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Bifunctional thiosialosides inhibit influenza virus

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Abstract

We have synthesized a panel of bivalent *S*-sialoside analogues, with modifications at the 4 position, as inhibitors of influenza virus. These first generation compounds show IC_{50} values ranging from low micromolar to high nanomolar in enzyme inhibition and plaque reduction assays with two intact viruses, Influenza H_1N_1 (A/California/07/2009) and H_3N_2 (A/Hongkong/8/68).

Keywords

Influenza virus; sialic acid; glycoconjugates; inhibitors; bifunctional

Influenza is an opportunistic pathogen that causes severe respiratory illnesses. The virus accounts for millions of infections worldwide each year, leading to significant morbidity and mortality. Senior citizens (over 65 years of age), young children, and individuals with underlying health problems are at increased risk for infection and subsequent secondary illnesses like pneumonia. [1] Along with vaccines, Oseltamivir®, Zanamivir®, and Premavir® (Figure 1B–D) are among the frontline drugs used to fight the infection. [2] These FDA approved transition state analogs inhibit the activity of the viral surface enzyme, Neuraminidase (NA), from cleaving the residual N-acetyl neuraminic acid (or sialic acid, Figure 1A) present on the infected host cell, consequently arresting the virus progeny from escaping the cell. However, some strains which include emerging, highly virulent strains that can potentially cause pandemics, have started to exhibit resistance to some of these inhibitors. In one recent surveillance study, 100% of all patients had a resistant strain to Oseltamivir®,[3] and another study identified a strain that was resistant to both, Zanamivir® and Oseltamivir®.[4] These studies emphasize the need for vigilance and continued development of novel drugs. Recently, a new class of mechanism based anti-viral compounds against NA has been reported to show broad spectrum anti-viral activity against all strains *in vitro* and in animal models. [5, 6](Figure 1E, F) Unlike transition state analogs, these compounds are similar to natural substrate, with modifications at the 3 and 4 positions,

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which enhance the binding activity. All of these recent reports are based on the natural substrate (sialic acid) with structure based drug design leading to increased and highly specific inhibition.

A slightly different strategy of developing inhibitors follows Nature's method of using multivalency that target Hemagluttinin (HA), a surface glycoprotein that binds to cell surface sialic acid to facilitate viral entry and NA. HA and NA are excellent targets for inhibition, because labeling experiments have shown that an individual viral particle has approximately 200–300 copies of trimeric HA and 50–100 copies of tetrameric NA, leading to over 800 binding sites per virion.[7, 8] Indeed, mucins, endogenous sialylated proteins released by respiratory epithelial cells, capture viral particles by virtue of their multiple sialic acid residues which bind to HA and NA, and flush them away by the natural process of sneezing and coughing.[9–11] A similar approach using glycopolymers and glycodendrimers with pendant sialic acids have been generated to capture the virus. It has been demonstrated that increase in the valency of the sialic acids increases the inhibitory effect significantly, from the micromolar IC_{50} value of a mono/di/tri saccharide, to the micromolar/submicromolar range. [12–14] These reports have focused on the architecture of the scaffolds, leaving the sialic acid unit unmodified.

In this report, we have increased the intrinsic binding affinity of a single sialic acid unit by introducing an amine/guanidine group at the 4 position of sialic acid. The basic amine/guanidine group fits perfectly into the binding pocket of viral NA as it interacts with the trio of amino acids present in the binding pocket. [2, 15] We introduced sulfur at the anomeric center, which provides additional advantages. First, replacing the *O*-sialoside with an *S*-sialoside makes the ligand more robust as we and others have demonstrated that *S*-sialosides are not readily cleaved by the virus. [16, 17] Second, the thiol group reacts with triflates and/or bromides present on multivalent scaffolds readily, thereby yielding rapid access to multivalent molecules. The combination of NA resistant sialosides combined with the multivalent presentation provides easy access to a new class of influenza virus inhibitors. Using this approach, we constructed a panel of bivalent compounds, (Schemes 1 and 2) that are similar to the natural substrate and evaluated their inhibitory activities with two viral NAs, N_1 and N_2, and intact viral strains. These first generation compounds show micromolar to nanomolar inhibition and could be further elaborated into potential therapies for influenza.

The design and synthesis of the compounds is shown in Scheme 1. Starting with the known azido compound, **1**,[18] hydrogen chloride was added across the double bond to produce the β-chloride **2**, followed by SN2 type replacement of the chloride by a thioacetate to introduce the sulfur moiety at the anomeric center in good yield. The alpha conformation in **3** was confirmed by ^1H and ^{13}C NMR spectroscopies. Reaction of **3** with a hydrophobic six carbon spacer in the presence of diethylamine yielded **4**. Next, the azide group of **4** was reduced using triphenylphospine to yield the amine **5**, which was subsequently protected with a t-butylcarbonyl group or reacted with a suitable protected guanidinium group to yield **6** and **7**, respectively. Removal of ester moieties was conducted under basic hydrolysis conditions, followed by removal of the acid sensitive t-butylcarbonyl groups using trifluoroacetic acid to yield analogs of sialic acid with an amine (**SA**) or guanidine group (**SG**) at the four

position. While not the focus of this report, we incorporated a thiol group at the terminus of the alkyl spacer in **SA** and **SG** as it provides facile conjugation to surfaces for capture of the virus or to a scaffold/protein to produce multivalent inhibitors.

We used a series of activated homobifunctional hydrophobic and hydrophilic linkers to produce the dimeric compounds, shown in Scheme 2. Different spacers were used because the bivalent molecules can interact with viral NA in three ways. Briefly, the bivalent molecules can crosslink adjacent NAs on the same tetramer or crosslink two NAs from two different NA tetramers on the same viral particle or crosslink NA's from two viral particles. The minimal distance between the active sites of the NAs in all three possibilities are ~ 16 Å, 30 Å, 50 Å, respectively.[19] Therefore, using a series of activated hydrophilic and hydrophobic spacers, we generated a panel of gemini compounds, **8–12** in decent yield. The synthesis of the activated spacers for coupling are presented in the supplementary data. The azides in **8–12** were deprotected as described previously to yield five bivalent S-sialoside analogs with an amine at the 4 position. (**C6-SA**, **C12-SA**, **TetraEG-SA**, **PentaEG-SA** and **HexaEG-SA**). We also introduced a guanidine group at the 4 position as described for the synthesis of the monomeric SG to yield five bivalent *S*-sialoside analogs. (**C6-SG**, **C12-SG**, **TetraEG-SG**, **PentaEG-SG** and **HexaEG-SG**). All intermediates and final compounds were characterized by NMR and mass spectroscopies (Supplementary data)

Next, we performed NA inhibition studies using commercially available 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) (Figure 1*G*) as the substrate. First, we used soluble viral NA as the enzymes before testing intact viruses. Since there is an initial binding component in the inhibitory mechanism, we premixed the compounds with NA and followed the cleavage of the substrate over two hours to obtain the IC_{50}s using the linear slope between 0–35 min. The IC_{50} values with three independent experiments for each compound are given in Table 1 and the selective raw data is presented in the supplementary data. The commercial drugs, Zanamivir® and Ostelimivir® are included as controls. As expected, sialic acid exhibits millimolar inhibition and the antivirals exhibit nanomolar inhibitory values. Introduction of an amine at the 4 position decreases the IC_{50} by 1000 fold to micromolar levels for both influenza viral enzymes, N1 and N2; the IC_{50} values for **SA** range from 60–180 μM. The bivalent sialosides (**C6-SA**, **C12-SA**, **TetraEG-SA**, **PentaEG-SA** and **HexaEG-SA**) all exhibit higher inhibition activity, approximately four to five fold increase in inhibition, especially for the N1 enzyme, when compared to the monovalent compound. There were exceptions, the compounds with the longer spacers (**PentaEG-SA** and **HexaEG-SA**), were not that efficacious compared to the monomeric compounds. With the N_2 enzyme, a similar trend was observed, with the bivalent compound exhibiting a two fold higher inhibition compared to the monomeric amine containing compound and the compounds with longer spacers not as effective. Introduction of the guanidine group led to increased inhibition with IC_{50} values ranging from 0.2–25 μM for **SG**, **C6-SG**, **C12-SG**, **TetraEG-SG**, **PentaEG-SG** and **HexaEG-SG**. The IC_{50} values for the bivalent compounds, irrespective of the spacers, are similar to **SG**, which is the monomeric compound and the values for both enzymes are similar, in the submicromolar range. This is because the soluble NAs are not membrane bound and are present as a mixture of monomers, dimers, trimers and some tetramers. To summarize this

subsection, these experiments confirm that the compounds with amine at the 4 position (**SA**, **C6-SA**, **C12-SA**, **TetraEG-SA**, **PentaEG-SA** and **HexaEG-SA**) are better inhibitors than the natural receptor The compounds with guanidine at the 4 position (**SG**, **C6-SG**, **C12-SG**, **TetraEG-SG**, **PentaEG-SG** and **HexaEG-SG**) are better inhibitors than compounds with amine at the 4 position. We also confirmed that both classes of NA, NA1 with the more open binding pocket and a flexible loop that closes upon introduction of sialic acid or an inhibitor and NA2, which has a preset binding pocket, [20] are inhibited very well by the synthetic compounds.

Since the compounds inhibited soluble NAs, we performed the same assay repeated with two intact viruses, Influenza H_1N_1 (A/California/07/2009) and H_3N_2 (A/Hongkong/8/68) to ensure that the compounds inhibit the transmembrane NAs present on the viruses. A similar trend as was observed for soluble NA. **SA**, the compound with an amine at the four position exhibits an IC_{50} value of 60 μM for both strains. The bivalent molecules, **C6-SA**, **C12-SA**, **TetraEG-SA**, **PentaEG-SA** and **HexaEG-SA** exhibit a 5 fold decrease in the IC_{50}s compared to the monomer to ~ 10 μM. The lone exception is the compound with the longest oligoethylene glycol spacer, which did not have a similar effect as the other bivalent molecules, presumably because the spacer is too flexible to exhibit a bidentate effect. In the case of the guanidine containing compounds, the monomer **SG** has an IC_{50} value of ~ 1 μM for both strains, which is a 50 fold decrease compared to **SA**. The bivalent compounds, **SG**, **C6-SG**, **C12-SG**, **TetraEG-SG**, **PentaEG-SG** and **HexaEG-SG** all show significant decreases in the IC_{50} values, ranging from 250 nm to 2.4 μM for both viruses, with **C6-SG** the best inhibitor of all compounds. However, since the values for all bivalent compounds are similar, it is not clear if the compounds bind to two NA's on the same virion or different virions. What is clear is that our hypothesis of increasing the intrinsic binding affinity of an individual sialoside analog coupled with bivalency decreases the IC_{50} values from the millimolar range by 10,000 fold to the nanomolar range.

We were also interested in testing the efficacy of these compounds to inhibit the virus in cell based systems. Towards that end, we performed a plaque assay for all the compounds. The virus was introduced to the cells for 30 minutes, followed by addition of the compounds to arrest the spread of the viruses. IC_{50} values based on 50% decrease in plaque size are given in Table 2. We were pleased to observe a similar trend for all compounds as was seen with the cell free system. Briefly, **SA**, the compound with an amine at the 4 position exhibited inhibition in the 10–1000 μM range for both, H_1N_1 and H_3N_2 strains, and the dimers **C6-SA**, **C12-SA**, **TetraEG-SA**, **PentaEG-SA** and **HexaEG-SA**, exhibiting a 10 fold decrease in the IC_{50} values (range from 1–100 μM) in comparison to **SA. SG**, the compounds with a guanidine at the 4 position, exhibited a 10 fold decrease in the IC_{50} values (1–10 μM) compared to **SA**. The bivalent derivatives**, C6- SG**, **C12-SG**, **TetraEG-SG**, **PentaEG-SG** and **HexaEG-SG,** all exhibiting lower IC_{50} values (10–1000 nM) than **SG**.

To summarize, we have combined robustness of *S*-sialosides, increased intrinsic affinity of a single unit and used bivalency to produce a panel of compounds that inhibit two different strains of influenza virus. The IC_{50} values range from the low micromolar to high nanomolar. We have also identified the appropriate length of the spacer for attachment to an appropriate scaffold, longer spacers such as a penta or hexa ethylene glycol do not seem

appropriate for effective inhibition. Finally, we note that these compounds are not as efficacious as Zanamivir® or Oseltamivir®. However, we are currently making structural modifications, e.g. introducing a fluorine at the 3 position and tethering the molecules polymeric/dendrimeric scaffold. We anticipate that these modifications will enhance the IC_{50} values to produce highly potent inhibitors. These results will be reported soon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and Notes

1. Fiore AE, Shay DK, Broder K, Iskander JK, Uyeki TM, Mootrey G, Bresee JS, Cox NJ. MMWR Recomm Rep. 2009; 58:1. [PubMed: 19644442]
2. von Itzstein M. Nat Rev Drug Discov. 2007; 6:967. [PubMed: 18049471]
3. Mai-Phuong HV, Hang Nle K, Phuong NT, Maile Q. Western Pac Surveill Response J. 2013; 4:25.
4. Hay AJ, Hayden FG. Lancet. 2013; 381:2230. [PubMed: 23809549]
5. Vavricka CJ, Liu Y, Kiyota H, Sriwilaijaroen N, Qi J, Tanaka K, Wu Y, Li Q, Li Y, Yan J, Suzuki Y, Gao GF. Nat Commun. 2013; 4:1491. [PubMed: 23422659]
6. Kim JH, Resende R, Wennekes T, Chen HM, Bance N, Buchini S, Watts AG, Pilling P, Streltsov VA, Petric M, Liggins R, Barrett S, McKimm-Breschkin JL, Niikura M, Withers SG. Science. 2013; 340:71. [PubMed: 23429702]
7. Murti KG, Webster RG. Virology. 1986; 149:36. [PubMed: 3511607]
8. Amano H, Uemoto H, Kuroda K, Hosaka Y. J Gen Virol. 1992; 73(Pt 8):1969. [PubMed: 1645138]
9. White MR, Helmerhorst EJ, Ligtenberg A, Karpel M, Tecle T, Siqueira WL, Oppenheim FG, Hartshorn KL. Oral Microbiol Immunol. 2009; 24:18. [PubMed: 19121065]
10. White MR, Crouch E, van Eijk M, Hartshorn M, Pemberton L, Tornoe I, Holmskov U, Hartshorn KL. Am J Physiol Lung Cell Mol Physiol. 2005; 288:L831. [PubMed: 15608147]
11. Lieleg O, Lieleg C, Bloom J, Buck CB, Ribbeck K. Biomacromolecules. 2012; 13:1724. [PubMed: 22475261]
12. Hidari KI, Murata T, Yoshida K, Takahashi Y, Minamijima YH, Miwa Y, Adachi S, Ogata M, Usui T, Suzuki Y, Suzuki T. Glycobiology. 2008; 18:779. [PubMed: 18621993]
13. Sakamoto J, Koyama T, Miyamoto D, Yingsakmongkon S, Hidari KI, Jampangern W, Suzuki T, Suzuki Y, Esumi Y, Nakamura T, Hatano K, Terunuma D, Matsuoka K. Bioorg Med Chem. 2009; 17:5451. [PubMed: 19592257]
14. Sakamoto J, Koyama T, Miyamoto D, Yingsakmongkon S, Hidari KI, Jampangern W, Suzuki T, Suzuki Y, Esumi Y, Hatano K, Terunuma D, Matsuoka K. Bioorg Med Chem Lett. 2007; 17:717. [PubMed: 17095224]
15. Von Itzstein M, YW, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Synthe ML, White HF, Oliver SW, Coleman PG, Varghese VJ, Cameron JM, Penn CR. Nature. 1993; 363:418. [PubMed: 8502295]
16. Wilson JC, Kiefel MJ, Angus DI, von Itzstein M. Org Lett. 1999; 1:443. [PubMed: 10822584]
17. Kale RR, Mukundan H, Price DN, Harris JF, Lewallen DM, Swanson BI, Schmidt JG, Iyer SS. J Am Chem Soc. 2008; 130:8169. [PubMed: 18529007]
18. Chandler M, Bamford MJ, Conroy R, Lamont B, Patel B, Patel VK, Steeples IP, Storer R, Weir NG, Wright M, Williamson C. Journal of the Chemical Society. Perkin Transactions. 1995; 1:1173.

19. Macdonald SJF, Watson KG, Cameron R, Chalmers DK, Demaine DA, Fenton RJ, Gower D, Hamblin JN, Hamilton S, Hart GJ, Inglis GGA, Jin B, Jones HT, McConnell DB, Mason AM, Nguyen V, Owens IJ, Parry N, Reece PA, Shanahan SE, Smith D, Wu WY, Tucker SP. Antimicrobial Agents and Chemotherapy. 2004; 48:4542. [PubMed: 15561823]

20. Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM, Hay AJ, Gamblin SJ, Skehel JJ. Nature. 2006; 443:45. [PubMed: 16915235]

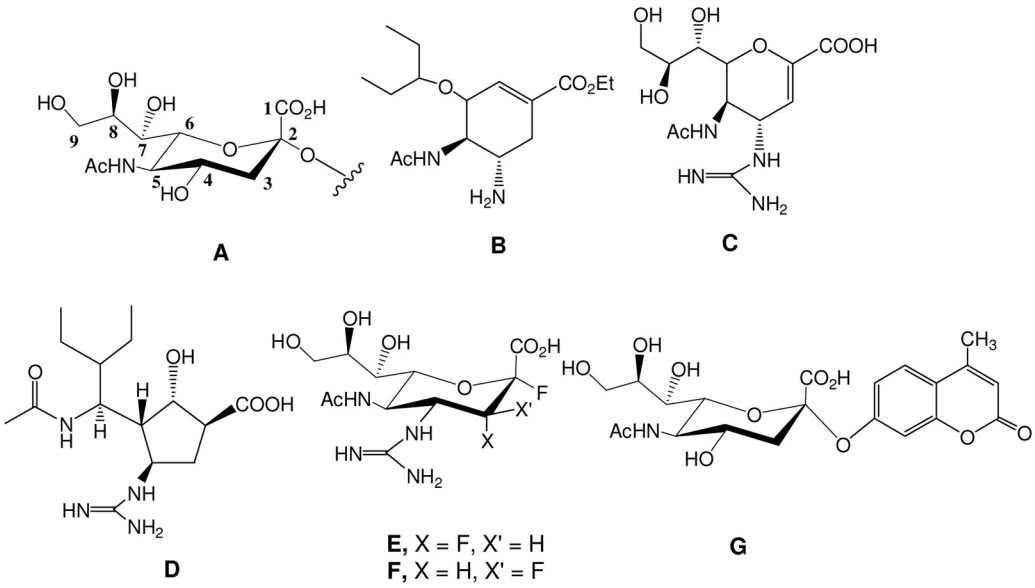
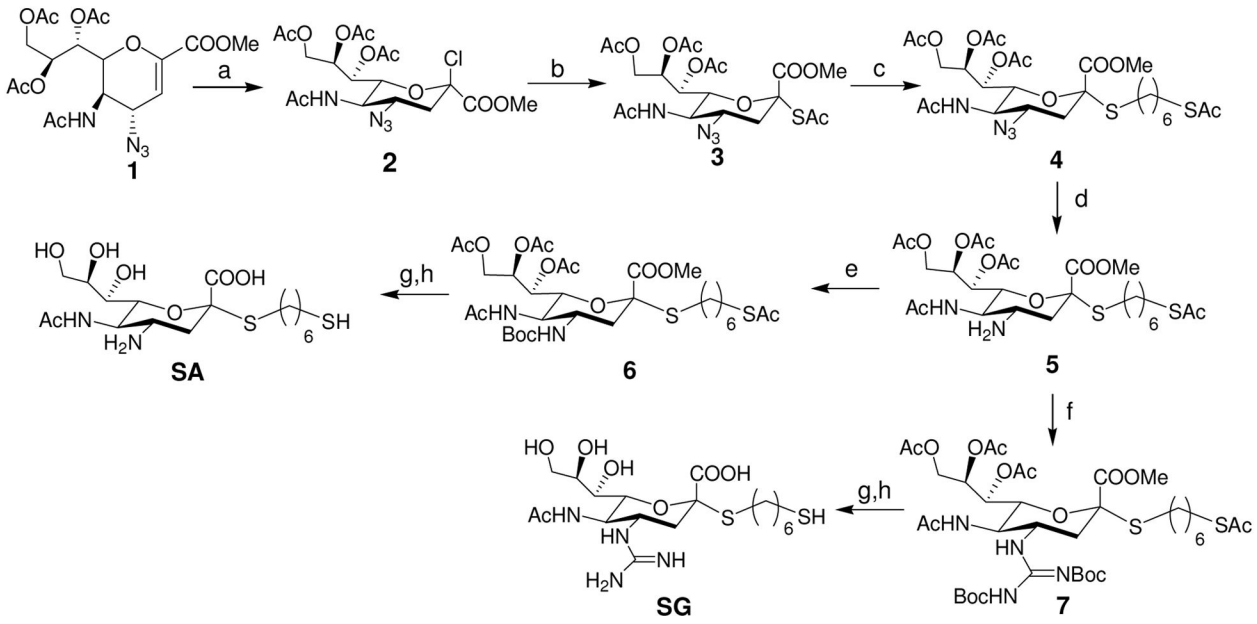


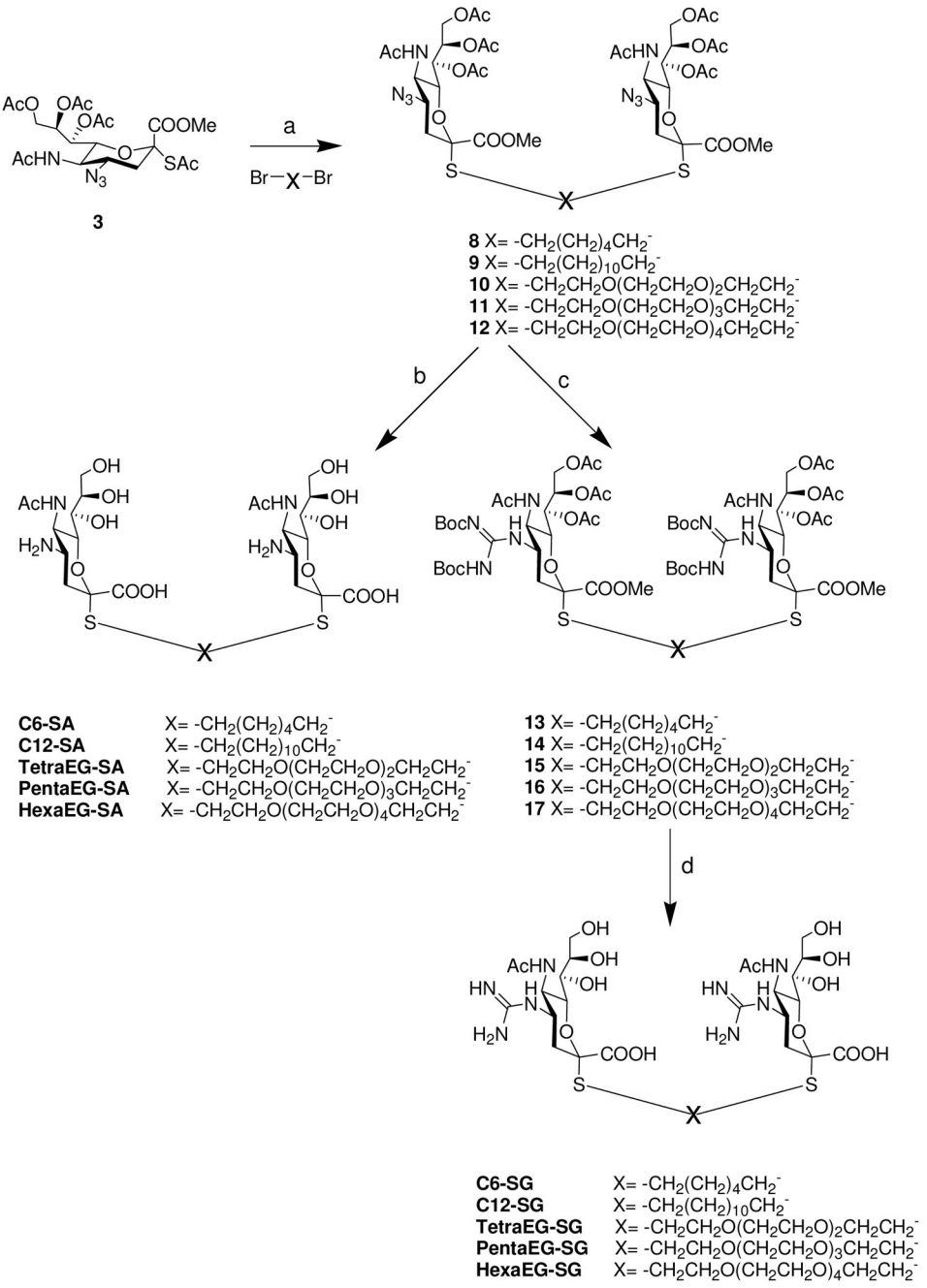
Figure 1.

Structures of N-acetyl Neuraminic Acid (sialic acid) analogs. **A.** Sialic acid with the numbering scheme. **B.** Oseltamivir ® **C.** Zanamivir®, **D.** Premavir® **E-F**. Flourinated analogs of Sialic acid. **G**. Fluorogenic substrate of sialic acid for obtaining IC_{50} values



Scheme 1.

Synthesis of **SA** and **SG**. *Reagents and Conditions:* a) HCl (g), LiCl, CH_3CN, 6 days; b) KSAc, TBAB (Tetrabutylammonium bisulfate), CH_2Cl_2/H_2O, overnight, 50% over 2 steps; c) $TosOC_6H_{12}SAc$, DEA, DMF, 65%; d) PPh_3, THF/H_2O, 12h; e) $(Boc)_2O$, TEA, THF, 60% over 2 steps f) MeS-(C=NBoc)NHBoc, $HgCl_2$, TEA, CH_2Cl_2, 80% over 2 steps; g) MeOH/NaOH (aq), 1 h; h) TFA/DCM (1:1). 90% over two steps for **SA** and **SG**, respectively.

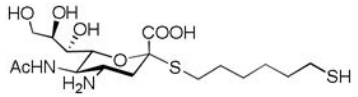
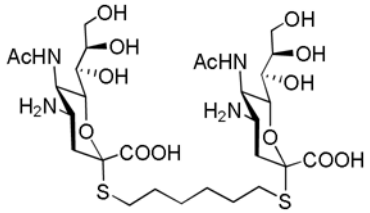
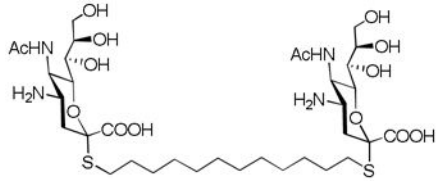
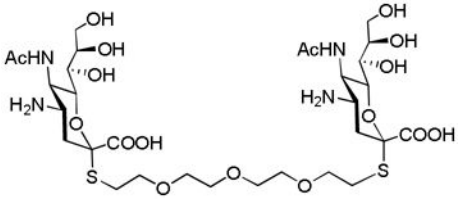


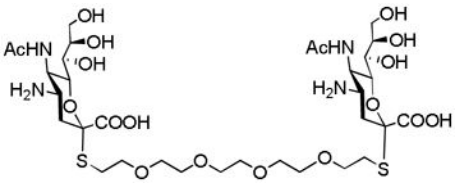
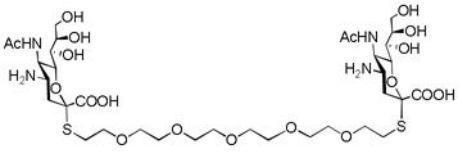
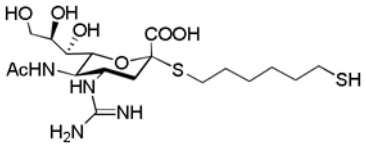
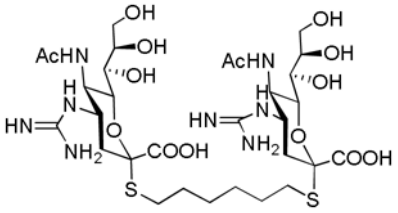
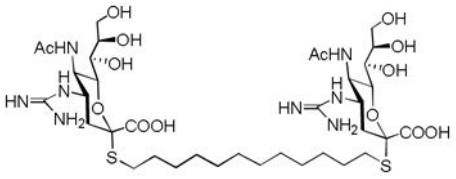
Scheme 2.

Synthesis of bivalent analogs of S-Sialosides. *Reagents and Conditions:* a) DEA, DMF; 76–85%; b) i) NaOH/CH_3OH (aq), ii) H_2/$Pd(OH)_2$; 90–92% % over 2 steps; c) i) H_2/$Pd(OH)_2$, ii)MeS-(C=NBoc)NHBoc, $HgCl_2$, TEA, CH_2Cl_2. 85–89% % over 2 steps; d) NaOH/CH_3OH (aq), 1 h; i) TFA/DCM (1:1), 80–85% over 2 steps.

Table 1

IC_{50} values for the inhibition of soluble NAs and intact virus by the gemini sialoside analogs. The influenza strains used in these studies were Influenza H_1N_1 (A/California/07/2009) and H_3N_2 (A/Hongkong/8/68).

Name	Structure	Virus		NA from Virus	
		H_3N_2 (μM)	H_1N_1 (μM)	H_5N_1 (μM)	H_3N_2 (μM)
SA		64±14	56±6	127±29	178±20
C6-SA		11.6±3.5	9.9±1.8	20±5	32±5
C12-SA		15±9	11±1	31±7	40±1
TetraEG-SA		11±5	9.8±2.4	28±3	39±14

Name	Structure	Virus		NA from Virus	
		H_3N_2 (μM)	H_1N_1 (μM)	H_5N_1 (μM)	H_3N_2 (μM)
PentaEG-SA		21±3	36±4	64±14	178±20
HexaEG-SA		33±15	45±1	85±12	216±56
SG		0.55±0.02	0.8±0.1	0.1±0.02	1±0.1
C6-SG		0.25±0.05	0.26±0.03	0.2±0.02	4.2±1.2
C12-SG		0.5±0.3	0.8±0.06	0.73±0.03	12±2

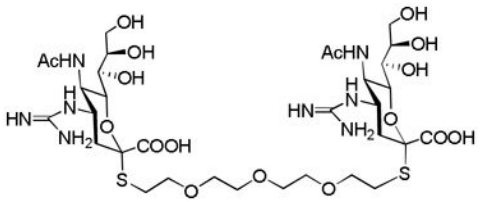
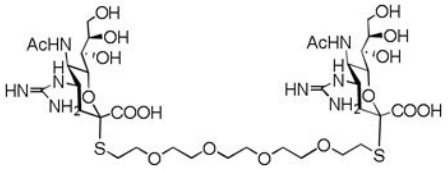
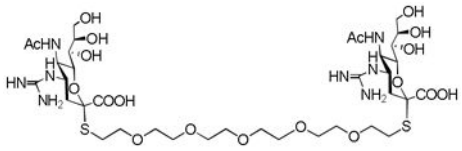
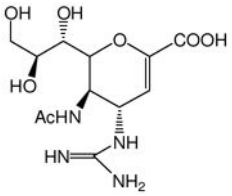
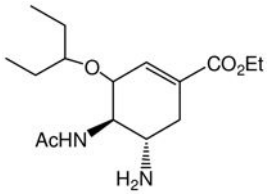
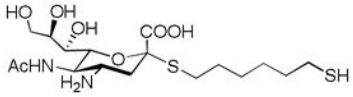
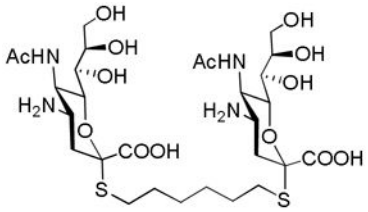
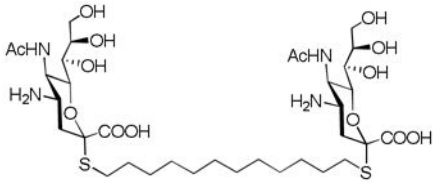
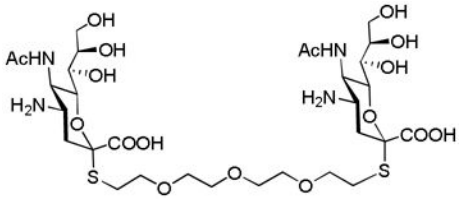
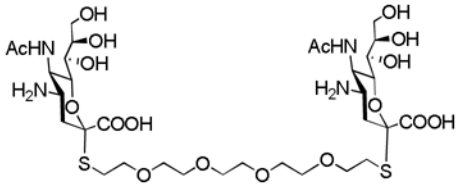
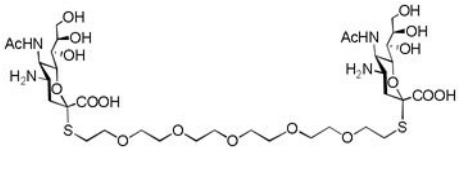
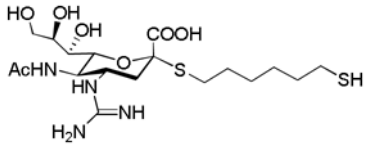
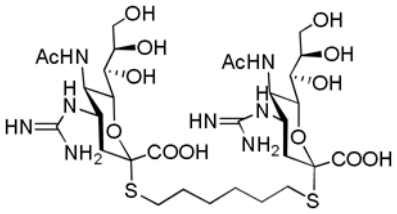
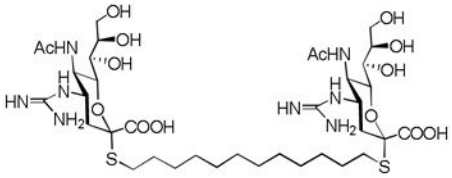
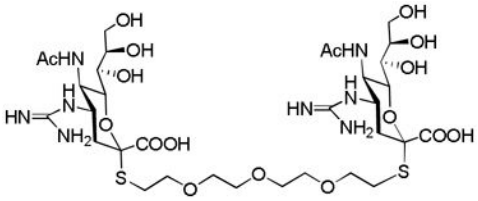
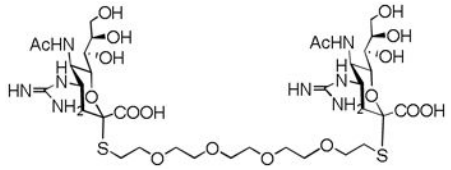
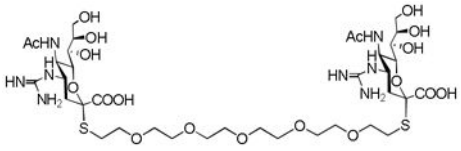
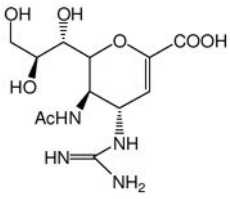
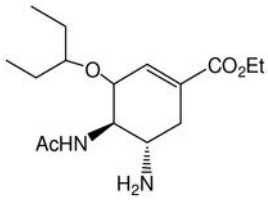
Name	Structure	Virus		NA from Virus	
		H_3N_2 (μM)	H_1N_1 (μM)	H_5N_1 (μM)	H_3N_2 (μM)
TetraEG-SG		1.78±1.3	2.4±0.4	2.5±0.1	10±1
PentaEG-SA		1±0.6	1.2±0.2	1.1±0.1	10±2
HexaEG-SA		0.5±0.2	0.9±0.1	0.57±0.06	11±2
Zanamivir®		~0.001	~0.01	~0.04	~0.04
Oseltamivir ®		~0.008	~0.01	~0.05	~0.05

Table 2

IC_{50} values of influenza strains by the gemini sialosides based on 50% decrease in plaque size. The strains used in these studies were Influenza H_1N_1 (A/California/07/2009) and H_3N_2 (A/Hongkong/8/68).

Name	Structure	Virus	
		H_3N_2 (μM)	H_1N_1 (μM)
SA		10–100	>100
C6-SA		1–10	100
C12-SA		1–10	100
TetraEG-SA		1–10	100
PentaEG-SA		1–10	100
HexaEG-SA		1–10	100

Name	Structure	Virus	
		H_3N_2 (μM)	H_1N_1 (μM)
SG		1–10	10
C6-SG		0.01–0.1	10
C12-SG		0.1	1.0
TetraEG-SG		0.1	1.0
PentaEG-SG		.01–0.1	1.0
HexaEG-SG		0.1–1.0	1.0
Zanamivir®		0.001	0.01

Name	Structure	Virus	
		H_3N_2 (μM)	H_1N_1 (μM)
Oseltamivir ®		0.001–0.01	0.001–0.01