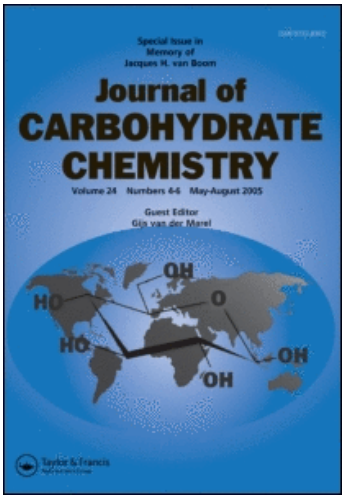

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CONFORMATIONAL ANALYSIS OF A COMPLEX BETWEEN *DOLICHOS BIFLORUS* LECTIN AND THE FORSSMAN PENTASACCHARIDE USING TRANSFERRED NOE BUILD-UP CURVES

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ABSTRACT

A complex between the Forssman pentasaccharide α-D-GalNAc-(1→3)-β-D-GalNAc-(1→3)-α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc and the seed lectin from *Dolichos biflorus* was studied using 2D transfer-NOESY (trNOESY) experiments. Experimental transfer-NOE (trNOE) build-up curves of the complex were compared to theoretical values from a complete relaxation and conformational exchange matrix (CORCEMA) calculation. The dissociation constant and the off-rate for the complex formation were determined by fitting experimental intra-glycosidic trNOE build-up curves to corresponding theoretical curves. Atomic coordinates for the complex were taken from a previous homology-modeling study of a complex between the disaccharide fragment α-D-GalNAc-(1→3)-β-D-GalNAc and *D. biflorus* lectin. The comparison of experimental and theoretical inter-glycosidic trNOEs expands our previous qualitative NMR study and allows further assessment of the quality of the homology modeling study.

INTRODUCTION

Specific interactions between carbohydrates and receptor proteins are the molecular basis of many biological recognition processes. An understanding of the specificity of protein-carbohydrate interactions requires a detailed analysis of the conformation of the bound carbohydrate ligand. Carbohydrate ligands are known to have low binding affinities, usually ranging between 10^3 and 10^6 M^{-1}. If the association rate is asumed to be diffusion controlled ($k_{on} \approx 10^8 M^{-1} s^{-1}$), off-rates in a range between 10^5 and 10^2 Hz are expected.[1] On the ^1H NMR relaxation time scale this corresponds to fast exchange, and, therefore, protein-carbohydrate complexes are ideal targets for so-called transfer-NOESY (trNOESY) experiments.[2-5] In principle observation of inter-glycosidic trNOEs provides an opportunity to determine the bioactive conformation of a bound carbohydrate ligand, if effects of spin diffusion are excluded. On the other hand, effects of spin diffusion contain valuable extra information, especially if relay protons from amino acid side chains in the binding pocket of the protein are involved. In order to treat spin diffusion quantitatively, several computational schemes based on a full relaxation matrix analysis have been designed.[2,6-9] A rather powerful algorithm that includes the treatment of chemical and conformational exchange, and explicitly treats protons located in the protein binding site has been put forward only recently by Krishna and coworkers.[10,11] Here, we applied this so called CORCEMA algorithm[10,11] for the analysis of a complex between the synthetic Forssman pentasaccharide, α-D-GalNAc-(1→3)-β-D-GalNAc-(1→3)-α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc[12] (Fig. 1), and the seed lectin from *D. biflorus*.[13]

RESULTS AND DISCUSSION

A conformational analysis of the free Forssman pentasaccharide in aqueous solution was reported in 1994 by Grönberg and coworkers,[14] and in a more recent study we described how trNOE and trROE experiments were used to map the size of the bound segment of the Forssman pentasaccharide in the binding site of *D. biflorus* lectin.[15] Especially from inter-molecular trNOEs between the saccharide ligand and the

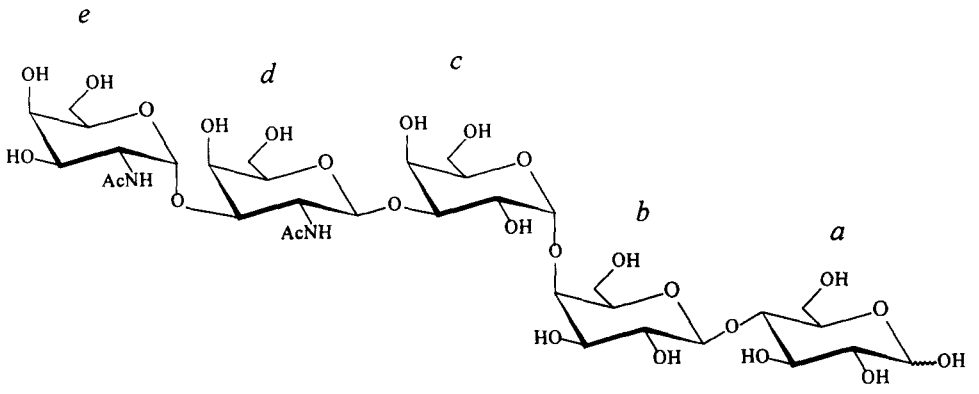

Figure 1. Schematic representation of the Forssman pentasaccharide (FPS) with the labelling of individual pyranose units as used in the text.

protein it is clear that the non-reducing disaccharide fragment α-D-GalNAc-(1→3)-β-D-GalNAc is in immediate contact with the protein binding site, whereas the remaining trisaccharide sticks out into solution. A model of the Forssman pentasaccharide *D. biflorus* complex was constructed on the basis of a previous homology modeling study[16] and the results of the conformational analysis of the free Forssman pentasaccharide.[14] Qualitatively, this model was in accordance with the trNOE and trROE experiments but no quantitative analysis had been performed.

To accomplish a quantitative assessment of the theoretical model of the complex trNOE build-up curves were measured at 315 K. At this temperature no NOEs for the free pentasaccharide are observed, and, therefore, in a quantitative evaluation contributions of the free ligand to the observed trNOE are neglected. A part of a trNOESY spectrum is shown in Fig. 2.

For the quantitative comparison, theoretical build-up curves were calculated using the program CORCEMA.[10,11] The program applies the full relaxation matrix approach and explicitly takes into account protons in the binding site of the protein, as well as effects of chemical and conformational exchange. For protein-carbohydrate complexes the full relaxation matrix approach also takes into account that chemical exchange has been already applied in the past (e.g.[17]). However, so far a complete

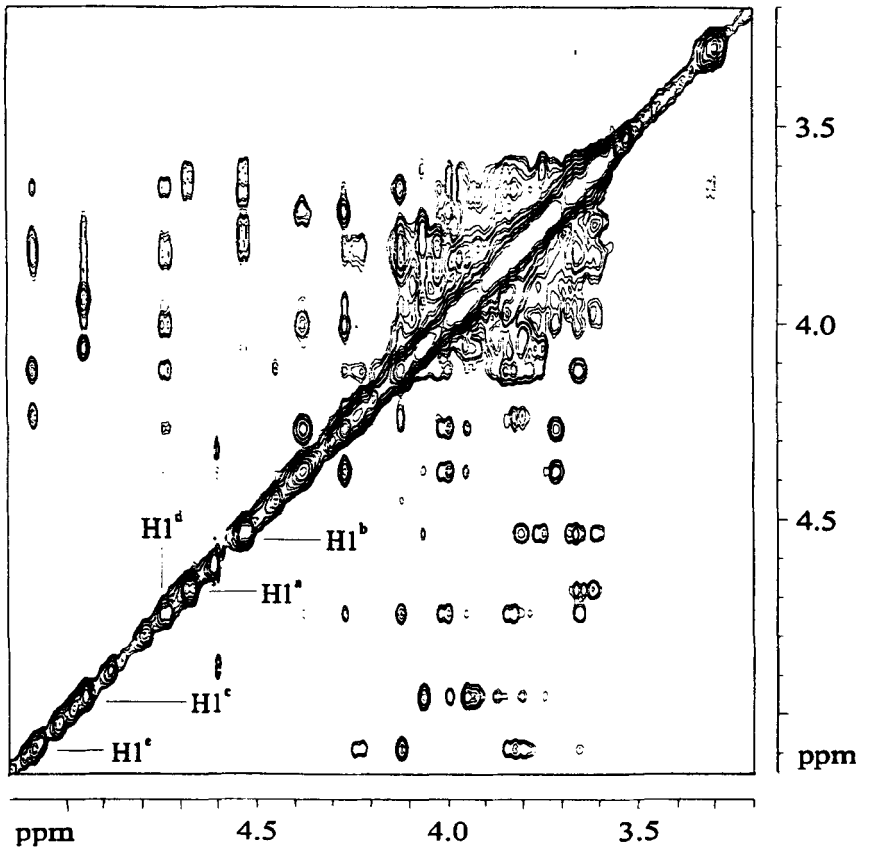

Figure 2. Contour plot of a 2D-transfer NOESY spectra of the complex FPS/DBL measured at 315 K in D_2O at 300 ms mixing time.

treatment that also explicitly includes binding site protons has only been performed for the blood group A trisaccharide bound to *D. biflorus* lectin.[18]

For the calculation of theoretical trNOEs overall isotropic motion of the complex with a correlation time τ_c of 50 ns was assumed, as this has been described before.[18,19] In general, free ligands have short correlation times ($\tau_c < 10^{-10}$ s), whereas bound ligands display the correlation time of the receptor protein ($\tau_c > 10^{-8}$ s). For the free pentasaccharide ligand a correlation time of 0.21 ns was used which is in accordance with all NOEs being very close to zero.

The conformation of the pentasaccharide was assumed to be fixed in the bound state and no internal motion of the trisaccharide fragment was considered. The off-rate k_{off} and the dissociation constant K_D were determined iteratively by fitting calculated intra-glycosidic trNOE curves against their experimental counterparts until a minimum R-factor was obtained (cf. experimental part). For the iterative fitting procedure the following intra-glycosidic trNOEs were chosen: $H1^d$-$H3^d$, $H1^c$-$H2^c$, $H1^c$-$H5^c$, $H1^b$-$H3^b$, $H1^b$-$H4^b$, and $H1^b$-$H5^b$.

Also, the lectin binding site protons described in the experimental part were included in the relaxation matrix during the fitting process. In Fig. 3 the corresponding experimental and theoretical build-up curves are displayed.

For the Forssman pentasaccharide three different types of trNOEs classified as intra-glycosidic, inter-glycosidic and protein-ligand trNOEs are observed. From these, the inter-glycosidic trNOEs are most important for the analysis of the bound conformation of the saccharide ligand. Protein-ligand trNOEs reflect the orientation of a ligand in the binding pocket of a protein, but there are only a few cases where such interactions have been observed for carbohydrate-protein complexes.[20,21] Unfortunately, the integration of the protein-ligand trNOEs of the *D. biflorus* lectin - Forssman pentasaccharide complex was rather difficult because of a poor signal to noise ratio, and, therefore we focused on the quantitative analysis of inter- and intra-glycosidic trNOEs. Moreover, a quantitative analysis of protein-ligand trNOEs would require the precise assignment of the protein protons involved which was not possible in the present case.

For a quantitative comparison all trNOEs were calculated using the parameters that have been derived from the fitting procedure described above. A quantitative comparsion between experimental and theoretical inter- and intra-glycosidic trNOEs was based on R-factors which were calculated as described in the experimental part, and which are summarized in Table 1.

For a visualization of the results Figs. 3 to 5 show the complete set of experimental and theoretical trNOE curves. For the majority of trNOEs a good correspondence between calculated and measured data is found with an average R-factor of 0.096 and 0.181 for intra- and inter-glycosidic trNOEs, respectively. Good correspondence between experimental and calculated inter-glycosidic trNOEs shows

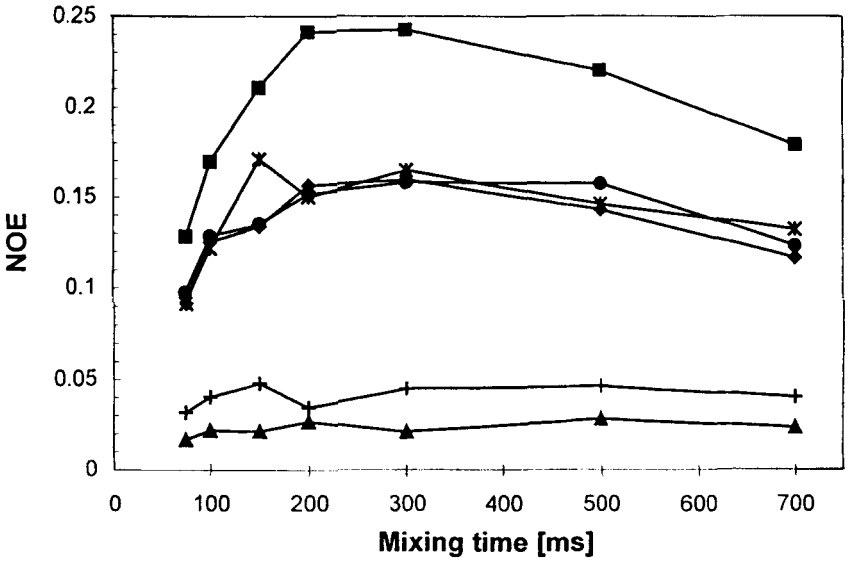

Figure 3a. NOE build-up curves of the complex Forssman pentasaccharide with *D. biflorus* (500 MHz, D_2O) for the intra-glycosidic part at 315 K. The curves represent experimental trNOEs $H1^d$-$H3^d$ (◆), $H1^c$-$H2^c$ (■), $H1^c$-$H5^c$ (▲), $H1^b$-$H3^b$ (✳), $H1^b$-$H4^b$ (✚) and $H1^b$-$H5^b$ (●).

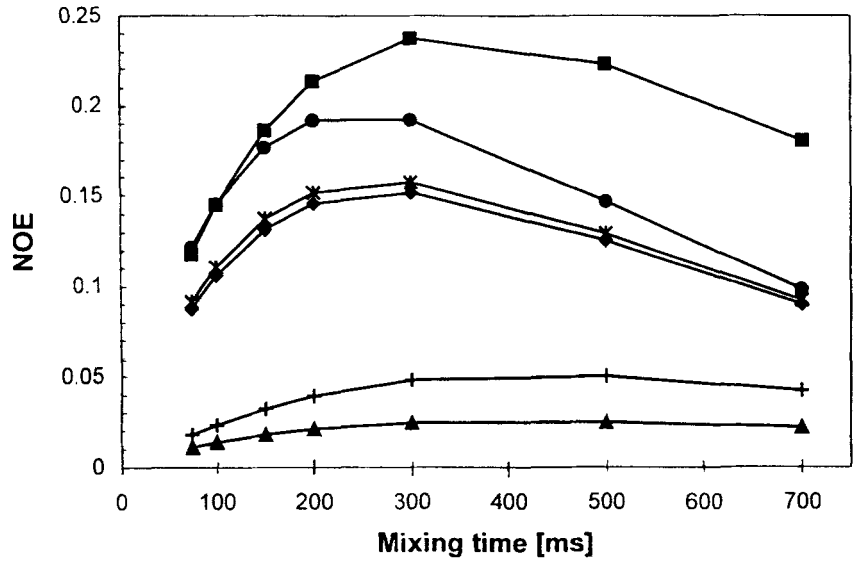

Figure 3b. Calculations of the corresponding intra-glycosidic part of the complex using CORCEMA with the anomeric proton of β-D-GalNAc ($H1^d$) as a reference peak at 300 ms. The signs of the trNOEs are as in Fig. 3a.

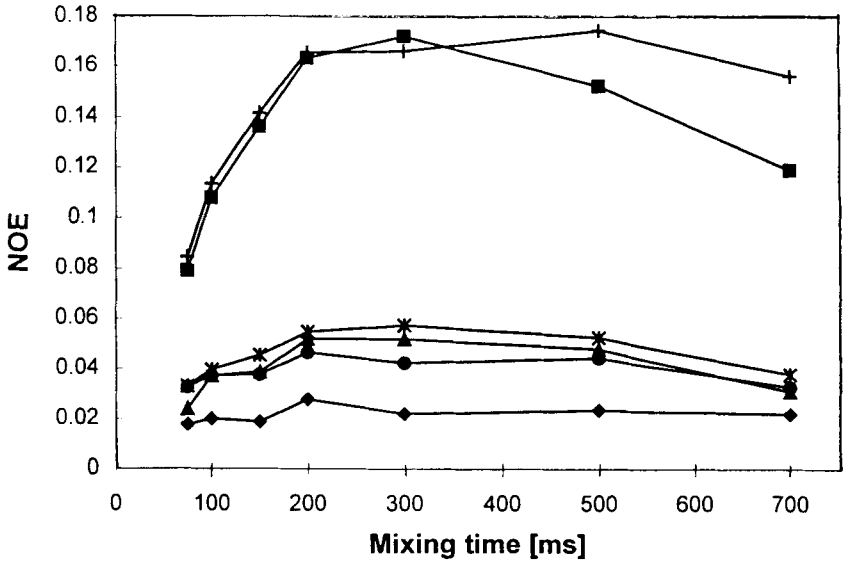

Figure 4a. NOE build-up curves of the complex Forssman pentasaccharide with *D. biflorus* (500 MHz, D_2O) for the inter-glycosidic part at 315 K. The curves represent experimental trNOEs $H1^e$-$H1^d$ (◆), $H1^e$-$H3^d$ (■), $H1^e$-$H5^d$ (▲), $H1^d$-$H4^c$ (✳), $H1^d$-$H5^c$ (●) and $H1^b$-$H4^a$ (✚).

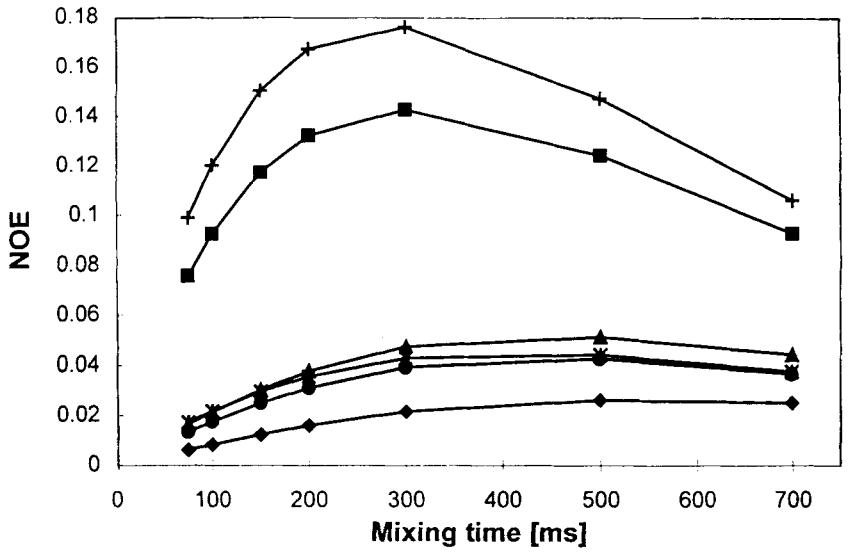

Figure 4b. Calculations of the corresponding inter-glycosidic part of the complex with CORCEMA with the anomeric proton of β-D-GalNAc ($H1^d$) as a reference peak at 300 ms. The signs for the cross-peaks are as in Fig. 4a.

Table 1. Calculated R-factors for the CORCEMA analysis

	NOE	R-factor	average R-factor
intra-glycosidic	$H1^d$-$H3^d$	0.098	
	$H1^c$-$H2^c$	0.064	
	$H1^c$-$H5^c$	0.158	0.096
	$H1^b$-$H3^b$	0.111	
	$H1^b$-$H4^b$	0.115	
	$H1^b$-$H5^b$	0.126	
	$H1^e$-$H2^e$	0.969	
	$H1^d$-$H5^d$	0.621	0.719
	$H1^c$-$H4^c$	0.567	
inter-glycosidic	$H1^e$-$H1^d$	0.234	
	$H1^e$-$H3^d$	0.164	
	$H1^e$-$H5^d$	0.119	0.181
	$H1^d$-$H4^c$	0.286	
	$H1^d$-$H5^c$	0.248	
	$H1^b$-$H4^a$	0.036	
	$H1^e$-$H4^d$	1.233	
	$H1^d$-$H3^c$	0.711	0.995
	$H1^c$-$H4^b$	1.042	

that the bound conformation of the Forssman pentasaccharide is very close to the conformation that we used in the present calculation. This bound conformation was constructed from coordinates of a previous homology modeling study[16] and from an NMR study of free Forssman pentasaccharide. The conformation of disaccharide e-d was fixed in the conformation given in reference 16, and for the residual trisaccharide c-b-a the coordinates given in reference 14.

This model implies that only disaccharide e-d is in close contact with the protein whereas the trisaccharide portion c-b-a is exposed to the solvent. In our recent study[15] we found protein-saccharide trNOEs that qualitatively supported the model. It is obvious that disaccharide e-d is most sensitive to specific orientations of amino acid

side chains in the binding pocket of *D. biflorus*. Therefore, it is not surprising that some intra- and inter-glycosidic trNOEs observed show deviations from the calculated values. From Table 1 it is seen that the largest deviations occur for $H1^e$ that has close contacts with an aliphatic amino acid. A further optimization of the model of the complex will probably improve the fit between experimental and theoretical data. Unfortunately, such an optimization would involve the optimization of the orientation of the ligand in the binding pocket, a task which is at best rather time consuming, if not impossible. To illustrate the deviations discussed Fig. 5 summarizes all corresponding trNOE curves.

Another source of error is the assumption of isotropic motion of the complex. Unfortunately, the use of an anisotropic model is not straightforward, and would require additional knowledge about the extent and the characteristics of such anisotropicities. Currently, we don't have access to corresponding experimental data for this complex.

CONCLUSION

In this study, we have performed an analysis of trNOEs using the CORCEMA program. In general, a good fit between theoretical and experimental trNOE curves was obtained. This adds quantitative support to the qualitative analysis that we performed recently.[15] The study also underlines the practicability of an extended full matrix analysis approach as provided by CORCEMA if a reasonable model for the complex is available. On the other hand, it would be desirable to perform an even more elaborate quantitative analysis which adds the optimization of protein and ligand geometry on the basis of calculated trNOEs. In principle such an analysis is possible but will require considerable computer power.

EXPERIMENTAL

General Methods. The Forssman pentasaccharide was available from chemical synthesis.[12] The model for the *D. biflorus* lectin-FPS complex was taken from our previous study[15] and subjected to a CORCEMA analysis. The ϕ/ψ angles at the glycosidic linkages in the Forssman pentasaccharide of this model were from a

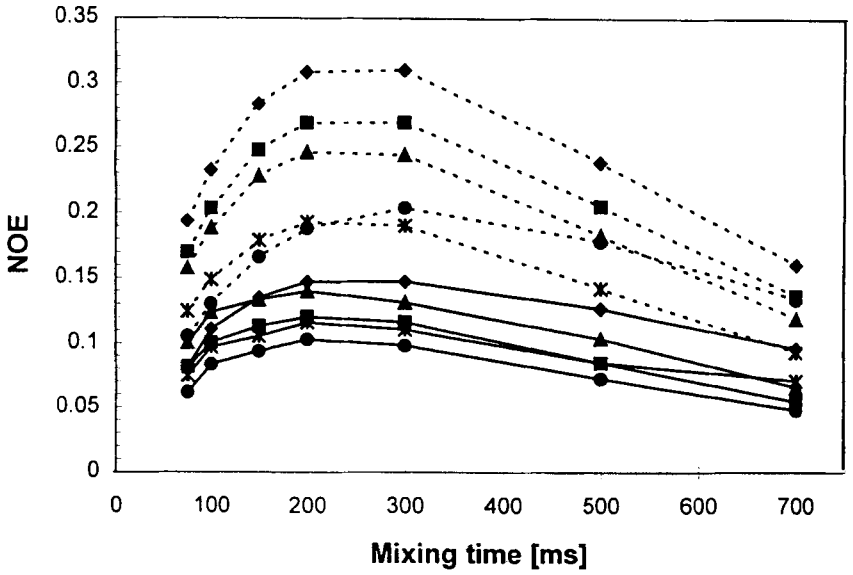

Figure 5. Comparison of experimental curves (—) with the ones calculated by CORCEMA (---) which differ in their intensities. The curves represent intra- and inter-glycosidic trNOEs of $H1^e$-$H2^e$ (●), $H1^d$-$H5^d$ (✱), $H1^e$-$H4^d$ (■), $H1^d$-$H3^c$ (▲) and $H1^c$-$H4^b$ (◆).

homology modeling study[16] (for disaccharide portion e-d) and from a conformational analysis[14] (for the pyranose units c-b-a). The torsion angles ϕ and ψ are defined as H-1–C-1–O-1–C-X and C-1–O-1–C-X–H-X, respectively. The ϕ/ψ values are compiled in table 2. The model implies that only disaccharide part e-d is in close contact with the protein whereas the trisaccharide portion c-b-a is exposed to the solvent.

NMR Experiments. NMR spectra of free FPS were recorded using a 5.1 mM solution of FPS in D_2O (99.998% D). For the complex a 0.0945 mM solution of *D. biflorus*, i.e., a 0.189 mM concentration of *D. biflorus* binding sites, in 0.01 mM deuterated Tris buffer was prepared. The concentration of FPS was 2.83 mM corresponding to the molar ratios of *D. biflorus* lectin binding sites to FPS of 1:15.

All NMR spectra were recorded on a BRUKER DRX 500 NMR spectrometer using XWINNMR software (Bruker). Phase-sensitive NOESY[22] experiments were performed using TPPI[23] with presaturation of the HDO signal. For all 2D-NOESY

Table 2. ϕ/ψ Torsional angles in the bioactive conformation of the Forssman pentasaccharide.[15] The values at the glycosidic linkage e-d are from reference 16, for the linkages d-c, c-b, and b-a from reference 14.

α-D-GalNAc-(1→3)-β-D-GalNAc-(1→3)-α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc				
e	**d**	**c**	**b**	**a**
ϕ	-43	48	-39	54
ψ	-26	6	-15	1

spectra, a $\pi/2$ -shifted squared sine bell-window function[24] was applied in both dimensions prior to the Fourier transformation. After zero filling in t_1, 2K (F_2) × 1K (F_1) data matrices were obtained.

Transfer-NOESY (trNOESY) spectra for the *D. biflorus* lectin-FPS complex were recorded at 315 K with mixing times of 75, 100, 150, 200, 300, 500, 700 ms and a relaxation delay of 1.5 s. Each experiment was performed with approximate 10 h measurement time. For the suppression of protein signals in trNOESY spectra a 10 ms spinlock pulse was applied after the first $\pi/2$ pulse. 32 scans were recorded for each and 512 experiments were performed in t_1 with 2 K data points in t_2. The resulting trNOEs were significant and negative.[15] The integration of cross-peak volumes was performed with the AURELIA program (Bruker), and trNOE curves were fitted to a double exponential function.

CORCEMA Calculations. Calculations were performed on a Silicon Graphics INDY workstation (R4400 CPU). For the CORCEMA calculations a two-state equilibrium involving a ligand and a protein forming a ligand-protein complex was assumed. The program requires the coordinates (in pdb format) of the pentasaccharide ligand in its free and bound forms (Table 2), overall rotational correlation times of the complex and the free ligand and the exchange rates, i.e., off- and on-rates. For the protein only the part of the binding pocket was used, i.e., the following amino acids are involved: Ser43, Phe83, Ala84, Asp85, Arg100, Asn101, Gly102, Gly103, Tyr104, Leu105, Asp109, Ser110, Asp111, Asp125, Thr126, Leu127, Ser128, Asn129, Ser130,

Gly131, Trp132, Asp133, Ile146, Thr211, Thr212, Gly213, Leu214, Ser215, Glu216, Gly217, Tyr218 and Ile219.

For the calculations an overall isotropic motion of the complex was assumed, but internal motion was neglected. A grid search was performed in which the dissociation constant varied from 10^{-3} to 10^{-4} M in $2*10^{-4}$ steps and the off-rate was incremented from 10^2 to 10^5 s^{-1} by 300 steps. These calculations were perfomed at different overall correlation times varying from 15 to 55 ns in 5 ns steps. Finally, an overall correlation time of 50 ns was found to correspond well within the experimental data. For the free ligand a correlation time of 0.21 ns yields a good approximation of experimental NOEs. For a comparison of theoretical and experimental trNOEs the intensity of the diagonal signal of $H1^d$ at a mixing time of 300 ms was used as a reference. Experimental and theoretical trNOE curves were normalized against this value. For the grid search only the intra-glycosidic peaks were considered. The program calculates cross peak intensities separately for direct NOESY cross-peaks and for exchange-mediated cross-peaks. For the comparison with experimental data these contributions were added. A comparison of theoretical trNOEs with experimental trNOEs allowed the determination of K_D and k_{off}. Values of $K_D = 8*10^{-4}$ M and an off-rate constant k_{off} of $3.3*10^2$ s^{-1} were found. R-factors were calculated using the following equation (1) proposed recently:[25]

$$R_{NOE} = \frac{\sum|NOE_o - NOE_c|}{\sum|NOE_o|} \qquad (1)$$

NOE_o and NOE_c denote observed and calculated NOEs, respectively. Equation (1) was used to calculate R-factors for each trNOE curve, taking the sum over individual data points.

After matching of theoretical and experimental intra-glycosidic trNOE curves CORCEMA calculations for the inter-glycosidic trNOEs of the Forssman pentasaccharide were performed. The trNOEs between the protein and the pentasaccharide were not calculated because of the broad and overlapped signals in the NOESY experiments.

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