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Synthesis and binding analysis of unique AG2 pentasaccharide to human Siglec-2 using NMR techniques

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ABSTRACT

Siglec-2 is a mammalian sialic acid binding protein expressed on B-cell surfaces and is involved in the modulation of B-cell mediated immune response. We synthesized a unique starfish ganglioside, AG2 pentasaccharide Gal*f*β(1–3)Gal*p*α(1–4)Neu5Acα(2–3)Gal*p*β(1–4)Glc*p*, and found that the synthetic pentasaccharide binds to human Siglec-2 by performing ^{1}H NMR experiments. Saturation transfer difference NMR experiments indicated that the C7–C9 side-chain and the acetamide moiety of the central sialic acid residue were located in the binding face of human Siglec-2. We determined the binding epitope of AG2 pentasaccharide to human Siglec-2, as the Gal*p*α(1–4)Neu5Acα(2–3)Gal*p* unit.

1. Introduction

Sialylglycans on proteins and lipids at cell surfaces play a key role in proper cell–cell interactions and signaling pathways in complex biological systems.[1] Mammalian sialylglycans mainly contain *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) with α(2–3/6) binding modes on penultimate galactose (Gal), *N*-acetylglucosamine (GlcNAc), or *N*-acetylgalactosamine (GalNAc) residues.[2] Another characteristic polysialic acid structure, which includes α(2–8) connected sialic acid polymer, exists in the vertebrate nervous system and immune system.[3] In mammalian sialylglycans, sialic acid residues are located at the non-reducing termini of the cell-surface glycans, and no further sugar modifications are present, except for the polysialic acid structure. Gangliosides, which are a major glycolipid species, are involved in cell recognition and signal transduction through lipid rafts and caveolae.[4,5] The biosynthetic pathways of mammalian gangliosides are currently being elucidated,[6] and their oligosaccharide chains were found to be terminated with sialic acid residue(s).

Meanwhile, unique echinoderm gangliosides with potent mammalian nerve cell stimulating activity have been identified from starfish and sea cucumbers.[7,8] One of these gangliosides, AG2 (isolated from the starfish *Acanthaster planci*), has an unusual glycan sequence involving an inner sialic acid residue with a galactofuranose capping moiety (Fig. 1).[8a] In addition to this unique structure,

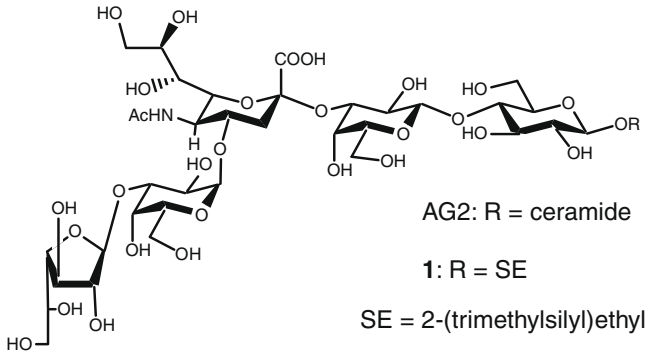

Figure 1. Structure of pentasaccharide moiety of the unique starfish ganglioside AG2 (**1**).

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AG2 exhibits potent nerve cell stimulating activity towards the mouse PC-12 cell line, which is comparable in magnitude to that of the mammalian GM1 ganglioside.[8b]

We were interested in whether this unique sialylglycan with an inner sialic acid residue was recognized by abundant mammalian sialic acid binding proteins to express biological phenomena, and conducted binding studies between AG2 pentasaccharide and Siglec-2 (sialic-acid-binding-immunoglobulin-like-lectin-2; CD22). Siglec-2 is a mammalian sialic acid binding protein expressed on B-cell surfaces, which negatively regulates the B-cell signaling pathway by associating with a sialylα(2–6)Gal unit on the other vicinal Siglec-2.[9,10] Siglec-2 is a 140 kDa transmembrane protein composed of seven extracellular immunoglobulin-like domains. The single V-set immunoglobulin domain located at the extracellular terminus contains the sialylglycan binding site, and six C2-set immunoglobulin domains connect the V-set domain and transmembrane domain. The cytosolic domain includes an immunoreceptor tyrosine-based inhibition motif (ITIM) for immune signaling. The sialylglycan ligand of Siglec-2 has been identified as a sialylα(2–6)Gal unit rather than a sialylα(2–3)Gal unit.[11] Recently, sulfated sialylLacNAc [sialylα(2–6)Galβ(1–4)-βGlcNAc(-6-*O*–SO_3H)–] was proposed as an endogenous ligand of Siglec-2 at the B-cell surface.[12] A distinguishable binding preference of the sialic acid species exists between human Siglec-2 (hSiglec-2) and mouse Siglec-2 (mSiglec-2). Namely, hSiglec-2 binds both *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), whereas mSiglec-2 binds Neu5Gc but not Neu5Ac.[11,13] The K_D values of Siglec-2 for sialylα(2-6)Gal on glycoproteins or polymers are reported to be 0.1–2.0 mM.[14,15]

Siglec-2 regulates B-cell functions depending on its ligand binding ability, and modulates the B-cell dependent immune response.[5] The inhibition of Siglec-2-endogenous ligand interactions therefore could potentially control B-cell function. Additionally, Siglec-2 is a drug delivery target for therapy in immune cell mediated disease.[16]

During an investigation to identify Siglec-2 binding partners, sialic acid derivatives having C-9-biphenylmethyl amide substitutions exhibited potent binding affinity to Siglec-2.[17] Furthermore, a dramatic increase in binding was exhibited by combined use of a C-9-biphenylmethyl amide substitution and an aromatic ring substitution at the reducing end (C-2),[18] which might suggest that the binding position of the sialic acid to the sugar residue is not crucial in terms of obtaining potent binding affinity.

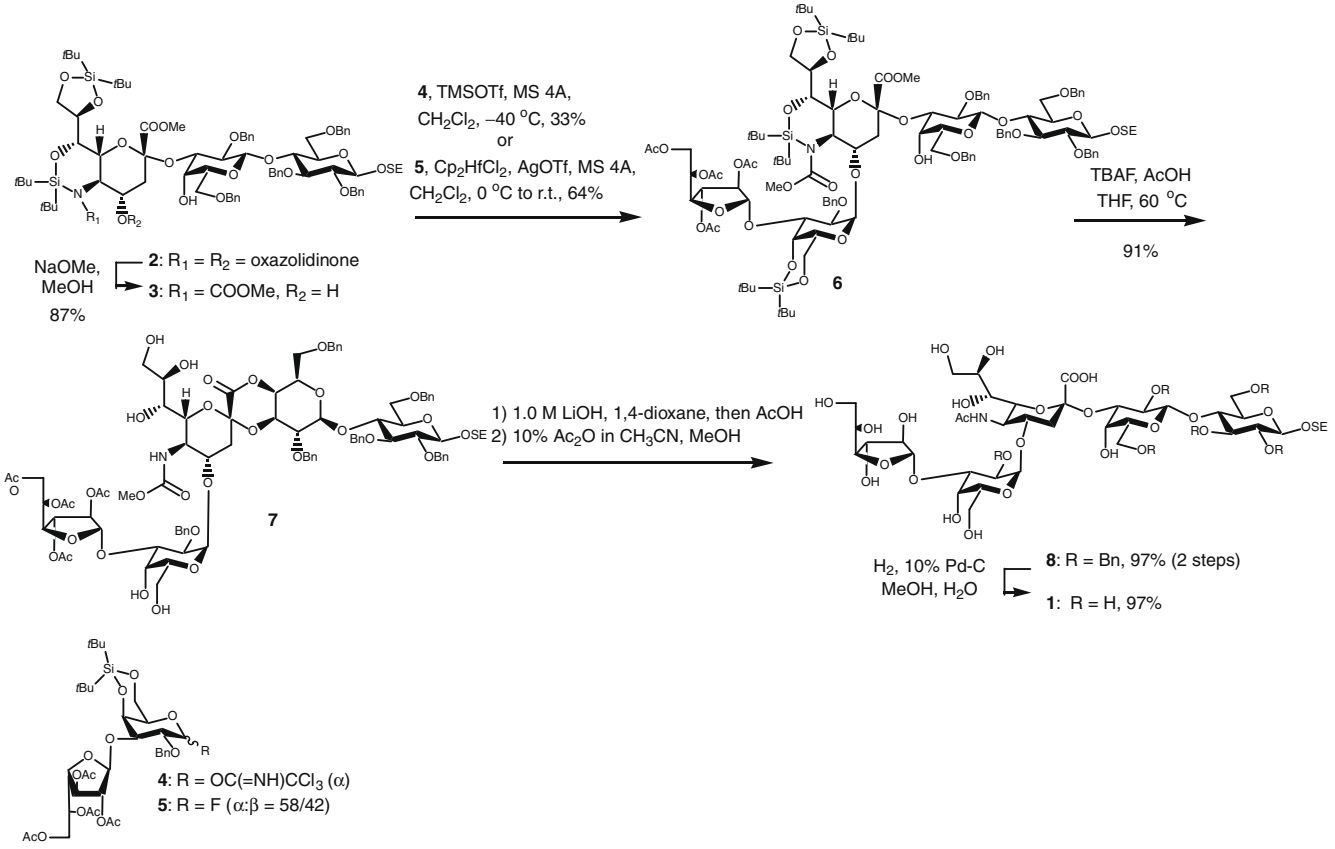
In this manuscript, we demonstrated that AG2 pentasaccharide (**1**; Fig. 1) bound to hSiglec-2 in spite of the C4-galactofuranosylgalactose modification on the sialic acid unit, which was confirmed by NMR binding studies. The binding epitope of AG2 **1** to hSiglec-2 was determined by STD-NMR experiments.

2. Results and discussion

2.1. Chemistry

First, we briefly describe about the synthesis of AG2 pentasaccharide **1** (Scheme 1).[19] The Neu5Acα(2–3)Galβ(1–4)Glcβ-*O*-SE trisaccharide unit **2**, which is a half part the desired AG2 pentasaccharide **1**, was synthesized as previously described.[20] Next, a single-step transformation from trisaccharide **2** to acceptor **3** by treatment with NaOMe was investigated and the desired trisaccharide acceptor **3** was provided upon careful optimization in 87% yield.[21]

To obtain fully protected AG2 pentasaccharide **6**, coupling reactions between trisaccharide acceptor **3** and disaccharide building blocks **4** or **5** were performed. Initially, we employed the highly reactive Schmidt type trichloroacetimidate **4**.[22] Although imidate



Scheme 1. Synthesis of the AG2 pentasaccharide **1**.

4 (2.0 equiv) was readily activated with a catalytic amount of TMSOTf at −40 °C, the desired pentasaccharide **6** was obtained in an unsatisfactory 33% yield. On the other hand, the best yield was obtained with the less reactive glycosylfluoride **5**. Upon activation of fluoride **5** with Cp_2HfCl_2 and AgOTf,[23] the desired and fully protected AG2 pentasaccharide **6** was readily obtained in 64% yield. The protecting groups on pentasaccharide **6** were simultaneously removed by treatment with TBAF and AcOH in THF at 60 °C to give lactone **7** in 91% yield. Next, the *N*-methoxycarbonyl group on **7** was hydrolyzed by 1.0 M aqueous LiOH with 1,4-dioxane at room temperature, then N-acetylation with Ac_2O in MeOH produced compound **8** in 97% yield (two steps). During treatment with base, the lactone moiety of **7** was simultaneously hydrolyzed to transform the carboxyl group at anomeric position of the sialic acid of **8**. The final removal of all benzyl protecting groups by hydrogenation with 10% Pd–C in aqueous media produced fully deprotected AG2 pentasaccharide **1** in 97% yield.

2.2. NMR binding study

First, we investigated whether synthetic AG2 pentasaccharide **1** having a central sialic acid residue binds to hSiglec-2 and mSiglec-2. hSiglec-2 and mSiglec-2 were prepared as chimeric proteins with the Fc fragment of human IgG1, which contains three exo-terminal Siglec-2 domains (see Section 4).[15]

In titration experiments monitored by ^{1}H NMR spectra, AG2 **1** was added into either hSiglec-2 or mSiglec-2 solutions in a stepwise manner (1–20 equiv), and ^{1}H NMR spectra were collected at each concentration (Supplementary Fig. S1). The ^{1}H NMR spectra of AG2 pentasaccharide **1** (a), 10 equiv of AG2 pentasaccharide **1** with mSiglec-2 (b), and 10 equiv of AG2 pentasaccharide **1** with hSiglec-2 (c) are shown in Figure 2. In the titration of AG2 **1** with mSiglec-2, the ligand signals exhibited no significant perturbations, and the signal pattern of the ligand **1** shown in Figure 2b was identical to the ^{1}H NMR pattern of AG2 alone (Fig. 2a). Based on this observation, no binding evidence between mSiglec-2 and AG2 pentasaccharide **1** was obtained. On the other hand, significant signal perturbation was observed in the presence of hSiglec-2. In the ^{1}H NMR spectrum, several ligand (**1**) signals became broader, but no significant chemical shift change was observed (Fig. 2c). The broadened ^{1}H NMR signals, indicated by arrows in Figure 2c, were assigned as H-1, H-2, and H-3 of α-Gal*p* (D), H-3 (equatorial) of Neu5Ac (C), and H-3 of β-Galp (B). The ^{1}H NMR spectrum clearly indicated the binding between AG2 pentasaccharide (**1**) and hSiglec-2. Furthermore, AG2 pentasaccharide (**1**) was bound specifically to the hSiglec domain of the Fc-chimeric protein and not to the stem domain because both hSiglec-2 and mSiglec-2 contain the same Fc stem-domains. The different binding property of AG2 to human and mouse Siglec-2 is considered to originate from the distinct preference of sialic acid species between hSiglec-2, which prefers both Neu5Ac and Neu5Gc, and mSiglec-2, which selectively binds to Neu5Gc.[11,13]

To obtain binding information of the ligand protons in contact with hSiglec-2, saturation transfer difference (STD) NMR experiments were performed.[24] To optimize the saturation transfer effect, STD-NMR experiments were carried out at 5 °C with 200 μM hSiglec-2 with various ligand concentrations. STD-amplification factors (STD-AF) were obtained for each ligand concentration,[24b,25] and the STD-AF of the acetamide proton on Neu5Ac (C) is shown in Figure 3. STD-AF increased with the addition of ligand **1**, and gradually approached the saturation point, at which the concentration of ligand **1** was estimated to be 12 mM. From the STD-titration curve, the K_D value of **1** to hSiglec-2 was estimated as 2 mM under the experimental conditions.[24b,25]

The STD-NMR spectrum of AG2 **1** (20 equiv) in the presence of hSiglec-2 is shown in Figure 4. The ^{1}H NMR spectrum is shown in Figure 4a, and the STD-NMR spectrum upon subtracting the on-resonance spectrum irradiated at 7.51 ppm from the off-resonance spectrum is shown in Figure 4b. To determine the binding epitope of AG2 **1** to hSiglec-2, the saturation transfer effect of STD-NMR was quantified by using the STD-AF of each proton signal, and the results are summarized in Figure 5.

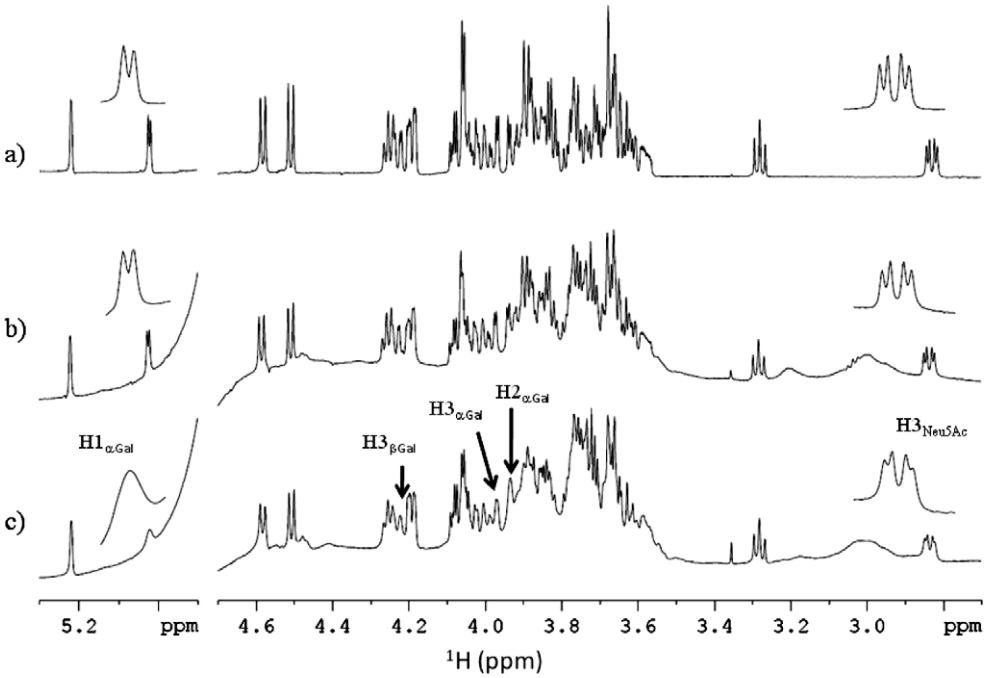


Figure 2. ^{1}H NMR spectra of AG2 pentasaccharide (**1**) in the presence of human and mouse Siglec-2. (a) AG2 alone; (b) in the presence of mSiglec-2, AG2/mouse Siglec-2 = 10:1; (c) in the presence of human Siglec-2, AG2/human Siglec-2 = 10:1. All ^{1}H NMR spectra were collected at 20 °C (probe temperature) using a 600 MHz spectrometer. Broadened signals in spectrum (c) are indicated by arrows.

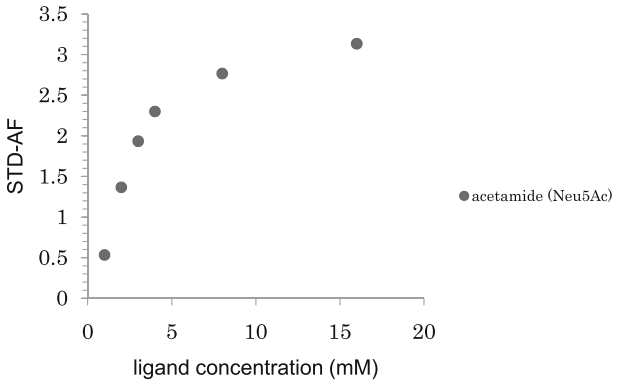


Figure 3. STD-titration data of acetamide proton of Neu5Ac residue of AG2 **1** using STD-amplification factor at each ligand concentration. STD-AF: $(I_0 - I_{sat})/I_0 \times$ ligand excess; I_0: signal intensity of off-resonance spectrum; I_{sat}: signal intensity of on-resonance spectrum.

The STD-NMR spectra showed that the acetamide singlet at 2.0 ppm and the side-chain (C7–C9: 3.7 and 3.9 ppm) of Neu5Ac exhibited relatively higher intensity (STD-AF > 2.3) than the other protons. STD-AF of the corresponding protons expressed significantly higher values (H-7: 3.4; H-8: 2.9; H-9: 2.9, 1.8; acetamide: 2.3). In contrast, the C-3 axial and equatorial protons of the Neu5Ac residue at 1.8 ppm and 2.8 ppm suffered a relatively weak saturation transfer effect even in the same sugar residue (ATD-AF: 1.8 and 2.0). These results indicate that hSiglec-2 preferentially binds to the flexible side-chain and acetamide moiety of the Neu5Ac residue. Previous computational modeling studies of hSiglec-2 with a sialic acid mimicking inhibitor suggested that two Trp residues (Trp2 and Trp106) interact with the side-chain and acetamide moiety of Neu5Ac.[18a] In terms of the Neu5Ac binding mode to hSiglec-2, our observation provided good agreement with the modeling results. Such interactions between aromatic amino acid and Neu5Ac side-chain/acetamide at sialic acid binding sites have also been reported in co-crystal structures of Siglec-5 (Tyr26 and Tyr133) and Siglec-7 (Tyr26 and Trp132) with corresponding sialylglycans.[26,27] From the STD-AF of the β-Gal (B) and α-Gal (D), both galactose residues also take part in the binding event. In the case of the β-Gal (B) residue, H-1 and H-3 directing axial expressed high AF (2.6 and 3.2). The evidence suggests the presence of amino acid residue(s) binding from the B-face of the Gal residue. Furthermore, H-2 and H-3 of the α-Gal residue (D) exhibited a potent AF (2.3 and 2.4), and H-5 also indicated relatively high AF (2.0).

On the other hand, weak saturation transfer effects were observed at H-1, H-2, and H-5 of the Glc residue (A) at the reducing end in addition to the galactofuranose (Gal*f*) residue (E) at the non-reducing terminus. The observed STD-AF of Glc (A) and Gal*f* (E), except for H-6 of Glc, were between 0.9 and 1.6. Furthermore, drastic diminishment of the STD signals was observed with the aglycon trimethylsilylethyl (SE) group at 1.0 and 1.1 ppm (STD-AF; 0.6 and 1.1) and 0.1 ppm (STD-AF; 0.2). These results indicated that the aglycon moiety is far from the binding pocket, and sugar residues at both termini make little contribution to the binding of hSiglec-2.

3. Conclusion

The pentasaccharide moiety of AG2 **1** was synthesized using novel sialic acid building blocks. Upon coupling reactions with trisaccharide **3**, fluoride **5** performed the best for producing the desired fully protected pentasaccharide **6**. Finally, synthesis of AG2 pentasaccharide **1** was achieved after global deprotection involving desilylation, introduction of an acetamide group, and total removal of acyl and benzyl protecting groups. We found that AG2 pentasaccharide **1** bound to human Siglec-2 by performing ^{1}H NMR and STD-NMR experiments. STD-NMR disclosed the binding epitope of hSiglec-2 to AG2 pentasaccharide **1**, which was a Galα(1–4)Neu5Acα(2–3)Gal unit. STD-NMR also indicated that hSiglec-2

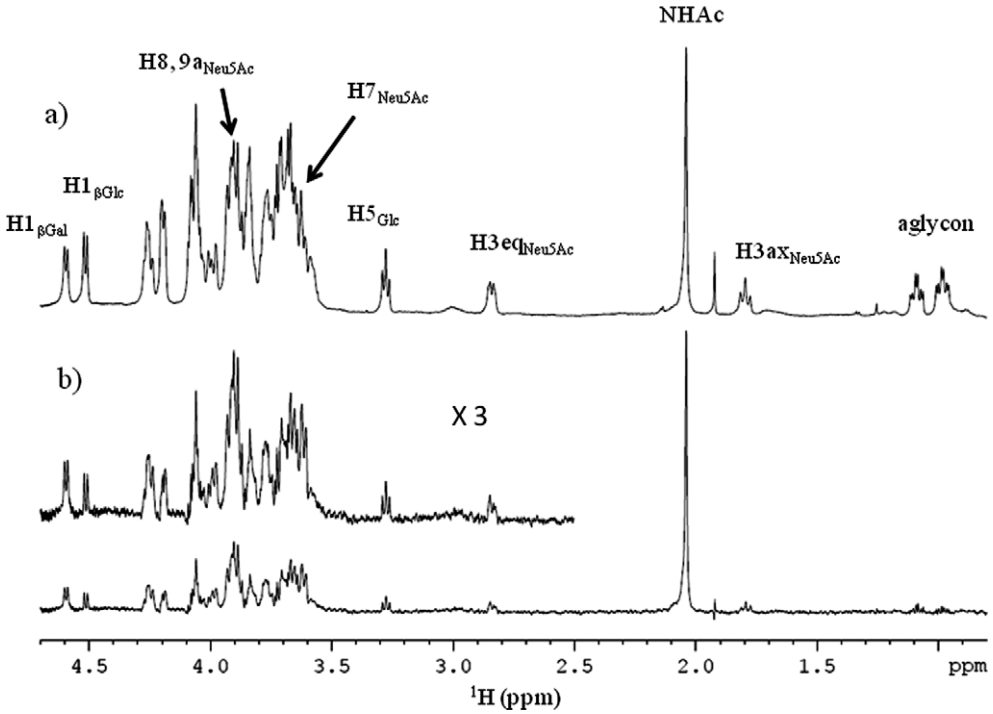


Figure 4. ^{1}H NMR spectrum of AG2 pentasaccharide **1** (a) and STD-NMR spectrum of AG2 pentasaccharide **1** with hSiglec-2 (b). Molar ratio was 20 (AG2) to 1 (hSiglec-2) in 10 mM sodium phosphate with 150 mM NaCl (final volume: 250 μL; pH 7.3 at 20 °C). Both ^{1}H NMR and STD-NMR spectra were measured at 5 °C. In STD-NMR, on-resonance irradiation of the protein was performed at a chemical shift of 7.51 ppm, and off-resonance irradiation was applied at 40 ppm.

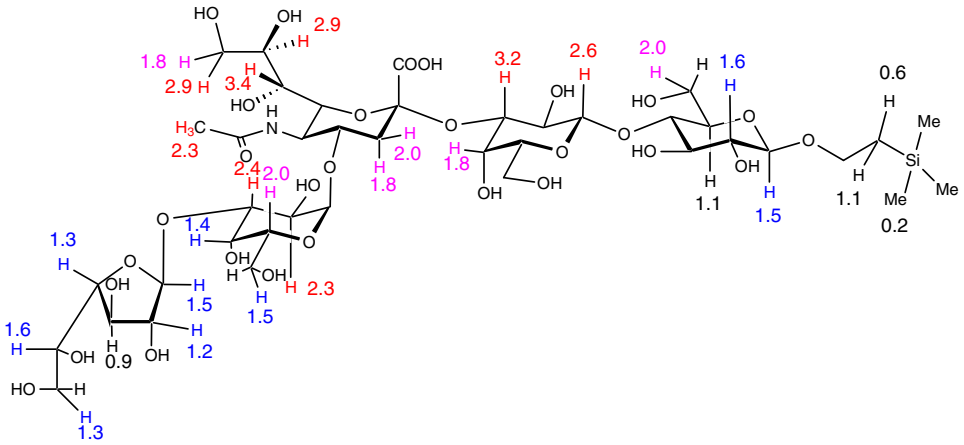


Figure 5. Binding epitope of AG2 pentasaccharide **1** to hSiglec-2 determined by STD-NMR. STD-amplification factor of each proton is shown in red (over 2.3), purple (1.8–2.2), blue (1.2–1.7), and black (under 1.2).

bound the C7-C9 side-chain and acetamide moiety of the central Neu5Ac residue. This information concerning the binding epitope of AG2 pentasaccharide **1** is of value toward the development of potent Siglec-2 inhibitors.

4. Experimental procedures

4.1. Expression of human and mouse Siglec-2

The chimeric protein containing N-terminal three domains of murine or human CD22 and the Fc fragment of human IgG1 (mSiglec-2-Fc and hSiglec-2-Fc, respectively) were prepared as reported.[15] Briefly, the chimeric proteins were produced in stably transfected Lec2 cells, a cell line deficient in protein sialylation. Siglec-Fc proteins were purified from the culture supernatant using Affi-Gel protein A columns (BioRad).

4.2. NMR experiments

In ^{1}H NMR titration experiments and STD-NMR experiments for Siglec-2 binding study, the spectra were recorded with Bruker AVANCE-600 spectrometer (600 MHz for ^{1}H resonance frequency). Chemical shifts of ^{1}H NMR were corrected with sodium 3-trimethylsilyl-1-propanesulfonate (DSS) as a standard signal at 0 ppm. Siglec-2 was dissolved in 10 mM sodium phosphate and 150 mM NaCl (pH 7.3) with 100% D_2O solution to be 20 μM (^{1}H NMR) or 200 μM (STD-NMR). ^{1}H NMR titration experiments were performed at 20 °C, and AG2 in same buffer system was added to the protein solution from 1 to 20 equiv. STD-NMR spectra were collected at 5 °C, and titrations were performed from equal amount to 80 times excess amount of AG2 (**1**) against human Siglec-2. On-resonance irradiation of the protein was performed at a chemical shift of 7.51 ppm, and off-resonance irradiation was applied at 40 ppm. The total number of scans was 512 in each with 16 dummy scans.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.062.

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