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Saturation Transfer Difference 1D-TOCSY Experiments to Map the Topography of Oligosaccharides Recognized by a Monoclonal Antibody Directed Against the Cell-Wall Polysaccharide of Group A *Streptococcus*

Margaret A. Johnson* and B. Mario Pinto*

Contribution from the Departments of Chemistry and of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

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Abstract: A new saturation transfer difference 1D-TOCSY NMR experiment that allows the investigation of complex ligands interacting with proteins and its application in the mapping of which portions of oligosaccharide ligands (epitope) interact with a complementary antibody are described. The interaction between trisaccharide and hexasaccharide ligands, corresponding to fragments of the cell-wall polysaccharide of *Streptococcus* Group A, and a monoclonal antibody directed against the polysaccharide is investigated at the molecular level. The polysaccharide consists of alternating α-(1→2) and α-(1→3) linked L-rhamnopyranose (Rha) residues with branching *N*-acetyl-D-glucopyranosylamine (Glc*N*Ac) residues linked β-(1→3) to alternate rhamnopyranose rings. The epitope is proven to consist not only of the immunodominant Glc*N*Ac sugar but also of an entire branched trisaccharide repeating unit. The experimental NMR data serve to check and validate the computed models of the oligosaccharide−antibody complexes.

1. Introduction

Current trends in vaccine strategies include the development of chemically well-defined, synthetic vaccines. The use of pure protein or glycoconjugate vaccines avoids potential adverse reactions due to contamination and the potential risks of live attenuated organisms. Group A *Streptococcus* (GAS) are widespread human pathogens, causing streptococcal pharyngitis (strep throat), some forms of pneumonia, streptococcal toxic shock syndrome (STSS), and necrotizing fasciitis or flesh-eating disease.[1] Streptococcal infections may lead to further complications such as rheumatic fever, scarlet fever, rheumatic carditis, heart valve disease, and acute glomerulonephritis.[2−5] A vaccine strategy would be greatly preferred over the current antibiotic strategy, to avoid the possible development of antibiotic resistance.[6,7] In addition, in the past decade, there has been an alarming increase in the incidence of rheumatic fever[8] and of the invasive infections such as necrotizing fasciitis, STSS, and myositis.[1b−d] Current therapy for these conditions involves high doses of antibiotics and intravenous administration of immunoglobulins. Despite the use of aggressive surgery, the mortality rate for these infections remains high.[1b]

The cell-wall polysaccharide of GAS is an ideal target for vaccine development, and the effectiveness of GAS polysaccharide conjugates as vaccines has also been demonstrated.[9] However, the use of chemically well-defined oligosaccharide topographies, presented at high density as a glycoconjugate, might be preferred to elicit discriminating immune responses.[10,11] We describe here the use of NMR methods to define the critical portions of the oligosaccharide recognized by a monoclonal antibody directed against the cell-wall polysaccharide. Such knowledge can be used to advantage in the design of the next-generation vaccines.

The cell-wall polysaccharide of GAS consists of a poly-α-L-rhamnopyranosyl backbone with alternating (1→2) and (1→3) glycosidic linkages. Branching β-D-*N*-acetylglucopyranosylamine residues are attached at the 3-position of alternate rhamnopyranose residues (Figure 1).[12,13] The synthesis of oligosaccharide fragments of this polysaccharide has been described.[14−16] These oligosaccharides and the corresponding

* To whom correspondence may be addressed. Telephone: (604) 291-4327/5650. Fax: (604) 291−5424. E-mail: bpinto@sfu.ca; johnsont@sfu.ca.

(1) (a) *Streptococcal Diseases and the Immune Response*; Read, S. E., Zabriskie, J. B., Eds.: Academic Press: New York, 1980. (b) Weiss, K. A.; Laverdiere, M. *Can. J. Surg.* **1997**, *40*, 18−25. (c) Stevens, D. L. *Immunol. Invest.* **1997**, *26*, 129−150. (d) Nowak, R. *Science* **1994**, *264*, 1665.
(2) Bisno, A. L. Nonsuppurative Poststreptococcal Sequelae: Rheumatic Fever and Glomerulonephritis; Mandell, G. L., Douglas, R. G., Bennett, J. E., Eds.; In *Principles and Practice of Infectious Diseases*, 2nd ed.; Wiley: New York, 1985; pp 1133−1142.
(3) World Health Organization: Community Control of Rheumatic Heart Disease in Developing Countries. 1. A Major Public Health Problem, *WHO Chron.* **1980**, *34*, 336−345. Markowitz, M. *Clin. Ther.* **1981**, *4*, 240−251.
(4) Wannamaker, L. W. *Rev. Infect. Dis.* **1979**, *1*, 967−975.
(5) Zabriskie, J. B. *Circulation* **1985**, *71*, 1077−1086.
(6) Wright, K. *Science* **1990**, *249*, 22−24.
(7) Rotta, J. Streptococci: Prospects for Vaccines; Bell, R., Torrigiani, G., Eds.; In *Towards Better Carbohydrate Vaccines*; John Wiley & Sons: New York, 1987; pp 203−218.
(8) Bronze, M. S.; Dale, J. B. *Am. J. Med. Sci.* **1996**, *311*, 41−54.
(9) Blake, M. S.; Zabriskie, J. B.; Tai, J. Y.; Michon, F. U.S. Patent 5,866,-135, 1999.
(10) Benaissa-Trouw, B.; Lefeber, D. J.; Kamerling, J. P.; Vliegenthart, J. F. G.; Kraaijeveld, K.; Snippe, H. *Infect. Immun.* **2001**, *69*, 4698−4701.
(11) Pozsgay, V. Oligosaccharide-Protein Conjugates as Vaccine Candidates Against Bacteria, *Adv. Carbohydr. Chem. Biochem.* **2001**, *56*, 153−199.
(12) Coligan, J. E.; Kindt, T. J.; Krause, R. M. *Immunochemistry* **1978**, *15*, 755−760.
(13) Huang, D. H.; Krishna, N. R.; Pritchard, D. G. *Carbohydr. Res.* **1986**, *155*, 193−199.

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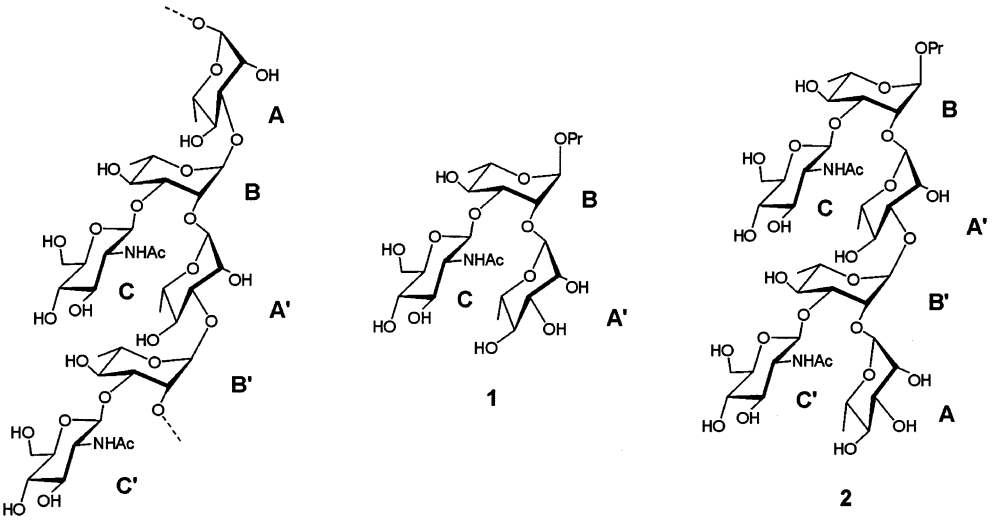

Figure 1. Structures of the Group A *Streptococcus* cell-wall polysaccharide and of branched trisaccharide (**1**) and hexasaccharide (**2**) fragments recognized by antibodies.

glycoconjugates[17−19] have been used to select and characterize polyclonal antibodies and to develop monoclonal antibodies.[17−21]

Glc*N*Ac has been characterized as the immunodominant monosaccharide component of the cell-wall polysaccharide,[21] defined as the sugar that contributes the majority of binding energy.[22] However, it is important to note that the immunodominant sugar is only part of a larger topographical surface.[22,23] Immunochemical studies have indicated that the branched trisaccharide unit, Rha-α-(1→2)-(Glc*N*Ac-*β*-(1→3)-)Rha, is required for even moderate binding to most anti-GAS antibodies (Abs), and optimal binding is achieved by the extended topography presented by tetra- to hexasaccharide ligands.[14,17,20,21] Correspondingly, although anti-GAS polyclonal and monoclonal Abs may bind weakly to Glc*N*Ac,[20,21,24,25] anti-GAS antibodies *are not elicited by immunization with glycoconjugates comprising less than a pentasaccharide*.[17]

The use of NMR spectroscopy allows an unambiguous characterization of protein−ligand interactions at the molecular level. Thus, a combination of transferred NOE (trNOE)[26] and saturation transfer difference (STD)[27−30] experiments has been used to probe the conformations of ligands bound to receptors and to define the critical epitope, that is, the portions of the ligand in close contact with the protein.[31] In addition, STD-NMR experiments have been used to screen complex mixtures of compounds for ligands that bind.[28] Vogtherr and Peters[32] have described the use of STD-NMR, in combination with chemical modification, for the simultaneous epitope mapping of a library of ligands, and the use of STD-2D-NMR methods, for example, STD-TOCSY and STD-HMQC.

We report here the design of a new STD-1D-TOCSY experiment that permits the rapid, selective investigation of enhancements in crowded regions of an NMR spectrum. The selective 1D analogue of 2D-TOCSY allows the selection of particular spin systems out of a complex spectrum. The use of a 1D-TOCSY sequence over the 2D variant offers a significant saving in experimental time. This feature is particularly important in the case of difference experiments such as STD-NMR in which observed enhancements are often weak, and a useful signal-to-noise ratio is only achieved by averaging a large number of scans. These experiments, together with trNOE experiments, are applied to the investigation of an important immunological problem, namely the interaction between an anti-GAS monoclonal antibody, Strep 9,[21] and the oligosaccharide ligands **1** and **2** (Figure 1). The problem is of significance because of the need for highly defined, targeted vaccines that avoid autoimmune reactions[2−5,25] to GAS.

(14) Pinto, B. M. Synthesis and Immunochemistry of Carbohydrate Antigens of the *β*-Hemolytic *Streptococcus* Group A; In *Carbohydrate Antigens*; Garegg, P. J., Lindberg, A. A., Eds.; ACS Symposium Series 519; American Chemical Society: Washington, DC, 1992; pp 111−131 and references cited.
(15) Marino-Albernas, J.-R.; Harris, S. L.; Varma, V.; Pinto, B. M. *Carbohydr. Res.* **1993**, *245*, 245−257.
(16) Auzanneau, F.-I.; Forooghian, F.; Pinto, B. M. *Carbohydr. Res.* **1996**, *291*, 21−41.
(17) Reimer, K. B.; Gidney, M. A. J.; Bundle, D. R.; Pinto, B. M. *Carbohydr. Res.* **1992**, *232*, 131−142.
(18) Auzanneau, F.-I.; Pinto, B. M. *Bioorg. Med. Chem.* **1996**, *4*, 2003−2010.
(19) Auzanneau, F.-I.; Christensen, M. K.; Harris, S. L.; Meldal, M.; Pinto, B. M. *Can. J. Chem.* **1998**, *76*, 1109−1118.
(20) Harris, S. L.; Craig, L.; Mehroke, J. S.; Rashed, M.; Zwick, M. B.; Kenar, K.; Toone, E. J.; Greenspan, N.; Auzanneau, F.-I.; Marino-Albernas, J.-R.; Pinto, B. M.; Scott, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2454−2459.
(21) Pitner, J. B.; Beyer, W. F.; Venetta, T. M.; Nycz, C.; Mitchell, M. J.; Harris, S. L.; Marino-Albernas, J.-R.; Auzanneau, F.-I.; Forooghian, F.; Pinto, B. M. *Carbohydr. Res.* **2000**, *324*, 17−29.
(22) Kabat, E. A. *Structural Concepts in Immunology and Immunochemistry*. Holt, Rinehart and Winston, Inc.: New York, 1968; pp 88−132.
(23) Jennings, H. J. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 155−208.
(24) Shikhman, A. R.; Cunningham, M. W. *J. Immunol.* **1994**, *152*, 4375−4387.
(25) Cunningham, M. W. In *Molecular Mimicry, Microbes, and Autoimmunity*; Cunningham, M. W., Fujinami, R. S., Eds.; ASM Press: Washington, DC, 2000; pp 39−56 and references therein.
(26) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1983**, *53*, 423−442. Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402−417. Albrand, J. P.; Birdsall, B.; Feeney, J.; Roberts, G. C. K.; Burgen, A. S. V. *Int. J. Biol. Macromol.* **1979**, *1*, 37−41. Balaram, P.; Bothner-By, A. A.; Dadok, J. *J. Am. Chem. Soc.* **1972**, *94*, 4015−4016. Balaram, P.; Bothner-By, A. A.; Breslow, E. *J. Am. Chem. Soc.* **1972**, *94*, 4017−4018.
(27) Mayer, M.; Meyer, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 1784−1788.
(28) Klein, J.; Meinecke, R.; Mayer, M.; Meyer, B. *J. Am. Chem. Soc.* **1999**, *121*, 5336−5337.
(29) Peters, T.; Meyer, B. German Patent No. DE19649359; Swiss Patent No. 690695; U.K. Patent No. GB23211401; U.S. Patent pending.
(30) Mayer, M.; Meyer, B. *J. Am. Chem. Soc.* **2001**, *123*, 6108−6117.
(31) Möller, H.; Serttas, N.; Paulsen, H.; Burchell, J. M.; Taylor-Papadimitriou, J.; Meyer, B. *Eur. J. Biochem.* **2002**, *269*, 1444−1455. Kooistra, O.; Herfurth, L.; Lüneberg, E.; Frosch, M.; Peters, T.; Zähringer, U. *Eur. J. Biochem.* **2002**, *269*, 573−582. Haselhorst, T.; Weimar, T.; Peters, T. *J. Am. Chem. Soc.* **2001**, *123*, 10705−10714. Meinecke, R.; Meyer, B. *J. Med. Chem.* **2001**, *44*, 3059−3065. Weimar, T.; Bukowski, R.; Young, N. M. *J. Biol. Chem.* **2000**, *275*, 37006−37010. Maaheimo, H.; Kosma, P.; Brade, L.; Brade, H.; Peters, T. *Biochemistry* **2000**, *39*, 12778−12788.
(32) Vogtherr, M.; Peters, T. *J. Am. Chem. Soc.* **2000**, *122*, 6093−6099.

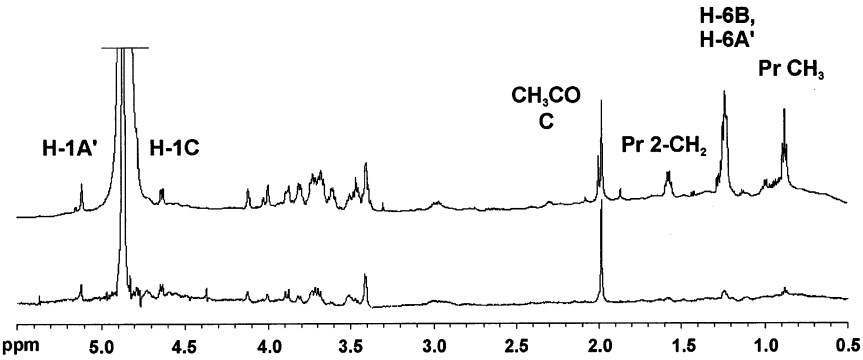

Figure 2. 1D ^{1}H NMR spectrum (top) and 1D STD-NMR spectrum (bottom) of trisaccharide **1** in the presence of the antibody. Intensities are given in Table 1.

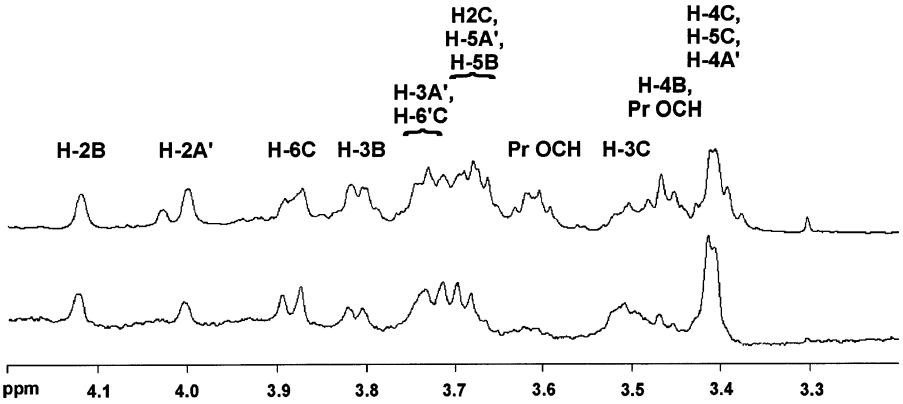

Figure 3. Expansion of a crowded region of the 1D ^{1}H NMR spectrum (top) and STD-NMR spectrum (bottom) of trisaccharide **1** in the presence of the antibody, containing enhancements to be investigated by STD-1D-TOCSY.

2. Results and Discussion

The saturation transfer difference NMR spectrum of the branched trisaccharide **1** in the presence of the antibody revealed numerous enhancements (Figures 2,3). The strongest enhancement occurred for the Glc*N*Ac *N*-acetyl methyl group, with an intensity of 15% in the saturated spectrum relative to a control spectrum. The intensity of this enhancement was normalized to 100%, and those of the other resonances measured relative to it, to facilitate a comparison of enhancements within the ligand.[32] The intensities are presented in Table 1.

The enhancements at 3.67−3.69 ppm, 3.71−3.73 ppm, 3.43−3.47 ppm, and 3.39−3.41 ppm are ambiguous in that multiple resonances are present at these chemical shifts (Table 1, Figure 3). To identify the contributions of each resonance to the observed enhancements, we developed an STD-1D-TOCSY experiment (Figure 4). The experiment is based on the 1D-TOCSY pulse sequence of Fäcke and Berger,[33] with gradient-enhanced selection of the resonance of interest using a 180° shaped pulse, isotropic mixing during a spin-lock period using the MLEV-16 composite pulse,[34] followed by rephasing. Selective saturation of the protein resonances is achieved by the application of a series of Gaussian pulses, at $\gamma B_1/2\pi \cong 100$ Hz. These pulses are applied at a frequency chosen to coincide with broad protein resonances without affecting ligand resonances, for example, 9−11 ppm or 0 to −1 ppm. The saturation is spread quickly through the protein by spin diffusion. Intermolecular NOE transfer then causes a decrease in the intensities of ligand resonances in close contact with the protein, observed as enhancements in the difference spectrum. In the case of 1D-TOCSY, the magnetization of a selectively excited resonance is distributed to coupled spins by isotropic mixing. If the intensity of this resonance is decreased by intermolecular NOE, lower intensities for the coupled spins will also result. This may be observed as enhancements in the difference spectrum, and the characteristic pattern of the spin system allows identification of the enhanced resonance observed in the simple 1D spectrum. Control experiments in the absence of antibody showed complete cancellation of signals in the difference spectra.

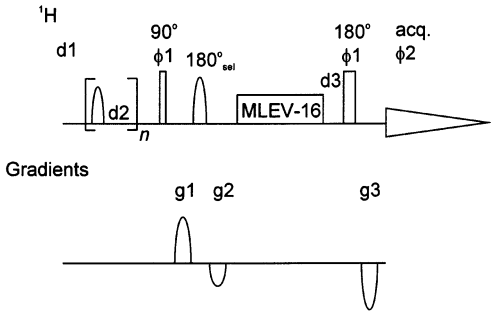

Figure 4. Pulse sequence for STD-1D-TOCSY. Selective saturation of the protein is performed with a series of Gaussian pulses separated by a short delay d2 (typically 1 ms). Selective excitation of the resonance of interest, and phase labeling by gradient pulses, is then accomplished by the series 90°−g1−180°$_{sel}$−g2, followed by isotropic mixing using the MLEV-16 composite spin-lock pulse. The gradient pulse strength ratio is 7:−3:−10. On alternate scans, saturation is applied far from protein resonances, and subtraction is accomplished by phase cycling, i.e., $\phi 1 = x, -x$; $\phi 2 = x, x$. d1 is an optional additional relaxation delay, while d3 is set equal to the length of the last gradient (typically 2 ms).

(33) Fäcke, T.; Berger, S. *J. Magn. Reson. A* **1995**, *113*, 257−259.
(34) Levitt, M. H.; Freeman, R.; Frenkiel, T. *J. Magn. Reson.* **1982**, *47*, 328−330.

Table 1. STD-NMR Intensities[a,b] of the Oligosaccharides **1** and **2** in the Presence of the Monoclonal Antibody Strep 9

	resonance	enhancement (%) in **1**	enhancement (%) in **2** (A′, A; B, B′; C, C′)
A′	H-1A′	55	82, 92
	H-2A′	32	87, 87
	H-6A′	6	41, 48
B	H-1B	n.d.[c]	81, 81
	H-2B	46	85, 79
	H-3B	32	*b*, 81
	H-6B	10	48, 47
C	H-1C	81	82, 77
	H-3C	74	*b*, *b*
	H-6C	66	*b*, 59
	CH_3CO C	100	100, 82[d]
Pr	Pr OCH	15	*b*
	Pr 2-CH_2	12	59
	Pr 3-CH_3	9	68
	multiplets (**1**)		
	H-3A′, H-6′C	48	
	H-5A′/B, H-2C	44	
	H-4B, Pr OCH	28	
	H-4A′, H-4/5C	68	
	multiplets (**2**)		
	H-3A/B, H-6C		81
	H-3A′, H-5A/B′, H-2C′, H-6′C/C′		72
	H-5B, H-2C		92
	H-5A′, Pr OCH		69
	H-4A/B/B′, H-3C/C′, Pr OCH		92
	H-4C/C′, H-5C/C′, H-4A′		84

[a] Intensities are given in %, with respect to a reference 1D spectrum, and normalized to the *N*-acetyl methyl group of the Glc*N*Ac C ring. [b] The resonances of these protons were coincident with other resonances; therefore, their enhancements could not be measured directly from the 1D STD-NMR spectrum. Enhancements for these multiplets are given separately. [c] Not determined because of interference by the HOD resonance. [d] CH_3CO methyl signals were not regiospecifically assigned to the C and C′ rings.

The enhanced multiplet at 3.39−3.41 ppm (Figures 2,3) could comprise contributions from one or all of H-4C (3.41 ppm), H-5C (3.40 ppm), and H-4A′ (3.39 ppm). A 1D-TOCSY experiment with selective excitation at this frequency identifies both C and A′ spin systems. The STD-1D-TOCSY experiment also identifies resonances of both spin systems, indicating that H-4/5C and H-4A′ both contribute to the enhancement (Figure 5B). The C ring resonances appear more strongly enhanced, while H-6A′ shows a weaker enhancement, and there is no visible enhancement of H-2A′ (Figure 5B, Table 2). Comparisons are made relative to the 1D-TOCSY spectrum without saturation of the protein, to correct for different resonance intensities.

The enhanced multiplet at 3.71−3.73 ppm (Figures 2,3) with possible contributions from H-3A′ and H-6′C was investigated in the same manner. As shown in Figure 5C, weak enhancements of H-6C, H-2A′, and H-6A′ are apparent, showing that both H-3A′ and H-6′C contribute to the weak enhancement in the 1D STD spectrum. Contributions from several resonances were also apparent in STD-1D-TOCSY spectra with selective excitation at 3.67−3.69 ppm and at 3.43−3.47 ppm (data not shown).

Figure 5D shows the 1D-TOCSY and STD-1D-TOCSY spectra resulting from the selective excitation of one of the 1-methylene protons of the propyl group, at 3.6 ppm. This enhancement is very weak, as shown in Figure 5A,D. Slightly stronger enhancements of the propyl methyl and 2-methylene

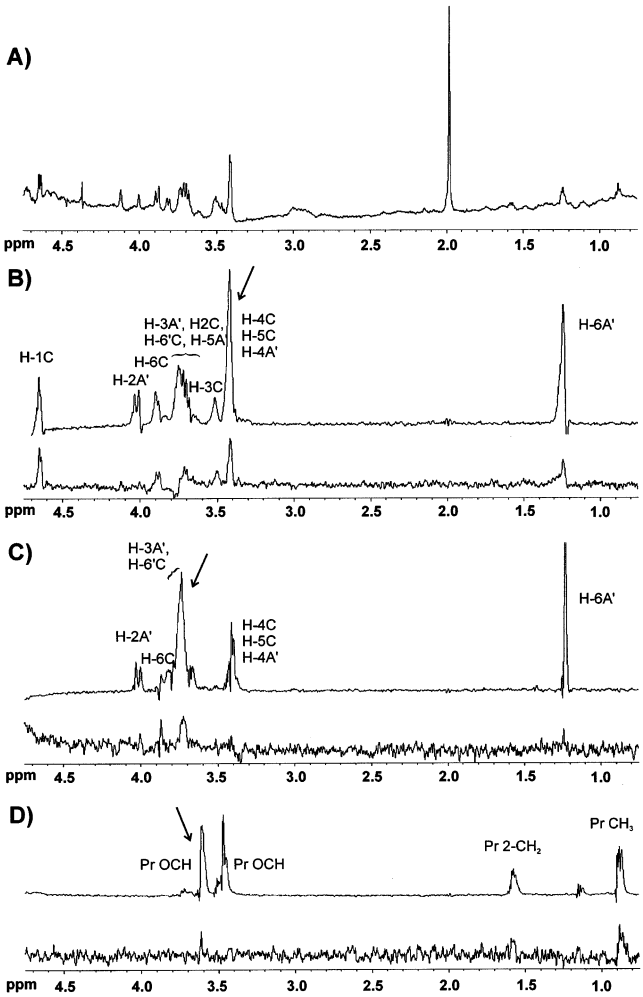

Figure 5. (A) 1D STD-NMR spectrum of trisaccharide **1** in the presence of the antibody. (B) 1D-TOCSY (top) and STD-1D-TOCSY (bottom) spectra with selective excitation at 3.4 ppm and a mixing time of 110 ms. The frequency of selective excitation is indicated by the arrow. Resonances of the C and A′ spin systems appear in both spectra, while enhancements of the C ring resonances are stronger in the STD-1D-TOCSY spectrum. (C) 1D-TOCSY (top) and STD-1D-TOCSY (bottom) spectra with selective excitation at 3.73 ppm and a mixing time of 62 ms. Enhancements of the A′ and C rings indicate that both H-3A′ and H-6′C contribute to the observed enhancement at this frequency. (D) 1D-TOCSY (top) and STD-1D-TOCSY (bottom) spectra with selective excitation of a propyl 1-methylene proton at 3.6 ppm and a mixing time of 62 ms. Enhancements of these methylene protons are very weak in the STD-1D-TOCSY spectrum, and TOCSY transfer to the 2-methylene protons (1.57 ppm) and methyl protons (0.87 ppm) reveal slightly stronger enhancements of these groups. Intensities are given in Table 2.

groups are observed, demonstrating that one can select a resonance that is not enhanced or only weakly enhanced and use TOCSY transfer to identify other resonances in the spin system that are more strongly enhanced (Table 2).

The observed enhancements were mapped onto the bound conformation of **1**, determined previously by trNOE NMR spectroscopy[35,36] (Figure 6A,B). The protons that contribute to the epitope have been colored according to the degree of enhancement in the STD spectra, corresponding to the extent of contact with the complementary antibody surface.

The hexasaccharide **2** in the presence of mAb Strep 9 was also investigated by STD-NMR spectroscopy. The STD-NMR

(35) Weimar, T.; Harris, S. L.; Pitner, J. B.; Bock, K.; Pinto, B. M. *Biochemistry* **1995**, *34*, 13672−13681.

Table 2. STD-1D-TOCSY Intensities[a] of the Branched Trisaccharide **1** in the Presence of the Monoclonal Antibody Strep 9, for the Spectra Shown in Figure 5

resonance (Figure 5B)	enhancement (%)	resonance (Figure 5C)	enhancement (%)	resonance (Figure 5D)	enhancement (%)
H-1C	235	H-2A′	222	**Pr OCH**	**100**
H-2A′	0	H-6C	535	Pr OCH	0
H-6C	181	**H-3A′/H-6′C**	**100**	Pr 2-CH_2	196
H-3A′/H-6′C	0	H-4C/H-5C/H-4A′	74	Pr CH_3	178
H-2C/H-5A′	119	H-6A′	45		
H-3C	167				
H-4C/H-5C/H-4A′	**100**				
H-6A′	72				

[a] Intensities in %, with respect to a reference 1D-TOCSY spectrum, and normalized to the intensity of the selectively excited resonance, indicated in bold.

Table 3. STD-1D-TOCSY Intensities[a] of the Hexasaccharide **2** in the Presence of the Monoclonal Antibody Strep 9, for the Spectrum Shown in Figure 8

resonance	enhancement (%)
H-1C′	53
H-1C	136
H-2A′	178
H-6C/C′	101
H-3A′, H-6′C/C′, H-2C/C′	93
H-5A′	143
H-3C/C′	80
H-4C/C′, H-5C/C′, H-4A′	**100**
H-6A′	159

[a] Intensities in %, with respect to a reference 1D-TOCSY spectrum, and normalized to the intensity of the selectively excited resonance, indicated in bold.

spectrum showed enhancements of all resonances (Figure 7), indicating much stronger binding than that of the trisaccharide **1**. Correspondingly, IC_{50} values for **1** and **2** are 135 μM and 27 μM, respectively.[21] STD-1D-TOCSY spectra recorded with selective excitation at several different frequencies (for example, Table 3, Figure 8) showed contributions from all resonances at that frequency. Therefore, as in the case of the trisaccharide, all residues contact the antibody and are required for binding. These data are consistent with immunochemical studies, which showed that strong binding to anti-GAS antibodies is achieved by the extended topography presented by a hexasaccharide.[17,20,21]

A similar pattern of STD-NMR intensities to the trisaccharide **1** was observed, in that resonances of the Glc*N*Ac (C, C′) rings were most strongly enhanced, while resonances of the rhamnopyranose (A, A′, B, B′) rings were more weakly enhanced. In particular, the Rha C-6 methyl groups and the propyl group showed the weakest enhancements in both **1** and **2**. These observations indicate that the two branched trisaccharide units behave similarly in binding to the antibody: the Glc*N*Ac methyl groups are involved in intimate contact with the binding site, while the Rha residues define a characteristic, extended surface, but with minimal contact to the Rha methyl groups. We suggest that the critical epitope of the Group A *Streptococcus* polysaccharide is provided by the trisaccharide **1** and that its presentation in an extended surface is advantageous.

A recent theoretical study[38] of the STD-NMR experiment showed that STD-NMR intensities exhibit a complex dependence on correlation times, relaxation times, exchange rates, and the detailed molecular structure of the protein−ligand complex, but that intensities more closely reflect exact ligand proton−protein proton distances when shorter saturation times (<1 s) are used.[38] The effect of varying saturation time and power on the STD-NMR intensities of both oligosaccharide ligands was investigated. No significant changes in the relative intensities of enhancements were found. Thus, when the saturation power was reduced to $\gamma B_1/2\pi = 34$ Hz or the saturation time was decreased to 500 ms, enhancements of all resonances of **2** were still observed. These observations demonstrate that the strong enhancements are unlikely to be caused by intraligand

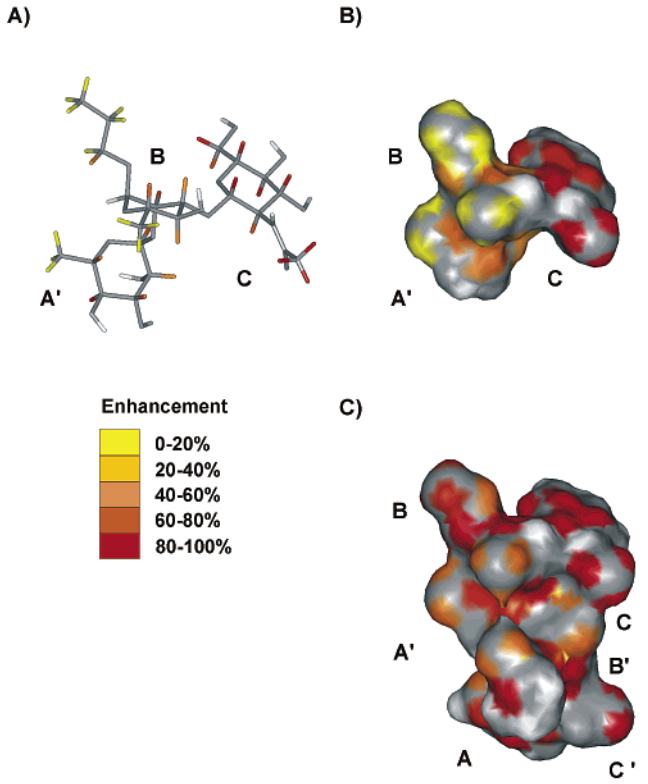

Figure 6. (A) Structure of trisaccharide **1** in the bound conformation derived by trNOE, with protons enhanced in the STD-NMR and STD-1D-TOCSY spectra, which form the epitope, colored according to the degree of enhancement in the STD spectra. (B) A molecular surface representation of the epitope. (C) A molecular surface representation of the hexasaccharide **2**, with the top branched trisaccharide unit in the same orientation as those in parts A and B, colored according to the degree of enhancement in the STD spectra.

(36) The torsional angles describing a local minimum conformation of **1**, which is bound by the antibody, were reported incorrectly in ref 35. Therefore, for the purposes of this study, the bound conformation of **1** was obtained from ref 35 and subjected to 100 steps of energy minimization, using the CVFF force field[37] within InsightII/Discover (Accelrys, Inc.). Agreement with experimentally derived distances was maintained, and the resulting torsional angles were (ϕ,ψ) = (41°,−31°) for the α-(1→2) linkage and (49°,−12°) for the β-(1→3) linkage (where ϕ = H1−C1−O*x*−C*x*, ψ = C1−O*x*−C*x*−H*x*, and *x* is the linkage position).

(37) Dauber-Osguthorpe, P.; Roberts, V. A.; Osguthorpe, D. J.; Wolff, J.; Genest, M.; Hagler, A. T. *Proteins: Struct., Funct., Genet.* **1988**, *4*, 31−47.
(38) Jayalakshmi, V.; Krishna, N. R. *J. Magn. Reson.* **2002**, *155*, 106−118.

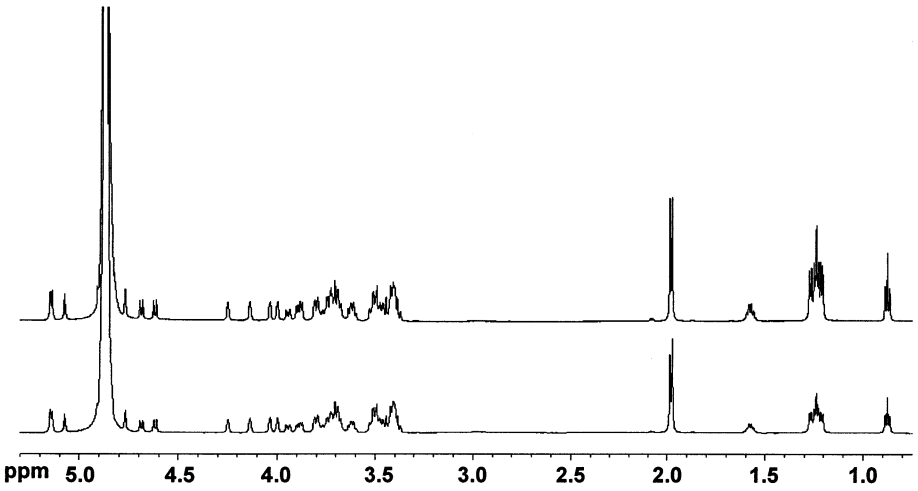

Figure 7. 1D ^{1}H NMR spectrum (top) and 1D STD-NMR spectrum (bottom) of hexasaccharide **2** in the presence of the antibody. All resonances are strongly enhanced.

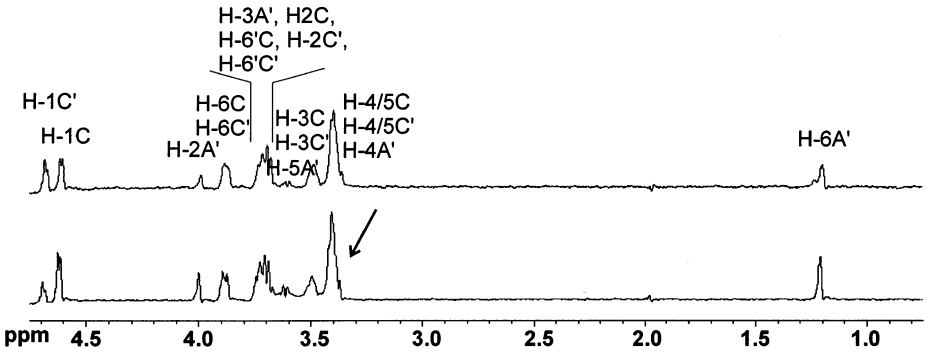

Figure 8. 1D-TOCSY (top) and STD-1D-TOCSY (bottom) spectra of hexasaccharide **2** in the presence of the antibody, with the selective excitation of H-4A′, H-4C/C′, and H-5C/C′ at 3.39−3.41 ppm (indicated by the arrow). Intensities are given in Table 3.

spin diffusion but rather reflect *intermolecular* spin diffusion indicative of close contact with the antibody combining site.

The bound conformation of **1**, derived from trNOE studies,[35,36] was used to construct a model of the hexasaccharide **2**, whose conformation could not be determined by trNOE because of unfavorable binding kinetics. The Rha-α-(1→3)-Rha (B′−A′) linkage was constructed using (ϕ,ψ) angles determined by Metropolis Monte Carlo[39] and molecular dynamics simulations[40] of the corresponding free oligosaccharides. The observed STD-NMR enhancements were mapped onto this model (Figure 6C). The molecular surface presented by the hexasaccharide and strong enhancements of the Glc*N*Ac residues, presented on one face of the molecule, are apparent.

The knowledge of the bound ligand conformation from trNOE NMR experiments, together with epitope mapping by STD-NMR experiments, can be used to guide the computer-simulated docking of ligands to proteins. A model of the complex between mAb Strep 9 and a heptasaccharide derived from the cell-wall polysaccharide exists.[21] The STD-NMR data reported here are consistent with this model, in which Glc*N*Ac residues are buried in deep pockets within the binding groove, and the rhamnopyranose rings contact the sides of the groove, while the Rha methyl groups are partially exposed. The present work confirms that the critical portion of the cell-wall polysaccharide, namely the branched trisaccharide, forms close contacts with complementary residues in the combining site. The data provide conclusive proof of the epitope or topography of the cell-wall polysaccharide of group A *Streptococcus* recognized by this antibody.

3. Experimental Section

NMR Spectroscopy. NMR spectra were recorded on a Bruker AMX-600 NMR spectrometer. Chemical shifts were referred to external 3-(trimethylsilyl)-1-propanesulfonic acid (DSS). The NMR assignments for **1** and **2** have been described in refs 41 and 42. NMR samples were prepared in phosphate-buffered saline solution (10 mM KPO_4, 50 mM KCl, 0.1 mM NaN_3, pH 7.2, 99.9% D_2O) with ratios of 14:1 **1**/antibody binding sites (2.2 mM **1**, 0.08 mM antibody)[35] and 16:1 **2**/antibody binding sites (4.2 mM **2**, 0.13 mM antibody). 1D spectra were recorded with 256 or 1024 scans and 32K data points and processed by zero filling to 64K points and multiplication by an exponential function, followed by Fourier transformation. After preliminary experiments, it was determined that STD-NMR intensities were greater at 284 K than at 295 K, and all 1D STD-NMR and STD-1D-TOCSY spectra were therefore recorded at 284 K. 1D STD-NMR spectra were recorded with 2048 or 4096 scans, with selective saturation of protein resonances at 9 ppm (30 or 50 ppm for reference spectra) using a series of 40 Gaussian shaped pulses (50 ms, 1 ms delay between pulses, $\gamma B_1/2\pi = 110$ Hz), for a total saturation time of 2.04 s. For experiments with **1**/antibody,

(39) Stuike-Prill, R.; Pinto, B. M. *Carbohydr. Res.* **1995**, *279*, 59−73.
(40) Kreis, U. C.; Varma, V.; Pinto, B. M. *Int. J. Biol. Macromol.* **1995**, *17*, 117−130.
(41) Pinto, B. M.; Reimer, K. B.; Tixidre, A. *Carbohydr. Res.* **1991**, *210*, 199−219.
(42) Reimer, K. B.; Harris, S. L.; Varma, V.; Pinto, B. M. *Carbohydr. Res.* **1992**, *228*, 399−414.

a 10 ms spin-lock pulse ($\gamma B_1/2\pi = 11$ kHz) was applied after excitation to reduce the intensity of broad protein resonances. Subtraction of saturated spectra from reference spectra was performed by phase cycling.[27,28] Measurement of enhancement intensities was performed by direct comparison of STD-NMR spectra and reference 1D spectra.

STD-1D-TOCSY spectra were recorded using the pulse sequence of Figure 4, with 4096 scans, and selective saturation of the protein as described earlier ($n = 40$, d2 = 1 ms). The MLEV-16 composite pulse[34] was used for isotropic mixing with a power level of $\gamma B_1/2\pi = 11$ kHz. Selective inversion of the resonance of interest was achieved using an 80 ms Gaussian pulse, with 70−71 dB attenuation ($\gamma B_1/2\pi = 11$ Hz). Gradient pulses were of duration 2 ms and strength ratio 7:−3:−10 (where 10% strength = 6.6 G/cm) and were followed by 100 μs ring-down delays. The additional relaxation delay was minimal (1 ms), and the rephasing delay d3 was set equal to the length of the last gradient and surrounding power-switching and ring-down delays (2.16 ms).

4. Conclusions

The oligosaccharides **1** and **2**, representing portions of the cell-wall polysaccharide of Group A *Streptococcus*, bind to mAb Strep 9 with an epitope comprising all monosaccharide residues. The strongest enhancements in trisaccharide **1** were observed for the C Glc*N*Ac residue, but enhancements of the A′ and B rhamnose rings were also apparent. The importance of the entire antigenic determinant, and not only the immunodominant sugar, in interaction with an antibody, has been demonstrated. A detailed characterization of the epitope has been performed by combining trNOE NMR spectroscopy, for the determination of the bound conformation, and STD-NMR, for the determination of the interacting atoms of the ligand. STD-1D-TOCSY allows the detailed mapping of resonances within crowded regions of an NMR spectrum.

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