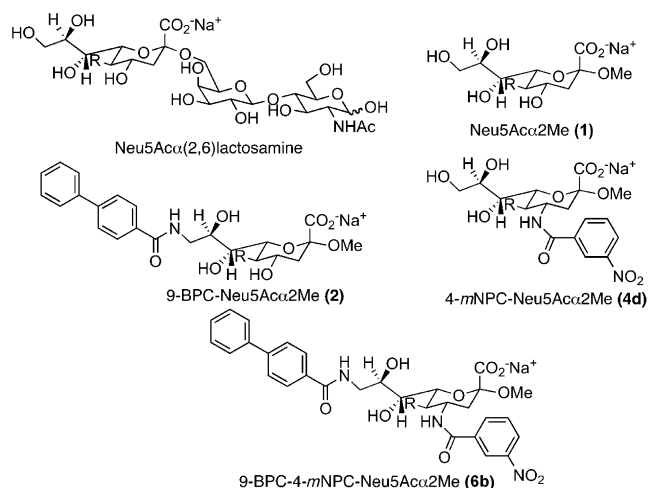


C-4 Modified Sialosides Enhance Binding to Siglec-2 (CD22): Towards Potent Siglec Inhibitors for Immunoglycotherapy**

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The regulatory functions of Siglecs (sialic acid binding immunoglobulin-like lectins) in the immune system provide opportunities for innovative therapeutic strategies for a wide range of immunological disorders or cancer (immunoglycotherapy).^[1] Siglec-2 (CD22), as a consequence of its pivotal role in B cell activation, has become an attractive target for therapies of autoimmune diseases and B cell-derived non-Hodgkin's lymphoma (NHL). NHL is among the ten most common cancers with over 20000 deaths in 2010 for the US alone.^[2] Siglec-2 binds with high preference to $\alpha(2,6)$ -linked sialic acids (Sia),^[3] such as Neu5Ac $\alpha(2,6)$ lactosamine (Scheme 1). Neu5Ac α 2Me (**1**) interacts with Siglec-2 mainly through 1) the negative charge on its carboxylate group, 2) the C-5 *N*-acetamido substituent, and 3) the glycerol side chain. Furthermore, replacement of the C-9 hydroxy group by an amino group did not interfere with binding to Siglecs.^[3] Crystallographic studies on Siglec-1 (sialoadhesin, Sn)^[4] demonstrated that acylation of this amino group enhances the overall affinity of the ligand for Siglecs by two to three orders of magnitude.^[5] The first breakthrough in the development of potent Siglec-2 inhibitors was the design of 9-biphenylcarboxamido Neu5Ac α 2Me (9-BPC-Neu5Ac α 2Me, **2**) which has a more than two orders of magnitude higher affinity to Siglec-2 than **1**,^[5d] and **2** has demonstrated potential to modulate signal transduction in B cells. Furthermore, based on **2**, compounds were developed, which kill B cell lymphoma cells.^[6] Structural studies^[4a,b] and modifications of the C-5 *N*-acyl substituent and the C-2 aglycon moiety of *N*-



Scheme 1. *N*-acetylneuraminic acid derivatives (R = NHAc).

acetylneuraminic acid (Neu5Ac) have led to further improvement in affinity.^[5a-c,f]

Herein we report, for the first time the design, synthesis, and evaluation of a novel class of disubstituted Neu5Ac derivatives that is modified at the C-4 and C-9 positions of **1**. Our structure-based design approach resulted in a promising novel lead compound 9-biphenylcarboxamido 9-biphenylcarboxamido-4-*m*-nitrophenylcarboxamido-4,9-dideoxy Neu5Ac α 2Me (9-BPC-4-*m*NPC-Neu5Ac α 2Me, **6b**) that has sub-micromolar affinity for Siglec-2 and may provide a pathway for immunoglycotherapy strategies.

An evaluation of our homology model (see Supporting Information) for Siglec-2 and other Siglecs led us to hypothesize that substituents at C-4 may provide additional interactions. To address this hypothesis we posed the following questions:

- 1) Can C-4 substituents enhance the interaction with Siglecs?
- 2) Do they interact specifically with the protein?
- 3) Do C-4 and C-9 modifications act synergistically?
- 4) Do the C-4 modified Neu5Ac derivatives bind to the same binding site as other Sia, such as **1**?

To see if C-4-modified derivatives of Neu5Ac α 2Me (**1**) enhance the interaction with Siglec-2 and other siglecs, novel C-4 functionalized compounds of **1** (**4b–4g**) were prepared. Thus, per-*O*-acetylated 4-amino-4-deoxy-Neu5Ac α 2Me (**3**) was readily synthesized using a literature method,^[7] and subsequent treatment of **3** with acyl chlorides or sulfonyl chloride followed by deprotection provided **4b–4g** (Table 1, Supporting Information).

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[**] We gratefully acknowledge the financial support of the Volkswagenstiftung (S.K.), Deutsche Forschungsgemeinschaft (S.K.), Tönjes-Vagt-Stiftung (S.K.), and the Australian Research Council (T.H., M.v.I.). S.K. is thankful for the Sir Allan Sewell Fellowship awarded by Griffith University. We thank Mary Murphy (Reichert Inc, Depew, NY (USA)) for the SPR analysis.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201207267>.

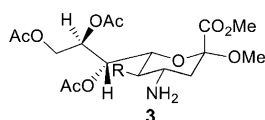


Table 1: Inhibition of Siglec-4 (MAG), Siglec-2 (CD22), and Siglec-9 by C-4-modified Sia.

		Siglec-2 (CD22)	Siglec-4 (MAG)	Siglec-9
	R = NHAc R'	rIC ₅₀ ^[a]		IC ₅₀ [mM] ^[b]
1	OH	1.0	1.0	n.i. ^[c]
4a	NH ₂	0.6	1.7	n.i. ^[c]
4b		2.2	0.2	n.i. ^[c]
4c		1.7	0.6	n.i. ^[c]
4d		15	0.4	6.5
4e		10	0.5	n.i. ^[d]
4f		0.5	n.i. ^[d]	n.i. ^[d]
4g		0.7	n.i. ^[d]	n.i. ^[d]

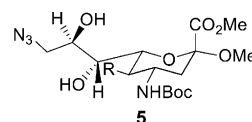
[a] rIC₅₀ values were calculated with **1** (IC₅₀ 2.0 mM) as reference compound from IC₅₀ values determined in at least three titrations, standard deviations were within 15%. [b] rIC₅₀ values could not be calculated, since **1** was not inhibitory at up to 20 mM. [c] Not inhibitory at 20 mM. [d] Not inhibitory at 2 mM.

Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein, MAG), and Siglec-9, were chosen to evaluate these compounds as potential inhibitors in hapten inhibition assays using immobilized fetuin as the target glycoprotein (Table 1). Compounds **1** and **4a**^[7c] that contain a hydroxy or an amino group, respectively, at the C-4 position bind only weakly to all three Siglecs (IC₅₀ ≈ 20 mM or more).

An *N*-acetamido moiety at C-4 (compound **4b**) led to an increase in affinity for Siglec-2 but did not improve the affinity for Siglec-9 and even decreases binding to Siglec-4. The largest increase in binding affinity for Siglec-2 was observed when a *meta*-nitrophenylcarboxamido moiety (*mNPC*) was installed to give **4d** (IC₅₀ = 0.13 mM; rIC₅₀ = 15). This result is in contrast to the moderate affinity for Siglec-9 (IC₅₀ = 6.5 mM) or Siglec-4 (IC₅₀ = 6.3 mM; rIC₅₀ = 0.4). In addition the results show that compounds **4d** and **4e** are specific inhibitors of Siglec-2. The presence of an aromatic residue (**4c**, **4f**, or **4g**) was not sufficient to enhance the

binding to Siglec-2, suggesting that the C-4 substituents in **4d** and **4e** interact specifically with amino acids positioned adjacent to the Sia binding site. The striking differences in inhibition potencies prompted us to evaluate the interaction of **4d** with Siglec-2, Siglec-4, and Siglec-9 by saturation transfer difference (STD) NMR spectroscopy (Figure 1).^[8] STD NMR signals of **4d** were normalized to the methyl protons of the *N*-acetamido group. The relative signal intensity for *Ho* proton of the *mNPC* moiety is almost double in Siglec-9:**4d** compared to that in Siglec-2:**4d** and three times that in the Siglec-4:**4d** complex. These results are in full agreement with the hypothesis that the C-4 *mNPC* functionality in **4d** significantly contributes to the compound's inhibitory potential observed in the inhibition assays. The protons of the O-methyl group of **4d** in Siglec-2:**4d** receives 80% saturation, whereas the same protons receive only 36% and 16% in Siglec-4:**4d** and Siglec-9:**4d**, respectively.

A number of bifunctionalized Neu5Acα2Me (**1**) derivatives with modifications at C-4 and C-9 were synthesized to investigate whether such modifications act synergistically. Initial protection of the amino group on C-4 of **3** with butoxycarbonyl (Boc) was undertaken to minimize the number of reaction steps. Thus, introduction of a C-9 azido group provided **5**,^[9] which, after reduction to the C-9 amino



precursor, enabled easy installation of a BPC group. Removal of the C-4 Boc protecting group enabled coupling of substituents to the free amine, providing the C-4/C-9 modified compounds (**6a–d**; Supporting Information).

Table 2 outlines the inhibition potencies of compounds **6a–d** in hapten inhibition assays showing that modifications at C-4 significantly alter binding to Siglec-2. A naphthylamido substituent (**6a**) enhances the binding to Siglec-2 approximately fourfold compared to **2** but the dibenzylamino derivative (**6d**) decreases the binding affinity to Siglec-2 by a factor of 13. The most significant increase in binding affinity was observed for 4-*mNPC* (**6b**; rIC₅₀ = 14) or 4-toluenesulfonylamido (**6c**; rIC₅₀ = 20) derivatives. This is in excellent agreement with the rIC₅₀ values determined for the Sia derivatives without the C-9 BPC moiety (Table 1) that show the *mNPC* moiety at C-4 (**4d**) increases the binding affinity by a factor of 15. Most striking is the synergistic effect of the C-4 and C-9 modifications resulting in an inhibition 9100 times stronger for **6b** compared to **1**.

Absolute binding affinities were also determined using surface plasmon resonance (SPR) measurements. Dissociation constants (*K_D*) were obtained for the key compounds **1** (*K_D* = 31 mM), **2** (*K_D* = 7 μM), and **6b** (*K_D* = 660 nM; see Supporting Information). STD NMR competition experiments were undertaken to explore whether C-4 and/or C-9 Sia derivatives bind to the same Siglec-2 Sia binding site as **1** and **2**. Compound **4d** was added incrementally to a mixture of

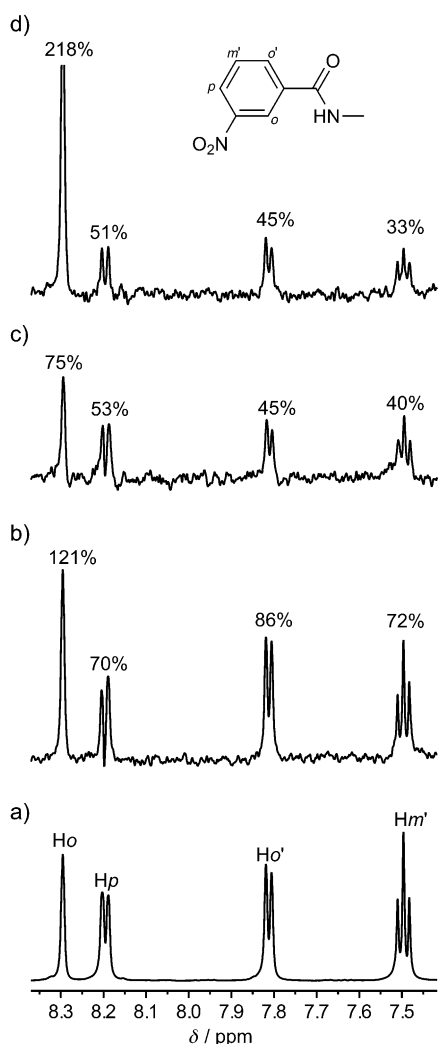


Figure 1. a) ^1H NMR spectrum of **4d** and STD NMR spectra of 500 μM **4d** in complex with b) 5 μM Siglec-2, c) Siglec-4, and d) Siglec-9.

5 μM Siglec-2 and 500 μM **1** (Figure 2a). STD NMR signal intensities for the methyl protons of the *N*-acetamido group of **1** reveal that a concentration of 200 μM of **4d** was sufficient to reduce the STD NMR signals of **1** by about 50%. At an equimolar ligand concentration, the STD NMR effects of **1** almost disappeared (Figure 2a). This result is in good agreement with a rIC_{50} value for **4d** of 15 times that of **1** (Table 1).

A second competition experiment was carried out using 5 μM of Siglec-2 and 500 μM of **2**, with a gradual increase in the concentration of **6b**. A very low **6b** concentration (5 μM) was sufficient to reduce the STD NMR signal of **2** by over 50% (Figure 2b). At a concentration of 10 μM of **6b** the STD NMR effects of **2** were decreased that they could hardly be detected, clearly demonstrating the very high affinity of **6b** for Siglec-2. This affinity of **6b** for Siglec-2 became even more clear in a competition experiment with **1** (data not shown). No binding of **1** (500 μM) to Siglec-2 was detected at a very low concentration (5 μM) of **6b**, suggesting that at a concentration

Table 2: rIC_{50} values of C-4/C-9-modified Sia for Siglec-2 (CD22).

Compound	R'	$\text{rIC}_{50}^{[a]}$	
		2	1
2	OH	1.0	667
6a		3.8	2500
6b		14	9100
6c		20	13 000
6d		0.1	50

[a] rIC_{50} values were calculated with **2** ($\text{IC}_{50} = 3.0 \mu\text{M}$) or **1** ($\text{IC}_{50} = 2.0 \text{ mM}$) as the reference compound from IC_{50} values determined in at least three titrations, standard deviations were within 15%.

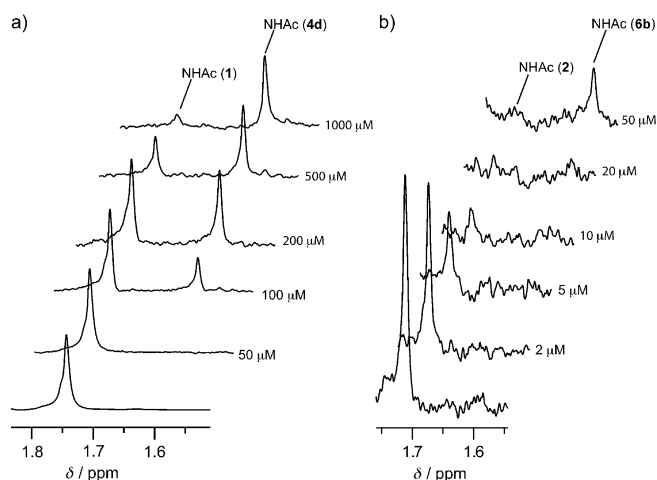
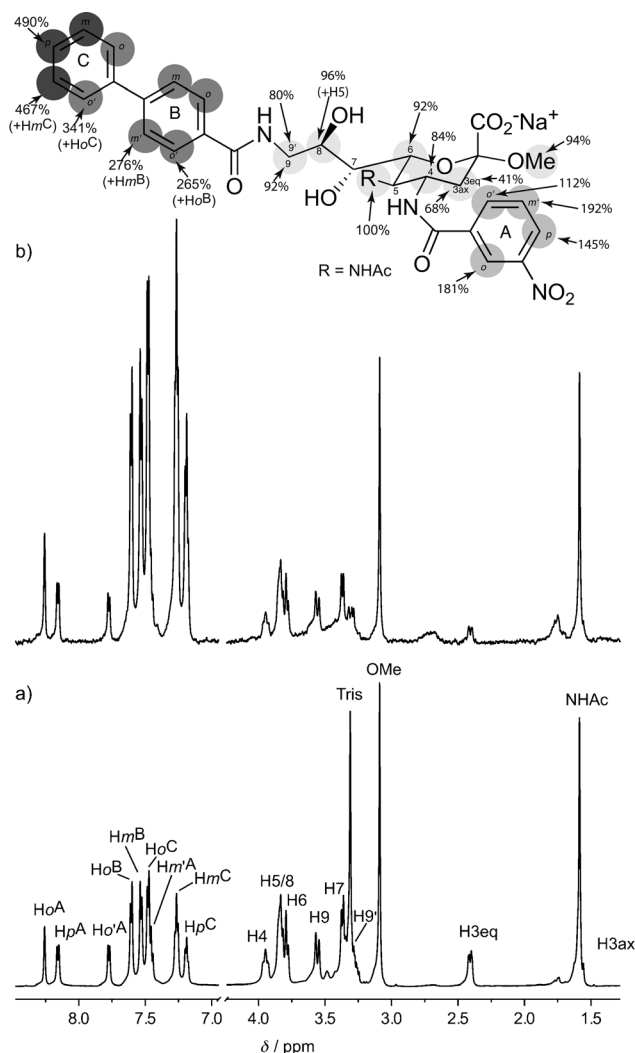


Figure 2. Competition STD NMR experiments of 5 μM Siglec-2 in complex with a) 500 μM **1** and an increasing concentration of **4d**, b) 500 μM **2** and an increasing concentration of **6b**. The ^1H NMR spectra of **1** and **2** are shown at the bottom of the respective panels.

equimolar to the protein, **6b** occupies essentially all the available Siglec-2 Sia binding sites.

The binding epitope of **6b** when in complex with Siglec-2 was determined by STD NMR spectroscopy (Figure 3). The STD NMR spectrum showed very strong STD NMR signals for the *mNPC* substituent at C-4. The Ho^A and Hm^A protons revealed a STD NMR effect of 181% and 192%, respectively, suggesting a close contact to the protein. Interestingly, these STD effects are stronger than those determined for **4d**



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