 μM. In our previous study, a liposome of mono-sialic acid conjugated with DLPE displayed a *K*i of 500 μM, and that of a trisaccharide **2** displayed a *K*i of 125 μM.[11] The formulation bearing *S*-linked Neu5Ac-α(2-6)-di-LacNAc designed in this study demonstrated the ability to inhibit the virus from binding with red blood cells (RBC) bearing sialic acid. This phenomenon suggests that our design of liposomal *S*-linked Neu5Ac-α(2-6)-di-LacNAc did have a stronger binding affinity with the virus than liposomal *S*-Neu5Ac-α(2-6)LacNAc (**2**) and liposomal mono-sialic acid. Since the sialic conjugation was homogenously displayed on the surface of liposome and yet still led to negative results in our virus entry inhibition assay, it is postulated that the binding to HA is unable to alleviate virus infectivity, a finding which merits further investigation. Perhaps the surface of the virus bears too high density of HA for its complete inhibition to be possible; and/or the binding of weaponized liposome to HA is simply insufficient for virus infectivity to be disabled.

3. Conclusion

Lipid conjugated *S*-linked NeuAc-α(2-6)-di-LacNAc (**5**) was successfully synthesized by [2+2] and [1+4] glycosylation methods. Liposomes bearing **5** at concentrations of 0.4 mol% and 0.8 mol% were prepared by combining **5** with DPPC and cholesterol. It was hoped that these liposomes would function as multivalent ligand inhibitors of HA, and all formulations/compounds were subjected to the HA inhibition assay by RBCs. **5-SL1** was determined to inhibit influenza virus binding with RBCs with a K_i of 15.6 μM. Disappointingly, however, none of the formulations tested could efficiently inhibit A/WSN/33/H1N1 influenza virus entry. The single layer liposome **5-SL1** (0.8 mol%) was found to impart superior inhibitory activity than the multilayer liposome **5-ML**, but both were inferior to the liposome encapsulated with Za (**5-SL1-Za**). Although *S*-linked Neu5Ac-α(2-6)-di-LacNAc (**4**) decoy liposome and micelles did not prove capable of inhibiting influenza entry, the synthesis of the sialyloligosaccharides used in this study is expected to useful in the future due to the homogenous nature of the products.

4. Experimental section

4.1. General methods

All reagents and solvents were of reagent grade, and were used without further purification unless otherwise stated. Reaction progress was monitored by analytical TLC on 0.25 mm E. Merck silica gel 60 F_{254} using *p*-anisaldehyde, ninhydrine, and cerium as visualizing agents. NMR spectra were recorded on Bruker-AV-400 (400 MHz) and Bruker-AV-600 (600 MHz) instruments, with a TCI cryo probe and a BACS 60 sample changer. Chemical shifts (δ) are given in parts per million relative to ^{1}H: 7.24 ppm, ^{13}C: 77.0 ppm for $CDCl_3$, ^{1}H: 4.80 ppm for D_2O, and ^{1}H: 4.78 ppm, ^{13}C: 49.2 ppm for methanol-D_4. 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 constants (*J*) are given in Hz. All peak assignments were confirmed using 2DNMR (COSY, HSQC) techniques. Exact mass measurements were performed using a VG platform electrospray ESI/MS or a BioTOF II (Taiwan). The DLS experiments used a BIC 90 PLUS device (Brookhaven Instrument Co. Holtsville, NY, USA).

4.2. Synthesis and characterization of compounds

4.2.1. 3-O-Benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-β-D-glucopyranoside (**12**)

Sodium cyanoboronhydride (2.2 g, 35.2 mmol) and 4 Å MS were added to a stirred solution of compound **16**[11] (2.4 g, 3.91 mmol), in dry THF (50 mL) at 0 °C over 15 min. After 8 h, 1.0 N HCl (in diethyl ether) was added slowly, until effervescence had ceased. The reaction was quenched by sat. $NaHCO_3$ solution, filtered to remove 4 Å MS, and concentrated *in vacuo* to give a residue that was extracted with dichloromethane. The organic layers were separated, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/ethyl acetate, 2/1) to afford **12** (1.7 g, 70% yield) as yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.02–7.27 (m, 10H, Ar-H), 5.66 (d, *J* = 9.6 Hz, 1H, N-H), 5.29 (t, *J* = 9.6 Hz, 1H, H-3), 4.75 (d, *J* = 12 Hz, 1H, Troc-CH), 4.71 (d, *J* = 8.4 Hz, 1H, H-1), 4.54 (q, *J* = 12 Hz, 2H), 4.46 (d, *J* = 12 Hz, 1H, Troc-CH), 4.33 (q, *J* = 16.8 Hz, 2H), 3.93 (q, *J* = 9.6 Hz, 1H, H-2), 3.87 (t, *J* = 9.6 Hz, 1H, H-4), 3.78 (d, *J* = 4.2 Hz, 2H, H-6), 3.72 (s, 3H, OCH_3), 3.60 (t, *J* = 4.2 Hz, 1H, H-5) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.3, 167.3, 154.7, 137.6, 133.5, 130.1, 129.1, 128.5, 128.4, 127.9, 127.7, 100.2 (C-1), 95.4 (Troc-CCl_3), 77.0, 76.2, 74.8, 74.3, 73.8, 70.6, 69.8, 64.7, 55.6, 52.0 (O$\underline{C}H_3$) ppm; HRMS (ESI) calcd. for $C_{26}H_{28}Cl_3NO_{10}Na$ $[M+Na]^+$ 642.0671, found *m*/*z* 642.0698.

4.2.2. 4-Methylphenyl-2,6-di-O-benzoyl-3,4-di-O-levulinoyl-1-thio-β-D-galactopyranoside (**18**)

Compound **11** (3.0 g, 5.6 mmol) was stirred in 60% acetic acid aqueous solution (30 mL) at 60 °C for 12 h. The reaction was quenched with sat. $NaHCO_3$ solution and brine and the organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give residue (2.8 g). The residue (2.8 g), EDCI (2.4 g, 12.3 mmol) and DMAP (12 mg, 0.112 mmol) in dichloromethane were added levulinic acid (1.3 mL, 12.3 mmol). After stirring for 2 h, the reaction was quenched with sat. $NaHCO_3$ solution and brine and the organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a residue that was purified by column chromatography (silica gel, toluene/ethyl acetate, 6/1) to afforded **18** (3.1 g, 80% yield in two steps) as a yellow solid; mp 121 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.00–6.92 (m, 14H, Ar-H), 5.57 (d, *J* = 3.2 Hz, 1H), 5.48 (t, *J* = 10 Hz, 1H), 5.20 (dd, *J* = 3.2, 10 Hz, 1H), 4.81 (d, *J* = 10 Hz, 1H, H-1), 4.52 (dd, *J* = 7.6, 11.2 Hz, 1H), 4.35 (dd, *J* = 5.2, 11.2 Hz, 1H), 4.11 (t, *J* = 6.4 Hz, 1H), 2.76–2.38 (m, 8H, –$COCH_2$-CH_2CO–), 2.25, (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.98 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.0 (2C), 171.9, 171.8, 166.0, 165.3, 138.3, 133.4, 133.2, 133.1, 129.9, 129.8, 129.6, 129.5, 129.3, 128.8, 128.5, 128.4, 87.3 (C-1), 74.7, 72.2, 67.8, 67.7, 62.3, 37.8, 37.7, 29.7, 29.4, 27.8, 27.8, 21.1 ppm; HRMS (ESI) calcd. for $C_{37}H_{38}O_{11}SNa$ $[M+Na]^+$ 713.2027, found *m*/*z* 713.2061.

4.2.3. 4-O-[2,6-Di-O-benzoyl-β-D-galactopyranosyl]-(1 → 4)-3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-β-D-glucopyranoside (**9**)

Thioglycoside **18** (1.7 g, 2.7 mmol) in dry dichloromethane (40 mL) was added 4 Å MS, BSP (848 mg, 4.05 mmol) and TTBP (1.3 g, 5.4 mmol). The solution was cooled to −60 °C, after which Tf_2O (0.8 mL, 4.59 mmol) was added slowly. After 10 min, **12** was then added into the solution. After stirring for 1.5 h, the cooling bath

was removed and the reaction diluted with dichloromethane. Then, the mixture was filtered to remove the 4 Å MS and washed with sat. $NaHCO_3$ and brine. The organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethyl acetate, 1/1) to afford a white foam (2.7 g, 85%). Hydrazine acetate (1.0 N solution in MeOH) was added to a solution of this residue (2.0 g, 1.7 mmol) in toluene (40 mL). After 10 min, the reaction was concentrated *in vacuo* to give a residue was purified by column chromatography (silica gel, hexanes/ethyl acetate, 1/1) to afford **9** (1.2 g, 70% yield) as a white solid; mp 97 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.05–7.26 (m, 20H, Ar-H), 5.58 (d, J = 8.8 Hz, 1H), 5.31 (t, J = 9.2 Hz, 1H), 5.16 (t, J = 8.8 Hz, 1H), 4.75 (d, J = 9.2 Hz, H-1), 4.61–4.44 (m, 4H), 4.3 (m, 3H), 4.10 (t, J = 9.2 Hz, 1H), 4.00 (m, 2H), 3.69 (s, 3H, OCH_3), 3.62–3.73 (m, 3H), 3.50 (q, J = 8.8 Hz, 2H), 3.40 (d, J = 10.8 Hz, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.3, 166.4, 166.2, 154.7, 1379, 133.5, 129.9, 129.8, 129.7, 129.5, 129.4, 128.6, 128.5, 128.4, 127.9, 127.8, 100.5 (C-1), 10.3 (C′-1), 95.4 (CCl_3), 75.1, 74.7, 74.3, 73.8, 73.5, 73.4, 72.5, 72.2, 68.5, 67.3, 64.7, 61.8, 55.8, 52.0 ppm; HRMS (ESI) calcd. for$C_{46}H_{46}Cl_3NO_{17}Na$ $[M+Na]^+$ 1012.1724, found *m/z* 1012.1766.

4.2.4. 4-Methylphenyl 3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside (**20**)

1.0 N HCl (in diethylether) was slowly added to a stirred solution of compound **15**[11] (4.0 g, 6.1 mmol), sodium cyanoboronhydride (3.5 g, 54.9 mmol) and 4 Å MS in dry THF (100 mL) at 0 °C for 15 min until the bubble disappeared. After 10 min, the reaction was quenched by sat. $NaHCO_3$ solution and the mixture was filtered to remove 4 Å MS, concentrated *in vacuo*, and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethyl acetate, 2/1) to afford **20** (2.8 g, 70% yield) as white foam; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.99–8.00 (d, J = 7.2 Hz, 2H, Ar-H), 7.18–7.57 (m, 10H, Ar-H), 7.06–7.07 (d, J = 7.98 Hz, 2H, Ar-H), 5.25–5.33 (m, 2H), 4.73 (d, J = 2.6 Hz, 1H), 4.71 (d, J = 4.3 Hz, 1H), 4.45–4.59 (m, 3H), 3.80–3.92 (m, 4H), 3.60–3.66 (m, 1H), 2.31 (s, 3H, STol-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz)δ 167.2 (COO), 154.2 (NCO), 133.6, 133.2, 130.0, 129.7, 128.5, 128.4, 127.9, 127.7, 95.3 (C-1), 87.4 (CCl_3), 78.4, 74.5, 73.8, 70.7, 60.6, 54.9, 29.8, 21.2; HRMS (ESI) calcd. for$C_{30}H_{30}Cl_3NO_7SNa$ $[M+Na]^+$ 676.0701, found *m/z* 676.0727.

4.2.5. 3-O-Benzoyl-6-O-benzyl-1-O-tert-butyldimethylsily-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside (**13**)

NBS (2.2 g, 12.4 mmol) was added to a stirred solution of compound **20** (2.0 g, 3.1 mmol) in acetone/H_2O (5/1, 20 mL) was added slowly at −25 °C. After 30 min, the reaction was quenched by 20% aqueous sodium thiosulfate, and organic solvent was evaporated. The mixture was diluted with dichloromethane, washed with sat. $NaHCO_3$ solution and brine. The organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The purified residue was added with TBDMSCl (561 mg, 3.72 mmol) and imidazole (498 mg, 7.44 mmol) in dichloromethane (40 mL). After stirred 12 h, the reaction was concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethyl acetate, 2/1) to afford **13** (1.4 g, 70% yield in two steps) as yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.07 (d, J = 7.8 Hz, 2H, Ar-H), 7.30–7.60 (m, Ar-H), 5.80 (t, J = 10.2 Hz, 1H), 5.44 (t, J = 9.6 Hz, 1H), 4.83 (d, J = 7.8 Hz, 1H, H-1), 4.56–4.70 (m, 4H), 3.89–3.96 (m, 2H), 3.83 (s, 2H), 3.71 (t, J = 4.8 Hz, 1H), 3.39 (s, –OH), 0.92 (s, -*t*-butyl, 9H), 0.17 (s × 2, 6H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ 167.4 (COO), 154.4 (NCO), 137.7, 133.5, 130, 129.1, 128.4, 127.7, 127.6, 96.4 (C-1), 95.3 (-CCl_3), 76.0, 74.3, 74.2, 73.7, 71.1, 70.3, 57.8, 25.5, 17.8 ppm; HRMS (ESI) calcd. for$C_{29}H_{38}Cl_3NO_8SiNa$ $[M+Na]^+$ 684.1324, found *m/z* 684.1353.

4.2.6. 4-O-[2,3-Di-O-Benzoyl-4,6-di-O-benzylidene-β-D-galactopyranosyl]-(1 → 4)-3-O-benzoyl-6-O-benzyl-1-O-tert-butyldimethylsily-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside (**22**)

NIS (0.95 g, 4.2 mmol) were added to a stirred solution of compound **13** (1.4 g, 2.1 mmol), **14** (2.46 g, 4.2 mmol) and 4 Å MS in dry dichloromethane (40 mL) at rt. After the solution was cooled to −60 °C, it was added TfOH (37 μL, 0.42 mmol) and warming to −45 °C. After 2 h later, the reaction was quenched by 20% aqueous sodium thiosulfate, and then the mixture was diluted with dichloromethane, washed with sat. $NaHCO_3$ solution and brine. The organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, toluene/ethyl acetate, 9/1) to afforded **22** (1.6 g, 70%) as white oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 (d, J = 7.2 Hz, 2H, Ar-H), 7.42–7.92 (m, 21H, Ar-H), 7.17 (d, J = 6.8 Hz, 2H), 5.69 (t, J = 10 Hz, 1H), 5.42 (t, J = 10.4 Hz,1H), 5.30 (s, 1H, CHPh), 5.25 (d, J = 9.6 Hz, 1H), 5.10 (d, J = 10.4 Hz, 1H), 4.84 (d, J = 8 Hz, 1H), 4.65 (m, 2H), 4.42 (d, J = 12 Hz, 1H), 4.33 (m, 2H), 4.15 (m, 2H), 3.83 (m, 2H), 3.57 (m, 4H), 2.98 (s, 1H), 0.84 (s, 9H, *t*-butyl), 0.09 (s, 3H, CH_3), 0.04 (s 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.1, 166.1, 164.1 (COO), 154.3 (NCO), 138.2, 137.4, 133.3, 133.2, 129.8, 129.8, 129.7, 129.5, 129.3, 128.9, 128.7, 128.5, 128.3, 128.3, 127.9, 127.6, 127.5, 126.3, 101.0 (C-1), 100.6 (C′-1), 96.5 (CHPh), 95.3 (CCl_3), 76.0, 74.5, 74.3, 73.8, 73.1, 72.7, 69.5, 68.1, 68.0, 66.4, 58.5, 25.5, 17.8, −4.18, −5.32 ppm; HRMS (ESI) calcd. for $C_{56}H_{60}Cl_3NO_{15}SiNa$ $[M+Na]^+$ 1142.2690, found *m/z* 1142.2743.

4.2.7. 4-O-[2,3-Di-O-benzoyl-4,6-di-O-benzylidene-β-D-galactopyranosyl]-(1 → 4)-3-O-[3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-(1 → 3)-4-O-[2,6-di-O-benzoyl-β-D-galactopyranosyl]-(1 → 4)-3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-β-D-glucopyranoside (**23**)

TBAF (0.4 mL, 1.32 mmol) and acetic acid (75 μL, 1.32 mmol) were added to a stirred solution of compound **22** (1.2 g, 1.1 mmol) in THF (20 mL). After the reaction completed, it was diluted with water and evaporated. After, the residue was extracted with dichloromethane, concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethyl acetate, 1/1) to afforded 1-OH disaccharide as a white solid. This disaccharide dissolved in dichloromethane (20 mL) was further reacted with trichloroacetonitrile (0.55 mL, 5.5 mmol) in the presence DBU (82 μL, 0.55 mmol). After stirring for 2 h, the reaction was concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes: ethyl acetate = 1:1) to afforded **10** (0.9 g, 70% yield over 2 steps) as yellow oil. Compound **10** (0.9 g, 0.77 mmol) and **9** (509 mg, 0.51 mmol) were dissolved in dry dichloromethane (20 mL) and stirred with 4 Å MS at −78 °C. After TMSOTf (7 μL, 0.077 mmol) was added, the reaction was stirred for 36 h and quenched by TEA (1 drop). The reaction was concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethyl acetate, 1/1) to afford **22** (1.2 g, 80% yield) as white foam; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.04–7.82 (m, 13H, Ar-H), 7.54–7.25 (m, 30H, Ar-H), 7.06–7.03 (m, 2H, Ar-H), 5.66 (t, J = 9.6 Hz, 1H), 5.26 (s, 1H, –CHPh), 5.33–5.25 (m, 2H), 5.08 (d, J = 9.6 Hz, 1H), 4.86 (s, 1H), 4.74 (d, J = 7.8 Hz, 1H, H-1), 4.69 (d, J = 12 Hz, 1H, H′-1), 4.57 (d, J = 12.6 Hz, 1H, H″-1), 4.52 (d, J = 7.8 Hz, 1H, H‴-1), 4.47–4.40 (m, 4H), 4.28–4.23 (m, 4H), 4.07–4.13 (m, 3H), 3.92–3.99 (m, 3H), 3.89 (s, 1H), 3.82–3.70 (m, 5H), 3.67 (s, 3H, OMe), 3.55–3.41 (m, 9H), 3.31–3.25 (m, 1H), 2.87–2.85 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.2, 166.3, 166.1, 165.8, 165.0, 164.4 (COO), 154.1 (NCO), 137.6, 137.4, 133.5, 133.2, 130.1, 129.9, 129.8, 129.6, 129.3, 129.0, 128.9, 128. 6, 128.5, 128.2, 128.0,

127.7, 126.3, 101.8 (C-1), 101.2 (C′-1), 100.6 (C″-1), 100.5 (C‴-1), 100.2 (CHPh), 95.5 (CCl_3), 95.2 (CCl_3), 76.2, 74.9, 74.6, 74.4, 74.2, 73.8, 73.5, 73.2, 73.1, 72.6, 72.2, 70.9, 69.6, 68.2, 68.0, 66.5, 56.5, 53.5, 52.1, 29.8, 25.7, 14.2 ppm; HRMS (ESI) calcd. for $C_{96}H_{90}Cl_6N_2O_{31}Na_2$ $[M+2Na]^{2+}$ 1012.1731, found *m/z* 1012.1742.

*4.2.8. S-[Methyl5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-(2 → 6)-4-O-[2,3-di-O-benzoyl-β-D-galactopyranosyl]-(1 → 4)-3-O-[3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-(1 → 3)-4-O-[2,6-di-O-benzoyl-β-D-galactopyranosyl]-(1 → 4)-3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-O-methylglycolate-β-D-glucopyranoside (**6**)*

Compound **23** (110 mg, 0.058 mmol) was suspended in 80% acetic acid and heated at 60 °C. After 12 h, the reaction was quenched by sat. $NaHCO_3$ solution and brine. The organic layers were collected, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a residue, which was purified by column chromatography (silica gel; hexanes/ethyl acetate, 1/1) to afford 4,6-dihydroxyl intermediate (68 mg, 0.036 mmol) which was dissolved in dry dichloromethane (1.2 mL) and pyridine (14.6 μL, 0.18 mmol, 5 equiv.) and stirred under N_2. The solution was cooled to −25 °C, then trifluoromethanesulfonic anhydride (7.3 μL, 0.043 mmol, 1.2 equiv.) was added dropwise over 1 min. After stirring at −25 °C for 1.5 h, the reaction mixture was diluted with dichloromethane (10 mL), quickly washed with 1.0 N HCl, saturated NaHCO3 solution, ice water, and brine. The organic layers were dried over MgSO4, filtered, and concentrated. The resting crude residue was added with **7** (20 mg, 0.036 mmol, 1 equiv.) in DMF (2 mL) and stirred under N_2. After cooled to −20 °C, diethylamine (37 μL, 0.36 mmol, 10 equiv.) was added and the solution was stirred for 3 h, the mixture was concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, hexanes/ethylacetate, 1/2) to afford **6** (60 mg, 43% yield for 3 steps) as white foam; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.10 (d, *J* = 7.2 Hz, 2H), 8.05–8.01 (m, 4H), 7.95–7.91 (m, 6H), 7.58–7.50 (m, 8H), 7.46–7.37 (m, 18H), 7.19–7.17 (d, *J* = 7.2 Hz, 2H), 5.53 (m, 2H), 5.37–5.34 (m, 3H), 5.28–5.24 (m, 3H), 5.18–5.17 (m, 1H), 4.94.93 (m, 2H), 4.82 (d, *J* = 10.2 Hz, 1H, H-1), 4.75 (d, *J* = 11.4 Hz, 1H, H′-1), 4.60 (d, *J* = 13.8 Hz, 1H, H″-1), 4.55 (d, *J* = 9 Hz, 1H, H‴-1), 4.53–4.47 (m, 4H), 4.37–4.31 (m, 3H), 4.27–4.22 (m, 3H), 4.17–4.11 (m, 3H), 4.08–4.05 (m, 1H), 4.00–3.96 (m, 3H), 3.86 (s, OMe), 3.83-0.753 (m, 4H), 3.73 (s, OMe), 3.67–3.59 (m, 4H), 3.56–3.50 (m, 6H), 3.42–3.38 (m, 1H), 2.75 (dd, *J* = 5.4, 13.2 Hz, 1H), 2.42–2.38 (m, 2H), 2.14 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.88 (s, 3H) ppm;^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.0, 170.5, 170.3, 170.2, 167.4, 166.4, 166.3, 165.7, 165.6, 165.1, 164.6, 164.4, 133.4, 130.1, 129.8, 129.7, 129.6, 128.6, 128.3, 128.2, 128.0, 127.9, 127.6, 101.9(C-1), 100.5(C′-1), 100.4(C″-1), 99.0(C‴-1), 95.6($\underline{C}Cl_3$), 95.2($\underline{C}Cl_3$), 75.1, 74.9, 74.8, 74.6, 74.4, 74.1, 74.0, 73.5, 73.4, 72.5, 72.3, 72.3, 71.8, 70.0, 69.4, 68.4, 68.2, 67.9, 67.5, 67.4, 67.3, 63.2, 62.5, 62.0, 56.2, 53.5, 52.8, 38.2, 37.9, 37.4, 37.2, 32.0, 29.8, 27.1, 23.3, 21.3 ppm; HRMS (ESI) calcd. for $C_{109}H_{113}Cl_6N_3O_{42}SNa$ $[M+Na]^+$ 2400.4512, found *m/z* 2400.4566.

4.2.9. S-[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-(2 → 6)-4-O-[β-D-galactopyranosyl]-(1 → 4)-3-O-[6-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-(1 → 3)-4-O-[β-D-galactopyranosyl]-(1 → 4)-6-O-benzyl-2-deoxy-2-acetamido-1-O-glycolic-2-deoxy-β-D-glucopyranoside (25)

Freshly acid washed activated Zn dust (60 mg) was added to a stirred solution of compound **6** (60 mg, 0.025 mmol), Ac_2O (6 μL, 0.063 mmol) and Et_3N (18 μL, 0.126 mmol) in dichloromethane (1.2 mL). The reaction vessel was sonicated in an ultrasonic cleaning bath at rt for 6 h. Then the mixture was filtered through Celite pad, and the filtrate was washed with $NaHCO_3$ solution, followed by brine. The organic layers were collected and evaporated *in vacuo* to give a residue which was dissolved in dry MeOH (2 mL) and then sodium methoxide (1 mg) was added. After stirring at rt for12 h, the solution was evaporated to give a residue which was treated with 0.1 N NaOH (0.5 μL) for 1 h. The solution was neutralized with Amberlite® IR 120H$^+$ resin, filtered and concentrated *in vacuo* to give a residue which was purified by Sephadex G-10 column chromatography to afford **25** (18 mg, 56% yield for 3 steps) as a yellow solid; ^{1}H NMR (D_2O, 600 MHz) δ 7.46–7.41 (m, 9H, Ar-H), 7.33 (m, 1H, Ar-H), 4.62 (d, *J* = 7.8 Hz, 1H, H-1), 4.57 (d, *J* = 8.4 Hz, 1H, H′-1), 4.52 (d, *J* = 12 Hz, 1H, H″-1), 4.33 (d, *J* = 8.4 Hz, 1H, H‴-1), 4.17–4.06 (m, 3H), 4.00 (d, *J* = 11.4 Hz, 1H), 3.95 (d, *J* = 7.8 Hz, 2H), 3.90–3.80 (m, 13H), 3.71–3.66 (m, 9H), 3.59–3.57 (m, 3H), 3.54–3.47 (m, 4H), 3.36–3.31 (m, 3H), 3.01–2.92 (m, 2H), 2.80 (dd, *J* = 5.4, 13.2 Hz, 1H, H⁗-3$_{eq}$), 2.05–2.03 (s × 3, 9H, CH_3), 1.77 (t, *J* = 12 Hz, H⁗-3$_{ax}$) ppm; ^{13}C NMR (D_2O, 150 MHz) δ 175.0, 175.0, 174.9, 174.9, 174.9, 137.3, 128.8, 128.6, 128.4, 128.3, 102.8 (C-1), 102.6 (C′-1), 102.3 (C″-1), 100.2 (C‴-1), 85.3, 81.5, 79.1, 76.9, 74.8, 74.0, 73.7, 73.4, 73.1, 72.9, 72.6, 72.4, 72.2, 71.8, 70.6, 69.8, 69.6, 69.3, 68.6, 68.3, 68.1, 67.8, 67.5, 62.6, 61.1, 55.2, 55.0, 51.7, 40.8, 29.6, 22.3, 22.2, 22.0 ppm; HRMS (ESI) calcd. for $C_{55}H_{79}N_3O_{30}SNa$ $[M+Na]^+$ 1316.4367, found *m/z* 1316.4374.

*4.2.10. S-[5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-(2 → 6)-4-O-[β-D-galactopyranosyl]-(1 → 4)-3-O-[2-deoxy-2-acetamido-β-D-glucopyranosyl]-(1 → 3)-4-O-[β-D-galactopyranosyl]-(1 → 4)-2-deoxy-2-acetamido-1-O-glycolic-2-deoxy-β-D-glucopyranosidedisodum (**26**)*

Compound **25** (18 mg, 0.014 mmol) in H_2O (1 mL) and methanol (1 mL) was added $Pd(OH)_2$ (20 mg) and stirred under H_2 (4 bar) for 12 h. The mixture was filtered through a pad of Celite and washed with water. The filtrates were combined and concentrated *in vacuo*. The residue was purified by Sephadex G-10 column chromatography to afford **26** (11 mg, 70% yield) as white foam; ^{1}H NMR (D_2O, 400 MHz) δ 4.40 (d, *J* = 6.9 Hz, 2H), 4.26 (t, *J* = 7.6 Hz, 3H), 3.95–3.97 (m, 4H), 3.75–3.91 (m, 5H), 3.31–3.66 (m, 31H), 3.17 (s, 1H), 2.36 (dd, *J* = 6.1, 12.2 Hz, 1H, H⁗′-eq), 2.17 (d, *J* = 12.2 Hz, 1H), 1.86 (s, 3H, CH_3), 1.85 (s, 3H, CH_3), 1.81(s, 3H, CH_3) ppm; ^{13}C NMR (D_2O, 100 MHz) δ 179.9, 179.9, 175.0, 175.9, 174.8, 103.0, 102.9, 102.7, 100.3, 82.1, 81.7, 78.9, 78.2, 75.2, 74.9, 74.8, 74.4, 73.9, 73.5, 72.7, 72.2, 72.1, 71.6, 71.2, 71.1, 70.8, 70.7, 70.6, 70.2, 70.0, 68.4, 68.4, 68.3, 68.3, 68.1, 68.0, 67.9, 63.0, 62.8, 62.5, 61.0, 59.95, 59.91, 55.1, 55.0, 52.57, 52.26, 52.23, 48.9, 48.3, 36.7, 34.3, 22.1, 22.0, 20.0, 15.4 ppm; HRMS (ESI) calcd. for $C_{41}H_{66}N_3Na_2O_{30}S$ $[M+2Na]^{2+}$ 1158.3242, found *m/z* 1158.3298.

*4.2.11. S-[5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-(2 → 6)-4-O-[β-D-galactopyranosyl]-(1 → 4)-3-O-[2-deoxy-2-acetamido-β-D-glucopyranosyl]-(1 → 3)-4-O-[β-D-galactopyranosyl]-(1 → 4)-2-deoxy-2-acetamido-1-O-(glycolic-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamido)-2-deoxy-β-D-glucopyranoside (**5**)*

Compound **26** (10 mg, 0.009 mmol) in methanol was added Amberlite®IR 120H$^+$ 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 (3 mg, 0.018 mmol, 2 equiv.), HOBt (6 mg, 0.045 mmol, 5 equiv.), DLPE (5 mg, 0.009 mmol, 1 equiv.), and NMM (5 μL, 0.045 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 **5** (7.6 mg, 50% yield) as brown oil; ^{1}H NMR (CD_3OD and $CDCl_3$, ratio = 4:1, 400 MHz) δ 5.32–5.30 (m, 1H), 5.20–5.19 (m, 1H), 4.88–4.85 (d, *J* = 18 Hz, 1H), 4.55–4.51 (m, 2H),

4.15–4.11 (m, 2H), 4.01–3.95 (m, 10H), 3.56–3.50 (m, 6H), 3.36–3.34 (m, 2H), 3.34 (d, J = 12 Hz, 1H), 3.15–3.05 (m, 6H), 2.71 (s, 1H), 2.67–2.65 (m, 1H), 2.52 (t, J = 12 Hz, 1H), 2.45 (t, J = 10 Hz, 1H), 2.39–2.38 (m, 2H), 2.31–2.31 (s, 2H) 2.28 (s, 3H, CH_3), 2.06–1.95 (m, 7H), 1.79–1.64 (m, 8H), 1.56–1.52 (m, 9H), 1.07 (t, J = 10.8 Hz, 3H), 085–0.82 (m, 15H) ppm; ^{13}C NMR (CD_3OD and $CDCl_3$, ratio = 4:1, 100 MHz) δ 181.6, 181.5, 174.9, 175.0, 174.8, 173.9, 173.5, 103.0, 102.9, 102.7, 100.3, 78.9, 75.9, 75.2, 75.0, 74.8, 74.5, 73.9, 73.5, 72.7, 72.2, 72.1, 71.6, 71.1, 70.7, 70.6, 70.5, 70.4, 70.3, 70.0, 69.95, 69.91, 69.8, 69.7, 69.3, 68.5, 68.5, 68.4, 68.1, 68.0, 68.0, 63.66, 63.62, 62.4, 61.66, 61.63, 61.0, 55.0, 52.5, 40.5, 40.4, 33.9, 33.8, 31.8, 29.5, 29.4, 29.2, 29.0, 24.7, 23.3, 22.5, 20.1, 13.5 ppm; HRMS (ESI) calcd. for $C_{70}H_{123}N_4NaO_{37}PS$ $[M+Na]^+$ 1697.7217, found m/z.1697.7259.

4.3. Liposome preparation

DLPE, or compound **5**, and cholesterol (CH) were dissolved with dipalmitoylphosphatidyl-choline (DPPC) in $CHCl_3$ (DPPC:CH:DLPE, **5** = 4:1:1, 0.008. The lipid sample mixtures were then concentrated *in vacuo* for 30 min at 40 °C. Film hydration were then reconstituted by gentle mixing in PBS buffer (5 mL, pH 7.4) to reach final lipid concentration of 20 mM. The contents of the vial were sonicated in a tip sonicator to give Lipo-control and **5-ML**. **5-ML** was pressed through extruder of membrane pore size 400 nm for 5 times and 100 nm for 5 times to give liposome formulation **5-SL1**. **5SL2** was prepared by the similar method, except for adding compound **5** in a ratio of 0.004 mol%. The zanamivir encapsulated liposome was also prepared by adding zanamivir (2 mg) to the **5-SL1** (5 mL) and which was pressed again through extruder of membrane pore size 400 nm and 100 nm to give liposome formulation, named **5-SL1-Za**. All these liposome-formulations were 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.

4.4. The virus entry inhibition assay

MDCK cells at 1×10^4/ml was added to each well. The 96 wells plates were incubated for 24 h at 35 °C. WSN virus (H1N1, provided by J.-T. Jan (GRC, Academic Sinica)) and inhibitors were mixed and preincubated for 1 h 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 h. 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 1 N H_2SO_4.

4.5. Hemagglutination inhibition assay

WSN H1N1virus (2^3 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.

Acknowledgements

We acknowledge the Ministry of Science and Technology (MOST 104-2320-B-002-008-MY3; 104-2627-M-002-011; 105-2627-M-002-009; 106-2627-M-002-001) and Academia Sinica in Taiwan for financial support. We gratefully thank Professor Y. S. Edmond Cheng at the Genomics Research Center of Academia Sinica for the influenza virus assay and valuable discussion.

A. Supplementary data

Supplementary data (synthesis of compound **11**, as well as physical parameter of liposomes and the calculation mol% of **5** on the liposome surface.) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.02.012.

References

1. Ito T, Couceiro JN, Kelm S, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Viol*. 1998;72:7367–7373.
2. Gamblin SJ, Haire LF, Russell RJ, et al. The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science*. 2004;303:1838–1842.
3. Shinya K, Ebina M, Yamada S, et al. Avian flu: influenza virus receptors in the human airway. *Nature*. 2006;440:435–436.
4. Spanakis N, Pitiriga V, Gennimata V, et al. A review of neuraminidase inhibitor susceptibility in influenza strains. *Expert Rev Anti Infect Ther*. 2014;12:1325–1336.
5. Hussain M, Galvin HD, Haw TY, et al. Drug resistance in influenza A virus: the epidemiology and management. *Infect Drug Resist*. 2017;10:121–134.
6. Khare MD, Sharland M. Influenza. *Expert Opin Pharmacother*. 2000;1:367–375.
7. Zeng LY, Yang J, Liu S. Investigational hemagglutinin-targeted influenza virus inhibitors. *Expert Opin Investl Drugs*. 2017;26:63–73.
8. Li F, Ma C, Wang J. Inhibitors targeting the influenza virus hemagglutinin. *Curr Med Chem*. 2015;22:1361–1382.
9. Mammen M, Choi S-K, Whitesides GM. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed Engl*. 1998;37:2754–2794.
10. Carlescu I, Scutaru D, Popa M, et al. Synthetic sialic-acid-containing polyvalent antiviral inhibitors. *Med Chem Res*. 2009;18:477–494.
11. Yeh HW, Lin TS, Wang HW, Cheng HW, Liu DZ, Liang PH. S-Linked sialyloligosaccharides bearing liposomes and micelles as influenza virus inhibitors. *Org Biomol Chem*. 2015;13:11518–11528.
12. Chandrasekaran A, Srinivasan A, Raman R, et al. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. *Nature Biotech*. 2008;26:107–113.
13. Maines TR, Jayaraman A, Belser JA, et al. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. *Science*. 2009;325:484–487.
14. Nycholat CM, McBride R, Ekiert DC, et al. Recognition of sialylated poly-N-acetyllactosamine chains on N- and O-linked glycans by human and avian influenza A virus hemagglutinins. *Angew Chem Int Ed Engl*. 2012;51:4860–4863.
15. Zheng XJ, Yang F, Zheng M, et al. Improvement of the immune efficacy of carbohydrate vaccines by chemical modification on the GM3 antigen. *Org Biomol Chem*. 2015;13:6399–6406.
16. Kiefel MJ, Beisner B, Bennett S, et al. Synthesis and biological evaluation of N-acetylneuraminic acid-based rotavirus inhibitors. *J Med Chem*. 1996;39:1314–1320.
17. Nazemi A, Haeryfar SM, Gillies ER. Multifunctional dendritic sialopolymersomes as potential antiviral agents: their lectin binding and drug release properties. *Langmuir*. 2013;29:6420–6428.
18. Mammen M, Dahmann G, Whitesides GM. Effective inhibitors of hemagglutination by influenza virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition. *J Med Chem*. 1995;38:4179–4190.
19. DeFrees SA, Phillips L, Guo L, et al. Sialyl Lewis x liposomes as a multivalent ligand and inhibitor of E-selectin mediated cellular adhesion. *J Am Chem Soc*. 1996;118:6101–6104.
20. de Paz JL, Ojeda R, Barrientos AG, et al. Synthesis of a Ley neoglycoconjugate and Ley-functionalized gold glyconanoparticles. *Tetrahedron Asymmetry*. 2005;16:149–158.
21. Szarek WA, Zamojski A, Tiwari KN, et al. A new, facile method for cleavage of acetals and dithioacetals in carbohydrate derivatives. *Tetrahedron Lett*. 1986;27:3827–3830.
22. Rich JR, Bundle DR. S-linked ganglioside analogues for use in conjugate vaccines. *Org Lett*. 2004;6:897–900.
23. Rowe T, Abernathy RA, Hu-Primmer J, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol*. 1999;37:937–943.
24. Ohuchi M, Asaoka N, Sakai T, et al. Roles of neuraminidase in the initial stage of influenza virus infection. *Microbes Infect*. 2006;8:1287–1293.
25. Hendricks GL, Weirich KL, Viswanathan K, et al. Sialylneolacto-N-tetraose c (LSTc)-bearing liposomal decoys capture influenza A virus. *J Biol Chem*. 2013;288:8061–8073.