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Graphical Abstract

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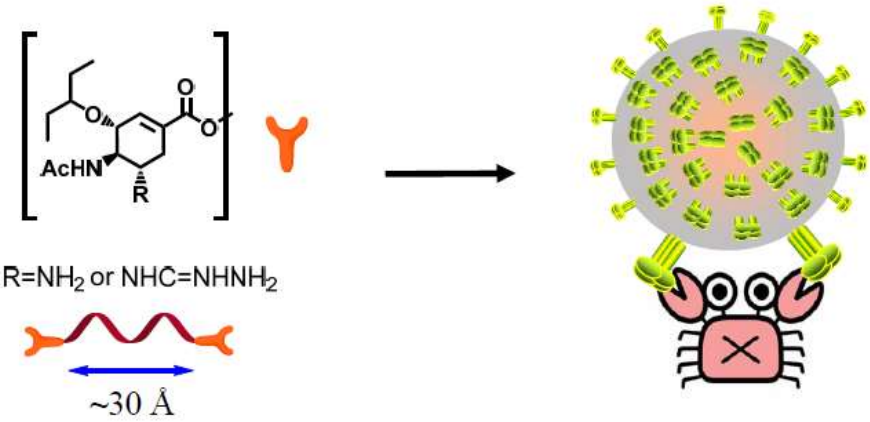

Highlights

- Novel divalent oseltamivir analogues were synthesized using Click chemistry.
- Distance between two oseltamivir monomers ~30 Å can enhance antiviral activity.
- Esterification of oseltamivir carboxylic acid can be further developed for improving efficacy.

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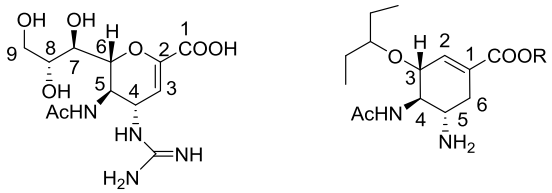
ABSTRACT

A panel of divalent oseltamivir and guanidino oseltamivir analogues with esterification on the carboxyl acid group as potent inhibitors of influenza virus neuraminidase was prepared *via* click reaction. The primary structure activity relationship study demonstrated that appropriate distance between two oseltamivir monomers around 30 Å can crosslink two adjacent neuraminidase tetramers on the virion surface and result in highly effective NA inhibitors against three strains of influenza virus and H7N9 virus like particle. This strategy also provides a basis for the multivalent modification on oseltamivir.

1. Introduction

Neuraminidases (NAs) or sialidases, catalyze the hydrolysis of α-glycosidic linkages of terminal sialic acid (SA) residues in glycoconjugates and are widespread in many tissues of animals, pathogens and viruses[1-2]. Several human pathologies are strongly associated with abnormal NAs activity[3], and some bacteria and viruses utilize their NAs for host cell infections[4]. Therefore, much attention has been paid on the design and synthesis of efficient NA inhibitors for the therapeutic applications and functional studies[5]. One of the most successful structure-based designs of potent NA inhibitor is the discovery of Zanamivir (Relenza™, **ZA**) (Fig. 1.), which mimics the transition state of the influenza NA-catalyzed sialoside hydrolysis reaction and has been used as the first-line drugs for the treatment of viral flu[6]. However, due to the low oral bioavailability and rapid renal elimination of **ZA**, Oseltamivir (Tamiflu™, **OS**) (Fig. 1.) was developed[7]. Compared with **ZA**, the pyran ring, C-4 guanidino group and glycerol side chain are replaced with cyclohexene, amine and 3-pentyl ether, respectively. After further esterification of the carboxyl group, **OS** as the orally available therapeutic prodrug is usually prescribed in the clinic for combat human influenza pandemics[8]. Unfortunately, the frequent mutations of NA, which leads to the emergence of new **OS**-resistant strains[9] has stressed the urgent need for the development of new antiviral agents in recent years.



Zanamivir, **ZA** (Relenza™)

R= Et, Oseltamivir, (Tamiflu™), **OS**
R= H, Oseltamivir Carboxylate, **OC**

Figure 1. Structures of two marketed NA inhibitors, **ZA** and **OS**.

It is now well known that the binding conformation of 3-pentyl ether side chain in **OS** affected by NA mutations is the main reason for the unbeneficial interactions, which results in drug-resistance[10]. In order to compensate this decreased binding affinity, many attempts of chemical modifications on C-5 amine[11], moving the location of C–C double bond[12] or replacing the C-3 pentyl group[13] have been applied. Generally speaking, this strategy more or less enhances the binding to NA in *vitro*, but does not lead to better inhibitors in *vivo*. Recently, *L*-amino acid ester linked Oseltamivir Carboxylate (**OC)** esters, which are

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facilitated by the peptide transporter–cellular activation as prodrugs show improved oral absorption and systemic availability[14]. In another example, **OC** hydroxamate and acyl sulfonamide derivatives[15] were also prepared and have been shown to have anti-influenza activity against wild-type H1N1 and **OS**-resistant virus. Building on these reports, we proposed that the esterification of the carboxyl group may be a target for the chemical structure modification and lead to obtain better NA inhibition activity.

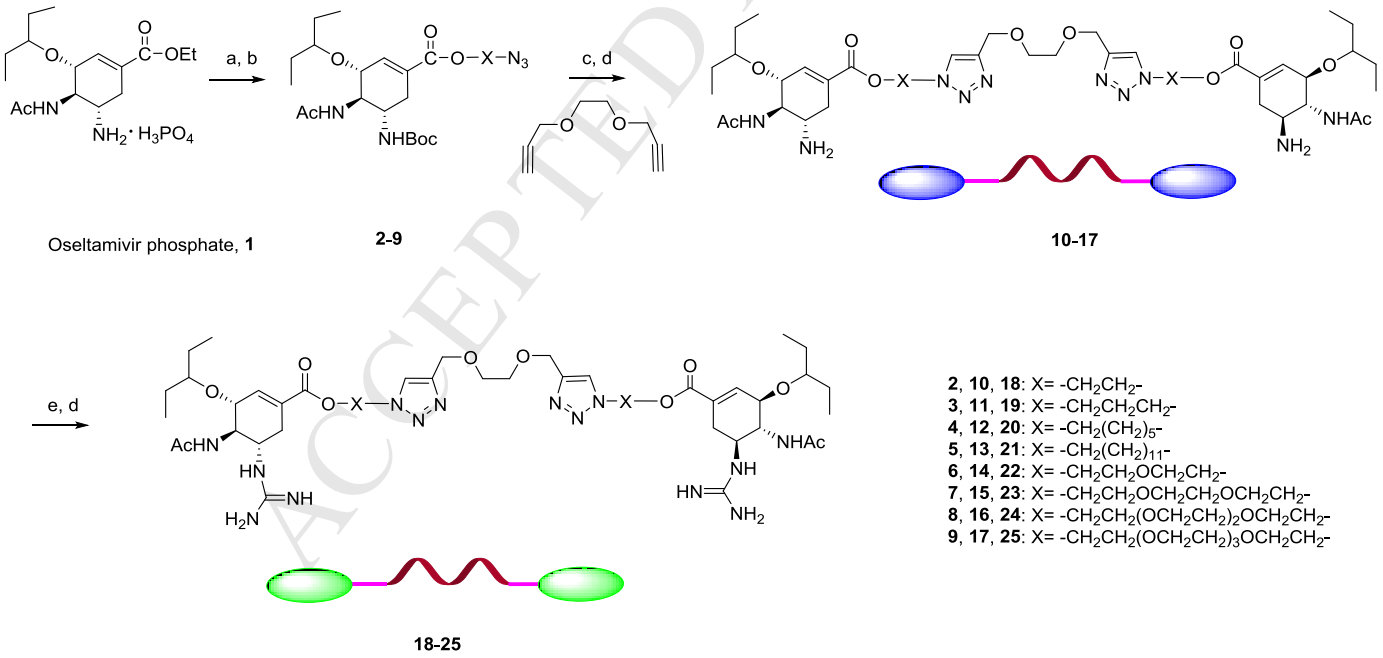
A different approach for the design of potent NA-inhibitors is to introduce "cluster effects"[16-18], which has been validated by the success of synthesizing multivalent **ZA** conjugates[19-29]. As four NA monomers form a mushroom-like tetramere anchored on viral surface[30], various **ZA** with appropriate distance attached on the multivalent backbones can simultaneously bind to more than one NA monomer within a single or different tetramers on the same or even separated virions. Structurally, these strategies use C-7 hydroxyl group of **ZA**, which has no direct interaction to the active site of NA for the linker attachment[31] and then assemble **ZA** on the multivalent scaffolds. However, due to the lack of hydroxyl group in the glycerol side chain of **OS**, multivalent **OS** conjugates have not been pursued.

Herein, we want to combine with "cluster effect"[32-34] and ester prodrug masked strategy together for increasing the binding affinity of **OS** to NA. As the initial investigation, a panel of different lengths of the linker carrying two **OC** or guanidino **OC** monomers was designed and synthesized. Furthermore, a primary relationship between the distance of two monomers in the divalent analogues and the NA inhibition activity is discussed.

2. Results and discussion

2.1. Chemical synthesis

The synthesis of divalent oseltamivir and guanidino oseltamivir analogues with different linker lengths were illustrated in Scheme 1. At first, **OS** was treated with di-tert-butyl dicarbonate $(Boc)_2O$ and further hydrolyzed to obtain the corresponding Boc protected carboxylic acid[35] (**OC**). Different lengths of hydrophobic or hydrophilic azidoalcohols were reacted with the acid under typical esterification condition to afford the azide moiety **2-9** in reasonable yields for the next Click chemistry[36-39]. Meanwhile, the alkyne part was prepared from ethylene glycol reacted with propargyl bromide under basic condition. With the two desired components in hand, 1, 3 dipolar Huisgen cycloaddition (Click reaction) was applied for the dimer synthesis. Typically, catalytic quantity of $CuSO_4 \cdot 5H_2O$ was added to the solution of bis-alkyne scaffold and different azide ester in tetrahydrofuran (THF) and water followed by sodium ascorbate. After purification by chromatography, the Boc group was removed with trifluoroacetic acid (TFA) to afford the final divalent **OC** analogues **10-17**. A Boc protected guanidino group was introduced by reaction with *N*, *N*'-bis-(tert-butoxycarbonyl)-*S*-methylisothiourea in the presence of $HgCl_2$ and Et_3N to provide divalent Boc-guanidino **OC** derivatives[40]. Treatment with TFA afforded free **18-25** in good yield. All intermediates and final products were characterized by spectroscopic properties.



Scheme 1. Synthesis of divalent **OC** analogues. Reagents and conditions: (a) (i) $(Boc)_2O$, trimethylamine (Et_3N), 98%; (ii) NaOH, tetrahydrofuran (THF)/H_2O 95%; (b) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), N, N-diisopropylethylamine (DIPEA), 85-89%; (c) $CuSO_4 \cdot 5H_2O$, sodium ascorbate (VcNa), THF/H_2O; (d) trifluoroacetic acid (TFA)/CH_2Cl_2 1:1; (e) MeSC(=NBoc)NHBoc, $HgCl_2$, Et_3N, CH_2Cl_2, rt, 12 h.

2.2. Biological Assays

With the divalent **OC** and guanidino **OC** analogues in hand, we evaluated NA inhibition activity using a typical fluorogenic substrate 2-(4-methylumbelliferyl)-α-*D*-N-acetyl neuraminic acid (MUNANA) against three viral strains [A/Hecheng, Hunan Province/SWL1331/2014(H1N1); A/Puerto Rico/8/1934 (H1N1); A/Chicken/Beijing/AT609/2014 (H9N2)] and H7N9 virus like particles (VLP)[41]. To compare the inhibition activity enhancement, **OS** and guanidino **OS** were selected as positive control, respectively. The IC_{50} values of each divalent analogues and potency per one corresponding **OS** monomer (γ)[29] are summarized in Table 1.

It is gratifying to note that the esterification on the carboxylate has little effect on NA inhibition activity, as all of the divalent analogues had comparable IC_{50} to their corresponding monomers. Additionally, introduction of the guanidino group increased the inhibition activities of **18**-**25**; decreased IC_{50} values in submicromolar than corresponding animo divalent analogues **10**-**17** were observed. This is due to the more basic gaunidino group, which has strong electrostatic interactions with the acidic peptide residues in the active site of NA[42].

We were very interested in whether these divalent **OC** analogues exhibit a bidentate binding effect similar to the reported dimeric **ZA**[26, 43-44] with ten to hundred times decreased EC_{50} or *S*-sialoside[40] clusters with ten times decreased IC_{50} compared to their corresponding monomers. Based on the X-ray crystal structure of influenza NA, it has been demonstrated that the distance of two NA active sites in a tetramer or two different neighboring tetramers on the same virion is approximately ~50 Å and ~30 Å, respectively (Fig. 2.)[19, 26]. Only the dimension of the two monomers in the divalent analogues, which matches the NA tetramer distribution can improve the binding affinity. To measure the distance between the two **OC** monomers in our synthetic dimers, the low-energy conformations of **10**-**17** and **18**-**25** were simulated using Discovery Studio 3.1 and the primary relationship between the lengths of the linker and the antiviral activity was determined (Table 1.).

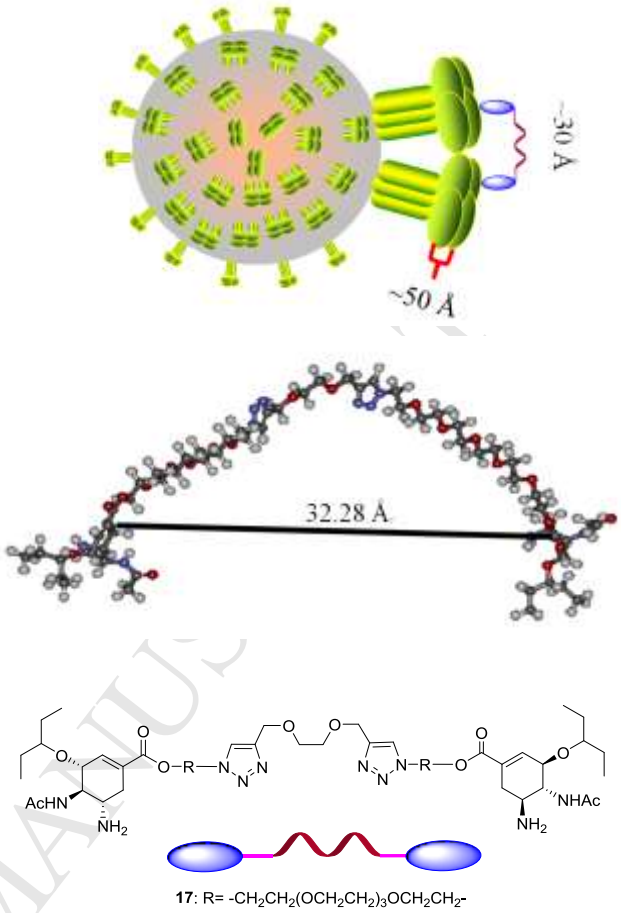


Figure 2. Schematic representation of distance between two NA monomer active sites on a same virion and the low-energy extended conformation of **17**.

Table 1. Neuraminidase inhibition activity of divalent **OC** and guanidino **OC** analogues

Compound number	Distance between two **OC** ananlogs monomer (Å)	IC_{50} (μM)			
		H1N1[a] (γ[d])	H1N1[b] (γ)	H7N9 VLP (γ)	H9N2[c] (γ)
10	19.30	1.10 (1)	1.30 (2)	0.73 (1)	0.18 (2)
11	24.60	3.63 (0.4)	5.41 (0.6)	2.10 (0.5)	0.59 (0.6)
12	25.90	0.52 (2)	0.73 (4)	0.35 (3)	0.11 (3)
13	28.86	3.79 (0.3)	4.76 (0.7)	1.80 (1)	0.71 (0.5)
14	27.48	0.84 (1)	1.21 (3)	0.45 (2)	0.13 (3)
15	32.80	0.22 (6)	0.37 (9)	0.11 (9)	0.03 (11)
16	25.30	0.93 (1)	1.48 (2)	0.55 (2)	0.14 (2)
17	32.28	0.24 (5)	0.048 (68)	0.10 (10)	0.034 (10)
OS		2.56 (1)	6.49 (1)	2.06 (1)	0.69 (1)
18	19.70	0.24 (4)	0.31 (9)	0.26 (2)	0.26 (7)
19	24.60	0.13 (8)	0.17 (17)	0.16 (3)	0.14 (13)
20	26.06	0.59 (2)	0.71 (4)	0.50 (1)	0.62 (3)
21	28.98	0.10 (10)	0.13 (22)	0.08 (6)	0.30 (6)
22	27.27	0.21 (5)	0.27 (11)	0.19 (2)	0.24 (8)
23	32.98	0.32 (3)	0.51 (6)	0.26 (2)	0.23 (8)
24	25.50	0.10 (10)	0.16 (18)	0.13 (4)	0.11 (17)
25	31.32	0.08 (12)	0.13 (22)	0.08 (6)	0.08 (23)
Guanidino OS		2.01 (1)	5.87 (1)	0.95 (1)	3.74 (1)

[a] A/Hecheng, Hunan Province/SWL1331/2014(H1N1);

[b] A/Puerto Rico/8/1934 (H1N1);

[c] A/Chicken/Beijing/AT609/2014 (H9N2);

[d] $\gamma = IC_{50\ \text{corresponding monomer}} / (2 \times IC_{50\ \text{divalent analogue}})$.

Our modeling study predicted that the distances between two **OC** monomers in the prepared divalent analogues are ranging from 20 Å to 33 Å. In **15**, **17** and **25**, the intermolecular space of the two **OC** monomeric analogues is ~32 Å, which can bind two neighboring NA resulting in bidentate binding effect and have stronger inhibition activity against all of the four strains, leading to decreased IC_{50} values. In contrast, the other divalent analogues with two **OC** monomer spacing less than 30 Å is not sufficient to achieve the bivalent binding requirement leading to less effective NA inhibition activity, which is in agreement with other published results[19, 26, 40]. To clearly compare the activity enhancement, a γ factor indicates the potency per **OC** monomer was used with **OS** and guanidino **OS** as positive control, respectively (Table 1). The most potent NA inhibitor **17** exhibits approximately 68-fold increase in inhibition against H1N1 strain compared with its **OS** monomer, which further stresses the importance of the linker for the dimer attachment.

3. Conclusions

We have introduced "cluster effect" principle to prepare a panel of divalent **OC** and guanidino **OC** ester analogues [45] with different linker lengths. A primary relationship between the length of the linker and NA inhibition activity was identified. The appropriate distance between two **OS** monomers to achieve divalent NA active site binding is ~32 Å, which is essential for the divalent NA inhibitor design. With these encouraging results, we are currently choosing new scaffolds to obtain multi or polyvalent **OC** or guanidino **OC** conjugates as more potent NA inhibitors. Furthermore, the inhibition activities of these synthetic dimers on cell or animal level are also under investigation in our lab. We believe that this research will provide a basis for investigating multivalent **OC** conjugates not only as therapeutics agents for wide strains of influenza virus, but also against drug-resistant strains.

4. Experimental

4.1. General methods

All the materials and solvents were obtained from commercial suppliers and used without further purification. Oseltamivir phosphate was purchased from Hangzhou Rongda Pharm & Chem Co, Ltd. China. Thin-layer chromatography (TLC) was purchased from EMD Co. Ltd. (German). All compounds were stained with iodine vapor. Flash column chromatography was performed on silica gel 200-300 mesh. All of the final compounds were purified using Sephadex™ LH-20 (GE Healthcare). NMR spectra were recorded on Bruker AVANCE III (400 MHz) instrument. Chemical shifts (δ) were reported in parts per million downfield from TMS, the internal standard; *J* values were given in Hertz. The molecular weights of the compounds were confirmed by electrospray ionization mass spectra (ESI-MS) on a hybrid IT-TOF mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan). Fluorescence intensity was measured using the Synergy™ H1/H1MF microplate reader (BioTek Instruments, Inc. USA).

The influenza viruses were obtained from National Institute for Viral Disease Control and Prevention, China CDC and propagated in 9 days old embryonated chicken eggs. The allantoic fluid was collected, then centrifuged at 3000 rpm/min for 10 min and the supernatant was stored at -80 ℃ before use.

4.2. Chemistry

4.2.1. General procedure for the linker attachment

To a solution of the Boc protected oseltamivir acid[35] (78 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) were added alcohol (0.24 mmol), EDCI (91 mg, 0.24 mmol), DIPEA (0.1 mL, 0.6 mmol) and DMAP (7.4 mg, 0.02 mmol). The mixture was stirred at room temperature for 10 h, diluted with CH_2Cl_2 and then washed with 1 M HCl, saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography to give Boc protected **OC** monomers with different length linkers as pale yellow oil.

*4.2.1.1. 2-azidoethyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**2**)*

According to the general procedure, **2** was synthesized in 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.06 (s, 1H), 5.22 (s, 1H), 4.31 (t, *J* = 4.1 Hz, 2H), 4.09–3.95 (m, 2H), 3.79 (dd, *J* = 10.2, 5.1 Hz, 1H), 3.55–3.46 (m, 2H), 3.37–3.27 (m, 1H), 2.77 (dd, *J* = 6.4, 18.7 Hz, 1H), 2.30 (dd, *J* = 17.7, 10.0 Hz, 1H), 1.97 (s, 3H), 1.52–1.44 (m, 4H), 1.41 (s, 9H), 0.87 (q, *J* = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87, 165.54, 156.36, 139.10, 128.53, 82.26, 79.67, 75.85, 63.69, 54.46, 49.80, 49.10, 30.91, 28.33, 26.10, 25.58, 23.34, 9.50, 9.14.

*4.2.1.2. 3-azidopropyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**3**)*

According to the general procedure, **3** was synthesized in 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (s, 1H), 6.40 (br, 1H), 5.36 (br, 1H), 4.23–4.16 (m, 2H), 4.08–3.94 (m, 2H), 3.81–3.71 (m, 1H), 3.37 (t, *J* = 6.6 Hz, 2H), 3.34–3.27 (m, 1H), 2.68 (d, *J* = 17.6 Hz, 1H), 2.27 (dd, *J* = 17.6, 10.1 Hz, 1H), 1.95 (s, 3H), 1.93–1.88 (m, 2H), 1.48–1.43 (m, 4H), 1.39 (s, 9H), 0.88–0.81 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.89, 165.72, 156.36, 138.33, 128.94, 82.31, 79.65, 75.90, 61.86, 54.51, 49.14, 48.19, 30.92, 28.35, 28.13, 26.13, 25.72, 23.33, 9.57, 9.21. HRMS (ESI): m/z calcd for $C_{22}H_{37}N_5O_6Na$ $[M+Na]^+$: 490.2636, found: 490.2624.

*4.2.1.3. 6-azidohexyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**4**)*

According to the general procedure, **4** was synthesized in 86% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69 (s, 1H), 6.47 (br, 1H), 5.38 (br, 1H), 4.09–3.97 (m, 4H), 3.72 (br, 1H), 3.30–3.22 (m, 3H), 2.65 (d, *J* = 17.4 Hz, 1H), 2.25 (dd, *J* = 17.4, 10.2 Hz, 1H), 1.93 (s, 3H), 1.62–1.37 (m, 21H), 0.90–0.73 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87, 165.90, 156.33, 137.89, 129.16, 82.24, 79.44, 75.83, 64.72, 54.55, 51.29, 49.31, 30.84, 28.71, 28.43, 28.32, 26.35, 26.13, 25.71, 25.54, 23.24, 9.54, 9.21. HRMS (ESI): m/z calcd for $C_{25}H_{43}N_5O_6Na$ $[M+Na]^+$: 532.3106, found: 532.3091.

*4.2.1.4. 12-azidododecyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**5**)*

According to the general procedure, **5** was synthesized in 86% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.73 (s, 1H), 6.22 (br, 1H), 5.25 (s, 1H), 4.12–3.95 (m, 4H), 3.80–3.71 (m, 1H), 3.36–3.28 (m, 1H), 3.23 (t, *J* = 6.9 Hz, 2H), 2.69 (dd, *J* = 17.7, 4.1 Hz, 1H), 2.26 (dd, *J* = 19.5, 11.8 Hz, 1H), 1.95 (s, 3H), 1.66–1.52 (m, 4H), 1.51–1.43 (m, 4H), 1.39 (s, 9H), 1.31–1.27 (m, 16H), 0.88–0.82 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.85, 165.98, 156.34, 137.69, 129.29, 82.20, 79.54, 77.39, 77.07, 76.75, 75.94, 65.07, 54.50, 51.47, 49.21, 30.94, 29.51, 29.48, 29.44, 29.24, 29.13, 28.82, 28.58, 28.32, 26.69, 26.14, 25.95, 25.73, 23.30, 9.54, 9.21.

*4.2.1.5. 2-(2-azidoethoxy)-ethyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**6**)*

According to the general procedure, **6** was synthesized in 89% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (s, 1H), 5.95 (br, 1H), 5.18 (br, 1H), 4.31 (br, 2H), 4.09–3.94 (m, 2H), 3.79–3.65 (m, 6H), 3.40–3.33 (m, 3H), 2.73 (d, *J* = 17.6 Hz, 1H), 2.30 (dd, *J* = 17.6, 9.6 Hz, 1H), 1.96 (s, 3H), 1.49–1.41 (m, 14H), 0.87 (q, *J* = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.75, 165.83, 156.27, 138.18, 128.92, 82.13, 79.60, 75.77, 70.07, 69.02, 63.87, 54.22, 50.60, 48.89, 30.79, 28.29, 26.04, 25.62, 23.31, 9.47, 9.14. HRMS (ESI): m/z calcd for $C_{23}H_{39}N_5O_7Na$ $[M+Na]^+$: 520.2742, found: 520.2726.

*4.2.1.6. 2-(2-(2-azidoethoxy)ethoxy)ethyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**7**)*

According to the general procedure, **7** was synthesized in 86% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (s, 1H), 5.87 (d, *J* = 9.1 Hz, 1H), 5.16 (d, *J* = 9.1 Hz, 1H), 4.30 (br, 2H), 4.07 (dd, *J* = 18.2, 9.1 Hz, 1H), 3.96–3.94 (m, 1H), 3.83–3.73 (m, 3H), 3.68– 3.65 (m, 6H), 3.45–3.26 (m, 3H), 2.74 (dd, *J* = 17.7, 4.4 Hz, 1H), 2.31 (dd, *J* = 17.3, 9.5 Hz, 1H), 1.98 (s, 3H), 1.52–1.41 (m, 13H), 0.89 (q, *J* = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.82, 165.89, 156.33, 138.15, 129.04, 82.19, 79.70, 75.89, 70.71, 70.70, 70.14, 69.16, 64.11, 54.34, 50.68, 48.91, 30.91, 28.33, 26.09, 25.69, 23.36, 9.53, 9.19. HRMS (ESI): m/z calcd for $C_{25}H_{43}N_5O_8Na$ $[M+Na]^+$: 564.3004, found: 564.2997.

*4.2.1.7. 14-azido-3,6,9,12-tetraoxatetradecyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**8**)*

According to the general procedure, **8** was synthesized in 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (s, 1H), 5.80 (d, *J* = 9.1 Hz, 1H), 5.14 (d, *J* = 9.1 Hz, 1H), 4.29 (t, *J* = 4.6 Hz, 2H), 4.06 (dd, *J* = 18.3, 9.3 Hz, 1H), 3.95 (br, 1H), 3.83–3.75 (m, 1H), 3.72 (t, *J* = 4.68 Hz, 2H), 3.68–3.64 (m, 14H), 3.39–3.33 (m, 3H), 2.73 (dd, *J* = 17.7, 4.9 Hz, 1H), 2.30 (dd, *J* = 17.7, 9.8 Hz, 1H), 1.97 (s, 3H), 1.55–1.44 (m, 4H), 1.41 (s, 9H), 0.88 (q, *J* = 7.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.78, 165.90, 156.32, 138.12, 129.06, 82.17, 79.69, 77.24, 75.90, 70.70, 70.67, 70.63, 70.60, 70.03, 69.06, 64.13, 54.32, 50.70, 48.86, 30.89, 28.33, 26.09, 25.68, 23.38, 9.53, 9.19.

*4.2.1.8. 17-azido-3,6,9,12,15-pentaoxaheptadecyl (3R, 4R, 5S)-4-acetamido-5-((tert-butoxycarbonyl) amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (**9**)*

According to the general procedure, **9** was synthesized in 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.80 (s, 1H), 5.87 (d, *J* = 8.9 Hz, 1H), 5.18 (d, *J* = 9.1 Hz, 1H), 4.28 (t, *J* = 4.64 Hz, 2H), 4.05 (dd, *J* = 18.2, 9.2 Hz, 1H), 3.95 (br, 1H), 3.82–3.74 (m, 1H), 3.71 (t, *J* = 4.64 Hz, 2H), 3.67–3.63 (m, 18H), 3.38–3.33 (m, 3H), 2.72 (dd, *J* = 17.8, 4.7 Hz, 1H), 2.29 (dd, *J* = 17.8, 9.5 Hz, 1H), 1.96 (s, 3H), 1.53–1.44 (m, 4H), 1.40 (s, 9H), 0.87 (q, *J* = 7.3 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.76, 165.89, 156.29, 138.09, 129.05, 82.16, 79.63, 77.25, 75.84, 70.69, 70.66, 70.63, 70.62, 70.60, 70.58, 70.56, 70.02, 69.05, 64.12, 54.28, 50.69, 48.88, 30.83, 28.33, 26.08, 25.69, 23.36, 9.52, 9.20.

4.2.2. General procedure for the preparation of divalent Oseltamivir derivatives

To a stirring solution of azide (2.5 eq.) and 1, 2-bis (prop-2-yn-1-yloxy) ethane (1eq) in THF/ water (1:1), $CuSO_4$ (cat.) was added along with sodium *L*-ascorbate (cat.). The reaction was stirred at r.t. for 12 h. Solvent was removed *in vacuum* and the residue was purified using flash column chromatography. The copper complex in **13** and **17** were further absorbed by CupriSorb®. After the purification step, TFA (5 ml) was added to a solution of click reaction product in CH_2Cl_2 (5 ml). The reaction mixture was stirred at 0 °C for 10 mins and another 1 h at room temperature, and then the solvent was evaporated. The resulting syrup was dissolved in distilled water and purified by Sephadex® LH-20 then lyophilisation to afford divalent Oseltamivir derivatives as foam and stored at 4 °C until use. No [M+Cu] ion peak of the final product in the mass spectrum was detected, and the potential content of Cu was not analyzed.

*4.2.2.1. 1, 2-Bis-[[(((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**10**)*

According to the general procedure, **10** was synthesized in 61% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.98 (s, 2H), 6.59 (s, 2H), 4.54–4.51 (m, 5H), 4.17 (d, *J* = 8.8 Hz, 2H), 3.93–3.88 (m, 2H), 3.59 (s, 4H), 3.48–3.36 (m, 4H), 2.76 (dd, *J* = 17.2, 5.5 Hz, 2H), 2.37–2.29 (m, 2H), 1.97 (s, 6H), 1.45–1.29 (m, 8H), 0.71 (t, *J* = 7.3 Hz, 12H). ^{13}C NMR (100 MHz, D_2O): δ 175.26, 166.13, 144.22, 138.77, 126.75, 125.23, 84.16, 75.01, 68.84, 63.28, 63.02, 52.55, 49.26, 49.03, 28.05, 25.47, 25.16, 22.37, 8.65, 8.58, 8.47, 8.41. HRMS (ESI): m/z calcd for $C_{40}H_{66}N_{10}O_{10}$ $[M+2H]^{2+}$: 423.2476, found: 423.2467.

*4.2.2.2. 1, 2-Bis-[[(((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)propyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**11**)*

According to the general procedure, **11** was synthesized in 51% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.97 (s, 2H), 6.64 (s, 2H), 4.56 (s, 2H), 4.48 (t, *J* = 6.52 Hz, 3H), 4.21–4.12 (m, 6H), 3.93 (dd, *J* = 9.2, 11.9 Hz, 2H), 3.62 (s, 4H), 3.51–3.41 (m, 4H), 2.79 (dd, *J* = 17.3, 5.5 Hz, 2H), 2.39–2.32 (m, 2H), 2.28–2.22 (m, 4H), 1.98 (s, 6H), 1.48–1.34 (m, 8H), 0.80–0.72 (m, 12H). ^{13}C NMR (100 MHz, D_2O): δ 175.30, 166.71, 162.99, 162.63, 138.37, 127.08, 117.79, 114.89, 84.13, 75.01, 69.02, 62.99, 62.85, 52.61, 49.08, 47.95, 28.19, 28.07, 25.51, 25.18, 22.40, 8.65, 8.48. HRMS (ESI) m/z calcd for $C_{42}H_{70}N_{10}O_{10}$ $[M+2H]^{2+}$ 437.2633, found 437.2624.

*4.2.2.3. 1, 2-Bis-[[(((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)hexyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**12**)*

According to the general procedure, **12** was synthesized in 55% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.93 (s, 2H), 6.75 (s, 2H), 4.56 (s, 2H), 4.36–4.24 (m, 6H), 4.08–3.94 (m, 6H), 3.62–3.44 (m, 10H), 2.87 (d, *J* = 16.9 Hz, 2H), 2.43 (br, 2H), 2.02 (s, 6H), 1.82 (br, 4H), 1.57–1.21 (m, 22H), 0.77 (s, 12H). ^{13}C NMR (100 MHz, D_2O): δ 175.27, 167.24, 138.07, 127.49, 84.08, 75.13, 68.69, 65.87, 63.01, 52.77, 50.32, 49.14, 29.25, 25.50, 25.21, 25.10, 24.64, 22.37, 8.66, 8.46. HRMS (ESI) m/z calcd for $C_{48}H_{82}N_{10}O_{10}$ $[M+2H]^{2+}$ 479.3102, found 479.3098.

*4.2.2.4. 1, 2-Bis-[[(((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) dodecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**13**)*

According to the general procedure, **13** was synthesized in 52% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (s, 2H), 6.77 (s, 2H), 6.34 (br, 2H), 4.67 (s, 4H), 4.32 (t, *J* = 7.2 Hz, 4H), 4.27–4.04 (m, 5H), 3.70–3.64 (m, 5H), 3.35–3.32 (m, 3H), 2.79 (br, 2H), 2.21 (br, 2H), 2.03 (s, 6H), 1.90–1.86 (m, 4H), 1.68–1.59 (m, 4H), 1.54–1.44 (m, 8H), 1.30–1.25 (m, 36H), 0.91–0.85 (m, 16H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.44, 166.30, 144.92, 137.93, 129.21, 122.48, 81.93, 69.69, 65.02, 64.68, 50.38, 30.27, 29.70, 29.40, 29.37, 29.30, 29.12, 28.94, 28.56, 26.48, 26.23, 25.90, 25.74, 9.56, 9.33. HRMS (ESI) m/z calcd for $C_{60}H_{106}N_{10}O_{10}$ $[M+2H]^{2+}$ 563.4041, found 563.4038

*4.2.2.5. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) -2-(2-ethoxy)-ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**14**)*

According to the general procedure, **14** was synthesized in 55% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.93 (s, 2H), 6.69 (s, 2H), 4.69–4.36 (m, 4H), 4.21–4.12 (m, 6H), 3.94–3.88 (m, 7H), 3.65– 3.40 (m, 14H), 2.80 (dd, *J* = 17.1, 4.8 Hz, 2H), 2.39 (d, *J* = 10.8 Hz, 2H), 1.98 (s, 6H), 1.46–1.34 (m, 9H), 0.78–0.70 (m, 12H). ^{13}C NMR(100 MHz, D_2O): δ 175.22, 166.62, 138.45, 126.98, 125.25, 84.10, 74.96, 68.78, 68.28, 64.44, 63.04, 52.50, 50.05, 49.04, 28.05, 25.41, 25.05, 22.31, 8.55, 8.39. HRMS (ESI) m/z calcd for $C_{44}H_{74}N_{10}O_{12}$ $[M+2H]^{2+}$ 467.2738, found 467.2740.

*4.2.2.6. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) 2-(2-(2-ethoxy)ethoxy)ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane, (**15**)*

According to the general procedure, **15** was synthesized in 50% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.96 (s, 2H), 6.75 (s, 2H), 4.64–4.44 (m, 6H), 4.28–4.10 (m, 6H), 3.94 (dd, *J* = 11.7, 9.0 Hz, 2H), 3.88–3.80 (m, 4H), 3.61–3.39 (m, 22H), 2.84 (dd, *J* = 17.2, 5.4 Hz, 2H), 2.41 (dd, *J* = 10.3, 6.9 Hz, 2H), 1.97 (s, 6H), 1.45–1.32 (m, 9H), 0.73 (q, *J* = 7.3 Hz, 12H). ^{13}C NMR (100 MHz, D_2O): δ 175.27, 166.86, 143.94, 138.49, 127.19, 125.42, 84.16, 75.05, 69.75, 69.66, 68.91, 68.75, 68.47, 64.67, 63.11, 52.65, 50.10, 49.11, 28.19, 25.47, 25.09, 22.38, 8.60, 8.45. HRMS (ESI) m/e calcd for $C_{48}H_{82}N_{10}O_{14}$ $[M+2H]^{2+}$ 511.3001, found 511.2987.

*4.2.2.7. 1, 2-Bis-[[3-(((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)-3,6,9,12-tetraoxatetradecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**16**)*

According to the general procedure, **16** was synthesized in 54% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.97 (s, 1H), 6.78 (s, 1H), 4.58–4.50 (m, 5H), 4.25–4.21 (m, 6H), 3.94 (dd, *J* = 11.6, 9.1 Hz, 2H), 3.85 (t, *J* = 4.96 Hz, 4H), 3.72–3.70 (m, 5H), 3.61–3.41 (m, 35H), 2.86 (dd, *J* = 17.2, 5.4 Hz, 2H), 2.45–2.38 (m, 2H), 1.98 (s, 6H), 1.48–1.31 (m, 8H), 0.78–0.71 (m, 12H). ^{13}C NMR (100 MHz, D_2O): δ 175.20, 166.87, 143.82, 138.43, 127.16, 125.44, 84.14, 75.03, 69.74, 69.61, 69.56, 69.54, 69.43, 68.84, 68.70, 68.47, 64.60, 63.03, 52.63, 49.99, 49.05, 28.19, 25.42, 25.03, 22.32, 8.57, 8.40. HRMS (ESI) m/z calcd for $C_{56}H_{98}N_{10}O_{18}$ $[M+2H]^{2+}$ 599.3525, found 599.3520.

*4.2.2.8. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5- amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)-3,6,9,12,15-pentaoxaheptadecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**17**)*

According to the general procedure, **17** was synthesized in 50% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.97 (s, 2H), 6.79 (s, 2H), 4.58–4.51 (m, 5H), 4.27–4.22 (m, 6H), 3.96 (dd, *J* = 11.6, 9.0 Hz, 2H), 3.92–3.79 (m, 6H), 3.78–3.66 (m, 6H), 3.61–3.42 (m, 52H), 2.87 (dd, *J* = 17.1, 5.1 Hz, 2H), 2.44 (dd, *J* = 10.1, 7.0 Hz, 2H), 1.98 (s, 6H), 1.48–1.31 (m, 8H), 0.84–0.64 (m, 12H). ^{13}C NMR(100 MHz, D_2O): δ 175.26, 166.92, 143.93, 138.49, 127.17, 125.48, 84.17, 75.06, 69.79, 69.68, 69.61, 69.50, 68.90, 68.76, 68.53, 64.67, 63.10, 52.59, 50.05, 49.12, 28.13, 25.49, 25.11, 22.38, 8.63, 8.46. HRMS (ESI) m/z calcd for $C_{60}H_{106}N_{10}O_{20}$ $[M+2H]^{2+}$ 643.3787, found 643.3774.

4.2.3. General procedure for the preparation of divalent guanidino oseltamivir derivatives

Divalent Oseltamivir analogs were dissolved in CH_2Cl_2. TFA/CH_2Cl_2 (1:1) was added dropwise under 0 ℃, the reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated to dryness and the crude products were dissolved in CH_2Cl_2 (5 ml), Et_3N (1 mL) was added and the solution was stirred at rt for 30 mins. $HgCl_2$ (0.6 eq.) and 1, 3-Bis (tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (3 eq.) was added. The reaction was stirred at r.t. for 12 h. The reaction mixture was washed with HCl (1M, 25 mL), extracted with CH_2Cl_2 (3×10 mL) and the product was purified by column chromatography to give the Boc protected divalent guanidino Oseltamivir derivatives, which were further dissolved in CH_2Cl_2. CH_2Cl_2/TFA (1:1) was added dropwise under 0 ℃, the reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated to dryness and the crude products were purified by Sephendex^{TM} LH-20. No [M+Cu] or [M+Hg] ion peak of the final product in the mass spectrum was detected, and the potential content of Cu or Hg was not analyzed.

*4.2.3.1. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5- guanidino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**18**)*

According to the general procedure, **18** was synthesized in 52% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.98 (s, 2H), 6.57 (s, 2H), 4.53–4.49 (m, 5H), 4.17 (d, *J* = 8.6 Hz, 2H), 3.79–3.60 (m, 5H), 3.57 (s, 4H), 3.39–3.34 (m, 2H), 2.63 (dd, *J* = 17.5, 4.9 Hz, 2H), 2.26–2.16 (m, 2H), 1.92 (s, 6H), 1.45–1.25 (m, 9H), 0.71 (q, *J* = 7.1 Hz, 12H). ^{13}C NMR (100 MHz, D_2O) δ 174.71, 166.41, 156.75, 138.61, 127.87, 125.24, 84.14, 75.29, 68.71, 63.12, 62.92, 54.74, 50.44, 49.23, 46.63, 29.73, 25.56, 25.23, 21.93, 8.59, 8.50, 8.18. HRMS (ESI) m/z calcd for $C_{42}H_{70}N_{14}O_{10}$ $[M+2H]^{2+}$: 465.2694, found: 465.2699.

*4.2.3.2. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)propyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**19**)*

According to the general procedure, **19** was synthesized in 51% yield. ^{1}H NMR (400 MHz, D_2O) δ 7.94 (s, 2H), 6.59 (s, 2H), 4.48 (s, 2H), 4.46 (t, *J* = 6.3 Hz, 3H), 4.19 (br, 2H), 4.10 (t, *J* = 5.6 Hz, 4H), 3.79–3.65 (m, 5H), 3.61 (s, 4H), 3.41–3.38 (m, 2H), 2.60 (dd, *J* = 17.4, 4.9 Hz, 2H), 2.26–2.15 (m, 6H), 1.93 (s, 6H), 1.47–1.30 (m, 8H), 0.78–0.71 (m, 12H). ^{13}C NMR (100 MHz, D_2O) δ 174.78, 167.12, 156.83, 138.23, 128.30, 84.12, 75.31, 68.96, 63.11, 62.09, 54.90, 50.53, 47.96, 29.79, 28.18, 25.66, 25.31, 22.02, 8.65, 8.56. HRMS (ESI) m/z calcd for $C_{44}H_{74}N_{14}O_{10}$ $[M+2H]^{2+}$: 479.2851, found: 479.2861.

*4.2.3.3. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)hexyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**20**)*

According to the general procedure, **20** was synthesized in 54% yield. ^{1}H NMR (400 MHz, D_2O) δ 6.68 (s, 3H), 4.47 (br, 2H), 4.34 (br, 4H), 4.22 (d, *J* = 8.0 Hz, 2H), 4.06– 3.99 (m, 4H), 3.82–3.69 (m, 9H), 3.42–3.38 (m, 2H), 2.73 (dd, *J* = 17.5, 4.4 Hz, 2H), 2.31–2.23 (m, 2H), 1.93 (s, 6H), 1.80 (br, 4H), 1.52–1.14 (m, 21H), 0.73 (q, *J* = 7.3 Hz, 12H). ^{13}C NMR (100 MHz, D_2O): δ 174.81, 167.61, 156.89, 138.03, 128.66, 84.05, 75.43, 65.83, 54.93, 50.64, 29.98, 29.14, 27.50, 25.70, 25.39, 25.17, 24.68, 22.05, 8.72, 8.58. HRMS (ESI) m/z calcd for $C_{50}H_{86}N_{14}O_{10}$ $[M+2H]^{2+}$: 521.3320, found: 521.3338.

*4.2.3.4. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) dodecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**21**)*

According to the general procedure, **21** was synthesized in 52% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.97 (s, 1H), 6.82 (s, 2H), 4.62 (s, 4H), 4.39 (t, *J* = 7.1 Hz, 4H), 4.28 (br, 2H), 4.16 (q, *J* = 6.3 Hz, 4H), 3.91 (br, 4H), 3.46–3.40 (m, 2H), 3.31–3.30 (m, 3H), 2.90–2.78 (m, 2H), 2.40–2.29 (m, 2H), 1.99 (s, 6H), 1.92–1.85 (m, 4H), 1.70–1.64 (m, 4H), 1.54–1.49 (m, 8H), 1.28 (br, 34H), 0.94–0.87 (m, 12H). ^{13}C NMR (100 MHz, MeOD) δ 172.75, 165.83, 157.17, 144.51, 137.57, 128.15, 123.61, 82.38, 74.69, 69.33, 64.85, 63.62, 54.46, 50.31, 49.97, 29.96, 29.87, 29.21, 29.17, 29.09, 28.64, 28.30, 26.05, 25.88, 25.70, 25.52,

21.50, 8.51, 8.31. HRMS (ESI) m/z calcd for $C_{62}H_{110}N_{14}O_{10}$ $[M+2H]^{2+}$: 605.4259, found: 605.4239.

*4.2.3.5. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) -2-(2-ethoxy)-ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**22**)*

According to the general procedure, **22** was synthesized in 51% yield. ^{1}H NMR (400 MHz, D_2O) δ 8.13 (br, 1H), 6.66 (s, 2H), 4.52 (br, 4H), 4.22 (d, *J* = 8.4 Hz, 2H), 4.12 (br, 4H), 3.98–3.53 (m, 17H), 3.44–3.38 (m, 2H), 2.66 (q, *J* = 4.6 Hz, 2H), 2.65 (dd, *J* = 4.8, 17.6 Hz, 2H), 2.27–2.20 (m, 2H), 1.93 (s, 6H), 1.48–1.30 (m, 8H), 1.17 (t, *J* = 7.3 Hz, 2H), 0.74 (q, *J* = 7.5 Hz, 12H). ^{13}C NMR (100 MHz, D_2O) δ 174.72, 167.01, 156.79, 138.33, 128.20, 84.11, 75.28, 68.71, 68.28, 64.36, 54.83, 50.50, 46.63, 29.78, 25.58, 25.21, 21.94, 8.56, 8.50, 8.18. HRMS (ESI) m/z calcd for $C_{46}H_{78}N_{14}O_{12}$ $[M+2H]^{2+}$: 509.2956, found: 509.2955.

*4.2.3.6. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy) 2-(2-(2-ethoxy)ethoxy)ethyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**23**)*

According to the general procedure, **23** was synthesized in 50% yield. ^{1}H NMR (D_2O, 400 MHz) δ 6.71 (br, 3H), 4.77 (br, 4H), 4.52 (br, 4H), 4.23–4.11 (m, 8H), 3.86–3.53 (m, 20H), 3.43–3.48 (m, 2H), 2.73 (d, *J* = 12.9 Hz, 2H), 2.30 (br, 2H), 1.92 (s, 6H), 1.52–1.29 (m, 13H), 0.83–0.64 (m, 12H). ^{13}C NMR (D_2O, 100 MHz) δ 174.78, 167.25, 156.84, 138.38, 128.38, 84.16, 75.36, 69.77, 69.66, 68.96, 68.75, 68.47, 64.59, 63.13, 54.87, 50.58, 50.14, 29.92, 25.64, 25.27, 22.02, 8.63, 8.55. HRMS (ESI) m/z calcd for $C_{50}H_{86}N_{14}O_{14}$ $[M+2H]^{2+}$: 553.3218, found: 553.3210.

*4.2.3.7. 1, 2-Bis-[[3-(((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)-3,6,9,12-tetraoxatetradecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**24**)*

According to the general procedure, **24** was synthesized in 48% yield. ^{1}H NMR (D_2O, 400 MHz) δ 8.02 (br, 2H), 6.75 (s, 2H), 4.55–4.52 (m, 4H), 4.24 (br, 6H), 3.86–3.42 (m, 44H), 2.77 (d, *J* = 17.3 Hz, 2H), 2.35–2.28 (m, 2H), 1.96 (s, 6H), 1.52–1.27 (m, 9H), 0.78–0.71 (m, 12H). ^{13}C NMR (D_2O, 100 MHz) δ 174.77, 167.33, 156.84, 138.37, 128.41, 84.16, 75.38, 69.82, 69.70, 69.61, 69.52, 68.95, 68.75, 68.53, 64.62, 54.88, 50.58, 50.12, 29.91, 25.65, 25.28, 22.02, 8.65, 8.57. HRMS (ESI) m/z calcd for $C_{58}H_{102}N_{14}O_{18}$ $[M+2H]^{2+}$: 641.3743, found: 641.3755.

*4.2.3.8. 1, 2-Bis-[[((3R, 4R, 5S)-4-acetamido-5-guanidino -3-(1-ethylpropoxy)-1-cyclohexene-1-carboxyl)oxy)-3,6,9,12,15-pentaoxaheptadecyl]-1H-1,2,3-triazol-4-yl-methoxyl]ethane (**25**)*

According to the general procedure, **25** was synthesized in 50% yield. ^{1}H NMR (D_2O, 400 MHz): δ 7.97 (s, 1H), 6.74 (s, 2H), 4.58–4.50 (m, 5H), 4.23 (br, 7H), 3.86–3.70 (m, 16H), 3.60–3.05 (m, 54H), 3.72 (q, *J* = 7.3 Hz, 6H), 2.76 (dd, *J* = 17.4, 4.3 Hz, 2H), 2.39–2.24 (m, 2H), 1.92 (s, 6H), 1.46–1.29 (m, 9H), 1.16 (t, *J* = 7.3 Hz, 9H), 0.78–0.70 (m, 12H); ^{13}C NMR (D_2O ,100 MHz): δ 174.69, 167.23, 163.11, 162.76, 156.76, 138.34, 128.34, 125.46, 117.80, 114.90, 84.12, 75.33, 69.74, 69.63, 69.57, 69.53, 69.46, 68.86, 68.71, 68.46, 64.54, 63.05, 54.82, 50.52, 50.00, 46.63, 29.87, 25.60, 25.22, 21.96, 8.61, 8.19. HRMS (ESI) m/z calcd for $C_{62}H_{110}N_{14}O_{20}$ $[M+2H]^{2+}$: 685.4005, found: 685.4022.

4.3. Neuraminidase Inhibition Assay

The virus used as the source of NAs is inactivated by the addition of β-propiolactone (β-PL). Enzyme inhibition assays were measured using 4-methylumbelliferyl -α-*D*-N –acetylneuraminic acid sodium salt (4-MUNANA) in the enzyme buffer (100 mM sodium acetate pH 5.5 and 10 mM $CaCl_2$) as the substrate. All compounds were dissolved in buffer and diluted to the corresponding concentrations. The enzyme inhibition assay was conducted in 384-well plates containing 15 μL diluted virus, 15 μL compounds in the buffer. The mixture was incubated for 30 min at 37 ^{o}C, and then added with 30 μL 4-MUNANA substrate per well in the buffer. The enzymatic reactions were carried out for 2 hours at 37 ^{o}C. The fluorescent signal was monitored using the kinetics function of Synergy™ H1/H1MF microplate reader with excitation and emission wavelengths of 360 and 440 nm, respectively. The buffer instead of the inhibitor was used as control and the concentration of the virus was determined as the hydrolysis reaction was completed in 30 min and the final fluorescent unit (FU) is stable at 10000. The IC_{50} was calculated as the concentration of inhibitor resulting in a 50% reduction in FU compared to the control.

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Supplementary Material

Supplementary data (^{1}H and ^{13}C spectra) related to this article can be found at http://

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