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Synthesis and Molecular Recognition Studies of the HNK-1 Trisaccharide and Related Oligosaccharides. The Specificity of Monoclonal Anti-HNK-1 Antibodies as Assessed by Surface Plasmon Resonance and STD NMR

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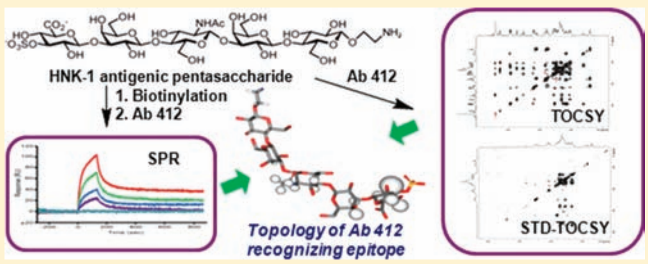
Supporting Information

ABSTRACT: The human natural killer cell carbohydrate, HNK-1, plays function-conducive roles in peripheral nerve regeneration and synaptic plasticity. It is also the target of autoantibodies in polyneuropathies. It is thus important to synthesize structurally related HNK-1 carbohydrates for optimizing its function-conducive roles, and for diagnosis and neutralization of autoantibodies in the fatal Guillain–Barré syndrome. As a first step toward these goals, we have synthesized several HNK-1 carbohydrate derivatives to assess the specificity of monoclonal HNK-1 antibodies from rodents: 2-aminoethyl glycosides of selectively O-sulfated trisaccharide corresponding to the HNK-1 antigen, its nonsulfated analogue, and modified structures containing 3-*O*-fucosyl or 6-*O*-sulfo substituents in the *N*-acetylglucosamine residues. These were converted, together with several related oligosaccharides, into biotin-tagged probes to analyze the precise carbohydrate specificity of two anti-HNK-1 antibodies by surface plasmon resonance that revealed a crucial role of the glucuronic acid in antibody binding. The contribution of the different oligosaccharide moieties in the interaction was shown by saturation transfer difference (STD) NMR of the complex consisting of the HNK-1 pentasaccharide and the HNK-1 412 antibody.



1. INTRODUCTION

Precise interaction of different cell types is essential to maintain neuronal network functions. During these processes, carbohydrate structures expressed on proteins represent recognition sites and cause structural diversity of carrier molecules, resulting in tight regulation of cell–cell interaction, recognition, and migration. One of the most characteristic carbohydrate epitopes of the nervous system are the HNK-1 (human natural killer-1) cell glycans. These oligosaccharides include a unique structure 1 (Figure 1) composed of a sulfated glucuronic acid attached to the nonreducing terminus of an *N*-acetyllactosamine residue.[1,2] This structure is involved in preferential motor reinnervation of the injured femoral nerve in mice[3−7] and nonhuman primates.[8] It binds to the $GABA_B$ receptor, thereby affecting synaptic activity and plasticity. The HNK-1 carbohydrate is the target of autoimmune antibodies that lead to autoimmune-based peripheral neuropathies, including the Guillain–Barré syndrome (for a review, see ref 9).

$3\text{-}SO_3^-$-β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p* **1**

$3\text{-}SO_3^-$-β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-[$6\text{-}SO_3^-$]-β-D-GlcNAc*p* **2**

$3\text{-}SO_3^-$-β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-GlcNAc*p* **3**

Figure 1. Structure of HNK-1 antigenic trisaccharide **1** and its 6-*O*-sulfated and 3-*O*-fucosylated derivatives **2** and **3**.

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Development and characterization of HNK-1-related structures have a tremendous impact on our knowledge on the regulation of nervous system functions. This also applies for modified HNK-1-like oligosaccharide structures as potential modulators of regeneration and synaptic activity. Also, these structures will help to define against which carbohydrate epitopes antibodies are specifically directed in polyneuropathies and thereby possibly allow performing diagnosis of distinct aspects of the disease. Precise knowledge of the epitope variation in different types of polyneuropathies will be decisive in generating carbohydrates that will allow neutralization of autoantibodies for therapy to be carried out.

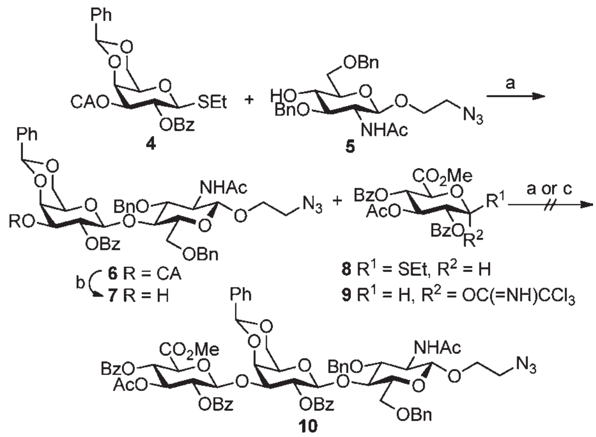
Several syntheses of HNK-1 oligosaccharides have been described including glycolipids with penta- and heptasaccharide carbohydrate chains,[10−13] trisaccharide octyl glycoside,[14] di- and pentasaccharides propyl[15] and 2-aminoethyl[16] glycosides. On the other hand, no modified analogues of HNK-1 oligosaccharides, natural or unnatural, have been synthesized thus far, except for our synthesis of an analogue of **1** containing 3,6-di-*O*-sulfoglucose instead of 3-*O*-sulfoglucuronic acid,[17] and the very recent chemo-bacterial synthesis of allyl 3-*O*-sulfoglucuronyl-3′-lactoside.[18]

In this contribution, we report the synthesis of 2-aminoethyl glycosides of the parent HNK-1 trisaccharide **1**, its nonsulfated analogue, and two structures modified in the *N*-acetylglucosamine residue: 6-*O*-sulfated trisaccharide **2** and 3-*O*-α-L-fucosylated tetrasaccharide **3**. The former disulfated trisaccharide was shown to be a constituent of carbohydrate chains of P0 glycoprotein.[19] The latter structure has not been found in nature yet. Taking into account the important role of the 3-*O*-α-L-fucosylation of lactosamine in various biological phenomena such as adhesion,[20] development,[21] cellular differentiation,[22] oncotransformation,[23] and some others, the 3-*O*-α-L-fucosylated tetrasaccharide could also be present in HNK-1 antigenic glycoproteins. The oligosaccharides described here and some related oligosaccharides synthesized previously[16,17,24] (Table 1) were transformed into biotin-tagged molecular probes and used in molecular recognition studies to assess the specificity of autoantibodies, which are involved in the development of acute immune-mediated neuropathies and chronic immune-mediated polyneuropathies, by surface plasmon resonance. The structural requirements for the carbohydrate binding to some HNK-1 antibodies were qualitatively estimated by ELISA using synthetic HNK-1 glycolipids,[25,26] but no quantitative measurements of these interactions have been made so far. To reveal the topology of the carbohydrate binding, the saturation transfer difference (STD) NMR studies of a complex of the HNK-1 pentasaccharide[16] with the HNK-1 412 antibody were carried out as well.

2. RESULTS AND DISCUSSION

2.1. Synthesis of Oligosaccharide Ligands Related to HNK-1 Antigens. Two approaches to the assembly of the key trisaccharide sequence **1** are possible, (1) by chain elongation from the reducing end ([1 + 2] scheme) and (2) from the nonreducing end ([2 + 1] scheme). We explored first the [1 + 2] synthetic scheme (Scheme 1). The NIS·TfOH-promoted glycosylation of 2-azidoethyl glycoside **5**[17] with thiogalactoside **4**[27] afforded disaccharide **6** in good yield; removal of the chloroacetyl group from **6** with thiourea provided disaccharide acceptor **7**. To introduce a glucuronic acid residue into **7**, two glucuronosyl donors **8**[28] and **9**[28] containing a selectively removable 3-*O*-acetyl group were tested. However, no formation of trisaccharide **10**

Scheme 1. Attempts at the Synthesis of Trisaccharide 10 by the [1 + 2] Scheme[a]



[a] Reagents and conditions: (a) NIS, TfOH, MS 4 Å, CH_2Cl_2, −30 °C, 70%; (b) $(H_2N)_2CS$, *sym*-collidine, MeOH, reflux, 90%; (c) $BF_3 \cdot Et_2O$, MS AW-300, CH_2Cl_2, 0 °C.

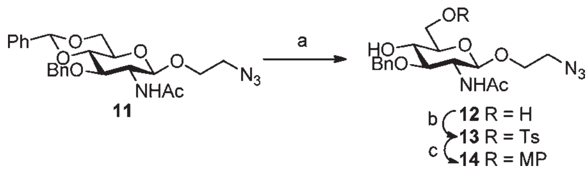
was detected upon the glycosylation of **7** with thioglycoside donor **8**, while imidate **9** provided **10** in an unsatisfactory yield of about 10%. Therefore, the [1 + 2] synthetic scheme was abandoned, and we focused our attention on the alternative [2 + 1] approach to prepare the target structures.

Glucosamine acceptor **5** is suitable for the preparation of the monosulfated structure **1**, whereas a glucosamine derivative with orthogonal protecting groups in the positions 3 and 6 was necessary as a glycosyl acceptor for the rational synthesis of the structures **2** and **3**. For this aim, the known benzylidene acetal **11**[17] was converted into diol **12**, and the diol was further transformed into 6-tosylate **13** by selective tosylation. (Scheme 2). The subsequent treatment of tosylate **13** with *p*-methoxyphenol in DMF in the presence of NaH afforded necessary glucosamine acceptor **14** with the orthogonal protecting group pattern.

Two different galactose acceptors **15**[29] and **16**[30] were examined to synthesize a glucuronosyl-(1→3)-galactose donor block (Scheme 3). The successful glycosylation of thioglycoside **15** would lead, without any intermediate transformations, to the necessary disaccharide donor **17**. However, the $BF_3 \cdot E_2O$-promoted reaction of **15** with imidate **9** produced target **17** in minor yield due to the predominant transfer of the SEt group to the uronic acid donor and some other side processes. The glycosylation of allyl galactoside **16** with the same donor **9** required the thorough optimization of reaction conditions with respect to promoter (TMSOTf, $BF_3 \cdot E_2O$), solvent (CH_2Cl_2, toluene), the molecular sieve type, and temperature. Under optimal conditions found (see Scheme 3), disaccharide **18** was obtained in 64% yield.

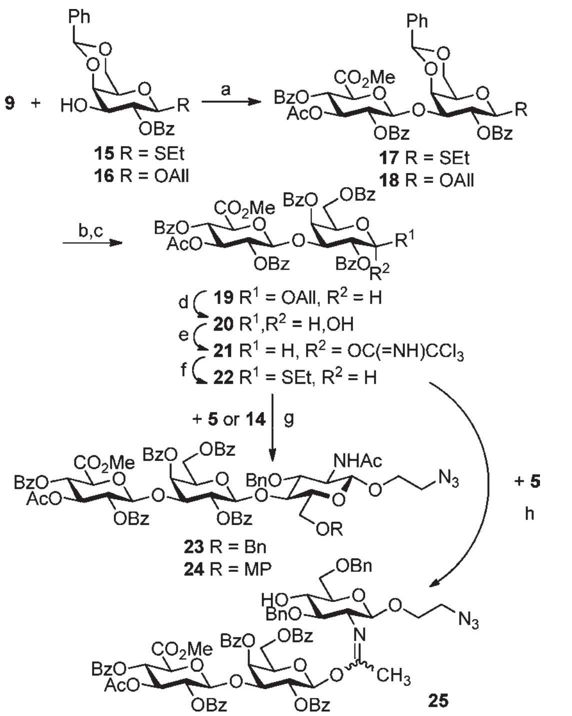
As the benzylidene group in **18** may be unstable under deallylation conditions, it was replaced by acid-stable benzoates to provide completely acylated disaccharide **19**. Removal of the anomeric allyl group produced hemiacetal **20**, which was then converted to imidate **21**. Surprisingly, the reaction of **21** with acceptor **5** afforded no trisaccharide **23**; only gradual destruction of the imidate was observed, while acceptor **5** was nearly quantitatively recovered from the reaction mixture. Then imidate **21** was transformed in thioglycoside **22**; its subsequent coupling

Scheme 2. Synthesis of Glycosyl Acceptor 14[a]



[a] Reagents and conditions: (a) 80% aq AcOH, 40 °C, 86%; (b) TsCl, Py, 0 °C → rt, 75%; (c) *p*-MeOC$_6$H$_4$OH, NaH, DMF, 60 °C, 91%.

Scheme 3. Synthesis of Trisaccharides 23 and 24[a]



[a] Reagents and conditions: (a) BF$_3$·Et$_2$O, MS AW-300, toluene, 0 °C → 4 °C, 64%; (b) PPTS, 90% aq CH$_3$CN, 80 °C; (c) BzCl, pyridine, 92%, two steps; (d) PdCl$_2$, AcONa, 95% aq AcOH, 64%; (e) CCl$_3$CN, DBU, CH$_2$Cl$_2$, 75%; (f) EtSH, TMSOTf, MS 4 Å, CH$_2$Cl$_2$, 98%; (g) NIS, TfOH, MS AW-300, CH$_2$Cl$_2$, −20 °C → −10 °C, 69% for **23**, 60% for **24**; (h) NIS, TfOH, MS 4 Å, CH$_2$Cl$_2$, −20 °C → −10 °C, 70%.

with acceptors **5** and **14** in the presence of NIS, TfOH, and MS AW-300 gave desired trisaccharides **23** and **24** in good yields. It is noteworthy that the replacement in the reaction with **5** of non-basic MS AW-300 with basic MS 4 Å resulted in the exclusive formation of the glycosylation product at the acetamido group, namely, imidate **25**. The formation of similar imidates upon glycosylation of *N*-acetylglucosamine acceptors has been reported earlier.[31,32]

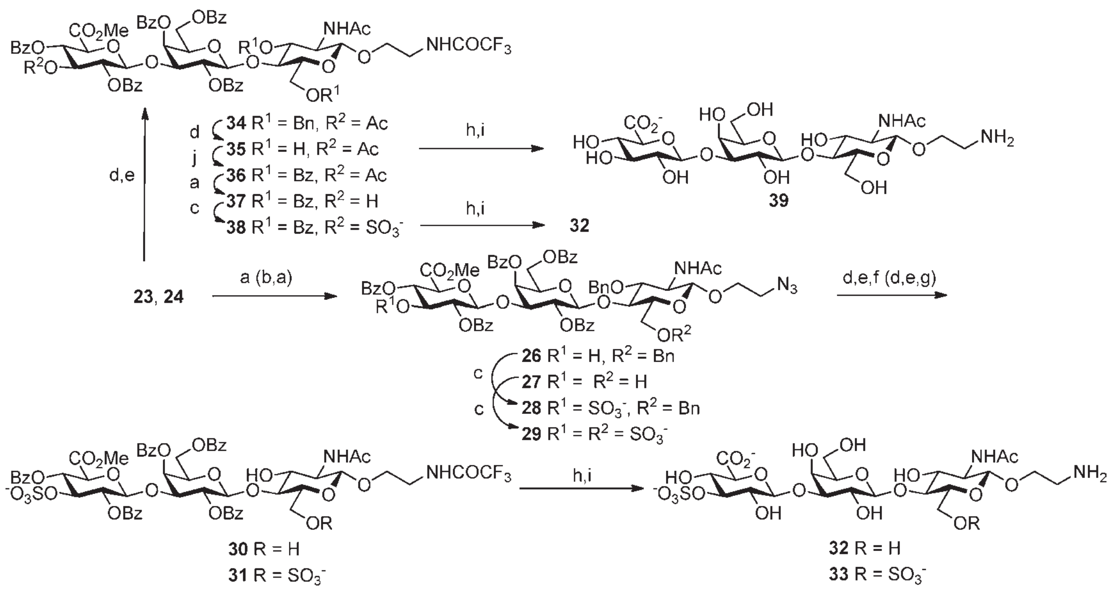
The only *O*-acetyl group in **23** was selectively removed by mild acidic methanolysis;[33] the splitting of the *p*-methoxyphenyl group by ceric ammonium nitrate (CAN) preceded acidic deacetylation in the case of **24** (Scheme 4). Monohydroxyl (**26**) and and dihydroxyl (**27**) derivatives obtained thereby were treated with the pyridine·SO$_3$ complex to provide the respective sulfates **28** and **29**. The location of the sulfate groups in **28** and **29** was confirmed by characteristic downfield shifts of the signals for C-3 of the uronic acid residue (δ 72.1 → 78.5 for **28**; δ 73.5 → 78.6 for **29**) and for C-6 of *N*-acetylglucosamine (δ 61.2 → 66.0) as compared to those of the parent hydroxyl compounds. Simultaneous reduction of the azide in the aglycon and removal of the benzyl group(s) by catalytic hydrogenolysis of **28** and **29** failed. After the fast reduction of the azido group, the hydrogenolysis of benzyl groups almost stopped, apparently due to poisoning of the catalyst by the amine formed.[16] Moreover, a considerable loss of the sulfate groups was observed on prolonged hydrogenolysis. To overcome this difficulty, two-step hydrogenolysis was used. At the first step, the amino group arising on azide reduction was blocked with a trifluoroacetyl group, and then hydrogenolysis was continued until complete debenzylation. Removal of the single benzyl group from **29** was thus achieved practically without competitive desulfation to give **31** in 77% yield. In the case of **28** having two benzyl groups, desulfation was much more pronounced, and buffering of the reaction solution with NaOAc was necessary to obtain debenzylated product **30** in reasonable yield. Following two-step saponification of **30** and **31** smoothly gave target free sulfates **32** and **33** corresponding to the structures **1** and **2**.

To avoid the loss of the sulfate upon catalytic hydrogenolysis, an alternative reaction sequence in which benzyl groups were replaced by benzoates before sulfation was also explored. The catalytic reduction of the azido group in **23** followed by *N*-trifluoroacetylation produced **34**. The latter was subjected to conventional catalytic hydrogenolysis, and the formed diol **35** was benzoylated with the formation of **36**. Benzoic anhydride was used at the latter step to avoid competitive *N*-benzoylation. Further transformations of monoacetate **36** included selective acidic deacetylation (→ **37**), sulfation (→ **38**), and final saponification to **32**. Nonsulfated trisaccharide **39**, necessary for the evaluation of carbohydrate specificity of anti-HNK-1 antibodies, was obtained by the saponification of diol **35**.

Trisaccharide **24** was used as the starting compound for the synthesis of the tetrasaccharide structure **3** (Scheme 5). The reduction of the spacer azido group and debenzylation as described above provided monohydroxyl trisaccharide acceptor **40**, which was coupled with thiofucoside **41**, obtained by conventional benzoylation of the corresponding 3,4-diol,[34] to yield tetrasaccharide **42**. The α-configuration of the fucoside bond was confirmed by the corresponding coupling constant value $J_{1,2}$ (3.4 Hz) in the ^{1}H NMR spectrum of **42**. As the removal of benzyl and *p*-methoxyphenyl groups from sulfated tetrasaccharide might be accompanied by desulfation (vide supra), these groups were replaced by benzoates as described above for **23**. The catalytic hydrogenolysis of **42** followed by the treatment with CAN afforded diol **43**, which was benzoylated to give **44**. The acidic deacetylation of **44** provided derivative **45**. It is noteworthy that the hydroxyl group in **45** displayed much lower reactivity toward sulfation than did the similar hydroxyl groups in trisaccharides **26** and **27**. Thus, the formation of only a minor amount of sulfate **46** was detected under the conditions used for the preparation of **28** and **29** (10-fold excess of the SO$_3$·pyridine complex, rt, 2 h); complete conversion of **45** to **46** needed a larger excess of the sulfating reagent (50 equiv) and prolonged reaction time (42 h). The saponification of **46** as described above produced target sulfate **47**.

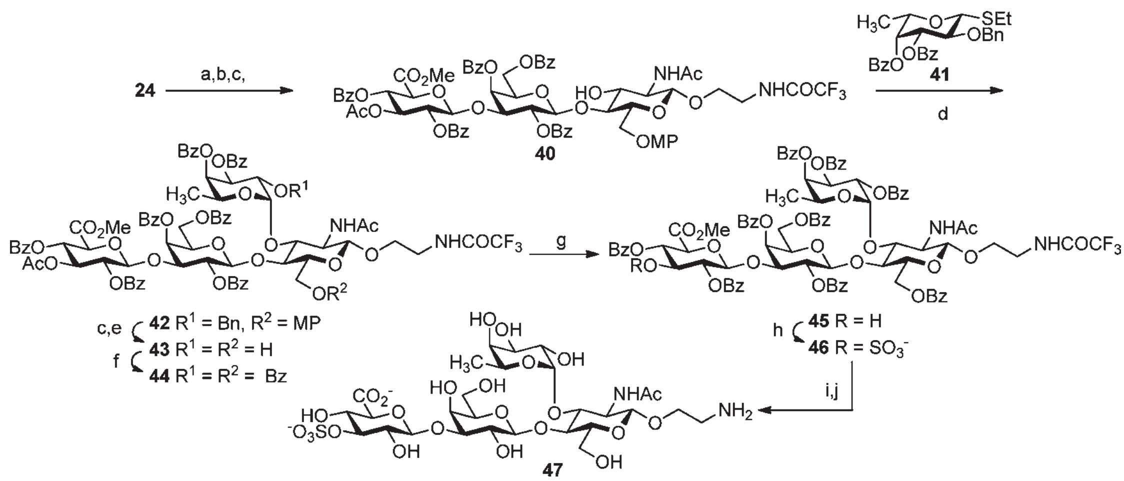
2-Aminoethyl 3-*O*-sulfoglucuronide **51** was also prepared (Scheme 6) to reveal a minimum carbohydrate element recognizable by anti-HNK-1 antibodies. The BF$_3$·Et$_2$O-catalyzed coupling of imidate **9** with 2-trifluoroacetamidoethanol afforded

Scheme 4. Synthesis of Sulfated Trisaccharides 32 and 33[a]



[a] Reagents and conditions: (a) 6% anhydrous HCl in MeOH, 86% for **26**, 85% for **37**, 71% for **27**, two steps; (b) CAN, aq CH_3CN, 0 °C; (c) complex SO_3·pyridine, DMF, 2 h, 86% for **28**, 81% for **29**, 96% for **38**; (d) H_2, PdO/C, MeOH, AcOH; 51% for **35**, three steps; (e) CF_3CO_2Et, Et_3N, MeOH; (f) H_2, PdO/C, MeOH, NaOAc, 45% for **30**, three steps; (g) H_2, PdO/C, MeOH, 77% for **31**, three steps; (h) LiOH, aq THF, −10 °C; (i) NaOH, aq MeOH, 72% for **32**, 83% for **33**, 82% for **39**; (j) Bz_2O, DMAP, pyridine, 40 °C, 96%.

Scheme 5. Synthesis of Sulfated Tetrasaccharide 47[a]



[a] Reagents and conditions: (a) H_2, PdO/C, MeOH, AcOH; (b) CF_3CO_2Et, Et_3N, MeOH; (c) H_2, PdO/C, MeOH, 57%, three steps; (d) NIS, TfOH, MS 4 Å, CH_2Cl_2, −20 °C → −10 °C, 52%; (e) CAN, aq CH_3CN, 0 °C, 64%, two steps; (f) BzCl, pyridine, −10 °C, 95%; (g) 6% anhydrous HCl in MeOH, 4 °C, 75%; (h) complex SO_3·pyridine, DMF·pyridine (3:1), 42 h, 82%; (i) LiOH, aq THF, −10 °C; (j) NaOH, aq MeOH, 92%.

glucuronide **48**, which was converted to **51** by deacetylation, sulfation, and saponification as described above for trisaccharide **36** and tetrasaccharide **44**.

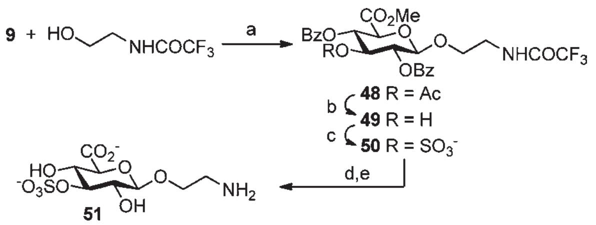
2.2. Synthesis of Biotin-Tagged Oligosaccharide Ligands. Surface plasmon resonance has become a powerful tool for evaluation and quantification of the carbohydrate−protein interaction (e.g., refs 35−40). Carbohydrate molecules are usually immobilized to the surface, and a solution of protein is allowed to run over the carbohydrates. The opposite case when low-molecular-mass carbohydrates (normally 200−2000 D) run over immobilized protein is less common, because it may not provide the necessary accuracy of measurements.[35,41] Several methods for immobilization of carbohydrate ligands to the surface have been reported,[37,38,41,42] but the application of biotin-tagged oligosaccharides[39,40,43,44] seems to be the most convenient because of the market appearance of streptavidin SPR sensor chips.

We have prepared a series of biotin-tagged HNK-1-related oligosaccharides in which the carbohydrate and biotin moieties are connected via a hydrophilic and flexible hexa(ethylene glycol) spacer. The spacer allows one to avoid undesirable hydrophobic interactions and ensures an optimal orientation of a carbohydrate ligand during the recognition process. The synthesis of a

Table 1. List of the Parent Saccharide 2-Aminoethyl Glycosides and Biotinylated Ligands Therefrom (see Scheme 7)

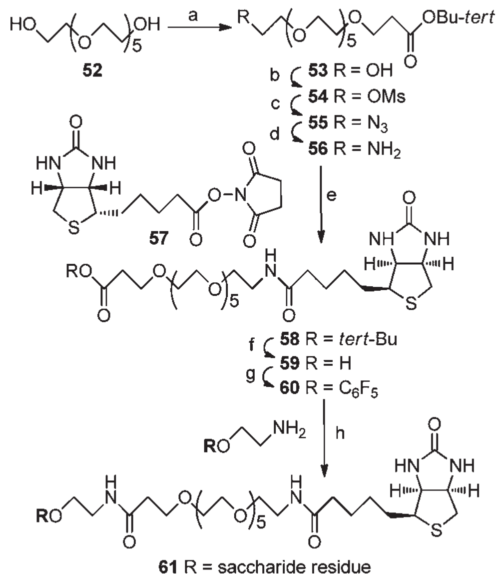
R	parent 2-aminoethyl glycoside	biotinylated ligand of type **61**
3-*O*-SO_3^--β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→	**32**	**66**
3-*O*-SO_3^--β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-(6-*O*-SO_3^--β-D-GlcNAc*p*)-(1→	**33**	**67**
3-*O*-SO_3^--β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→	**62**	**68**
3-*O*-SO_3^--β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-[(α-L-Fuc*p*-(1→3)]-β-D-GlcNAc*p*-(1→	**47**	**69**
3-*O*-SO_3^--β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→	**63**	**70**
β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→	**64**	**71**
β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→	**39**	**72**
3-*O*-SO_3^--β-D-GlcA*p*-(1→	**51**	**73**
3,6-di-*O*-$(SO_3^-)_2$-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→	**65**	**74**

Scheme 6. Synthesis of 3-*O*-Sulfoglucuronide **51**[a]



[a] Reagents and conditions: (a) $BF_3 \cdot Et_2O$, CH_2Cl_2, −15 °C, 62%; (b) 6% anhydrous HCl in MeOH, 84%; (c) complex $SO_3 \cdot$pyridine, pyridine, 91%; (d) LiOH, aq THF, −10 °C; (e) NaOH, aq MeOH, 84%.

Scheme 7. Synthesis of Biotin-Tagged Oligosaccharides[a]



[a] Reagents and conditions: (a) $CH_2{=}CHCO_2Bu$-*t*, NaH, THF, 78%; (b) MsCl, Et_3N, CH_2Cl_2; (c) NaN_3, DMF, 75%, two steps; (d) H_2, $Pd(OH)_2$/C, MeOH, 96%; (e) **57**, DMF, pyridine, 76%; (f) TFA, CH_2Cl_2, 90%; (g) $CF_3CO_2C_6F_5$, pyridine, CH_2Cl_2, 92%; (h) Et_3N, DMF.

reagent for the introduction of the biotin tag is depicted in the Scheme 7.

A reaction sequence similar to that described for tetra(ethylene glycol)[45] was used to functionalize hexa(ethylene glycol) **52**. The base-catalyzed addition of **52** to *tert*-butyl acrylate furnished monoester **53**. Then the hydroxyl group was mesylated (→ **54**) followed by substitution with azide (→ **55**). The catalytic reduction of the azido group provided amino ester **56**, which was acylated with biotin active ester **57** to afford **58**. The removal of the *tert*-butyl group from **58** and the subsequent treatment of acid **59** formed with pentafluorophenyl trifluoroacetate resulted in the formation of pentafluorophenyl ester **60**.

Further acylation of oligosaccharide 2-aminoethyl glycosides with active ester **60** produced necessary biotin-tagged oligosaccharides of general formula **61**. Trisaccharides **32** and **33**, their disaccharide fragment **62**,[16] tetrasaccharide **47**, pentasaccharide fragment of HNK-1 glycolipids **63**,[16] its tetrasaccharide fragment **64**[24] devoid of the glucuronic acid, nonsulfated trisaccharide **39**, 3-*O*-sulfoglucuronide **51**, and trisaccharide **65**[17] containing 3,6-di-*O*-sulfoglucose instead of 3-*O*-sulfoglucuronic acid were subjected to this transformation to provide ligands **66**-**74** for SPR measurements (Table 1).

The ligands were characterized by ^{1}H NMR spectra, which were essentially the superposition of the spectra of the corresponding saccharide and acid **59**, and HRMS data. Then molecular recognition studies to determine the precise carbohydrate specificity of two different HNK-1 antigen-recognizing antibodies by using of the synthesized oligosaccharide derivatives and both SPR and NMR methods were performed.

2.3. SPR Investigation of the Interaction of the Oligosaccharides with Monoclonal Antibodies. The interaction of synthetic oligosaccharides with two different HNK-1 carbohydrate-specific antibodies was studied: with HNK-1 antibody (mouse monoclonal IgMκ) and HNK-1 412 antibody first known as L2 antibody (rat monoclonal IgG2aκ). It is known that both antibodies recognize specifically sulfated HNK-1 carbohydrate structures, while the HNK-1 412 antibody is also able to recognize the nonsulfated epitope.[25,26] We have shown that monosaccharide **73**, disaccharide **68**, trisaccharides **66** and **67**, fucosylated tetrasaccharide **69**, and pentasaccharide **70** bind to both antibodies in a concentration-dependent manner, whereas HNK-1 412 antibody interacts also with nonsulfated trisaccharide **72**. Typical sensorgrams of oligosaccharide−antibody interactions exemplified by trisaccharide **66** are given in Figure 2 (for a complete set of sensorgrams of interactions studied, please see Supporting Information [SI].). None of the antibodies recognized tetrasaccharide **71** devoid of the uronic acid and trisaccharide **74** in which 3-sulfated glucuronic acid is replaced by 3,6-disulfated glucose.

Two kinetic models were employed to calculate equilibrium constants K_D: (a) the Langmuir model (1:1 bimolecular interaction),

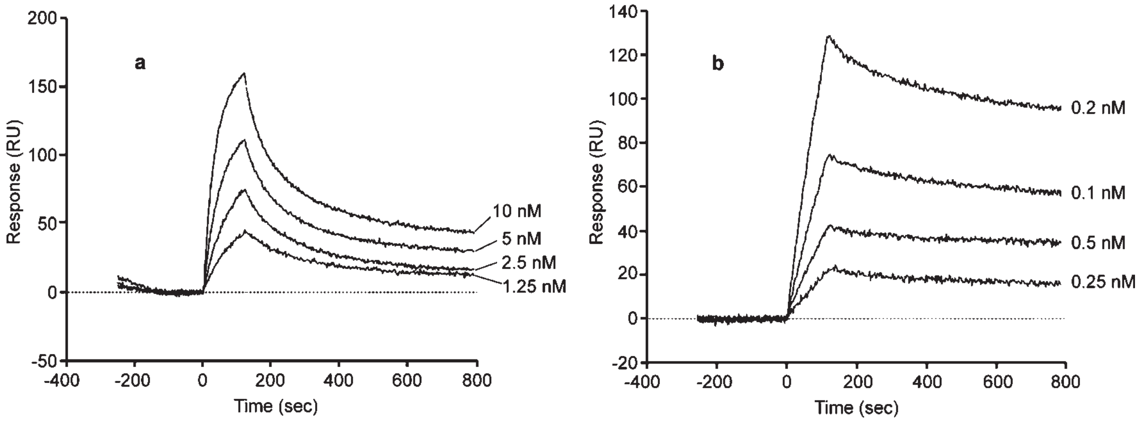


Figure 2. Sensorgram of the binding of trisaccharide **66** to HNK-1 412 antibody (a) and HNK-1 antibody (b).

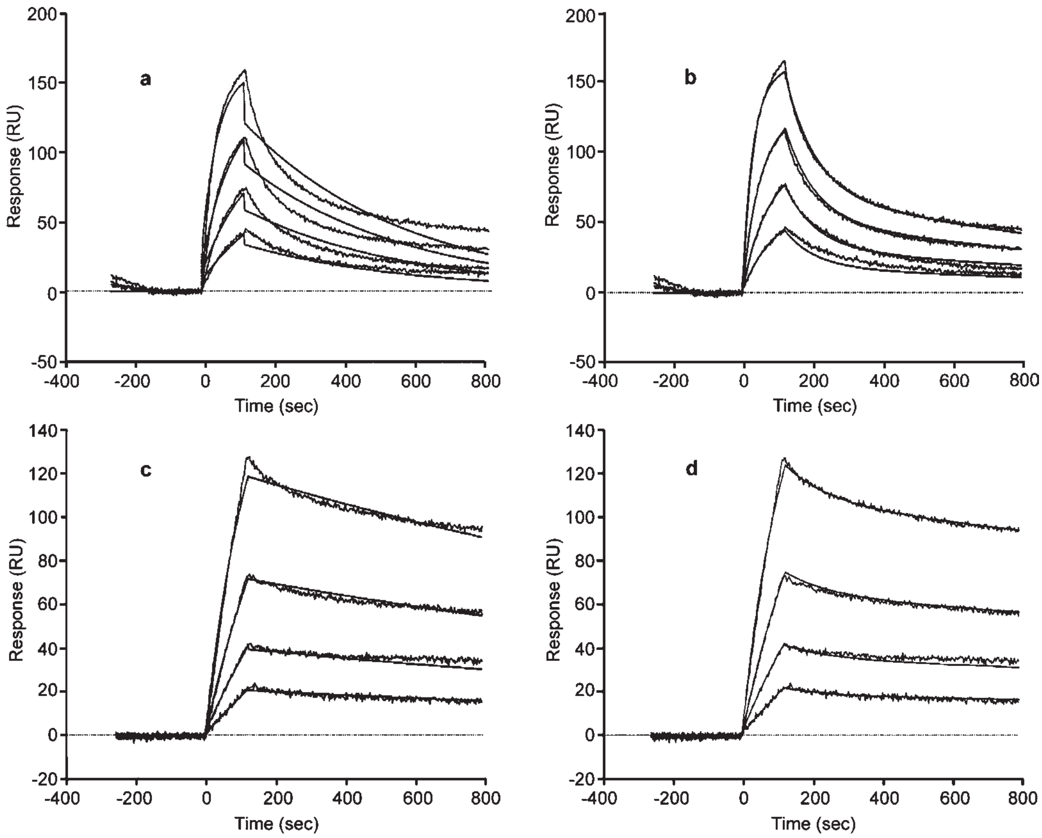


Figure 3. Fitting of the data of the interaction of trisaccharide **66** with HNK-1 412 (a, b) and HNK-1 (c, d) antibodies. Fitted data obtained using the Langmuir model (a, c) and the two-state model (b, d) are represented by smooth curves.

and (b) the two-state model, which assumes that the ligand—analyte complex undergoes a conformational change. As it is obvious from Figure 3, the two-state model provides better fit of experimental and calculated curves especially in the case of the HNK-1 412 antibody (a, b); the difference in the accuracy of two kinetic models is less pronounced for the HNK-1 antibody (c, d). These data show that the binding of carbohydrates to HNK-1-related antibodies may be a more complicated process than a simple 1:1 interaction. The bivalent analyte kinetic model was tested as well, taking into account that HNK-1 412 antibody has two binding sites and HNK-1 antibody, which is a hexamer, has 12 binding sites. However, application of this model to both antibodies showed even worse fitting than the Langmuir model (data not shown). Equilibrium constants K_D calculated using the two-state model are given in the Table 2 (for complete kinetic data, including association and dissociation constants, and standard errors, see Tables 1 and 2 in SI).

The $K_D(1)$ values of interactions with HNK-1 412 antibody are approximately 2 orders of magnitude higher than those with HNK-1 antibody (except nonsulfated structure **72**), thus indicating the higher affinity of the latter toward the carbohydrate antigens studied. The minimum carbohydrate fragment recognizable by both antibodies was proven to be sulfated glucuronic acid **73**. The presence of the glucuronic acid is the prerequisite for the recognition of an oligosaccharide by both antibodies, whereas structures **71** and **74** devoid of this monosaccharide showed no binding. The case of **74** is noteworthy. Although both trisaccharides **66** and **74** are terminated with the *gluco*-configured monosaccharides bearing two negatively charged groups, only **66** is recognized by the antibodies. Apparently, the different spatial arrangement of the $CH_2OSO_3^-$ and CO_2^- groups at C-5 of the terminal monosaccharides in **74** and **66** and different properties of the sulfate and carboxylate groups, such as basicity and ability to bind metal cations, are responsible for the dramatic difference in the recognition of **66** and **74**. Unlike the HNK-1 antibody that recognizes only sulfated structures, the HNK-1 412 antibody is able also to bind nonsulfated trisaccharide **72** although much weaker than the corresponding sulfated counterpart **66**. In other respects, the carbohydrate specificities of the antibodies studied are similar, although the HNK-1 412 antibody seems to be more sensitive to the structure of the carbohydrate backbone.

Table 2. Calculated (Two-State Kinetic Model) Equilibrium Binding Constants of Oligosaccharides to HNK-1 412 and HNK-1 Antibodies

	HNK-1 412 antibody		HNK-1 antibody	
oligosaccharide	$K_D(1) \times 10^{-8}$ (M)	$K_D(2)$ (M)	$K_D(1) \times 10^{-10}$ (M)	$K_D(2)$ (M)
66	0.371	0.37	0.490	0.15
67	1.45	0.10	2.04	0.12
68	10.9	0.12	8.92	0.06
69	7.25	0.10	34.2	0.04
70	0.153	0.37	1.61	0.10
72	22.2	0.05	no binding	
73	91.5	0.07	12.8	0.06

Examination of binding abilities of HNK-1 antibodies to linear structures revealed that both antibodies displayed the weakest binding to sulfated uronic acid **73**. Elongation of the carbohydrate chain to di- (**68**), tri- (**66**), and pentasaccharide (**70**) decreased $K_D(1)$ values in the case of HNK-1 412 by almost 3 orders of magnitude. The contribution of the oligosaccharide chain to the binding was confirmed by STD NMR investigation of a complex of pentasaccharide **63** with the HNK-1 412 antibody (vide infra). The HNK-1 antibody demonstrated the same trend within the series of monosaccharide **73**, disaccharide **68**, and trisaccharide **66**, although the decrease in the $K_D(1)$ values was not as substantial as in the case of the HNK-1 412 antibody. Unlike the HNK-1 412 antibody, weakening of the binding of pentasaccharide **70** as compared to trisaccharide **66** was observed in the case of the HNK-1 antibody. 6-*O*-Sulfatation (**67**) or 3-*O*-fucosylation (**69**) in the *N*-acetylglucosamine residue of trisaccharide **66** caused similar increase in the $K_D(1)$ values for both antibodies. The $K_D(2)$ values characterizing a conformational change within an antigen−antibody complex showed only slight variations and ranged from 0.04 to 0.37.

2.4. STD NMR Investigation of a Complex of Pentasaccharide 63 with the HNK-1 412 Antibody. Saturation transfer difference (STD) NMR methods[46] were then employed to access residue-specific binding information. STD has widely been applied to investigate ligand−receptor biomolecular interactions,[47] including antigen−antibody recognition events.[48] We exploited this methodology to explore, at atomic resolution,

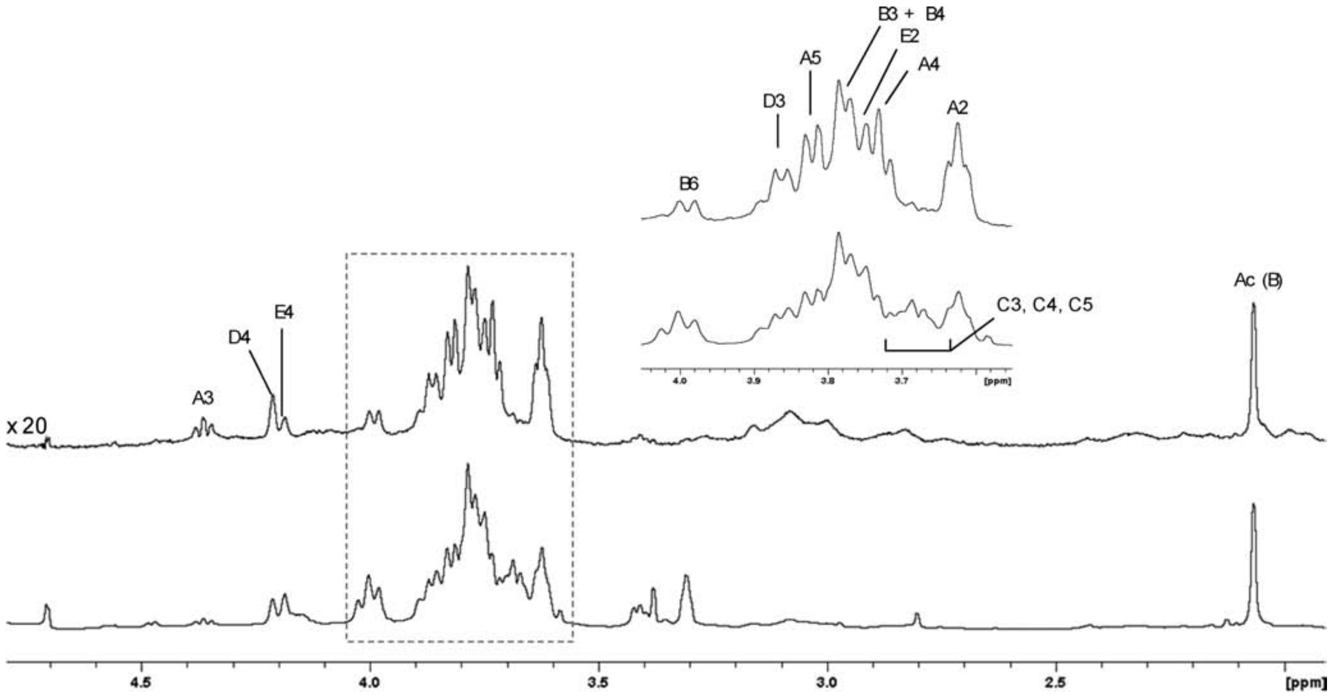


Figure 4. ^{1}H NMR (600 MHz) STD spectrum (saturation time 2 s with on-resonance irradiation at the aromatic region) of a 20:1 mixture of **63**/HNK-1 412 antibody at 310 K. The bottom trace shows the off-resonance spectrum, while the upper trace shows the STD signals. The region between 3.6 and 4.0 ppm is enlarged. Different responses from the different residues are observed, which were further analyzed with STD-TOCSY.

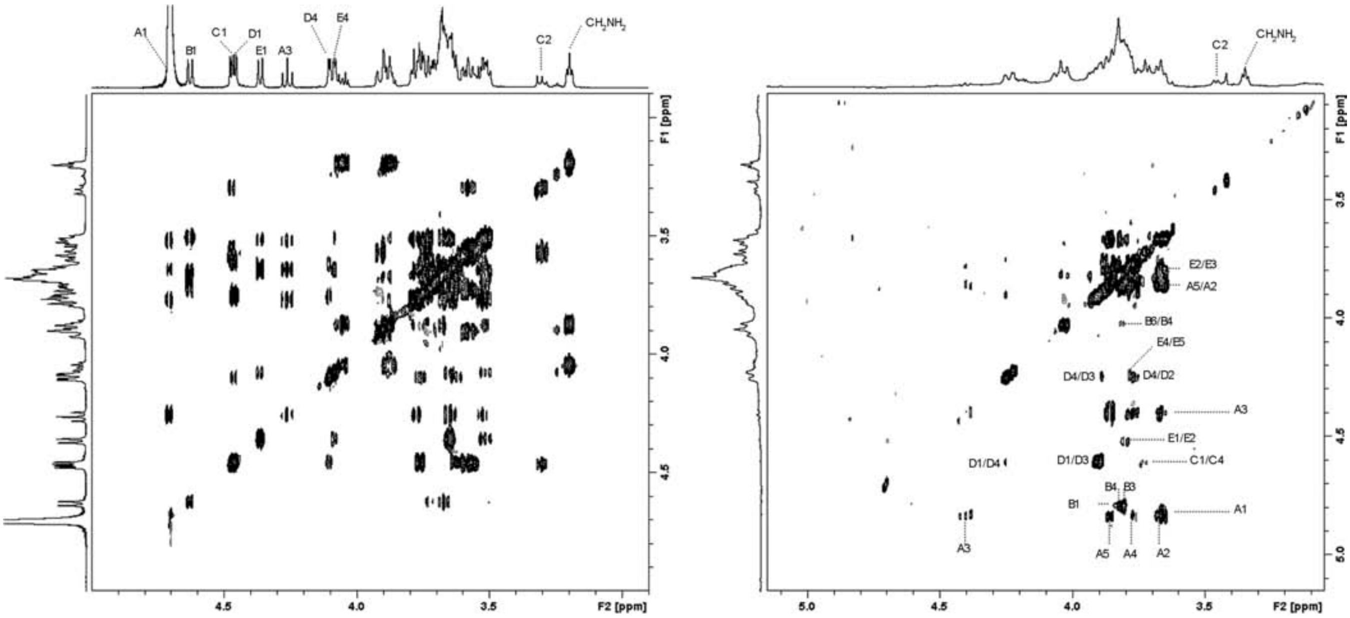


Figure 5. NMR (500 MHz) regular 2D-TOCSY spectrum (left), and STD-2D-TOCSY spectrum (right), with saturation time 2 s, mixing time 60 ms, and on-resonance irradiation at the aromatic region of a 20:1 mixture of **63**/HNK-1 412 antibody at 310 K.

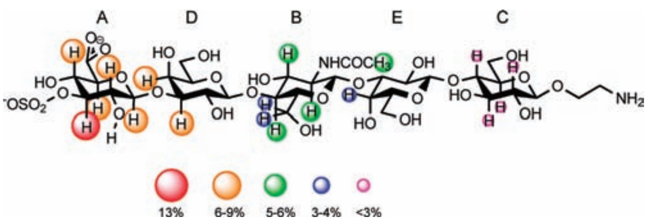


Figure 6. Values of STD signals in the complex of pentasaccharide **63** with HNK-1 412 antibody.

the binding epitope of pentasaccharide **63** for the HNK-1 412 antibody. Various experimental conditions were employed to obtain the best results.[49] Finally, a sample containing HNK-1 412 antibody (30 μM) in the presence of a 20 molar excess of **63** (600 μM) was chosen. In the presence of the HNK-1 412 antibody, some of the signals of **63** were broadened and, especially, H5 of the sulfated GlcA moiety was significantly upfield shifted. The most intense STD signals were observed at 310 K. The results (Figure 4) indicated the existence of a clear binding epitope, since not all of the ^{1}H NMR signals of the pentasaccharide were displayed in the STD spectrum.[50,51] Moreover, stronger STD intensities were observed for the signals belonging to the GlcA moiety than for the other residues.

At this stage, due to the heavy overlapping observed between 3.6 and 3.9 ppm, no fine details of the contributing moieties could be obtained in an unambiguous manner. Therefore, a STD-TOCSY experiment[52] was performed to resolve the STD signals in a second dimension (Figure 5). In this manner, a linear binding epitope was clearly defined, starting from the sulfated GlcA unit, which showed the major interaction with HNK-1 412 antibody, followed by its vicinal Gal residue and the subsequent GlcNAc moiety. The terminal lactose fragment provided very weak STD effects, whereas the linker atoms gave no STD intensity at all, indicating that they mainly remain outside of the binding site. Thus, the major contributions to binding may be schematized as displayed in Figure 6.

Blank experiments were performed for the pentasaccharide **63** in the absence of the HNK-1 412 antibody, as well as for tetrasaccharide **64** devoid of the nonreducing sulfated GlcA unit in the presence of the HNK-1 412 antibody. No STD effects were observed in the first case, while very weak effects (maximum 0.4%) were observed for the tetrasaccharide **64**, and only at a very high ligand:antibody (200:1) molar ratio, indicating nonspecific binding under these experimental conditions. No STD was observed for **64** at a molar ratio of 20:1 employed for the study of the interaction of pentasaccharide **63** with the antibody. Moreover, DOSY experiments were also performed for both **63** and **64** oligosaccharides in the absence and in the presence (5:1 molar ratio) of the HNK-1 412 antibody. No variation of the diffusion coefficient of the sugar signals of **64** were observed upon addition of the antibody, while large changes were evidenced for the pentasaccharide sample when the HNK-1 412 antibody was present.[53] Thus, the NMR data nicely complement the SPR results described above and provide a detailed structural view of the interaction process. In fact, these experimental approaches provide an alternative protocol to that previously described[54] using molecular modeling approaches to study the recognition of carbohydrate HNK-1 antigens and their mimetics by protein receptors.

3. CONCLUSION

To gain insights into the specificity of HNK-1 carbohydrate binding antibodies, three sulfated and one nonsulfated oligosaccharides related to the HNK-1 antigen were synthesized and converted, together with some other oligosaccharides of this type, to biotin-tagged molecular probes. They were used to determine the carbohydrate specificity of HNK-1 412 and HNK-1 monoclonal antibodies by SPR. It has been shown that the presence of 3-*O*-sulfated glucuronic acid is a decisive feature for the binding of oligosaccharides to both antibodies. Prior to this study, a little was known about the role of further monosaccharides of the HNK-1 glycan chain in the binding. Here we have shown that the monosaccharides of the contiguous *N*-acetyllactosamine moiety are also involved in the molecular recognition process of the HNK-1 glycan by the antigen-binding sites of the studied antibodies. In contrast, the lactose fragment at the "reducing end" of the HNK-1 pentasaccharide only barely interacts with the carbohydrate epitope binding site. This fact was clearly evidenced from the STD NMR study of the HNK-1 pentasaccharide—HNK-1 412 antibody complex.

Our results illustrate that HNK-1 antigenic trisaccharide or longer chains are preferentially detected by the examined HNK-1 antibodies. The use of synthesized HNK-1 related structures alone or in combination with carbohydrate ligands of other types will allow one in the future and after necessary validation studies to screen autoantibodies in the series of patients with polyneuropathies in order to identify different types of this disease and to develop new therapeutic strategies using selected HNK-1 structures for neutralization of HNK-1 autoantibodies in the affected patients. It should be also pointed out that HNK-1 antigenic carbohydrate ligands are carried by several adhesion molecules[55,56] that determine their ability to bind to the neurite outgrowth promoting the extracellular matrix molecule laminin.[57] Thus, the described results can also form the rationale for the selection of optimal oligosaccharides for enhancing peripheral nerve regeneration and GABAergic synaptic transmission, the importance of which is implicated in down-tuning of the central nervous system overactivity, as seen in epilepsy. Initial studies in this direction have been started by us[58] previously and will be continued with the use of the glycoconjugate tools described here.

4. EXPERIMENTAL SECTION

For general analytical procedures and synthetic protocols used to prepare oligosaccharides and their biotin-tagged derivatives, please see the SI.

4.1. Antibodies. Both monoclonal anti-HNK-1 antibodies (412 and HNK-1) were prepared as described.[55] The purified IgG or IgM fractions, respectively, were used in SPR studies.

4.2. SPR Measurements. SPR experiments were performed using a ProteOn XPR-36 instrument (Bio Rad). PBST (phosphate-buffered saline, 10 mM phosphate, 150 mM NaCl, pH 7.40 containing 0.005% Tween 20) was used as a running buffer. The biotinylated oligosaccharides (150 μL of 0.5—100 nM solutions in PBST depending on the saccharide structure) were immobilized at a flow rate of 30 μL/min on neutravidin-coated NLC-chips. One horizontal ligand channel was employed per oligosaccharide. One of the six horizontal channels was loaded with biotin (150 μL of 10 μM solution in PBST) and used as a reference channel. Interaction was measured by running the antibody solutions vertically over the immobilized carbohydrate structures using one analyte channel per antibody concentration (200 μL of antibody solution in PBST, flow rate 100 μL/min, duration of the association step 120 s, duration of the dissociation step 400—600 s). Four concentrations were measured for the calculation of kinetic constants. After each analysis, the surface was washed with a regeneration buffer (10 mM NaOH) and equilibrated with the running buffer. Data processing was performed with the ProteOn Manager software.

STD NMR Experiments. These spectra were recorded at 310 K in a phosphate buffer (D_2O) at pH 7.3, uncorrected for isotope effects, on Bruker AVANCE 500- and 600 MHz spectrometers equipped with a triple-channel cryoprobe, as described.[47] The STD (10k scans) and STD TOCSY experiments (mixing time 60 ms with 256 increments of 80 scans each) were performed as described, using a 20:1 ligand to receptor molar ratio with a 600 μM concentration of oligosaccharides in Shigemi NMR tubes. One-dimensional STD experiments were performed with 0.5, 1, and 2 s saturation times (by concatenation of 50-ms Gaussian pulses separated by 1 ms), whereas the STD TOCSY experiment was performed only with 2 s. In all cases, the on-resonance frequency was adjusted at 6.8 ppm.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic protocols used to prepare oligosaccharides and their biotin-tagged derivatives. Sensorgrams of interaction of biotin-tagged oligosaccharides with mAbs and kinetic data. Copies of NMR spectra. Complete references 49 and 54. This material is available free of charge via the Internet at http://pubs.acs.org.

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