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N-Acetyl-6-sulfo-D-glucosamine as a Promising Mimic of *N*-Acetyl Neuraminic Acid

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Abstract—6-Sulfo-D-GlcNAc with a molecular geometry close to that of *N*-acetylneuraminic acid (Neu5Ac) was hypothesized to serve as a simple Neu5Ac mimic possessing high potential in biochemical and medicinal applications. The hypothesis was evidenced with a neuraminidase inhibition assay using *p*-nitrophenyl (*p*NP) 3-, 4-, and 6-sulfo-β-D-GlcNAc (**4**, **5** and **2a**) and 6-sulfo-β-D-Glc **6**, in which only *p*NP 6-sulfo-β-D-GlcNAc **2a** was found to show substantial activity.

Sialic acids, bound to *exo*-terminal β-galactosyl residues in cell surface glycoproteins and glycolipids, play a key role in the carbohydrate-protein recognition events leading to cell adhesion, trafficking of leukocytes, and infection of influenza viruses to host cells.[1] They are recognized by many types of sialic acid-binding proteins such as sialoadhesins (siglecs), selectins, and influenza hemagglutinins. Therefore, the sialyl linkage particularly of the most abundant *N*-acetylneuraminic acid (Neu5Ac **1**, Fig. 1), possesses high potential in applications to medicinal agents and bio-mimetic materials. However, the practical application has been hampered by the property of Neu5Ac-glycoside linkages labile to chemical and enzymatic degradations as well as by the difficulty of their large-scaled preparations. Therefore, many different approaches have been designed for their mimic synthesis aiming at the development of anti-inflammation drugs[2] and anti-influenza virus agents.[3] For the anti-inflammation drugs working as selectin antagonists, the linkage of sialyl Lewis^X (sLe^X) tetrasaccharide has been replaced with a simple anionic group such as carboxylate, phosphate, and sulfate groups.[4] This approach works effectively when the receptor proteins recognize the sialyl linkage mainly at the carboxylate group, as in the case of selectin/sLe^X interactions.[5] Otherwise, the mimic synthesis should be performed in such a way as to simulate the key recognition structure of the sialyl linkage exactly. This holds true for the sialyl linkages potentially useful as therapeutic agents against many types of infectious diseases.

Along with our continuous studies on the design and application of polyvalent glycoconjugates, we have investigated practical syntheses of human oligosaccharides and their mimics.[6] In our recent effort to establish a chemical pathway based on the carbohydrate module,[7] we found that an acylamido copolymer carrying a cluster of *N*-acetyl-6-*O*-sulfo-β-D-glucosaminide (6-sulfo-β-D-GlcNAc) possesses potent activity to block L-selectin/sLe^X binding ($IC_{50}=3$ μM).[8] The activity is much higher than that of a copolymer carrying a cluster of 3′-sulfo-Le^X mimics. This finding, indicating the high biological potential of 6-sulfo-D-GlcNAc, allows us to

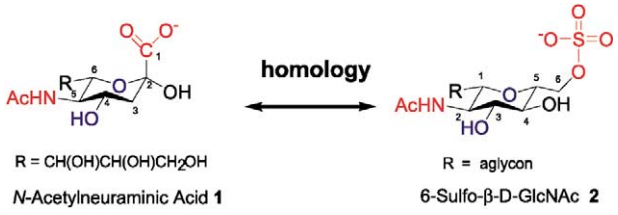

Figure 1. A homology of molecular structures between Neu5Ac **1** and 6-sulfo-D-GlcNAc **2.** The structure of **2** is viewed from a ring frame along the C2–C3–C4 bonds. The C5–C6 conformation is given in *gt* (+60 for the dihedral angle of C5–C6/C6–O6).

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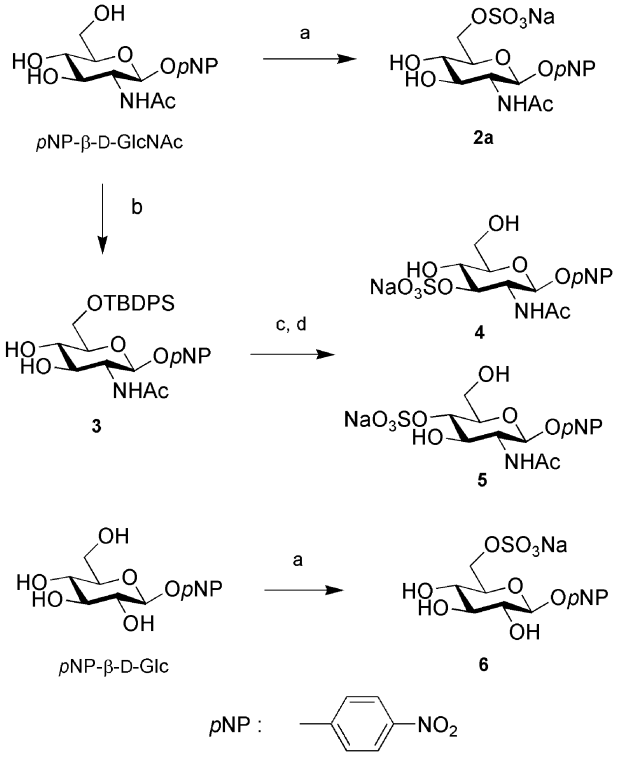
speculate that the L-selectin may recognize the structure of 6-sulfo-D-GlcNAc as a mimic of the Neu5Ac linkage. Recently, Schwörer and Schmidt[9] reported a homology in the structure between D-GlcNAc and Neu5Ac and applied it to the design of sialyltransferase inhibitors. In this paper, we describe a similar correlation between 6-sulfo-D-GlcNAc **2** and Neu5Ac **1** and provide experimental evidence that the 6-sulfo-D-GlcNAc **2** serves as a simple Neu5Ac mimic.

When the structure of 6-sulfo-D-GlcNAc **2** is viewed from a ring frame along the C2–C3–C4 bonds as depicted in Figure 1, the homology with Neu5Ac **1** becomes obvious. The structure **2** locates the NHAc, OH, and anionic functions along the frame close to those of NeuNAc. A difference exists in the aglycon structure, which will be, however, modulated by chemical methods. Though the location of the 6-*O*-sulfate group in **2** changes by the free rotation of a C5–C6 single bond, the ^{1}H NMR analysis[10,11] of *p*NP 6-sulfo-D-GlcNAc **2a** showed that the exocyclic moiety takes the time-averaged population of *gg:gt:tg* = ca. 60:40:0 (%). The *gt*-conformation locates the sulfate group close to the position of a carboxylate anion in Neu5Ac **1**.

To verify our working hypothesis as described above, three position isomers of *p*NP sulfo-D-GlcNAc (**2a**, **4**, and **5**) were prepared in a chemical pathway as shown in Scheme 1. As referential compounds, *p*NP 6-sulfo-D-Glc **6** and *p*NP D-GlcNAc lacking an NHAc and a sulfate group, respectively, were also used. These substances were subjected to an inhibitory assay in the reported way[12] using a neuraminidase (EC: 3.2.1.18) of *Vibrio cholerae* and 2′-(4-methylumbelliferyl)-*N*-acetylneuraminic acid. In this assay, the enzyme reaction (K_m = 1.5 mM for the fluorescent substrate and 1.2 mM for sialyllactose[12]) liberates a fluorescent 4-methylumbelliferone from the non-fluorescent substrate. The results summarized in Figure 2 indicate that neither the referential *p*NP 6-sulfo-D-Glc **6** nor *p*NP D-GlcNAc showed inhibition activity at concentrations below 10 mM. This means that the *p*NP chromophore commonly applied to the substrates does not affect the florescence assay. The assay indicated that *p*NP 6-sulfo-D-GlcNAc **2a** shows notable activity to inhibit the neuraminidase (IC_{50} = 2 mM). The above data indicate that both of the sulfate and NHAc groups play a key role in the neuraminidase inhibition activity of **2a**. Among the three isomers, the 6-sulfo-isomer **2a** showed the highest activity in the order of 6-sulfo **2a** > 4-sulfo **5** > 3-sulfo **4**, indicating that the position of 6-sulfate is another major factor. The overall biological data support that the neuraminidase recognizes the absolute structure of *p*NP 6-sulfo-D-GlcNAc **2a** as a competitor of Neu5Ac.



Scheme 1. Syntheses of *p*NP sulfo-D-GlcNAc and sulfo-D-Glc. Reaction conditions: (a) $Me_3N{\cdot}SO_3$ (6 equiv), DMF, 40 °C, 3 h, Dowex (Na^+ form), 52% for **2a**, 42% for **6**; (b) TBDPSCl, pyridine, DMAP, rt, 24 h, 95%; (c) $Me_3N{\cdot}SO_3$ (6 equiv), DMF, 40 °C, 10 h, Dowex (Na^+ form), 63% (**4**/**5** = 1.4/1); (d) TBAF, THF, rt, 24 h, 98%. TBDPSCl = *tert*-butyldiphenylsilylchloride, DMAP = 4-dimethylaminopyridine, TBAF = tetrabutylammonium fluoride.

Our hypothesis was supported also by a molecular modeling of **2a** compared with the X-ray crystallographic data of a Neu5Ac-neuraminidase complex[13] (Fig. 3). The molecular modeling using an AMBER force field showed that the 2-NHAc and 6-sulfate groups in **2a** prefer to take a spatial geometry (distance and helical pattern) matching with that in Neu5Ac in the enzyme complex rather than free Neu5Ac with a typical $^1C_4$(chair) conformation. In the crystalline data, the Neu5Ac ligand takes a conformation deviated from a $^1C_4$(chair) one and analogous to that of an oxo-carbenium ion at a transitional state. Thus it is probable

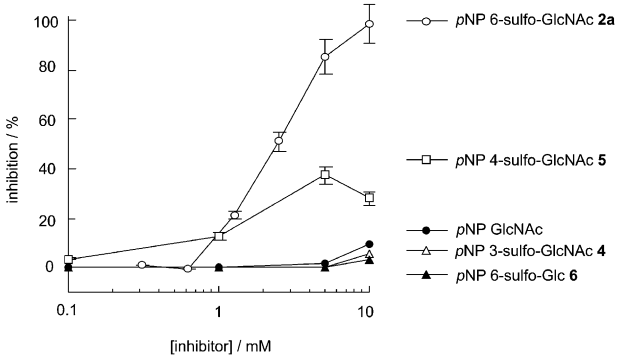


Figure 2. Inhibitory activities of *p*NP sulfo-sugars against a neuraminidase of *V. cholerae*. The degree of inhibition (%, deviation ± 5%) was obtained as an averaged value in three experiments using an equation as follows. Inhibition (%) = $100 \times F_i/F_o$, where F_i and F_o indicates the strength of fluorescence at em. 450 nm (ex. 365 nm) in the presence and the absence of inhibitors, respectively. A typical assay protocol: A reaction buffer (pH 4.6) was prepared with 100 mM sodium acetate, 150 mM NaCl, and 4 mM $CaCl_2$ in pure water (Milli-Q, Millipore). A mixture of 2′-(4-methylumbelliferyl)-*N*-acetylneuraminic acid (0.1 mM), a sialidase (30 μU) (*V. cholerae*, Sigma), and an inhibitor (10–0.1 mM) was dissolved in the reaction buffer (250 μL) and incubated in a micro tube at 37 °C for 30 min. The enzyme reaction was quenched by adding a glycine buffer (133 mM, pH 10.5, 1 mL) containing 60 mM NaCl and 83 mM Na_2CO_3. The fluorescence at 450 nm excited at 365 nm was measured for the solution.

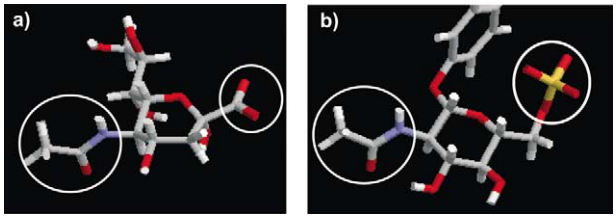


Figure 3. Conformational homology between Neu5Ac and *p*NP 6-sulfo-GlcNAc **2a**: (a) A crystal structure of Neu5Ac in a complex with neuraminidase;[13] (b) a minimized structure of **2a** (MM calculation with an AMBER force field in a MOE program system).

that the inhibitory activity of **2a** may be ascribed to the homology to the transitional intermediate.

In conclusion, we have presented experimental evidences supporting the hypothesis that 6-sulfo-D-GlcNAc may serve as a simple Neu5Ac mimic. Though the inhibitory activity may not be strong enough for practical applications, chemical tuning at the aglycon and C4 positions in the 6-sulfo-D-GlcNAc skeleton as well as multivalent design are expected to integrate biological activity. Since D-GlcNAc (or D-GlcNH$_3^+$ Cl$^-$) is one of the most abundant carbohydrates occurring in nature, the hypothesis and experimental evidences presented in this study will provide a practical basis for the development of anti-virus agents. Recent biological studies[14] have disclosed that GlcNAc-6-*O*-sulfotransferases are expressed widely in human endothelial tissues. The enzymes recognize an *endo*-type GlcNAc residue as the acceptor substrate and contribute possibly to the functional modulation of human oligosaccharides. The present study has suggested also that the sulfotransferases may have a certain role complementary to that of sialyltransferases, which recognize an *exo*-terminal β-D-*galacto*-residue.

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10. Selected data for the compound **2a**; ^{1}H NMR (δppm, 300 MHz, D_2O, room temp) 5.28 (d, 1H, $J_{1,2}=8.7$ Hz, H-1), 4.41 (dd, 1H, $J_{\text{H-5, H-6}proS}=2.1$ Hz, $J_{\text{H-6}proS,\ \text{H-6}proR}=11.1$ Hz, H-6*proS*), 4.25 (dd, 1H, $J_{\text{H-5, H-6}proR}=5.4$ Hz, $J_{\text{H-6}proR,\ \text{H-6}proS}$ 11.1 Hz, H-6*proR*), 4.05 (dd, 1H, $J_{1,2}=8.7$ Hz, $J_{2,3}=10.1$ Hz, H-2), 3.94 (m, 1H, H-5), 3.74 (dd, 1H, $J_{2,3,=}10.1$, $J_{3,4}=10.1$ Hz, H-3), 3.63 (dd, 1H, $J_{3,4}=10.1$, $J_{4,5}=10.1$ Hz, H-4). The population of *gg:gt:tg* (60:40:0) was estimated with an equation optimized for D-*gluco*-series (vs D-*galacto*-series) sugars (ref 11) as follows, $J_{\text{H-5, H-6}proS}=2.2\ gg+2.4\ gt+11.1\ tg$; $J_{\text{H-5, H-6}proR}=1.7\ gg+10.8\ gt+4.1\ tg$; $100=gg+gt+tg$.
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