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Lectin-bound conformations and non-covalent interactions of glycomimetic analogs of thiochitobiose

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ABSTRACT

The bound conformations of five *S*-glycoside analogs of *N,N′*-diacetylchitobiose as well as their non-covalent interactions with two lectins, *Phytolacca americana lectin* (PAL) and *wheat germ agglutinin* (WGA), are reported. The conformations of the ligands were examined by trNOESY experiments and compared with the free, solution-state conformations and molecular modeling data obtained by force field calculations. In the case of *S*-aryl, *S*-glycosides with exclusively *S*-glycosidic linkages, similar free and lectin-bound conformations and non-covalent interactions were found, whereas they differed for mixed glycosides and for a thiazoline derivative. In addition, STD (saturation *t*ransfer *d*ifference) NMR magnetization transfer efficiencies at three different temperatures were determined and assessed with respect to the structural differences of these pseudosaccharides. The binding epitopes of each substrate with PAL and WGA were also determined.

1. Introduction

Oligosaccharides composed of β-(1,4)-linked *N*-acetylglucosamine, so called chitooligosaccharides, arise naturally by enzymatic degradation of chitin. Increasing evidence indicates that these compounds function as signaling molecules in several fundamental biological processes, such as pattern formation in vertebrates[1] and the synthesis of hyaluronic acid (for reviews, see Refs. 2 and 3). Thus, the interaction of hydrolytically stable *S*-glycosidic sugars with sugar binding proteins is of considerable interest. For pseudo-di- and pseudo-trisaccharides, to be biologically active, is suggested that their conformational behavior should be analogous to their *O*-analogs. However, since sulfur has longer C–X bonds than oxygen, *S*-glycosides with respect to their *O*-analogs are conformationally more flexible due to reduced interresidue steric hindrance and altered stereoelectronic effects. Consequently, *S*-glycosides may exhibit bound states that are conformationally different to their *O*-glycosides. Also of interest is the influence of any aromatic moiety, as a hydrophobic entity, on the *S*-glycoside conformation and any subsequent binding interaction with proteins.

We report now on the lectin-bound conformations of three *O*- and *S*-glycosides of *N,N′*-diacetylthiochitobiose **2**–**4**[4] as well as the known analog **1**[5] and the thiazoline derivative **5**[4] (Scheme 1) and compare the results to the free, solution-state conformational equilibria of these compounds,[4] which were based on NMR spectroscopic measurements supported by molecular modeling force field calculations. The lectins to which **1**–**5** were bound were *Phytolacca americana lectin* (PAL) and *wheat germ agglutinin* (WGA).

NMR spectroscopy is a very effective method for assessing the structures of oligosaccharides free in solution as well as in a protein-bound state.[6] Specifically, NOE experiments provide a means to determine the conformation of the free ligand states whereas trNOE experiments, which are dependent on the exchange rate between the free and the bound state, can supply information on the conformations of a molecule in a protein-bound state. Hence, the lectin-bound states were examined by trNOESY and compared with the conformational equilibria of the free states.

STD NMR[7] was used to investigate the binding epitopes of **1**–**5** toward PAL and WGA. STD NMR[8–10] is a broadly applicable and efficient method to assess protein carbohydrate interactions, which can also reveal those parts of a ligand, which have a close, a minor or, especially for larger ligands, no contact to the protein. In STD NMR, the protein is selectively excited by irradiation in a spectral region, that is, devoid of ligand resonances or, especially for small proteins, well away from such resonances. By spin diffusion, magnetization is transferred from the protein protons to the proximal protons in the bound ligand during the saturation time and then subsequently identified after the ligand has dissociated from the enzyme by reduction of the signal intensity of these ligand protons. The degree of magnetization transfer to the ligand resonances is correlated to the proximity of the protein and ligand protons, thus providing a means to assess their spatial relationships. It is worth

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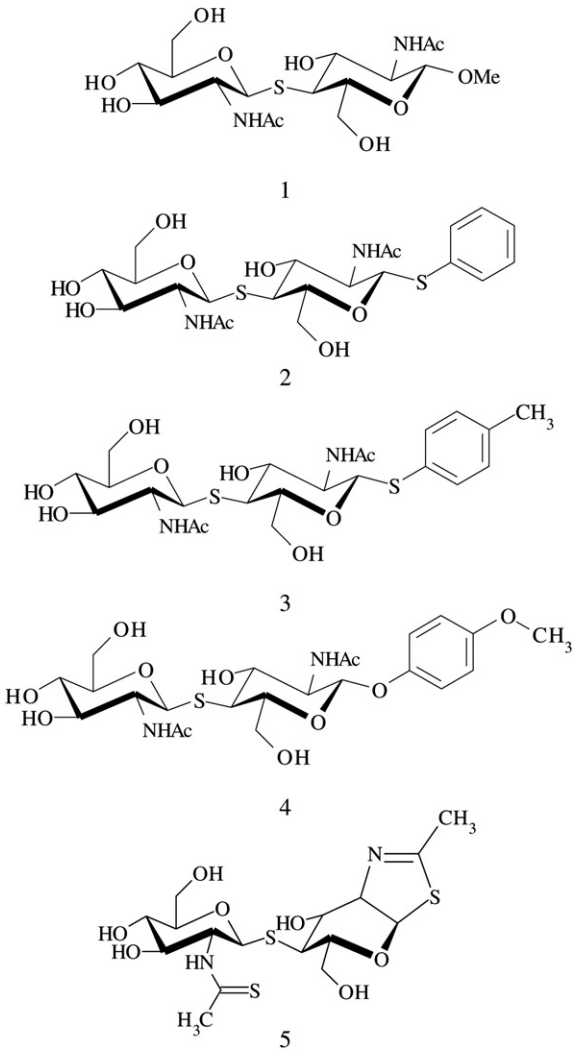


Scheme 1. The structures of the compounds **1**–**5.**

noting that several parameters play a critical role in the efficient transfer of the magnetization to the bound ligand, and thus ultimately the success of the STD experiment. For example, since the STD experiment is promoted by an accumulative effect on the signal intensity, the ligand:protein molar ratio is one such significant parameter.[7] In particular, the ligand off-rate is of dominating influence on the sensitivity of the experiment,[11] and since typically the ligand is usually in high excess over the protein, the binding must neither be too tight nor too weak. Hence, the off-rate has to be high enough, though the contact time long enough, to effect efficient magnetization transfer. Additionally, the temperature can greatly influence STD experiments since both kinetic and thermodynamic parameters can be changed to an eminent degree by temperature variation with the resulting STD sensitivity either possibly enhanced or reduced. Only limited studies on the effect of temperature on STD have been reported[12,13] and thus we present variable temperature STD signal intensity results of the carbohydrate systems **1**–**5** with PAL.

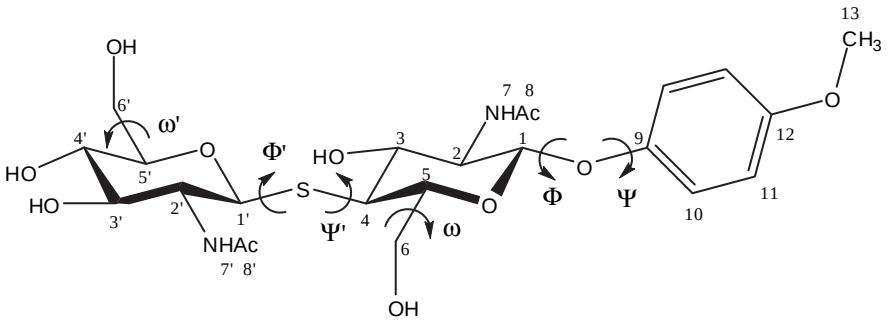
2. Results and discussion

The syntheses and detailed ^{1}H and ^{13}C NMR spectral assignments as well as the conformational analyses of the free states of **1**–**5** in solution have been previously reported.[4] The conformational analyses regarding the glycosidic linkages were made based exclusively on interresidue NOEs whereby observed contacts were considered to represent the population-weighted averages of interresidue NOEs for conformers participating in a dynamic conformational equilibrium. The NOEs explicitly characterized the pyranosylglycosidic *synΦ*/*synΨ* (by H-1′,H-4 and H-1′,H-6 NOE contacts), *synΦ*/*antiΨ* (H-1′,H-3 and H-1′,H-5) and *antiΦ*/*synΨ* (H-2′,H-4) conformations as well as the arylglycosidic *synΦ* (H-1,H-10) conformation. The torsion angles at the glycosidic linkages are commonly defined as: Φ ($H_{1'}$–$C_{1'}$–S–C_4) and Ψ ($C_{1'}$–S–C_4–H_4) for **1** and **5** and Φ' ($H_{1'}$–$C_{1'}$–S–C_4), Ψ' ($C_{1'}$–S–C_4–H_4), Φ (H_1–C_1–X–C_9) and Ψ (C_1–X–C_9–C_{10}) for **2**–**4** with X=O or S (Scheme 2).

2.1. The free-state conformations

For the free states of **2**–**5**, it was not possible to ascribe all observed interresidue NOEs to just one conformation and thus compounds **1**–**5** are present in more than one pyranosyl and arylglycosidic conformation whereby they participate in rapidly interconverting dynamic conformational equilibria.[4] Due to signal overlap and limited conformational information (concerning, for instance, the population of involved conformers), additional theoretical calculations using molecular modeling force field calculations had also been conducted to provide a more detailed picture of the systems.[4] By this approach, population-weighted averaging over all participating conformers based on the relative energies were then able to yield interglycosidic H,H distances that satisfied the distance constraints of the experimentally observed NOEs.

Specifically, from the molecular modeling results and the one, weakly, detected H-1′,H-4 NOE, it was assessed that for **1**, the *antiΦ*/



Φ = H1 - C1 - O1 - C9 Ψ = C1 - O1 - C9 - C10
Φ' = H1' - C1' - S1' - C4 Ψ' = C1' - S1' - C4 - H4
ω = O5 - C5 - C6 - O6 ω' = O5' - C5' - C6' - O6'

Scheme 2. Numbering of atoms and torsion angles.

antiΨ and *antiΦ*/*synΨ* conformers dominate (71%) over the *synΦ*/*synΨ* conformation. For the exclusively *S*-glycosidic compounds, analysis indicated that the pyranosylglycosidic *synΦ*/*antiΨ* (**2**–84%) and *synΦ*/*synΨ* conformers (**3**–82%) are heavily populated in solution. Akin to compound **1**, the dominating pyranosylglycosidic conformers of the mixed glycoside **4** (74%) are the *antiΦ*/*antiΨ* and *antiΦ*/*synΨ* conformers whereas for thiazoline derivative **5**, with *synΦ*/*antiΨ* and *antiΦ*/*synΨ* conformers (72%) dominating, a different conformational preference is evident. In addition to these dominant conformers for **1**–**5**, several other minor free-state conformations also participate in the conformational equilibria as exemplified by the seven energetically lowest conformers calculated for **2** (Fig. 1).

Thus, the type of glycosidic linkage as well as the type of sugar unit and aglycon present prove to be of considerable influence on the conformational equilibria of these glycomimetic analogs of thiochitobioses.

2.2. The lectin-bound conformations

To assess the bound-state conformations, trNOEs, which represent the conformer(s) participating in the lectin-bound state, were measured for **1**–**5** with PAL and, due to limited sample amounts, **1** and **2** with WGA. For all *S*-glycosides, the bindings to both PAL and WGA were sufficiently loose and the exchanges between the free and bound states of the ligand sufficiently slow to provide only true trNOEs, i.e., negative contacts. H,H distance constraints for the trNOEs were determined in an analogous fashion to the free-state NOE distance[4] ranges using intramolecular gauges whereby the distances between H-10 and H-11 (set to 2.48±0.01 Å) and that between H-1 and H-5 (set to 2.24±0.01 Å) were used to semi-qualitatively assess NOE intensities, thereby enabling a comparison of the free- and bound-state NOE results. Measurements were also made with various mixing times, 100, 200 and 300 ms, to provide spectra with strong and negative NOEs. Though sugar–lectin inter-trNOEs were not observed, in some cases, intralectin NOEs were present.

2.3. The PAL-bound conformations

Only negative trNOEs were detected (Table 1) for all carbohydrates bound to PAL except for the thiazoline derivative **5**, which showed both positive and negative NOEs. A comparison of the observed interresidue NOEs between NOESY spectra with and without PAL showed clear differences. Several cross peaks present in the NOESY spectra of the free ligand were not found in the trNOESY spectra of the PAL-bound ligand. In the trNOESY spectra, only the trNOEs, which are characteristic for the *synΦ*/*synΨ* conformation (H-1′,H-4) and a few H-1′,H-6 transferred NOE contacts were present. All other interresidue NOEs found in the

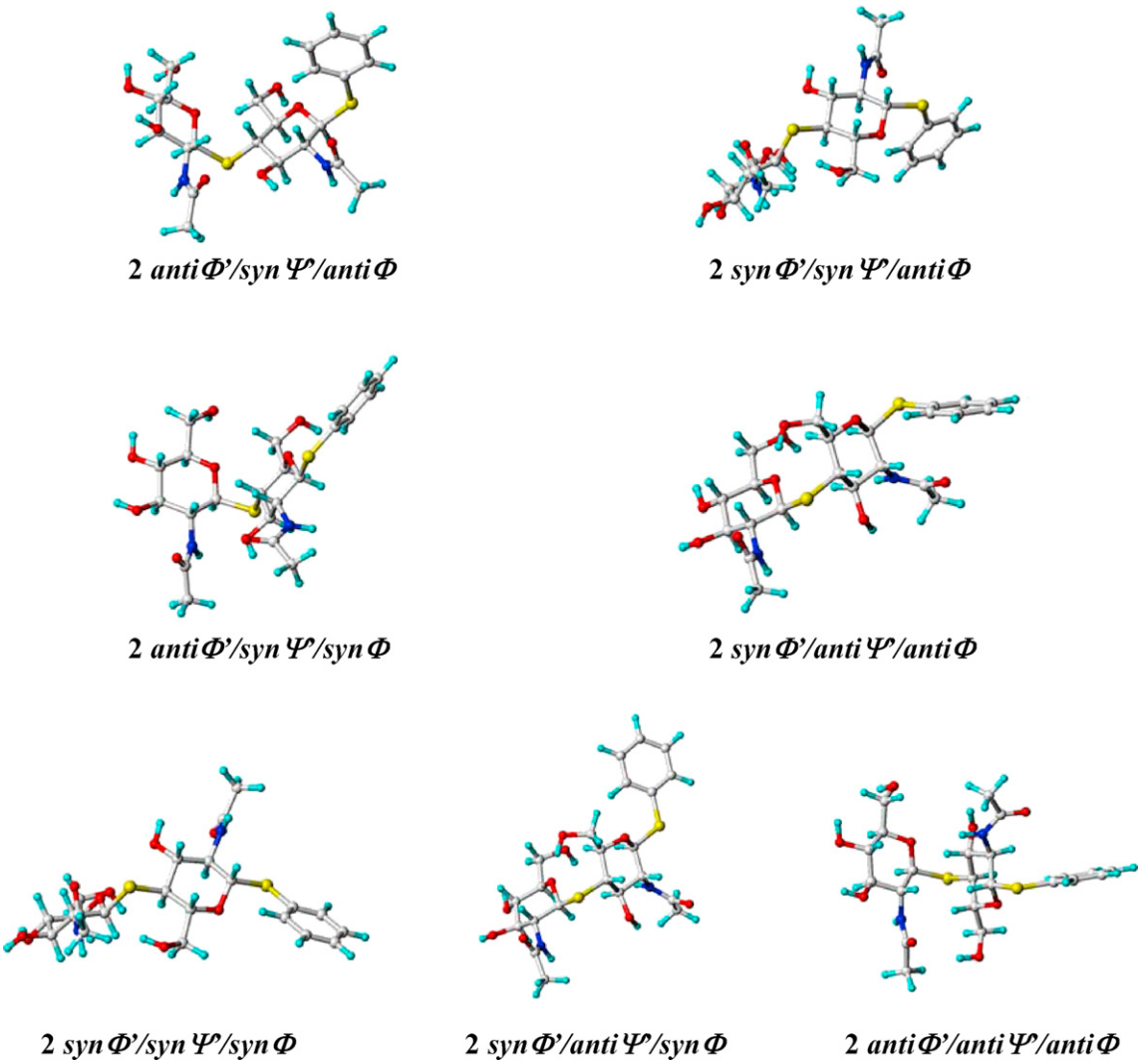


Figure 1. The seven energetically lowest conformers of the conformational equilibrium calculated for **2** in the free state.

Table 1
Relative intensities of interresidue trNOEs for bound states of **1**–**4** with PAL

PAL	H,H	Obs. signal[a]	Conformation	PAL	H,H	Obs. signal[a]	Conformation
1	H-1′,H-4	s-vs	*synΦ/synΨ*	**3**	H-1′,H-4	m	*synΦ′/synΨ′*
	H-1′,H-3	n.d.	*synΦ/antiΨ*		H-1′,H-3	n.d.	*synΦ′/antiΨ′*
	H-1′,H-5	n.d.	*synΦ/antiΨ*		H-1′,H-5	n.d.	*synΦ/antiΨ*
	H-1′,H-6*pR*	w	*synΦ/synΨ*		H-1′,H-6*pR*	n.d.	*synΦ/synΨ*
	H-1′,H-6*pS*	n.d.	*synΦ/synΨ*		H-1′,H-6*pS*	n.d.	*synΦ/synΨ*
	H-1,OMe	vs			H-1,H-10	n.d.	*synΦ*
	H-2′,H-4	n.d.	*antiΦ/synΨ*		H-2′,H-4	n.d.	*antiΦ′/synΨ′*
2	H-1′,H-4	m	*synΦ′/synΨ′*	**4**	H-1′,H-4	vs	*synΦ′/synΨ′*
	H-1′,H-3	n.d.	*synΦ′/antiΨ′*		H-1′,H-3	n.d.	*synΦ′/antiΨ′*
	H-1′,H-5	n.d.	*synΦ/antiΨ*		H-1′,H-5	n.d.	*synΦ/antiΨ*
	H-1′,H-6*pR*	n.d.	*synΦ/synΨ*		H-1′,H-6*pR*	m	*synΦ/synΨ*
	H-1′,H-6*pS*	n.d.	*synΦ/synΨ*		H-1′,H-6*pS*	w	*synΦ/synΨ*
	H-1,H-10	n.d.	*synΦ*		H-1,H-10	n.d.	*synΦ*
	H-2′,H-4	n.d.	*antiΦ′/synΨ′*		H-2′,H-4	n.d.	*antiΦ′/synΨ′*

[a] Legend: intensities of the NOE cross peaks were assessed as very strong (vs, $d_{H,H}<2.8$ Å), strong (s, 2.8 Å$<d_{H,H}<3.2$ Å), medium (m, 3.2 Å$<d_{H,H}<3.6$ Å) or weak (w, 3.6$<d_{H,H}<4.0$ Å)[14,15]; n.d., not detected.

free state were absent and, furthermore, additional NOEs were not observed. The results prove that neither the *synΦ/antiΨ* nor the *antiΦ/synΨ* pyranosylglycosidic conformation nor a conformation not present in the free-state conformational equilibrium is bound by the lectin. Hence, PAL binds exclusively only the *synΦ/synΨ* pyranosylglycosidic conformation. Since a H-1,H-10 trNOE for **2**–**4** indicative of the *synΦ* conformation was not detected and, with respect to the molecular modeling results, it is concluded that the PAL-bound arylglycosidic energetically favored conformation for **2**–**4** is *antiΦ*. The *synΦ/synΨ* H-1′,H-4 NOE contact, which is present only at *weak* intensity for both **1** and **4** as free ligands, rises in intensity to *strong–very strong* for **1** and *very strong* for **4** in the trNOESY spectra (Fig. 2). On the other hand, the intensities of the *synΦ/synΨ* H-1′,H-4 NOE contacts for **2** and **3** reduce from *strong* and *medium–strong* for the free state to *medium* for the *synΦ/synΨ* population of the bound state. Since minor conformational changes of the glycosidic linkage for the *synΦ/synΨ*-conformation interchanging between the free and lectin-bound states will only cause slight changes in the interresidue NOE intensities, these results clearly indicate distinct variations for **1** and **4** of the predominantly bound, or even exclusively bound, *synΦ/synΨ* conformations (Fig. 2c).

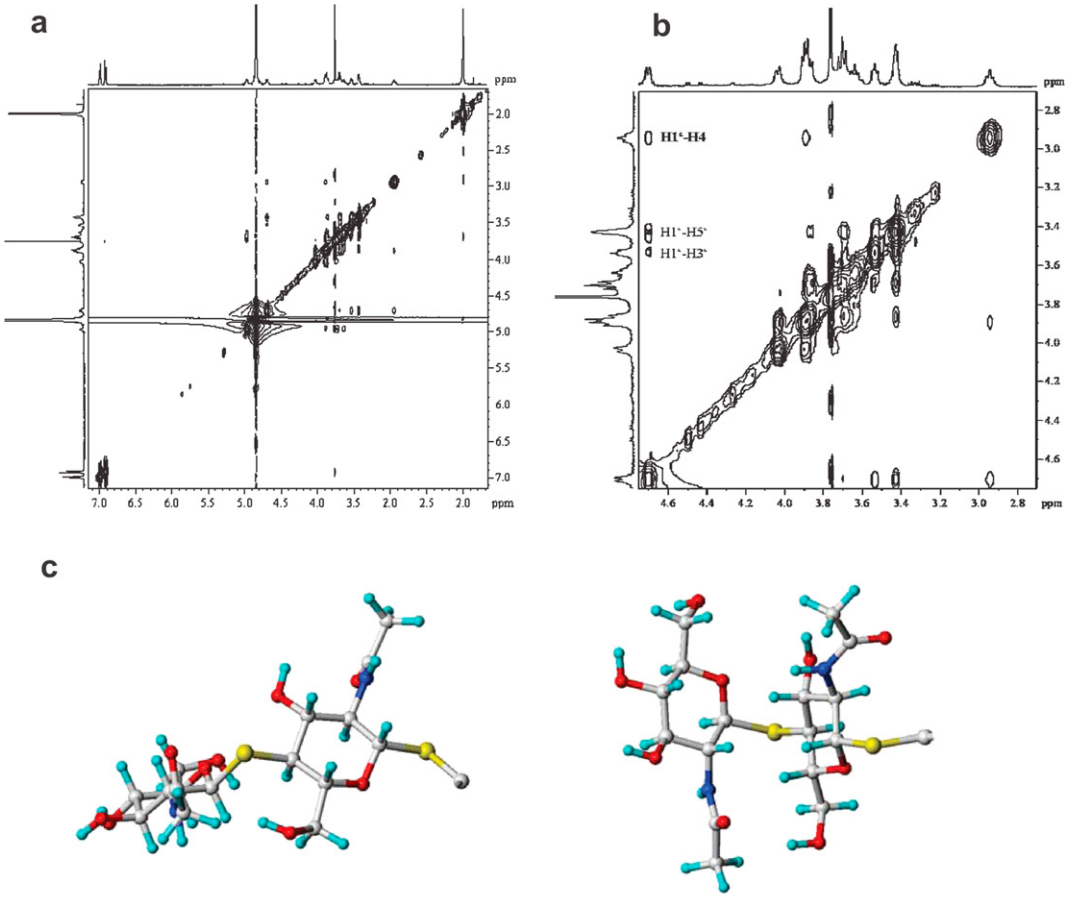


Figure 2. (a) 2D-trNOESY spectrum of **4** with PAL with a mixing time of 200 ms, (b) expanded region from 2.7–4.7 ppm, and (c) theoretically determined *synΦ′/synΨ′* (left) and *antiΦ′/antiΨ′* (right) *S*-pyranosylglycosidic conformations for the free state.

By contrast, compounds **2** and **3** seem to bind to PAL by an additional conformation which is neither the *synΦ′/antiΨ′* nor the *antiΦ′/synΨ′* pyranosylglycosidic conformation, though it must be a conformation belonging to the free-state conformational equilibrium. Therefore, the additional bound-state conformation has to be assigned as the pyranosylglycosidic *antiΦ′/antiΨ′* conformation which was not observed[4] experimentally in the free state but, which was theoretically predicted for **1**–**5** in the free state. Based on the relative intensities of the H-1′,H-4 NOE contact in the free and lectin-bound states, the population of the *antiΦ′/antiΨ′* conformation in the bound state has to be much higher than in the free state.

Thiazoline derivative **5** exhibited both positive and negative NOEs. Though this result clearly indicates a binding of **5** to PAL, analysis of the trNOEs in a similar manner to **1**–**4** is not possible thus precluding identification of the bound-state conformation of **5**.

These results show that PAL recognizes a local minimum pyranosylglycosidic conformation of the *S*-glycosides **1**–**4**, which corresponds to the global minimum in the free state and the same conformation as adopted by the protein-bound states of *O*-glycosides.[14–21] The pseudo-trisaccharidic *S*-pyranosyl, *S*-arylglycosides **2** and **3** can also bind to PAL in a further conformation, viz. the *antiΦ′/antiΨ′* conformation. By contrast, the pseudo-trisaccharidic *S*-pyranosyl *O*-arylglycoside **4** showed a dominating, perhaps even exclusively, pyranosylglycosidic *synΦ′/synΨ′* bound state, most likely caused by the *O*-arylglycosidic linkage and not by the substituent on the aromatic moiety. Thus, the experimental free and bound states as well as the calculated free state H,H distances clearly show differences between the compounds with exclusively *S*-glycosidic linkages. The bound *S*- and *O*-arylglycosidic conformation of **2**–**4** is *antiΦ*.

2.4. The WGA bound conformations

The trNOE experiments were also conducted to deduce the bound conformations of **1** and **2** with WGA; results presented in Table 2. The comparison of the interresidue NOEs between the NOESY spectra with and without WGA also showed clear differences. In the trNOESY spectra of **1** and **2** with WGA, only the pyranosylglycosidic *synΦ/synΨ* H-1′,H-4 and some H-1′,H-6 trNOEs appeared. On the other hand, as found for the trNOESY spectra with PAL, all other interresidue NOEs present in the free state were not detected and, furthermore, additional NOEs did not appear. Hence, these results indicate that neither the *synΦ/antiΨ* nor the *antiΦ/synΨ* pyranosylglycosidic conformation nor a conformation outside of the free-state conformational equilibrium is bound to WGA. Since the intensity of the H-1′,H-4 trNOEs rise to *very strong*, therefore it can be concluded that WGA selects predominantly, or even exclusively, the *synΦ/synΨ* pyranosylglycosidic conformation for interaction. Since a *synΦ* H-1,H-10 trNOE contact was not detected for **2** and, with respect to the molecular modeling results, the energetically favored arylglycosidic conformation has to be *antiΦ*.

These results indicate that WGA recognizes more selectively than PAL the *synΦ/synΨ* pyranosylglycosidic conformation of **1** and **2**, which corresponds to the global minimum in the free state in concert to the known protein-bound states of *O*-glycosides.[14–21]

2.5. STD NMR spectroscopy at 298 K

STD NMR Spectra were recorded for the complexes of **1**–**5** with both PAL and WGA. Like the trNOESY experiments, the STD NMR experiments also proved that all carbohydrates bind to PAL and WGA. With both PAL and WGA being relatively small proteins, they are expected to show a distinct influence on the STD signal intensity with respect to the on-resonance positions, in particular for on-resonances near to ligand signals. Since preliminary experiments indicated this to be the case, two high-field on-resonances (10.0 and 12.0 ppm) and two low-field on-resonances (−2.0 and −0.1 ppm) were therefore assessed with respect to their influence on the STD signal intensity to discount these types of effects. STD measurements on samples without carbohydrate present at the four on-resonance positions, which were well away from the ligand signals, showed highly similar influences on the protein signal intensities and thus STD effects at the four on-resonance positions for each carbohydrate–protein complex were measured for comparison.

Difference spectra were obtained by subtraction of the on- from the off-resonance spectra and the intensities of the off- and difference spectra used to obtain a difference:off-resonance integral ratio (hereafter, diff:off ratio). By using the same parameters for each lectin probe of **1**–**5**, compound-specific information was garnered via variations in the STD diff:off ratios. The signal intensity of the difference spectra in these cases depends mainly on the nature of the ligand structure and hence on both the thermodynamic and kinetic parameters of the carbohydrate–lectin systems at a given temperature. Thus, the integrals of the difference and off-resonance spectra for one significant ligand proton resonance were compared using the same integral limits in both spectra to reduce errors due to such variations. The ^{1}H signals of the NHAc group methyls were selected because of the high intensity in the difference spectra of this signal and, conveniently, because all five compounds **1**–**5** possess this group, or a similar methyl in the case of **5**, thereby facilitating comparison between them.

Firstly, the STD effects of **1**–**5** with PAL at 298 K were investigated. Table 3 presents the diff:off ratios of the ^{1}H NHAc methyl signals of **1**–**5** for the four on-resonance positions as well as the mean value of the four measurements. The data generally only show a slight influence on the diff:off ratio due to the frequency of the on-resonance irradiation and thus, to limit measuring times, only two on-resonance frequencies, −2.0 and 12.0 ppm, were used for subsequent investigations. The results also reveal clear influences on the diff:off ratio due to structural differences within **1**–**5**. For example, the very similar, exclusively *S*-glycosidic-linked pseudo-trisaccharides **2** and **3** exhibit very similar ratios, which are much lower than those of the mixed glycosidic pseudo-trisaccharide **4** and the non-aromatic carbohydrates **1** and **5**. Because of the high STD difference signal intensities for **2** and **3**, and consequently the low diff:off ratios, it can be assumed that both the off-rate and contact time lead to efficiently

Table 2
Intensities of interresidue trNOEs for bound states of **1** and **2** with WGA

WGA	H–H	Obs. signal[a]	Conformation	WGA	H–H	Obs. signal[a]	Conformation
1	H-1′,H-4	vs	*synΦ′/synΨ′*	**2**	H-1′,H-4	vs	*synΦ′/synΨ′*
	H-1′,H-3	n.d.	*synΦ′/antiΨ′*		H-1′,H-3	n.d.	*SynΦ′/antiΨ′*
	H-1′,H-5	n.d.	*synΦ′/antiΨ*		H-1′,H-5	n.d.	*synΦ′/antiΨ*
	H-1′,H-6*pR*	m	*synΦ/synΨ*		H-1′,H-6*pR*	n.d.	*synΦ/synΨ*
	H-1′,H-6*pS*	w	*synΦ/synΨ*		H-1′,H-6*pS*	n.d.	*synΦ/synΨ*
	H-1,OMe	vs			H-1,H-10	n.d.	*synΦ*
	H-2′,H-4	n.d.	*antiΦ′/synΨ′*		H-2′,H-4	n.d.	*antiΦ′/synΨ′*

[a] Legend: intensities of the NOE cross peaks were assessed as very strong (vs, $d_{H,H}$<2.8 Å), strong (s, 2.8 Å<$d_{H,H}$<3.2 Å), medium (m, 3.2 Å<$d_{H,H}$<3.6 Å) or weak (w, 3.6<$d_{H,H}$<4.0 Å)[14,15]; n.d., not detected.

Table 3
STD diff:off ratios for the ^{1}H NHAc methyl signals of **1**–**5** with PAL at 298 K

On resonance positions (ppm)	1	2	3	4	5
−2.0	1:130	1:29	1:40	1:72	1:117
−0.1	1:99	1:25	1:26	1:75	1:126
10.0	1:133	1:30	1:29	1:81	1:106
12.0	1:134	1:25	1:29	1:82	1:96
Mean value	1:124	1:27	1:29	1:78	1:112

transferred magnetization during the saturation time and a high accumulation of molecules with binding information. Due to structural variation, the difference signal intensities for **1**, **4**, and **5** are smaller as a result of reduced magnetization transfer or accumulation compared to **2** and **3**. In general, the aromatic pseudo-trisaccharides **2**–**4** show much stronger STD effects than the non-aromatic carbohydrates **1** and **5** thereby confirming the important role that an aromatic moiety plays in the interactions of **1**–**5** with PAL.

The STD effects of **1** and **2** with WGA at 298 K were also investigated (Table 4) via diff:off ratios of the ^{1}H NHAc methyl signals whereby different values were obtained in comparison to PAL. The differences between the intensities of the signals in the STD difference spectra of **1** and **2** are less distinct than were found for PAL, probably due to the different binding regions of the two lectins and hence different ligand–protein interactions. The non-aromatic compound **1** shows a lower diff:off ratio with WGA in comparison to PAL, whereas the aromatic compound **2** shows a higher diff:off ratio with WGA than with PAL. This may indicate that hydrophobic interactions play a major role for binding to PAL compared to WGA. This is in agreement with the known binding regions from X-ray analysis of PAL[22] and WGA[23] in complexation with their natural ligands. For WGA complexed with di-*N*-acetylchitobiose, several 'binding points' were found. The first sugar unit of di-*N*-acetylchitobiose has a closer contact to the amino acids of the binding region than the second sugar unit, whereas for PAL complexed with tri-*N*-acetylchitotriose, two

Table 4
STD diff:off ratios for the ^{1}H NHAc methyl signals of **1** and **2** with WGA at 298 K

On resonance positions (ppm)	1	2
−2.0	1:90	1:43
12.0	1:107	1:81
Mean value	1:99	1:62

binding sites have been reported at which all three subunits show close contact to the amino acids. Thus, the third subunit of the carbohydrates—for **2**, the aromatic moiety—can interact much more strongly with adjacent hydrophobic amino acids in binding to PAL than in the case of binding to WGA (see Figs. 3 and 4).

2.6. The effect of temperature on STD NMR

As is widely appreciated, temperature can greatly influence the non-covalent binding between a carbohydrate and a lectin[12,13] and it was evident in this work that the structures of the carbohydrates **1**–**5** exhibited a strong influence on the off-rate and contact time and thus consequently on the difference signal intensity. Although for carbohydrates **1**–**5** literature values for binding and kinetic parameters were not available and it was not possible to determine herein the dissociation constants or off-rates with PAL and WGA, nonetheless the effect of temperature on the STD spectra and hence the diff:off ratio was investigated by recording STD spectra of **1**–**5** with PAL at 283 and 313 K for comparison to the data obtained at 298 K. The results are presented in Table 5.

For all compounds, variation of temperature showed a clear influence on the diff:off ratio resulting from both variations in the off-rates and contact times.

Changing the temperature from 298 K to either 283 or 313 K reduced the signal intensities in the difference spectra in all cases except for **1** at 283 K. Thus, it can be assumed that at 298 K, both the off-rates and contact times lead to optimally transferred

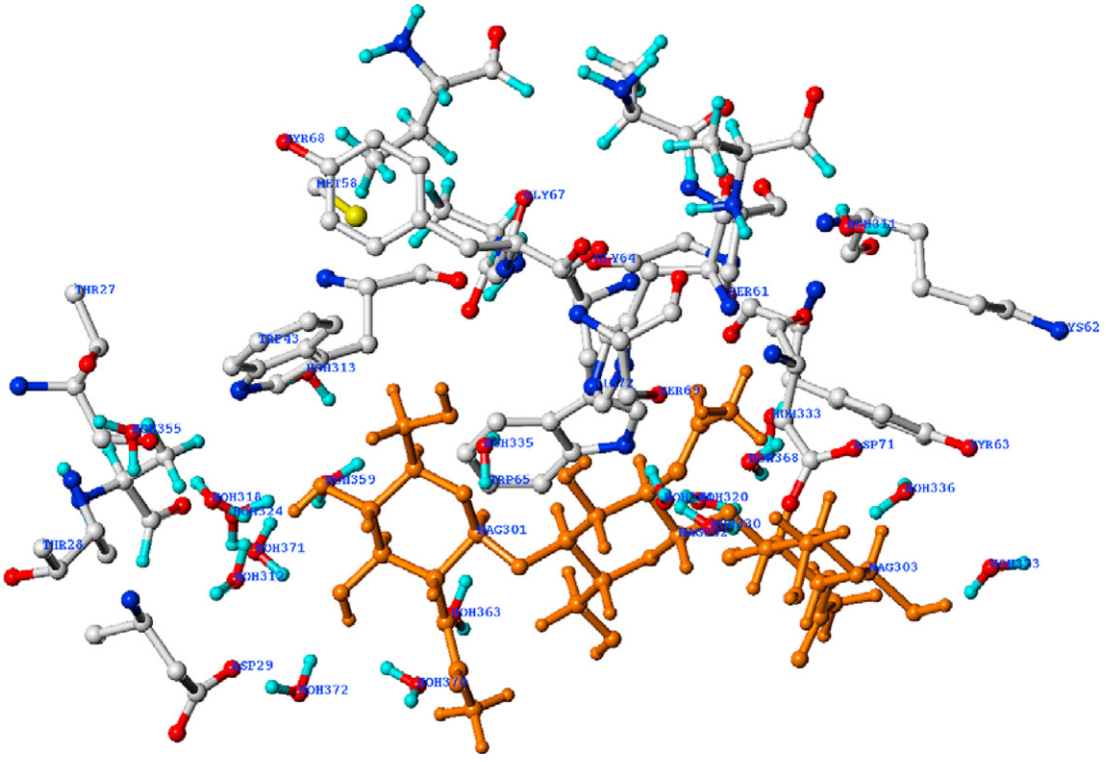


Figure 3. Binding site of PAL (PDB-ID:1ULM) with a complexed ligand (orange); amino acids within 6 Å of the ligand are shown.

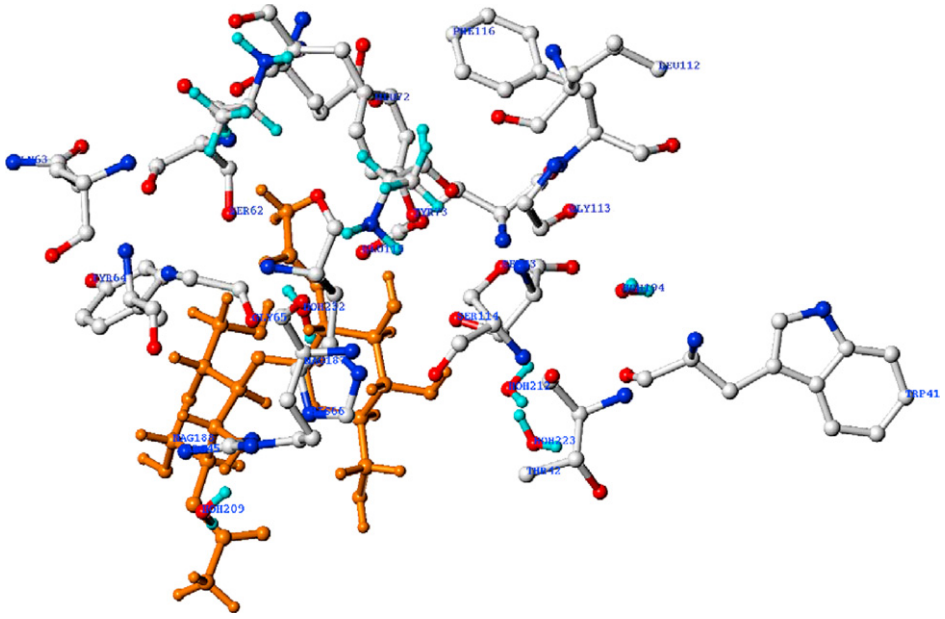


Figure 4. Binding site of WGA (PDB-ID:1K7U) with a complexed ligand (orange); amino acids within 6 Å of the ligand are shown.

magnetization and/or high accumulation of molecules with binding information during the saturation time (with only the one aforementioned exception). Hence, lowering or raising the temperature leads to a reduced magnetization transfer and/or an accumulation of molecules with binding information. Generally, it was found that raising the temperature by 15 K has a stronger effect on the diff:off ratio than a reduction of the temperature by 15 K.

Table 5
STD diff:off ratios for the ^{1}H NHAc methyl signals of **1**–**5** with PAL at 283, 298 and 313 K

On resonance positions (ppm)	1	2	3	4	5
283 K					
−2.0	1:113	1:46	1:35	1:91	1:151
12.0	1:105	1:54	1:47	1:117	1:179
Mean value	1:109	1:50	1:41	1:104	1:165
298 K					
−2.0	1:130	1:29	1:40	1:72	1:117
12.0	1:134	1:25	1:29	1:82	1:96
Mean value	1:132	1:27	1:35	1:77	1:107
313 K					
−2.0	1:193	1:121	1:101	1:142	1:241[a]
12.0	1:179	1:146	1:102	1:151	1:364[a]
Mean value	1:186	1:134	1:102	1:147	1:303

[a] Signal reduced probably due to hydrolysis.

Structural differences within **1**–**5** were also evident by variable temperature STD measurements. The *S*-glycosidic-linked pseudo-trisaccharides **2** and **3** exhibited very similar properties and showed only a modest change in the diff:off ratio at 283 K in comparison to 298 K, but a very strong change in the ratio upon raising the temperature to 313 K. The mixed glycosidic-linked pseudo-trisaccharide **4** also only showed a modest diff:off ratio change at 283 K in comparison to 298 K whilst a stronger change occurred at 313 K. However, compound **1** displayed its lowest ratio at 283 K with increasing ratios attained upon increasing the temperature and overall it possessed higher ratios in comparison to **2**–**4**. Compound **5** showed the highest ratios at 283 and 313 K of all the compounds indicating that the thiazoline moiety is disadvantaged in its binding toward PAL at these temperatures more than a compound containing a NHAcGlc sugar unit. The non-aromatic carbohydrates **1** and **5** showed much lower signal intensities in the difference spectra, leading to the conclusion that an aromatic moiety, and hence hydrophobic interactions, promotes binding between PAL and *S*-glycosidic carbohydrates. The solely *S*-glycosidic-linked pseudo-trisaccharides **2** and **3** exhibit, at all three temperatures, higher signal intensities in the difference spectra than the mixed pseudo-trisaccharide **4**, thereby indicating stronger interactions to PAL for the former compounds.

Since at lower temperatures rotational and translational energies are consequently reduced, it can be assumed that binding at 283 K is therefore tighter enabling more magnetization to be transferred from the lectin to the ligand in comparison to measurements at 298 K. On the other hand, the accumulation of substrate signal intensity with binding information is accordingly reduced. From the results, it seems that an enhancement of the magnetization transfer confers a reduction in the diff:off ratio for **1**, whereas a disproportionate loss of signal accumulation results in enhancement of the diff:off ratio for **2**–**5**. The behavior of **2**–**5** can also be rationalized from the perspective that, at 283 K, fewer ligand molecules possess the activation energy required to bind to the protein. In these systems here, the reduction in magnetization transfer seems to overwhelm the gain in signal accumulation and hence the diff:off ratio for **1**–**5** binding with PAL rises at 313 K.

From the measurement of the diff:off ratio at various temperatures, an approximation of the thermodynamic and kinetic data can be attained. Furthermore, since a number of parameters—with many of them unknown—affect the final STD effect, it is difficult to prepare the perfect sample or to readily locate optimal conditions. However, the demonstrated strong temperature influence for these systems and others[12,13] imply the advantage of conducting variable temperature STD measurements. By doing so, the ready optimization and extension of STD applications to a given problem may be more easily facilitated.

2.7. Epitope mapping

Table 6 presents the relative STD effects for various ^{1}H signals of **1**–**5** when bound to PAL at 298 K. The ^{1}H signals of the NHAc group methyls were selected for referencing purposes because of their

high signal intensity in the difference spectra and, since all five compounds have this group (or a similar methyl in the case of **5**), to facilitate comparison between the compounds. The STD effects for each ligand were calculated for four on-resonance irradiations and with similar integral limit constraints applied.

Table 6
Relative STD effects of **1**–**5** with PAL at 298 K

On resonance positions (ppm)	H-1	H-6	H-6/6′	H-6′/2′	H-2/5	H-3/3′	OMe/H-5′/4′	H-4	NHAc
1+PLA									
−2.0	65	42	19	56	32	22	16	67	100
−0.1	67	61	67	82	126	56	48	68	100
10.0	56	24	18	57	31	21	22	57	100
12.0	53	61	51	65	67	66	52	90	100
Mean value	60	47	39	65	64	41	35	71	100

2+PLA	H-10	H-11/12	H-6*pS*	H-6*pR*/6′*pS*	H-2	H-5/2′/6′*pR*	H-3	H-3′	H-4′/5′	H-4	NHAc
−2.0	88	88	47	42	57	43	46	52	58	83	100
−0.1	106	107	27	24	38	34	45	39	52	76	100
10.0	108	107	29	39	60	39	49	64	57	74	100
12.0	93	88	23	40	51	70	59	46	55	57	100
Mean value	99	98	32	36	52	47	50	50	56	73	100

3+PLA	H-10	H-11	H-6*pS*	H-6*pR*/6′*pS*	H-2′/2/6′*pR*	H-5/3/3′	H-4′/5′	H-4	Me	NHAc
−2.0	94	88	34	43	73	21	42	80	92	100
−0.1	70	71	32	37	52	87	60	124	78	100
10.0	97	102	42	32	40	43	50	75	93	100
12.0	99	94	27	31	30	34	53	67	94	100
Mean value	90	89	34	36	49	46	51	87	89	100

4+PLA	H-10	H-11	H-1	H-6*pS*	H-6*pR*/6′*pS*/2	H-5/2′/6′*pR*	OMe	H-3	H-3′	H-4′/5′	H-4	NHAc
−2.0	82	72	34	29	36	49	58	50	54	54	66	100
−0.1	88	92	49	27	35	58	50	67	46	74	84	100
10.0	106	100	43	23	38	91	62	49	43	48	58	100
12.0	91	92	34	29	34	74	62	60	48	51	73	100
Mean value	92	89	40	27	36	68	58	57	48	57	70	100

5+PLA	H-1	H-2	H-6′	H-6	H-6′	H-3′	H-6	H-4′/5′	H-5	H-4	H-8	NHAc
−2.0	87	80	43	53	56	82	61	57	77	80	115	100
−0.1	94	100	36	38	28	45	53	47	58	60	105	100
10.0	75	76	43	40	36	41	42	31	50	87	101	100
12.0	99	93	33	36	30	60	46	41	70	83	85	100
Mean value	89	87	39	42	38	57	51	44	64	78	102	100

Since the ^{1}H signals of the NHAc group methyls and the aromatic moieties show large STD effects, there must be close contact between these groups and the protein. In the case of the NHAc groups, these interactions are probably hydrogen bonds, whereas for the aromatic moieties they are likely to be hydrophobic interactions. On the other hand, the low STD effects of the protons of the exocyclic hydroxyl methyl group indicate a minor role for these groups with regards to binding and an incoherent contact to the lectin. The pyranose ring protons generally show half the amount of STD effects relative to the reference except for the H-4 protons, which are slightly more intense.

Overall, the structurally related pseudo-trisaccharides **2**–**4** show very similar STD effects, which may be due to the domination of the *synΦ′*/*synΨ′* conformation in the bound conformer in all three cases. So it can therefore be concluded that these three carbohydrates are geared toward the binding site in a related manner. Whilst **1** revealed slightly higher STD effects for the ring protons, altogether, because of only slight deviations, a similar binding toward PAL can be assumed for **1** as for **2**–**4**. The thiazoline derivative **5** shows similar STD effects for the NHthioAcGlc sugar unit as detected for **1**–**4**, but different STD effects for the thiazoline moiety. Therefore it can be presumed that a different orientation of the thiazoline moiety is in effect compared to a NHAcGlc moiety placed at the same position. Thus, the resulting differing distances to the amino acid residues at the binding site would account for these variations in STD effects.

Table 7 presents the relative STD effects of **1** and **2** bound to WGA. In general, the results of the epitope mapping for **1** and **2** with PAL and WGA are similar but there are also some differences.

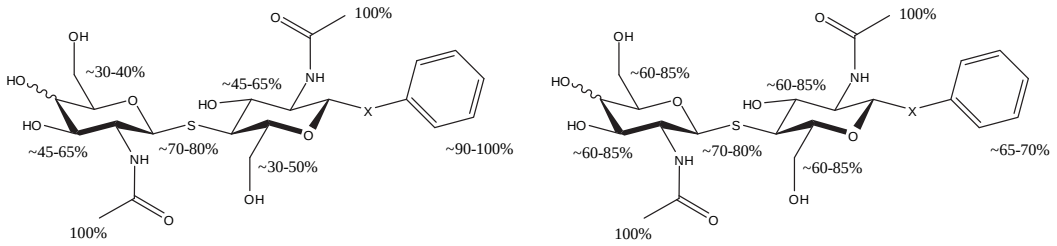
The ^{1}H signals of the NHAc group methyls again exhibit strong STD effects, whereas they are smaller for the ring protons. The STD effects of the ring protons bound to WGA are stronger than in the case of PAL binding indicating a closer relative contact to WGA. On the other hand, the STD effects for the aromatic protons of **2** are weaker when bound to WGA in comparison to PAL. This is maybe due to the fact that the aromatic moiety is the third moiety and has only a slight interaction to the binding point of WGA with relatively large distances between the unit and the amino acid residues, whereas PAL has a binding site where all three moieties are in close contact to the lectin (cf. Figs. 3 and 4).

Table 7
Relative STD effects of **1** and **2** with WGA

1+WGA	H-1′	H-1	H-6	H-6/6′	H-6′/2′	H-2/5	H-3/3′	OMe/H-5′/4′	H-4	NHAc
−2.0	66	50	61	76	86	56	70	53	69	100
12.0	73	71	74	85	87	71	85	64	89	100
Midpoint	70	61	68	81	87	64	78	59	79	100

2+WGA	H-10	H-11	H-6	H-6/6′	H-2	H-5/2′/6′	H-3	H-3′	H-4′/5′	H-4	NHAc
−2.0	66	59	56	46	80	56	73	79	68	115	100
12.0	73	69	61	49	86	57	78	88	71	92	100
Midpoint	70	64	59	48	83	57	76	84	70	104	100

The STD effects of the various groups are summarized in Scheme 3. Notably, the NHAc group methyls reveal close contact to both lectins whereas the aromatic moiety reveals close contact only in the case of PAL. The pyranose ring protons clearly show less contact, in particular, the protons of the exocyclic hydroxyl methyl groups. Hence, it is clear



Scheme 3. Summary of the relative STD effects of **1**–**5** with PAL and **1** and **2** with WGA.

that the NHAc groups play a major role for the interaction of **1**–**5** toward the lectins PAL and WGA.

3. Conclusions

Compounds **1**–**5** were shown to bind to the two lectins PAL and WGA. The comparison of the free- and bound-state conformations and the non-covalent interactions of **1**–**5** with PAL and WGA showed both similarities and differences. Differences between the carbohydrate–PAL and carbohydrate–WGA complexes follow from differences in lectin structure and binding region. Similarities result from the fact that both lectins have nearly the same size, similar natural binding partners and related functions.

The conformation of the bound state of the *S*-glycosides was found to be *synΦ*/*synΨ* for all complexes in concert with the known preferred bound conformations of *O*-glycosides. For the PAL complexes of the *S*-pyranosyl, *S*-arylglycosides **2** and **3**, a further conformation, *antiΦ*/*antiΨ*, was determined to also occur. The *S*- and *O*-arylglycosidic conformation of the bound state was found to be *antiΦ*.

The STD difference resonance intensities of **1**–**5** vary with temperature, though for highly similar molecular structures, very similar STD effects result. Measuring the STD difference resonance intensities at different temperatures can afford rough thermodynamic and kinetic data and thus, if unknown, can help to extend and optimize STD applications. The NHAc group methyls of **1**–**5** almost always showed the strongest interaction to both lectins. Due to the different binding regions of PAL and WGA, the aromatic moiety only revealed strong interaction in the case of PAL.

4. Experimental

4.1. General

NMR samples of saccharides **1**–**5** (0.008–0.091 μmol) were prepared in D_2O (0.7 mL). 2D-NOESY and 2D-trNOESY spectra were recorded at ambient temperature without sample spinning using the HDO signal as internal reference (4.70 ppm). In general, 2D-NOESY experiments utilized a mixing time of 500 ms since intensity build up curves indicated that NOEs attained a maximal value at this value. Additional NOESY spectra were also recorded with mixing times of 250, 650 and 800 ms. 2D-trNOESY spectra were recorded for 1:50 molar ratios of lectin:ligand with mixing times of 100, 200 and 300 ms.

STD NMR was performed for 1:100 molar ratios of lectin:ligand with a saturation time of 2.0 s. On-resonance spectra were recorded with selective irradiations at −2.0, −0.1, 10.0 or 12.0 ppm, with off-resonance spectra recorded with selective irradiation at 40.0 ppm. The relaxation delay was set to 5.0 s, the pre-scan-delay to 10.0 μs and the spectral width to 9.0 ppm with the center of the spectral window at 4.7 ppm. 64k data points were acquired with 512, 1k or 2k scans depending on saccharide concentration. Subtraction of the STD spectra was performed via phase cycling together with the change of irradiation frequency between on- and off-resonance. No influence on the STD difference signal intensity due to variation of the on-resonance position was evident as control spectra without saccharide and with on-resonance irradiations at −2.0, −0.1, 10.0 or 12.0 ppm showed similar signal intensity changes of the protein signals. The intensities of the STD effects were evaluated by division of the integrals by their respective signals in the reference spectra. The STD effect of the NHAc group methyl signal in each sample was set to 100% and the intensities of all the other signals were determined relative to this.[24] The STD effects were calculated with identical integral limits in each particular case for all on-resonance irradiations to eliminate errors due to integral limit variation.

Calculation of the molar ratios of the lectins to ligands was based on a molecular weight of 32 kDa and one sugar binding site for PAL and a molecular weight of 36 kDa and two binding sites for WGA. Since PAL and WGA both showed pH stable D_2O solutions with the sugars **1**–**5** and were announced already to be pH resistent[25] (pD c7.43 at 298 K), addition of buffer was not required. Our first experiments were performed by solving the lectin and the substrate in a solution of Na_2HPO_4/H_3PO_4 in D_2O, which resulted in a pH* (pH value in D_2O) of 7.8. Privat et al.[26] found the K_d value of a WGA–substrate complex remaining nearly constant in the pH range from 4 up to 9. Without H_3PO_4/Na_2HPO_4 buffer the pH* of all investigated enzyme–substrate solutions was 7.3, which fully lies in the above mentioned range and because the buffer produced some problems esp. in the trNOESY experiments, we decided to renounce using it.

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