Carbohydrate Polymers 81 (2010) 330–334

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

One-step synthesis of efficient binding-inhibitor for influenza virus through multiple addition of sialyloligosaccharides on chitosan

Myco Umemura[a,*], Yutaka Makimura[b], Masae Itoh[c], Takeshi Yamamoto[d], Toshiki Mine[d], Seiji Mitani[e], Ichiro Simizu[b], Hisashi Ashida[e], Kenji Yamamoto[e]

[a] Department of Chemistry, Graduate School of Science, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan
[b] Faculty of Dentistry, Asahi University, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan
[c] Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan
[d] Glycotechnology Business Unit, Japan Tobacoo Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan
[e] Graduate School of Biostudies, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan

ARTICLE INFO

Article history:
Received 9 November 2009
Received in revised form
21 December 2009
Accepted 12 February 2010
Available online 15 March 2010

Keywords:
Binding inhibitor
Influenza virus
Chitosan
Sialic acid
Multivalent effect

ABSTRACT

We have succeeded in one-step synthesis of an efficient binding-inhibitor for influenza virus, which is composed of only sugar chains. This binding-inhibitor utilizes the carbohydrate recognition of influenza virus, thus it can prevent the virus from infection. We modified chitosan with multiple sialyl saccharides, α2,6-sialyllactose or free sialyl glycan, using reductive amination reaction. The resulting inhibitors showed sufficient inhibitory activity against influenza virus infection in MDCK cells compared to that of α2,6-sialyllactose or free sialyl glycan. Unlike the other binding-inhibitors of influenza virus, this virus inhibitor of sugar chains requires only one step in its synthesis. Therefore this inhibitor is suitable for use in products such as filters and masks.

1. Introduction

Influenza virus is one of the most threatening pathogens for human especially due to its high ability to mutate (Suzuki, 2005). Emergence of new strains amongst human can lead to a pandemic and bring severe social and economic damages. There have already been efficient anti-influenza agents such as oseltamivir and zanamivir, however, they can only prevent the progeny virion from spreading after infection (Kim et al., 1998; Kim, Chen, & Mendel, 1999; Wade, 1997). To remove or alleviate the threat of the viral infection, other methodology is required. Influenza virus infects host cells via its protein, hemagglutinin (HA), which recognizes a specific saccharide structure containing sialic acid at the end of the *N*- or *O*-glycans on the host cells. This HA recognition is the beginning of the viral entry into host cells (Cross, Burleigh, & Steinhauer, 2001; Stevens & Donis, 2007). Utilizing this carbohydrate recognition of the virus, we previously reported an effective binding-inhibitor of the virus, CDO-chitosan (Makimura et al., 2006; Umemura et al., 2008). CDO-chitosan possesses multiple sialic acids hanging from a polymer backbone, chitosan, and can trap the virus before infection by the so-called glycosidic cluster effect (Sashiwa, Shigemasa, & Roy, 2000a; Sashiwa, Makimura, Shigemasa, & Roy, 2000b; Sashiwa, Shigemasa, & Roy, 2001a; Sashiwa, Shigemasa, & Roy, 2001b; Sashiwa, Shigemasa, & Roy, 2001c; Sun, 2007). Although the compound exhibits an efficient inhibitory activity, it would be difficult to manufacture because of elaborate procedures in the synthesis. In this report, we describe a quite simple and economical way to synthesize binding-inhibitors for influenza virus. We synthesized this compound with only sugar chains, that is, a polymer derived from natural marine product, chitosan, and α2,6-sialyllactose (6SL) or free sialyl glycan (FSG). We modified chitosan with multiple side chains including *N*-acetylneuraminic acid (Neu5Ac) linking to galactose (Gal) by α2,6-linkages that is recognized by the viral HA (Ito et al., 1997; Rogers et al., 1983; Ryan-Poirier et al., 1998; Varki et al., 1999). As the resulting inhibitor demonstrated sufficient inhibitory activities against influenza virus infection through a quite simple procedure, it is one of the most practical methods to prevent the influenza virus infection. Although there are several compounds aimed at binding inhibitors of influenza virus (Sigal, Mammen, Dahmann, & Whitesides, 1996; Totani et al., 2003), the compound reported herein has advantages in its quite simple synthesis and because its constituents are limited to saccharides. In addition, as the compound is composed of chitosan fibrils that are easy to be processed

* Corresponding author. Tel.: +81 75 753 4003; fax: +81 75 753 4000.
E-mail address: umemura-m@kuchem.kyoto-u.ac.jp (M. Umemura).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.02.014

(Auxenfans et al., 2009; Masotti & Ortaggi, 2009), it can be utilized in a filter, a face mask, or clothes, to prevent the virus from entering our body or environment.

2. Materials and methods

2.1. Synthesis of binding-inhibitors

2.1.1. Synthesis of 6SL-chitosan

The synthesis of the present compound is quite simple and easy. We chose 6SL as the hand to trap the virus, which is one of the trisaccharides including the minimum structure, Neu5Acα2,6Gal, recognized by viral HA. The reducing agent opened the reducing terminal of saccharides, and made the amino groups of chitosan acylated with the reducing sugar (Sashiwa & Shigemasa, 1999; Sashiwa et al., 2003; Yalpani & Hall, 1984). Using this procedure, we dangled the trisaccharide at the amino groups in chitosan only in one step (Fig. 1). To estimate the optimum amount of 6SL in the synthesis, we tested two conditions: 0.5 or 1 molar equivalent of 6SL to the glucosamine unit of chitosan. Chitosan (Wako, DP 392, deacetylation degree 81.8%) was dissolved with stirring in 1% acetic acid at a concentration of 50 mg/mL, followed by neutralization with 3 M NaOH. Twenty microliters of the resulting solution (1.0 mg of chitosan, 6.2 μmol of glucosamine (GlcN)) was diluted in 100 μL of distilled water containing 3.9 or 2.0 mg of 6SL (1 or 0.5 molar equivalent of chitosan GlcN, respectively). After adding 12.4 μL of 2 M sodium cyanoborohydride (Sigma–Aldrich Co.), reaction proceeded for 24 h at 60 °C. The reaction product was ultra-filtrated on MWCO 30K membrane devices (Millipore) and lyophilized. The yields with 1.0 and 0.5 equiv. of 6SL (6SL-chitosan-1 and 6SL-chitosan-0.5, respectively) were 2.8 and 2.4 mg, respectively. ^{1}H NMR data of 6SL-chitosan-0.5 was (600 MHz, D_2O, RT): δ 4.53 (br d, H-1 of chitosan-GlcN), 4.48 (br d, H-1 of Gal), 3.46–3.90 (br m, 34.66H, -NH-C*H*2-, H-4,5,6,7,8,9 of Neu5Ac, H-2,3,4,5,6 of Gal, Glc and chitosan-GlcN) 2.70 (br dd, 0.93H, H-3*eq* of Neu5Ac), 2.00, 2.02 (2 s, 4.44H, AcN) and 1.67 (t, 1.00H, $J_{gem} = J_{3ax,4}$ = 11.0 Hz, H-3*ax* of Neu5Ac). That of 6SL-chitosan-1 was similar to 6SL-chitosan-0.5.

2.1.2. Preparation of FSG

The method described above can be applied to any saccharides in principle. Therefore we synthesized another binding-inhibitor using a free sialyl glycan (FSG) to compare with 6SL-chitosan. FSG is a sialyloligosaccharide moiety of a sialylglycopeptide from hen egg yolk (SGP, kindly donated by Taiyo Chemical Industry Co. Ltd.) (Seko et al., 1997). SGP (71.6 mg, 25 μmol) was hydrolysed by the recombinant endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M, 200 mU) in 0.1 M sodium phosphate buffer (pH 6.0, 1 mL) at 30 °C for 16 h. Purification by high-performance liquid chromatography gave 45.5 mg of FSG (yield 90%): ^{1}H NMR (600 MHz, D_2O, RT) δ 5.09 (d, 0.6H, H-1α of GlcNAc-1), 5.01 (s, 1H, H-1 of Man-3), 4.93 (d, 0.4H, H-1β of GlcNAc-1), 4.83 (s, 1H, H-1 of Man-3′), 4.72 (s, 1H, H-1 of Man-2), 4.49 (2d, 2H, $J_{1,2}$ = 7.6 Hz, H-1 of GlcNAc-4,4′), 4.33 (2d, 2H, $J_{1,2}$ = 7.6 Hz, H-1 of Gal-5,5′), 4.14 (s, 1H, H-2 of Man-2), 4.03 (s, 1H, H-2 of Man-3), 4.00 (s, 1H, H-2 of Man-3′), 3.36–3.88 (br m, 49H, H-4,5,6,7,8,9 of Neu5Ac-6,6′, H-3,4,5,6 of Man-2,3,3′ and H-2,3,4,5,6 of Gal-5,5′ and GlcNAc-1,4,4′), 2.55 (2dd, 2H, H-3*eq* of Neu5Ac-6,6′), 1.95, 1.94, 1.92, 1.91 × 2 (5 s, 15H, AcN) and 1.60 (2t, 2H, $J_{gem} = J_{3ax,4}$ = 12.37 Hz, H-3*ax* of Neu5Ac-6,6′).

2.1.3. Synthesis of FSG-chitosan

Using FSG, we synthesized another binding-inhibitor, FSG-chitosan (Fig. 2). The procedure was the same as in 6SL-chitosan. To a solution of chitosan (2.5 mg, GlcN 15 μmol) with FSG (9.1 mg, 4.5 μmol) 2 M $NaBH_3CN$ aqueous solution (20 μL) was added, and gave 6.6 mg of FSG-chitosan: ^{1}H NMR (600 MHz, D_2O, RT) δ 4.99 (s, 0.98H, H-1 of Man-3), 4.81 (s, H-1 of Man-3′), 4.61 (br d, H-1 of chitosan-GlcN), 4.31 (2d, 1.96H, $J_{1,2}$ = 8.2 Hz, H-1 of Gal-5,5′), 4.06 (s, H-2 of Man-2′), 4.03 (s, H-2 of Man-3), 3.92 (s, H-2 of Man-3′), 3.39–3.88 (br m, 129.06H, –NH–C*H*2–, H-4,5,6,7,8,9 of Neu5Ac-6,6′, H-3,4,5,6 of Man-2,3,3′ and H-2,3,4,5,6 of Gal-5,5′, GlcNAc-1,4,4′ and Chitosan-GlcN), 2.53 (2dd, 2.00H, H-3*eq* of Neu5Ac-6,6′), 1.93, 1.92, 1.89 × 2, 1.87, 1.80 (6 s, 27.32H, AcN) and 1.57 (2t, $J_{gem} = J_{3ax,4}$ = 14.43 Hz, H-3*ax* of Neu5Ac-6,6′).

2.1.4. Synthesis of CDO-chitosan

CDO-chitosan was prepared according to the previously described procedure (Makimura et al., 2006; Umemura et al., 2008): ^{1}H NMR (600 MHz, D_2O, RT) δ 7.23 (d, 2.12H, $J_{2,3} = J_{6,5}$ = 6.9 Hz, H-2 and H-6 of Ph), 6.94 (d, 2.11H, $J_{3,2} = J_{5,6}$ = 7.6 Hz, H-3 and H-5 of Ph), 5.04 (d, $J_{1,2}$ = 12.4 Hz, of H-1 of GlcNAc-1), 5.03 (s, H-1 of Man-4), 4.81 (s, 0.83H, H-1 of Man-4′), 4.50 (br d, H-1 of GlcNAc-2,5,5′, chitosan-GlcN), 4.34 (2d, 1.96H, $J_{1,2}$ = 8.3 Hz, H-1 of Gal-6,6′), 4.15 (s, 0.91H, H-2 of Man-3′), 4.09 (s, 0.91H, H-2 of Man-4), 4.01 (s, 0.91H, H-2 of Man-4′), 3.38–3.92 (br m, 130.16H, –NH–C*H*2–, H-4,5,6,7,8,9 of Neu5Ac-7,7′, H-3,4,5,6 of Man-3,4,4′ and H-2,3,4,5,6 of Gal-6,6′, GlcNAc-1,2,5,5′ and Chitosan-GlcN), 2.56 (2dd, 1.74H, H-3*eq* of Neu5Ac-7,7′), 1.99, 1.97 × 3, 1.96 × 2, 1.92 (7s, 23.98H, AcN) and 1.61 (2t, 2.00H, $J_{gem} = J_{3ax,4}$ = 12.3 Hz, H-3*ax* of Neu5Ac-7,7′).

2.1.5. DS estimation

The degree of substitution (DS) of sialic acid side chains over D-glucosamine residues of chitosan was determined from the relative integrated values of ^{1}H NMR signal areas between the axial or equatorial H-3 protons in Neu5Ac and the methyl protons in *N*-acetyl group that exist in Neu5Ac and *N*-acetyl-D-glucosamine (GlcNAc). As chitosan is obtained by *N*-deacetylation of chitin, the

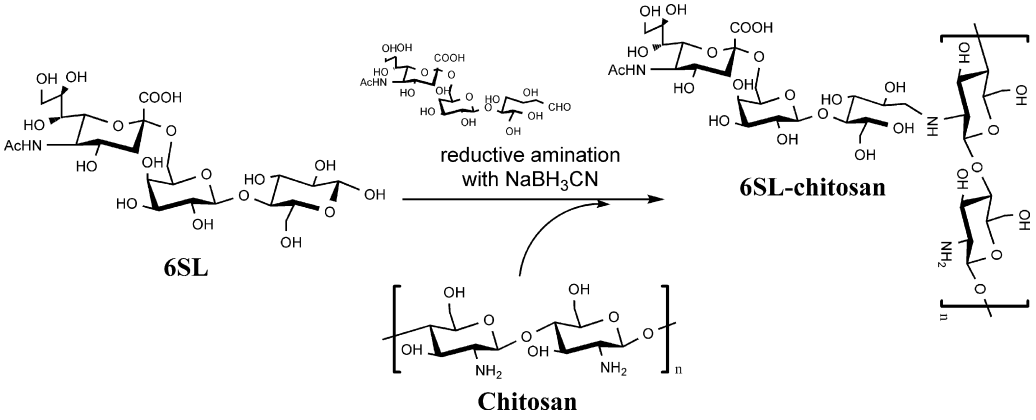

Fig. 1. Synthesis of 6SL-chitosan.

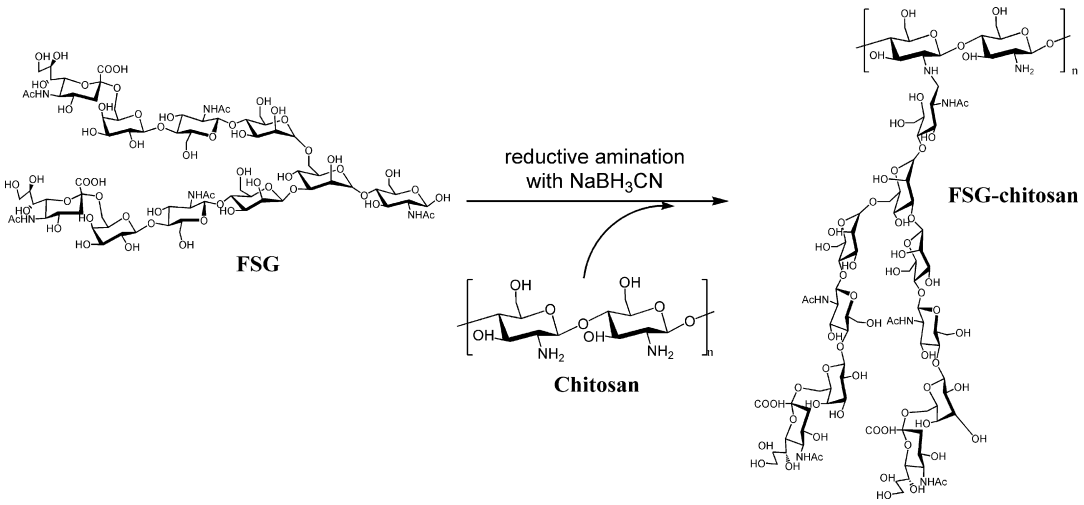

Fig. 2. Synthesis of FSG-chitosan.

homo-polymer of GlcNAc, GlcNAc residues remain in chitosan. We can estimate the ratio of remained GlcNAc in chitosan from the deacetylation percentage of the chitosan (DDS) as 81.8%. Therefore, we can derive the DS value (%) from the following relation:

$$I_{\text{H-3}} : I_{\text{AcHN}} = \text{DS} \times N_{\text{sialic}} : (\text{DS} \times N_{\text{AcHN}} + 100 - \text{DDS}) \times 3$$

where $I_{\text{H-3}}$ and I_{AcHN} indicate the peak area of axial or equatorial H-3 proton in sialic acid and the methyl protons in *N*-acetyl groups, respectively, and N_{sialic} and N_{AcHN} do the number of sialic acid and *N*-acetyl group in one side chain, respectively. In the case of 6SL-chitosan, both N_{sialic} and N_{AcHN} are 1, while those are 2 and 5, respectively, in FSG-chitosan.

2.2. Inhibitory assay against influenza virus infection

2.2.1. Viruses

We tested the inhibitory activity of each binding-inhibitor against influenza virus infection using a virus strain A/New Caledonia/20/99 (H1N1). The virus was inoculated in Madin–Darby canine kidney (MDCK) cells and incubated in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co. Ltd.) supplemented with 2.5 μg/mL purified trypsin (Sigma–Aldrich Co., St. Louis, MO) at 34 °C for 3 days. Culture medium was harvested and stored at −80 °C after centrifugation at 3000 rpm for 10 min. Titers of the virus stocks were estimated by indirect immunofluorescent method using MDCK cells and expressed as cell-infecting units (CIU)/mL (Kashiwazaki, Homma, & Ishida, 1965). Antibody used was anti-influenza A virus nucleoprotein mouse monoclonal antibody (Serotec) followed by FITC-conjugated anti-mouse IgG goat serum (Medical and Biological Laboratories Co. Ltd., MBL).

2.2.2. Procedure of inhibitory assay

Inhibitors were dissolved in phosphate-buffered saline (PBS) at a concentration of 4 mg/mL and serially diluted by 1:4, 1:16, 1:64, and 1:256. We used fetuin (Sigma–Aldrich), 6SL (Japan Tobacco Industry), and FSG as control inhibitors from the concentration of 20 (fetuin and 6SL) or 5 (FSG) mg/mL. A volume of 50 μL of each diluted inhibitor was mixed with an equal volume of influenza virus solution containing 4.0×10^5 CIU/mL and incubated at 25 °C for 1 h. Forty microliters of each inhibitor-virus mixture was then inoculated in duplicate on a monolayer of MDCK cells in a 96-well plate. After 1 h adsorption at 37 °C in 5% CO_2, 150 μL of DMEM supplemented with 5 μg/mL of soybean trypsin inhibitor (Sigma–Aldrich) was overlayed. The infected cells were incubated at 37 °C in 5% CO_2 for 14 h, and were then fixed by 1% paraformaldehyde in PBS for 1 h followed by treatment with 1% Triton X-100 for 15 min. After washing with PBS, cells were stained by the indirect immunofluorescent method. Antibodies used for each virus were the same as those used in the virus titration. The positive cells were counted using a fluorescent microscope (Carl Zeiss, Inc.) ($N = 4$, two fields per each of two wells). The residual infectivity was expressed as a percentage over the number of infected cells without inhibitor.

3. Results and discussion

After only one step of procedure, we obtained the binding-inhibitor of influenza virus, 6SL-chitosan that have multiple Neu5Acα2,6Gal components recognized by the viral HA (Fig. 1). We synthesized the binding-inhibitor with both 0.5 and 1 molar equivalent of 6SL to the glucosamine unit of chitosan. Under each condition, we obtained the intended compounds, 6SL-chitosan-0.5 and 6SL-chitosan-1, respectively, with the different degrees of substitution (DS) of opened 6SL for OH in chitosan glucosamine residues. From NMR analysis, we estimated the DS of 6SL-chitosan-0.5 as 37.9% while that of 6SL-chitosan-1 as 31.7% (Table 1). It might be difficult to add 6SL on chitosan more than DS of 40% due to the chitosan structure. It is interesting that the DS values of 6SL-chitosan-0.5 and -1 were in inverted order of the amount of 6SL in reaction solution. It is considered that the 0.5 equiv. of 6SL was sufficient for this synthesis, and excessive amount of 6SL might have caused too crowded condition for the reductive amination of chitosan glucosamine.

Then we tested the inhibitory activity of each 6SL-chitosan against influenza virus infection in MDCK cells. The used virus strain was A/New Caledonia/20/99 (H1N1), which is one of the most representative human influenza viruses. The compound 6SL-

Table 1
IC_{50} values of compounds used in the inhibition assay against A/New Caledonia/20/99 (H1N1) in MDCK cells.

Compound	DS (%)	MW	IC_{50} (mg/mL)	IC_{50} (Sia μM)[a]
6SL	–	634	5	7890
6SL-chitosan-0.5	37.9	155,000	0.2	192
6SL-chitosan-1	31.7	140,000	0.5	444
FSG	–	2021	*n.d.*[b]	*n.d.*[b]
FSG-chitosan	4.4	98,100	1	352
CDO-chitosan	15.0	199,000	0.02	12
Fetuin	–	48,000	>20	>1600

[a] Molar concentration of sialic acid.
[b] Not detected.

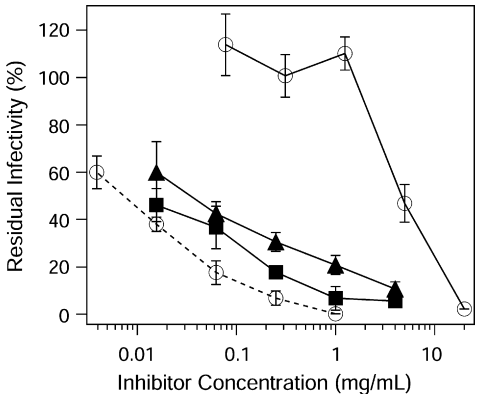

Fig. 3. Inhibitory activity of 6SL-chitosan against the virus strain A/New Caledonia/20/99 (H1N1). The solid lines with filled square and triangle denote 6SL-chitosan-0.5 and -1, respectively. The other two lines indicate the results of monomeric 6SL (solid with open circle) and our another binding-inhibitor, CDO-chitosan (dashed with open circle), respectively.

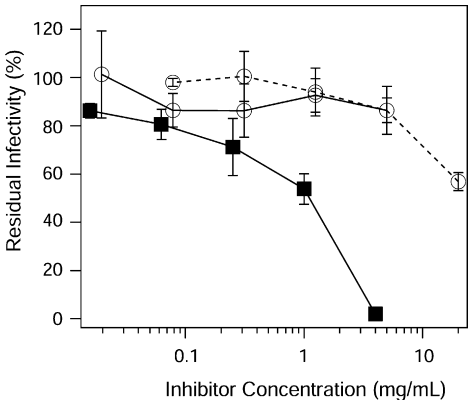

Fig. 4. Inhibitory activity of FSG-chitosan against the strain A/New Caledonia (H1N1). The solid line with filled square denotes FSG-chitosan. The other two lines indicate the results of monomeric FSG and fetuin (solid and dashed with open circle, respectively).

chitosan-0.5 or -1 showed much higher inhibitory activity than monomeric 6SL (Fig. 3). The IC_{50} values of 6SL-chitosan-0.5 and -1 were 0.2 and 0.5 mg/mL, respectively, which were 25–10 times lower than that of 6SL (Table 1). As the molecular weight of 6SL (634) is quite lower than 6SL-chitosan-0.5 (155,000) or 6SL-chitosan-1 (140,000), the IC_{50} value of 6SL in sialic acid molar concentration reached 41–18 times higher than those of 6SL-chitosan-0.5 or -1. Although 6SL-chitosan was less effective than our previous binding-inhibitor, CDO-chitosan (Makimura et al., 2006; Umemura et al., 2008), it efficiently inhibited the virus infection.

The compound 6SL-chitosan-0.5 showed higher inhibitory activity than 6SL-chitosan-1 in accordance with its DS, thus the inhibition ability of 6SL-chitosan depends on the amount of sialic acid in chitosan. Taking into account that CDO-chitosan has DS of 15%, however, the inhibitory activity of 6SL-chitosan is still low despite its high DS over 30%. This result indicates other determining factors of the inhibition than the amount of sialic acid in chitosan. One of the considerable factors is the length of the sialic acid side chain. Sidechains that are too short to reach and fit in the receptor-binding pocket of HA may increase steric hindrance for the sialic acid recognition of HA, which leads to the low inhibition.

To compare with the inhibitory activity of 6SL-chitosan, we synthesized another binding-inhibitor, FSG-chitosan, by replacing FSG for 6SL (Fig. 2). FSG is a biantennary decasaccharide containing two sialic acid at each non-reducing terminal (Seko et al., 1997). The highest DS value reached only 4.4% with the resulting compound (Table 1). The low DS of FSG-chitosan is probably because the sizable decasaccharide interrupted the reductive amination reaction in the solution. As observed with 6SL-chitosan, excessive amount of FSG decreased the DS (data not shown). The inhibitory activity of FSG-chitosan was higher than FSG and fetuin, a protein containing α2,6sialylgalactose (Fig. 4), but lower than 6SL-chitosan-0.5 and -1 as shown by IC_{50} (mg/mL), probably due to its low DS (Table 1). It is notable, however, that the IC_{50} value of FSG-chitosan in sialic acid molar concentration was located between 6SL-chitosan-0.5 and -1. This means that the ability of FSG-chitosan per sialic acid to trap the virus was close to that of 6SL-chitosan despite its quite low DS of 4.4%, one-fourth amount of sialic acid in 6SL-chitosan. The result indicates that the length of the side chains is quite important to achieve the high inhibitory activity in addition to the amount of sialic acid conjugated to the backbone. Influenza virus was reported to recognize the *N*-acetylglucosamine or glucose subsequent to Neu5Acα2,6(3)Gal (Suzuki et al., 1986; Suzuki et al., 1992). Therefore, the inhibition behavior of the compounds should depend on the component of the side chain. While FSG-chitosan contains above trisaccharide structure, 6SL-chitosan remains only Neu5Acα2,6Gal because the glucose at the reducing end was opened by the reducing agent. This may be the reason why the inhibitory activity of 6SL-chitosan was rather low despite its high DS over 30%.

4. Conclusion

We have herein reported a notable simple synthesis of binding-inhibitors against influenza virus. These influenza virus binding-inhibitors, 6SL-chitosan and FSG-chitosan, intensified the inhibitory activity of respective monomeric 6SL and FSG by assembling on a chitosan backbone. These are the first binding-inhibitors of influenza virus composed of only sugar chains to our knowledge. Taking advantage of this simpleness and therefore high cost-performance, there is a possibility of developing binding-inhibitors with various sugar components for practical and commercial use. Recently, sialyllactose has become available in abundance as a result of biogenetic engineering (Endo, Koizumi, Tabata, & Ozaki, 2000; Fierfort & Samain, 2008; Gilbert et al., 1998). The biotechnology provides us the gram quantity production of the pure sialyllactose from *Escherichia coli* expressing the appropriate recombinant glycosyltransferase. Although sialyllactose has long been considered as a favorable material for inhibitors against influenza virus, no trial has succeeded yet. One of the main reasons for this must be the unavailability and expensiveness of sialyllactose. Short length of sialyllactose has disadvantage for the desirable high inhibitory activity expressed by longer side chains, but on the other hand, 6SL-chitosan maintained high DS because of the shortness of 6SL and therefore its sufficient inhibitory activity against influenza virus infection. We expect that 6SL-chitosan will become a promising inhibitor against influenza virus infection with stable supply of 6SL.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.02.014.

References

Auxenfans, C., Fradette, J., Lequeux, C., Germain, L., Kinikoglu, B., Bechetoille, N., et al. (2009). Evolution of three dimensional skin equivalent models reconstructed in vitro by tissue engineering. *European Journal of Dermatology*, *19*, 107–113.
Cross, K. J., Burleigh, L. M., & Steinhauer, D. A. (2001). Mechanisms of cell entry by influenza virus. *Expert Reviews in Molecular Medicine*, *6*, 1–6.

Endo, T., Koizumi, S., Tabata, K., & Ozaki, A. (2000). Large-scale production of CMP-NeuAc and sialylated oligosaccharides through bacterial coupling. *Applied Microbiology and Biotechnology*, *53*, 257–261.

Fierfort, N., & Samain, E. (2008). Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides. *Journal of Biotechnology*, *134*, 261–265.

Gilbert, M., Bayer, R., Cunningham, A. M., DeFrees, S., Gao, Y., Watson, D. C., et al. (1998). The synthesis of sialylated oligosaccharides using a CMP-Neu5Ac synthetase/sialyltransferase fusion. *Nature Biotechnology*, *16*, 769–772.

Ito, T., Suzuki, Y., Takada, A., Kawamoto, A., Otsuki, K., Masuda, H., et al. (1997). Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. *Journal of Virology*, *71*, 3357–3362.

Kashiwazaki, H., Homma, M., & Ishida, N. (1965). Assay of Sendai virus by immunofluorescence and hemadsorbed cell-counting procedures. *Proceedings of the Society for Experimental Biology and Medicine*, *120*, 134–138.

Kim, C. U., Chen, X., & Mendel, D. B. (1999). Neuraminidase inhibitors as anti-influenza virus agents. *Antiviral Chemistry & Chemotherapy*, *10*, 141–154.

Kim, C. U., Lew, W., Williams, M. A., Wu, H., Zhang, L., Chen, X., et al. (1998). Structure-activity relationship studies of novel carbocyclic influenza neuraminidase inhibitors. *Journal of Medicinal Chemistry*, *41*, 2451–2460.

Makimura, Y., Watanabe, S., Suzuki, T., Suzuki, Y., Ishida, H., Kiso, M., et al. (2006). Chemoenzymatic synthesis and application of a sialoglycopolymer with a chitosan backbone as a potent inhibitor of human influenza virus hemagglutination. *Carbohydrate Research*, *341*, 1803–1808.

Masotti, A., & Ortaggi, G. (2009). Chitosan micro- and nanospheres: Fabrication and applications for drug and DNA delivery. *Mini-Reviews in Medicinal Chemistry*, *9*, 463–469.

Rogers, G., Paulson, J., Daniels, R., Skehel, J., Wilson, I., & Wiley, D. (1983). Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature*, *304*, 76–78.

Ryan-Poirier, K., Suzuki, Y., Bean, W., Kobasa, D., Takada, A., Ito, T., et al. (1998). Changes in H3 influenza A virus receptor specificity during replication in humans. *Virus Research*, *56*, 169–176.

Sashiwa, H., Kawasaki, N., Nakayama, A., Muraki, E., Yajima, H., Yamamori, N., et al. (2003). Chemical modification of chitosan. Part 15: Synthesis of novel chitosan derivatives by substitution of hydrophilic amine using *N*-carboxyethylchitosan ethyl ester as an intermediate. *Carbohydrate Research*, *338*, 557–561.

Sashiwa, H., Makimura, Y., Shigemasa, Y., & Roy, R. (2000). Chemical modification of chitosan: Preparation of chitosan-sialic acid branched polysaccharide hybrids. *Chemical Communications*, 909–910.

Sashiwa, H., & Shigemasa, Y. (1999). Chemical modification of chitin and chitosan 2: Preparation and water soluble property of *N*-acylated or *N*-alkylated partially deacetylated chitins. *Carbohydrate Polymers*, *39*, 127–138.

Sashiwa, H., Shigemasa, Y., & Roy, R. (2001a). Preparation and lectin binding property of chitosan-carbohydrate conjugates. *Bulletin of the Chemical Society of Japan*, *74*, 937–943.

Sashiwa, H., Shigemasa, Y., & Roy, R. (2001b). Chemical modification of chitosan. 10. Synthesis of dendronized chitosan-sialic acid hybrid using convergent grafting of preassembled dendrons built on gallic acid and tri(ethylene glycol) backbone. *Macromolecules*, *34*, 3905–3909.

Sashiwa, H., Shigemasa, Y., & Roy, R. (2001c). Highly convergent synthesis of dendrimerized chitosan-sialic acid hybrid. *Macromolecules*, *34*, 3211–3214.

Sashiwa, H., Shigemasa, Y., & Roy, R. (2000). Chemical modification of chitosan. 3. Hyperbranched chitosan-sialic acid dendrimer hybrid with tetraethylene glycol spacer. *Macromolecules*, *33*, 6913–6915.

Seko, A., Koketsu, M., Nishizono, M., Enoki, Y., Ibrahim, H. R., Juneja, L. R., et al. (1997). Occurrence of a sialylglycopeptide and free sialylglycans in hen's egg yolk. *Biochimica et Biophysica Acta*, *1335*, 23–32.

Sigal, G. B., Mammen, M., Dahmann, G., & Whitesides, G. M. (1996). Polyacrylamides bearing pendant α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus: the strong inhibition reflects enhanced binding through cooperative polyvalent interactions. *Journal of the American Chemical Society*, *118*, 3789–3800.

Stevens, J., & Donis, R. O. (2007). Influenza virus hemagglutinin—structural studies and their implications for the development of therapeutic approaches. *Infectious Disorders - Drug Targets*, *7*, 329–335.

Sun, X. (2007). Recent anti-influenza strategies in multivalent sialyloligosaccharides and sialylmimetics approaches. *Current Medical Chemistry*, *14*, 2304–2313.

Suzuki, Y. (2005). Sialobiology of influenza: Molecular mechanism of host range variation of influenza viruses. *Biological & Pharmaceutical Bulletin*, *28*, 399–408.

Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K., et al. (1986). Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. Specificity for oligosaccharides and sialic acids and the sequence to which sialic acid is attached. *Journal of Biological Chemistry*, *261*, 17057–17061.

Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Guiyun, X., et al. (1992). Structural determination of gangliosides that bind to influenza A, B, and C viruses by an improved binding assay: Strain-specific receptor epitopes in sialo-sugar chains. *Virology*, *189*, 121–131.

Totani, K., Kubota, T., Kuroda, T., Murata, T., Hidari, K. I.-P. J., Suzuki, T., et al. (2003). Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. *Glycobiology*, *13*, 315–326.

Umemura, M., Itoh, M., Makimura, Y., Yamazaki, K., Umekawa, M., Masui, A., et al. (2008). Design of a sialylglycopolymer with a chitosan backbone having efficient inhibitory activity against influenza virus infection. *Journal of Medicinal Chemistry*, *51*, 4496–4503.

Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., & Marth, J. (1999). *Essentials of glycobiology*. New York: Cold Spring Harbor Laboratory Press.

Wade, R. C. (1997). 'Flu' and structure-based drug design. *Structure*, *5*, 1139–1145.

Yalpani, M., & Hall, L. D. (1984). Some chemical and analytical aspects of polysaccharide modifications. III. Formation of branched-chain, soluble chitosan derivatives. *Macromolecules*, *17*, 272–281.