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Consistent Bioactive Conformation of the Neu5Acα(2→3)Gal Epitope Upon Lectin Binding

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Dedicated to Prof. Hans Paulsen on the occasion of his 86th birthday.

The injured adult mammalian central nervous system has no capacity for axon regeneration,[1] predominantly due to specific inhibitors expressed on residual myelin and on astrocytes recruited to the injury site.[2–6] Several of these inhibitory proteins have been identified, including the myelin-associated glycoprotein (MAG).[7,8] MAG is a transmembrane glycoprotein[9] that belongs to a family of sialic acid-binding immunoglobulin-like lectins, the so-called siglecs.[10] There are two classes of well-defined axonal targets of MAG on the surface of neurons: sialylated glycans, specifically the gangliosides GD1a and GT1b,[10–13] and proteins of the NgR family.[14,15] Although the relative roles of gangliosides and NgRs as MAG ligands have yet to be resolved, in some systems MAG inhibition is completely reversed by sialidase treatment, suggesting that MAG uses sialylated glycans as its major axonal ligands.[16] Therefore, potent glycan inhibitors of MAG may be a valuable therapeutic approach to enhance axon regeneration.

The native carbohydrate ligand with the highest affinity to MAG is the ganglioside GQ1bα.[17] As a starting point for our search for MAG antagonists, data about the minimal binding epitope of **1** and its bioactive conformation are required, since the most abundant solution conformation does not necessarily represent the bound conformation. Recently, the MAG-affinity of partial structures of GQ1bα (**1**), namely derivatives of tetrasaccharide **2** and trisaccharide **5**, was clearly correlated with their ability to reverse MAG-mediated inhibition of axon outgrowth (Scheme 1).[18,19] Both saccharides **2** and **5** contain a flexible α(2→3)-glycosidic linkage between the sialic acid (Neu5Ac) and the central galactose (Gal) residue, and it is unknown which of the solution conformations is recognized by the receptor protein. The objective of this study is to analyze the conformation of these α(2→3)-glycosidic linkages when bound to MAG. It is known that conformational preorganization of ligands may significantly improve binding affinities, and this information is crucial for the design of potent antagonists. A prominent example is conformationally preorganized E-selectin antagonists based on the bioactive conformation of sialyl Lewis^x.[20,21]

In general, carbohydrate–protein interactions are characterized by exchange reactions that are fast on the NMR chemical shift and relaxation timescales. Therefore, transferred NOE (trNOE) experiments are ideally suited for the analysis of bioactive conformations of protein-bound carbohydrates.[22] Flexible glycosidic linkages have drawn special attention since different bound conformations are possible. Here, we analyze the bioactive conformations of Neu5Acα(2→3)Galβ(1→3)[Neu5Ac α(2→6)]Gal*N*Ac (**2**), Neu5Acα(2→3)Galβ(1→3)[Neu5Acα(2→6)]Gal (**3**), Neu5Acα(2→3)Galβ(1→3)Gal*N*Ac (**5**)[23] and Galβ(1→3)[Neu5Acα(2→6)]Gal (**6**),[23] as well as the tetrasaccharide mimic **4** when bound to MAG (Scheme 1; for the syntheses of **2**, **3** and **4** see the Supporting Information). This study complements an accompanying paper that describes the analysis of the binding epitopes of trisaccharide **5** and tetrasaccharide **2** when bound to MAG using saturation transfer difference (STD) NMR.[24]

From the change in the pattern of specific interglycosidic NOEs between tetrasaccharide **2** free in solution (Figures 1 A and C) and in the bound form (Figures 1 B and D, namely the disappearance of the NOEs H3′′–H3ax′′′ and H3′′–H3eq′′′), it is concluded that the terminal α(2→3)-linked Neu5Ac residue is bound in a minus-gauche orientation, as it has been observed before for sialyl Lewis^x binding to E-selectin.[25–27] The thorough quantitative analysis of the bioactive conformations of the structurally related ganglioside derivatives **2** to **5** (see the Supporting Information) reveals that the "sialyl Lewis^x-type binding mode" is a common theme among the carbohydrate-protein interactions studied (Table 1, see also Table S7). Interestingly, binding of trisaccharide **6** was too weak to give sizeable trNOEs (Supporting Information), supporting the assertion that an α(2→3)-linked Neu5Ac residue is absolutely required for MAG binding.[28,29] Based on a full relaxation and exchange matrix analysis of the trNOE data employing the program CORCEMA,[30,31] we deduced a docking model (Supporting Information) for the interaction of the ganglioside derivatives with MAG.

A complete set of NOESY spectra of oligosaccharides **2** to **5** in the absence and presence of MAG is found in the Supporting Information (Figures S1 and S2). As an example, Figure 1

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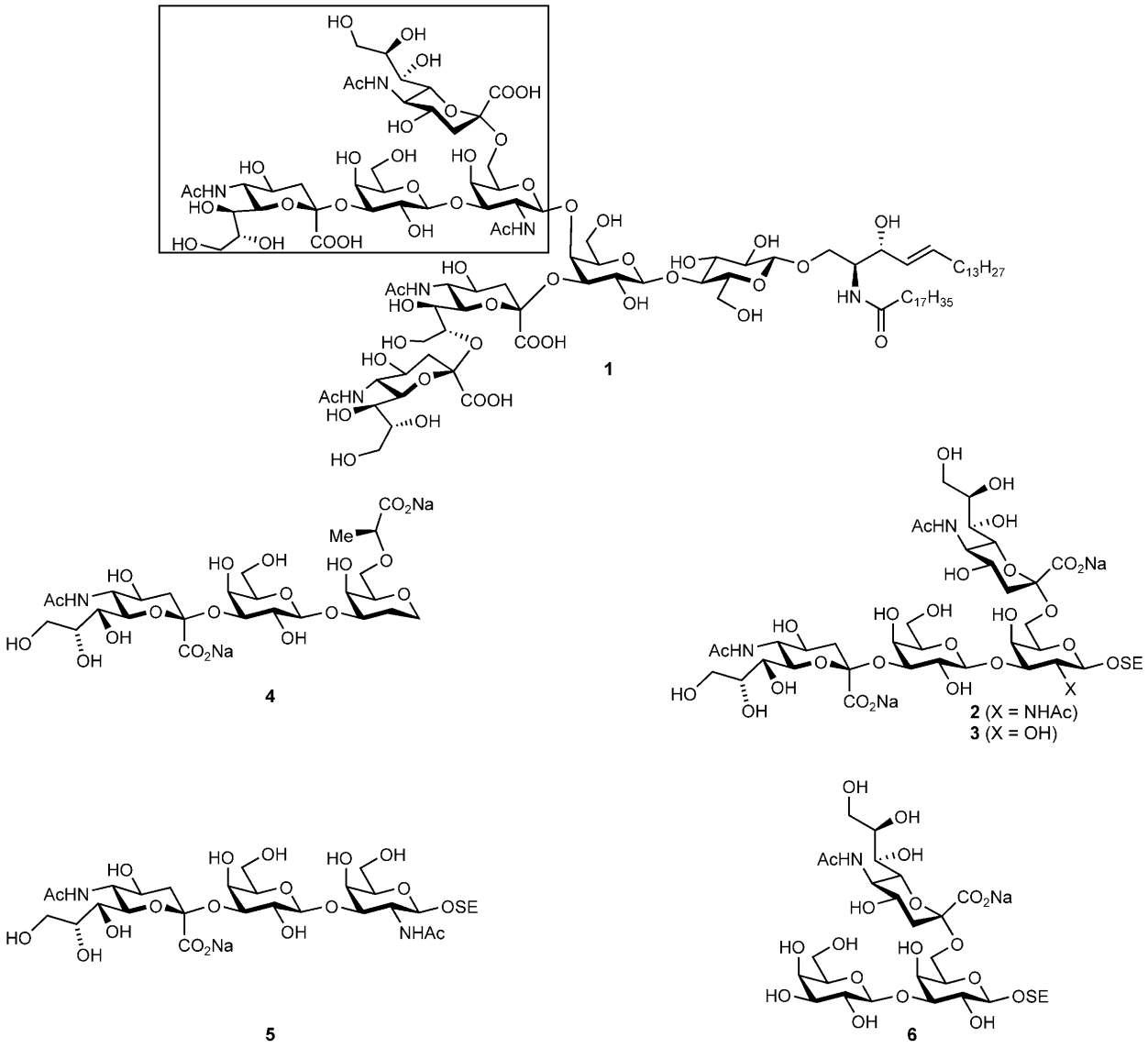
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: inter alia: full relaxation matrix calculations and docking procedures.



Scheme 1. Structures of the ganglioside GQ1bα (**1**) and the oligosaccharides **2**–**6** (OSE = 2-trimethylsilylethoxy).

displays NOESY spectra of tetrasaccharide **2**. For all saccharides except pseudo-tetrasaccharide **4**, the acquisition of build-up curves was required in order to quantify trNOEs. For **4**, in the absence of MAG, NOEs were close to zero and a distinction between NOEs and trNOEs was in this case straightforward. Details of the NOE experiments can be found in the Supporting Information.

The trNOE patterns in the presence of MAG were clearly different from the NOE patterns observed for the free saccharides as highlighted for tetrasaccharide **2** in Figure 1. In comparison to the corresponding NOEs, the trNOEs between H3 of Gal and $H3_{ax}$ of Neu5Ac at the α(2→3)-glycosidic linkage were considerably weakened in the presence of MAG for all saccharides investigated. At the same time, the trNOEs between H3 of Gal and H8 of Neu5Ac gained intensity in all cases as compared to the corresponding NOE. This pattern is only compatible with the so-called "*syn*" conformation at the α(2→3)-glycosidic linkage (Table 1 and Figure 2). The relative sizes of NOEs across the Galβ(1→3)-glycosidic linkages were almost identical in the absence and in the presence of MAG, indicating that no significant conformational changes occur around these linkages upon binding.

For a quantitative analysis of the trNOEs, a homology model of MAG was constructed based on the crystal structure of the N-terminal V-set domain of sialoadhesin[32] (Figure S4). Ligands **2** to **5** were docked to this homology model with the program AutoDock 3.0,[33] and the docking models were further energy minimized with Sybyl.[34] For the docking experiments, ligands were assumed to be rigid using qualitative restraints from interglycosidic trNOEs (Figure 1).

For the α(2→6)-linkage, manual docking of the tetrasaccharides with Sybyl yielded a gt-orientation for the ω dihedral angle around the C5–C6 bond of the reducing galactose moiety. The dihedral angles at the α(2→3)-glycosidic linkages are shown in Table 1. The complete set of glycosidic angles of all docked ligands can be found in the Supporting Information

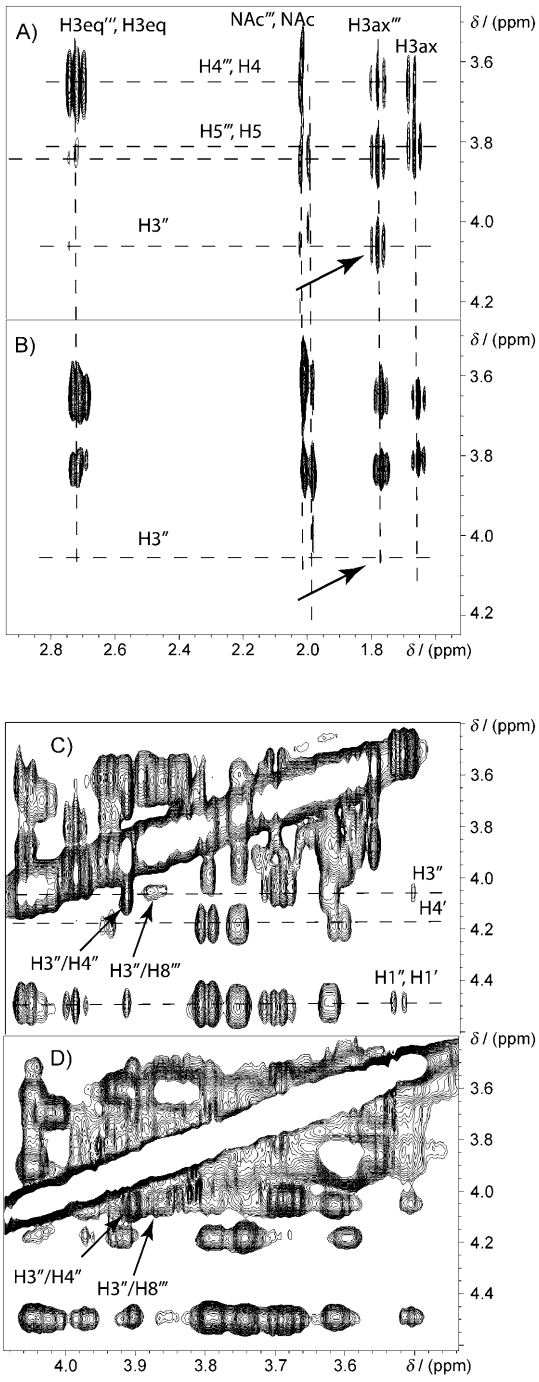


Figure 1. Portions of NOESY spectra of tetrasaccharide **2** in the absence (see A and C); 700 MHz, 288 K, mixing time 500 ms) and in the presence of MAG (see B and D); 700 MHz, 288 K, mixing time 200 ms). NOEs and trNOEs are negative, and therefore build-up curves have been obtained for a more detailed analysis (Supporting Information). The spectra show that there are important changes between the trNOE and the NOE pattern. Most importantly, the NOE between H3″ and H3ax‴ (see A, arrow) is almost extinct in the presence of MAG (see B, arrow). In contrast, the NOE between H3″ and H8‴ (C) is more pronounced in the presence of MAG (D). This "pattern" of interglycosidic transfer NOEs (see B and D) reveals the so called minus-gauche conformation at the α(2→3)-glycosidic linkage of **2** as the bioactive conformation. The same effects have been observed for the other ligands investigated (Supporting Information). ′ refers to the GalNAc residue, ″ to Gal, and ‴ to α(2→3)-Neu5Ac. For a comparison of the trNOE and NOE build-up curves of the critical effects shown in this Figure see Figure S3 in the Supplemental Information.

Table 1. ϕ/ψ values of the α(2→3) Neu5Ac-Gal motif in saccharides **2** to **5** as compared to the same motif as part of sLe^x bound to E-selectin.

Ligand	ϕ (C1‴-C2‴-O3″-C3″)	ψ (C2‴-O3″-C3″-H3″)
2, 3	−58°	−25°
4	−59°	−26°
5	−64°	−17°
sLe^x [25–27]	−43° to −76°	+6° to −22°

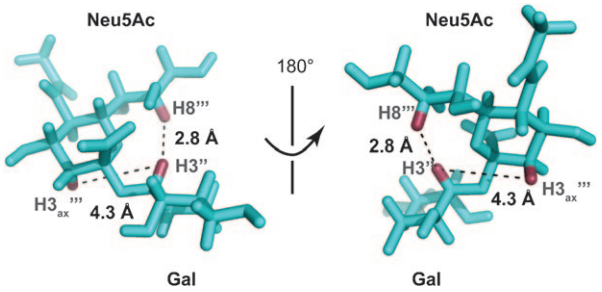


Figure 2. Bioactive conformation of the Neu5Acα(2→3)Gal moiety of tetrasaccharide **2** bound to MAG as determined by trNOE experiments. The orientation of this linkage in the bound form is very similar for the saccharides **2** to **5** investigated here (see Table 1). Upon binding to MAG, the trNOE between H3″ and H3$_{ax}$‴ is considerably attenuated indicating a rather large distance between the two protons (4.3 Å). At the same time, the distance between H3″ and H8‴ becomes 2.8 Å, which is consistent with a significant trNOE. In solution, this conformation is in equilibrium with a different conformational family in which the distance between H3″ and H3$_{ax}$‴ is rather short and leads to a large NOE as shown in Figure 2A. The image was produced with the program PyMOL (http://www.delanoscientific.com).

(Table S3). Based on the docking results, it is predicted that the carboxyl group of the α(2→3)-linked Neu5Ac residue forms a salt bridge with Arg118, and that the carboxyl group of the α(2→6)-linked Neu5Ac is in contact with the amino group of Lys67 (Figure 3).

A full relaxation matrix analysis of the trNOE build up curves was then performed for saccharides **3**, **4** and **5** employing the program CORCEMA (Supporting Information). Due to severe overlap of the anomeric protons of Gal (H1″) and Gal*N*Ac (H1′), tetrasaccharide **3** rather than **2** was employed for a detailed quantitative analysis of trNOEs. The calculated trNOEs were in very good agreement with the experimental values. For the α(2→6)-linkage, experimental trNOEs are rather sparse and cannot "prove" the proposed bound conformation. On the other hand, site-directed mutagenesis of Lys67 (K67A) leads to a significant drop in binding activity for tetrasaccharide **2**, but not for trisaccharide **5**, which further indicates the significance of the proposed salt bridge and substantiates our model (unpublished results, for further details see the Supporting Information).

It is interesting to note that using K_D values obtained from the STD NMR titrations as described in the accompanying paper[24] yields k_{on} and k_{off} rate constants through CORCEMA calculations (Table S4) that correspond very well with surface plasmon resonance data obtained for the binding of sialyl Lewis^a to a monoclonal antibody.[35] In the present study k_{on} and k_{off} values were obtained by fitting experimental and cal-

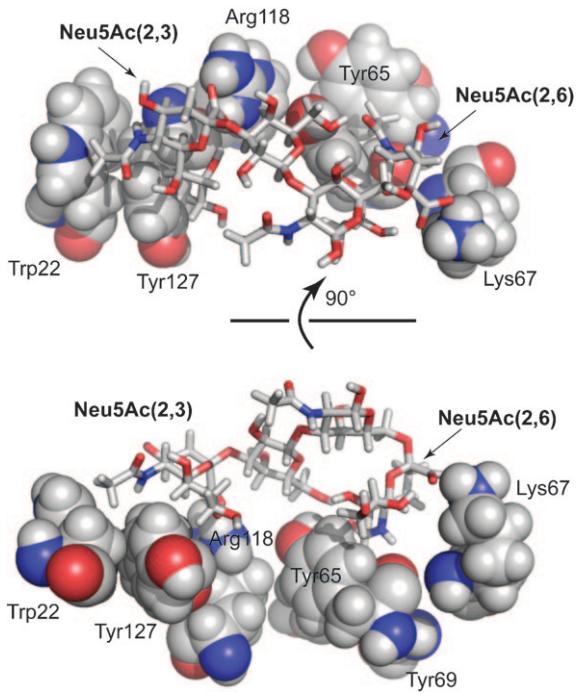


Figure 3. NOE-based docking model of tetrasaccharide **2** in the binding site of MAG. Only selected amino acids of the binding pocket are shown. The α-(2→3)-glycosidic linkage prefers a "*syn*" orientation. It is seen that the carboxy groups of the α(2→6)-linked and the α(2→3)-linked Neu5Ac residues are close to the side chains of the amino acids Lys67 and Arg118, respectively, allowing for corresponding salt bridges. The image was produced with the program PyMOL (http://www.delanoscientific.com).

culated trNOE curves. The data suggest that association of the saccharides and MAG is significantly slower than would be expected for a diffusion-controlled process. It is an open question whether this is due to conformational changes of MAG, the ligand, or both during the binding process.

To summarize, our study shows that the Neu5Acα(2→3)Gal moiety, present in compounds **2** to **5**, binds to MAG in a "sialyl Lewis^x-type binding mode." So far, from the data available it appears that this is the preferred binding conformation for the Neu5Acα(2→3)Gal disaccharide moiety, independent of the glycosylation pattern present at the reducing end (Table S7). As a representative example, Figure 3 shows the docking model of tetrasaccharide **2** bound to MAG. For the future design of conformationally preorganized and thus more potent MAG antagonists, the knowledge of the bioactive conformation of the Neu5Acα(2→3)Gal moiety will be extremely valuable.

Experimental Section

Synthesis: The synthesis of the compounds **2**–**4** is described in the Supporting Information. The synthesis of compounds **5** and **6** has been published.[23]

NMR sample preparations for free ligands: NMR samples were prepared in deuterated phosphate buffer (600 μL, 99.98% D) containing sodium phosphate (10 mM), NaCl (150 mM), and NaN_3 (0.1%) at pH 7.4.

NMR sample preparation in the presence of MAG: MAG concentrations were determined using UV absorbance at 280 nm ($\varepsilon = 1.44\ M^{-1}\,cm^{-1}$). The exchangeable protons of the protein were exchanged into a deuterated phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) by repeated washing using a microconcentrator with a 10 kDa molecular weight exclusion limit (Sartorius, Göttingen, Germany). Protein concentrations in the NMR samples ranged between 30 and 60 μM. The concentrations of trisaccharide **5**, tetrasaccharide **2**, tetrasaccharide **3**, pseudo-tetrasaccharide **4**, and trisaccharide **6** were 540, 800, 800, 814 and 840 μM, respectively.

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Keywords: bioactive conformation · docking · inhibitors · myelin-associated glycoprotein · NMR spectroscopy

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