Application of NMR Based Binding Assays to Identify Key Hydroxy Groups for Intermolecular Recognition

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Abstract: In general, few functional groups are sufficient for the binding of ligands to receptor proteins. For carbohydrates, characteristically spaced hydroxy groups often determine the binding affinity. Key functional groups may be identified by testing derivatives that have the potential sites blocked, which is most effectively achieved by simultaneous testing of all these derivatives. We employed such a parallel approach to identify the binding specificity of *Sambucus nigra* agglutinin (SNA), a lectin from elderberry. Compound libraries of randomly methylated *O*-methyl glycopyranosides were screened for binding activity toward SNA using trNOESY experiments, as well as 1D- and 2D-STD NMR experiments. The individual compounds have identical molecular weights and display rather similar physicochemical properties. Nevertheless, fast and reliable identification of the active compounds directly from the mixture was possible. The results are consistent with earlier biochemical work, showing that SNA has a binding specificity for D-galactose with the hydroxy functions at carbon atoms C3 and C4 of methyl β-D-galactopyranoside being critically important for binding. Finally, we show that the screening protocol can be extended to heteronuclear STD pulse sequences if ^{13}C-labeled derivatives are present in the library.

Introduction

Understanding the molecular details of intermolecular recognition between two molecules is of utmost importance for the characterization of binding processes, and is an essential step in the design of bioactive compounds that bind specifically to a target of pharmaceutical significance. Any binding process is the result of a fine balance between different contributions.[1] As a simplification, the key-and-lock model[2] focuses on the relative spatial arrangement of key functional groups. Identification of these key functional groups is a crucial step in exploiting the binding pocket of a protein and deriving a lead structure for further optimization. Obviously, it is advantageous to use a parallel approach for this task. In nucleic acid biochemistry, such a parallel technique, known as modification interference, is widely used. A pool of randomly modified nucleic acid sequences, which can be synthesized easily, is dissected into batches of active and inactive species, and the location of modifications in both pools is determined by sequencing. However, this approach is not transferable to other substance classes. Therefore, synthesis and testing of compounds must usually be done in a sequential manner (e.g., ref 3).

For a long time, NMR has been used as a powerful tool to monitor molecular interactions because a number of NMR parameters change in a characteristic manner upon binding. Recently, several NMR methods for screening of substance libraries for binding activity have been developed,[4] based on T_2 filtering,[5] diffusion,[5,6] transferred NOEs,[7,8] or saturation transfer.[9,10] One large advantage of NMR-based methods is that they allow direct elucidation of structural information. Here, we show that substance libraries from random chemical modification can be screened for binding activity using transferred NOEs (trNOEs)[7] and saturation transfer difference (STD) NMR.[9] The observation of trNOEs is based on the dramatically different NOE properties[11] of free ligand molecules (NOEs are small and positive) and ligand molecules bound to a receptor protein (NOEs are large and negative). The STD NMR protocol[9] takes advantage of saturation transfer from a large receptor protein to ligand molecules that bind. Saturation transfer is achieved by selectively irradiating protein protons. Acquisition of on- and off-resonance spectra with subsequent subtraction generates difference spectra that contain only signals of ligand molecules that bind to the protein.

Here, we study the binding of carbohydrate derivatives to *Sambucus nigra* agglutinin (SNA), a lectin from European elderberry. The 140 kDa protein possesses two binding sites that bind to the trisaccharide α-D-NeuNAc(2→6)β-D-Gal(1→4)-Glc (sialyl lactose) with a K_D value in the micromolar range.[12,13]

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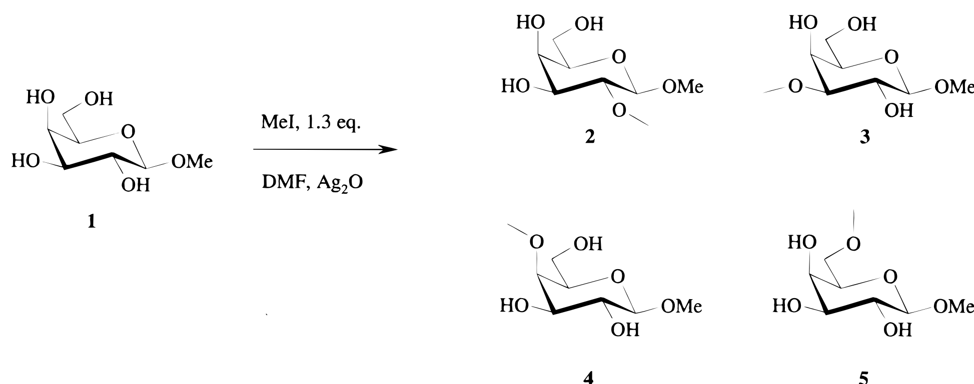
Scheme 1. Synthesis of Randomly *O*-Methylated Galactopyranosides[a]



[a] For the synthesis of ^{13}C-labeled derivatives, ^{13}C-labeled methyl iodide is used.

Lactose and galactose are also recognized by SNA, although with significantly reduced binding affinity (K_D in the millimolar range). The binding of galactose is in the fast-exchange regime on the NMR relaxation time scale,[14] which is a prerequisite for trNOE studies. Available biochemical[13] and NMR[14] data indicate the importance of an axial OH at C4, and an equatorial OH at C3. It follows that *O*-methylated derivatives of methyl *β*-D-galactopyranoside **1** with an *O*-methyl group in position 2 or 6 (compounds **2** and **5**, Scheme 1) should retain binding activity, while compounds with an *O*-methyl group in position 3 or 4 (compounds **3** and **4**) should be inactive, as this has been reported previously.[13] Here we demonstrate that simple random *O*-methylation of *O*-methyl glycosylpyranosides and subsequent NMR screening of the resulting compound mixtures provides a fast and robust protocol that allows rapid identification of the bioactive pyranosides. For the synthesis of randomly *O*-methylated monosaccharides standard protocols were applied[15] (Scheme 1). In the following, the analysis of a mixture of four regioisomeric galactopyranosides **2**−**5** will be discussed first, demonstrating the principles of the approach. Application to a more complex mixture of 20 monosaccharides and an extension to heteronuclear STD-NMR protocols using ^{13}C labeled *O*-methylated derivatives follows.

Results

Synthesis. Applying a standard protocol[15] different random-*O*-methylated libraries were synthesized. Library A was derived from *β*-D-*O*-methyl-galactopyranoside **1**, and contains all regioisomeric mono-methylated derivatives **2** to **5**. Library B is equivalent to library A with the only difference being that ^{13}C-labeled methyl iodide was employed for methylation yielding compounds **2** to **5** with ^{13}C labeled *O*-methyl groups. These compounds are labeled **2***−**5***. Library C (Scheme 2) was obtained from *β*-D-*O*-methyl-galactopyranoside **1**, α-D-*O*-methyl-galactopyranoside **6**, α-D-*O*-methyl-mannopyranoside **7**, *β*-D-*O*-methyl-glucopyranoside **8**, and α-D-*O*-methyl-glucopyranoside **9**. The different components in all libraries were present in approximately equimolar amounts.

NMR Spectroscopic Assignments. All regioisomers **2** to **5** of libraries A and B yielded rather similar NMR spectra. Nevertheless, assignment of all ^{1}H and ^{13}C NMR signals of each individual isomer in the mixture was straightforward using standard techniques. As an example, Figure 1 shows homonuclear correlation spectra of the mixture of all regioisomeric *O*-methylated *O*-methyl-*β*-D-galactopyranosides **2** to **5**. Assignment was facilitated by the fact that *O*-methylation causes a high-field shift of ca. 0.3 ppm for the corresponding ring proton and a low-field shift of ca. 10 ppm for the respective carbon. In addition to chemical shift arguments, heteronuclear one- and multiple-bond correlations provided a link between methyl protons and ring protons, enabling an assignment of the *O*-methyl proton signals. Proton and carbon chemical shifts for the *O*-methyl *β*-D-galactopyranosides **2**−**5** are compiled in Table 1 and are consistent with literature data.[16] For the more complex library C unambiguous assignment of all individual components in the mixture was impossible.

NOESY and trNOESY Experiments. Library A was subjected to NOESY experiments in the presence and absence of SNA. In the absence of SNA, positive NOEs were observed as this is expected for small molecules. NOEs involving the anomeric protons are displayed in Figure 2. NOE cross-peaks between the anomeric protons and the *O*-methyl groups at C1 allowed a straightforward assignment of the anomeric *O*-methyl groups in compounds **2** to **5**. Upon addition of SNA, two of the four anomeric signals displayed trNOEs. Since for small molecules trNOEs have an opposite sign as compared to NOEs, discrimination between NOEs and trNOEs was simple. trNOEs (negative signals, right spectrum in Figure 2) were observed for the regioisomers **2** and **5** only, with *O*-methyl groups attached to carbon atoms C2 and C6, respectively. Compounds **3** and **4** with the *O*-methyl groups in positions 3 and 4 displayed no trNOEs (Figure 2). It follows that **2** and **5** were recognized by the lectin, whereas **3** and **4** were not bound. This result is in perfect agreement with previous biochemical data.[13]

The trNOE approach was successful because the monosaccharides **2** to **5** displayed a well-dispersed fingerprint region consisting of the anomeric signals. In more complex situations, the detection of trNOEs may suffer from signal overlap, as this has already been described.[8] Therefore, we also applied the more versatile and robust STD NMR protocol.[9]

STD NMR Experiments. 1D STD NMR experiments for library A in the presence of SNA (ca. 50-fold excess of the library) provided already most of the information necessary to identify the components with binding activity (Figure 3).

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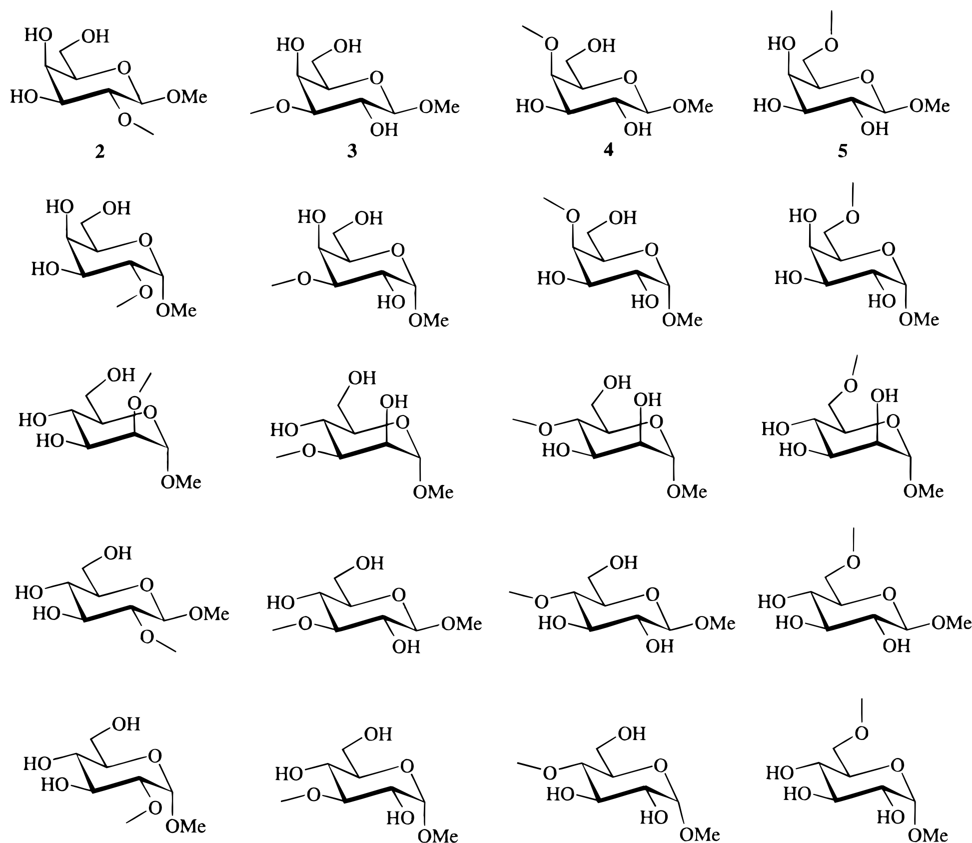
Scheme 2. Compounds of Library C[a]



[a] First row: β-D-galactopyranosides **2**−**5**. Second row: α-D-galactopyranosides. Third row: α-D-mannopyranosides. Fourth row: β-D-glucopyranosides. Fifth row: α-D-glucopyranosides. All compounds are present in approximately equimolar amounts.

Prominent signals in the 1D STD NMR spectrum were the anomeric protons, protons H4, and the anomeric *O*-methyl groups of compounds **2** and **5**. Proton H2 of **2** is also easily identified. It appeared that the saturation transfer was especially efficient for protons H4 indicating that these protons must be close to the protein surface making intimate contact with protons in the binding pocket. Correspondingly, line broadening upon binding to the lectin was most significant for protons H4 in compounds **2** and **5** as this had been observed before.[14] The 1D STD NMR did not allow unambiguous assignments for the ring proton region (ca. 3−4 ppm).

To better resolve the spectral region of the ring protons STD TOCSY and STD COSY spectra were acquired.[9] In the case of library A, the complete spin systems of the two bioactive components **2** and **5** were seen in the STD TOCSY spectrum (Figure 4). The assignment is indicated for galactopyranoside **2**.

STD HMQC Spectra. All inverse heteronuclear pulse sequences start with proton magnetization. Therefore, it is straightforward to apply the concept of STD NMR to, e.g., HMQC spectra. Because of the better dispersion in the ^{13}C dimension the interpretation of STD HMQC spectra is extremely simple. The STD HMQC pulse sequence was applied to library B in the presence of SNA, and Figure 5 shows a comparison of the HMQC spectrum with the corresponding STD HMQC experiment. It was immediately obvious that two of the four cross-peaks that arose from the *O*-methyl groups in derivatives **2*** to **5*** disappeared in the STD HMQC spectra. Only the cross-peaks for the compounds **2*** and **5*** were visible indicating that these two compounds bound to SNA. A STD HMBC experiment (data not shown) directly linked the *O*-methyl groups with the corresponding protons in the pyranose ring and thus allowed an assignment of the *O*-methyl groups without previous knowledge of their position.

STD NMR Spectra of Library C. Library C contained 20 different compounds that were very similar in their chemical and physical properties. Spectral overlap was severe, and therefore it was very difficult to assign individual compounds in the mixture. Even for the anomeric protons no individual signals could be separated, as this is seen from a TOCSY spectrum (Figure 6, left spectrum). Addition of SNA to library C (50-fold excess) and acquisition of a STD TOCSY spectrum yielded a rather simple cross-peak pattern that was readily attributed to compounds **2** and **5** (Figure 6, right spectrum). Therefore, the STD TOCSY experiment discriminated well between components with and without binding activity despite the severe signal overlap. In the STD TOCSY spectrum no signals were observed for the α-anomers of compounds **2** and **5**. In 1D STD NMR experiments with 1K scans weak signals were observed for the anomeric protons of the α-anomers of **2** and **5** suggesting that these compounds had a very low affinity to SNA, probably in the submillimolar range.

Discussion

From recent work in the field it is obvious that NMR spectroscopic techniques are gaining substantial weight in drug discovery.[4] The general advantage of NMR is its direct access to detailed structural information. Here, we applied NMR methods that are based on dipolar interactions between the receptor protein and the ligand to identify binding. The target investigated was a lectin (SNA) that specifically binds to D-galactose. To identify the key hydroxy groups for binding activity newly developed parallel NMR screening protocols were

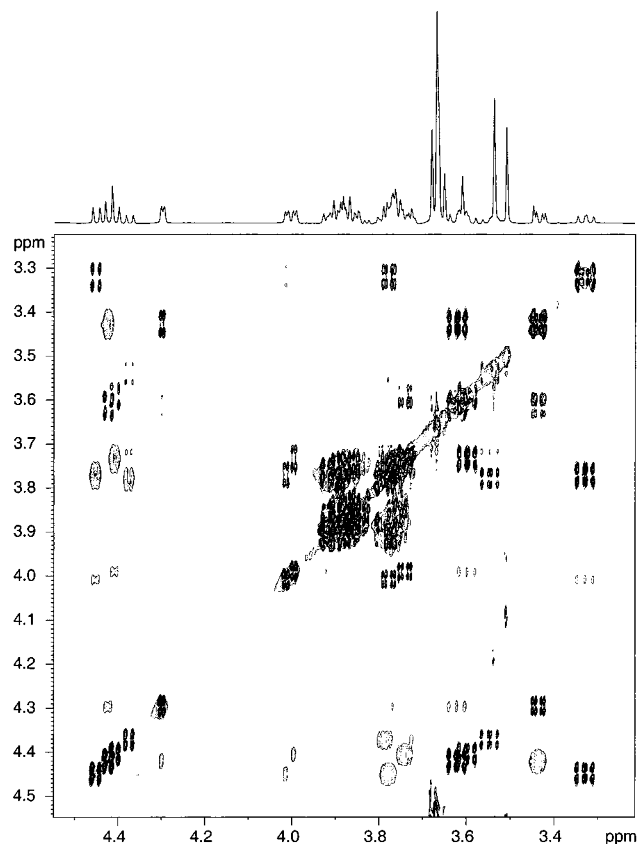


Figure 1. Homonuclear correlation spectra of library A (gray = TOCSY; black = COSY) enable the assignment of the individual components **2**−**5**. Anomeric ^{1}H resonance signals are labeled.

Table 1. Proton and Carbon Chemical Shifts [ppm] for Compounds **2**, **3**, **4**, and **5** in D_2O (310 K)

	2	**3**	**4**	**5**
H1	4.363	4.334	4.286	4.319
H2	3.240	3.522	3.459	3.509
H3	3.690	3.346	3.697	3.653
H4	3.926	4.211	3.635	3.906
H5	3.648	3.675	3.697	3.825
H6/H6′	3.8	3.77	3.8	3.68
O-methyl-1	3.586	3.575	3.570	3.557
O-methyl	3.576	3.444	3.517	3.415
C1	104.2	104.3	104.2	104.3
C2	81.1	71.2	70.2	71.6
C3	73.8	82.5	72.9	73.3
C4	69.3	64.7	79.6	69.5
C5	75.5	75.6	75.8	73.9
C6	61.6	61.5	61.1	72.2
O-methyl-1	57.6	57.7	57.8	57.6
O-methyl	60.7	56.7	62.0	59.0

applied. Instead of sequentially synthesizing compounds with the hydroxyl functions selectively blocked, our approach is based on simple random methylation.

For library A (Scheme 1), trNOE-based screening and STD NMR both allowed unambiguous assignment of the bioactive components **2** and **5** in the mixture. For the more complex library C, trNOESY spectra were obscured by serious resonance overlap, whereas STD NMR led to clean 1D STD and 2D STD TOCSY spectra. An assignment of the bioactive components was straightforward. It has been shown already that overlap problems in trNOE-based screening protocols can be overcome by applying homonuclear 3D NMR experiments, e.g., 3D-TOCSY-trNOESY.[17] This approach is rather time-consuming, and therefore will be limited to cases where sequence informa-

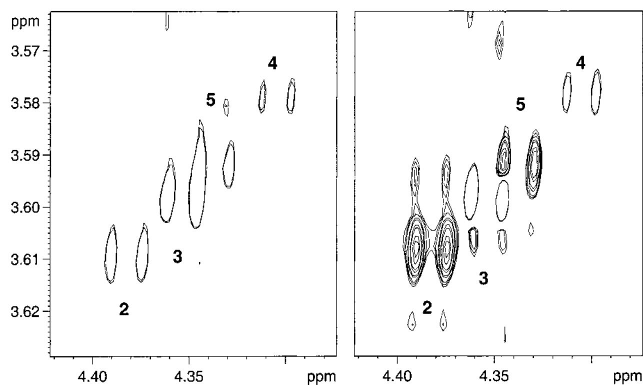


Figure 2. NOESY spectrum (500 MHz) showing the region of anomeric protons (positive contour levels = open; negative contour levels = filled) of library A. Left spectrum: Free library A (mixing time 900 ms). Right spectrum: Library A in the presence of SNA at a molar ratio of approximately 15:1, library to protein (mixing time 150 ms). The numbers correspond to the compounds in Scheme 1.

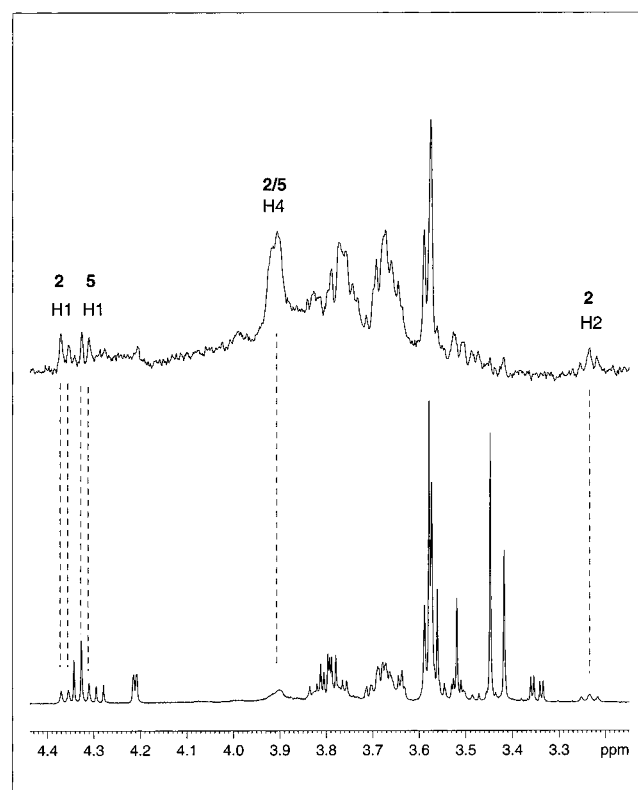


Figure 3. One-dimensional STD spectrum (top) in comparison to a normal proton NMR spectrum (bottom) of library A in the presence of SNA (molar ratio of approximately 1:50, library to protein). The presaturation time was 2 s. Inspection of the anomeric region shows that compounds **2** and **5** are bound to SNA. Protons H4 give rise to a strong response in the STD spectrum and show significant line broadening in the proton NMR spectrum.

tion is required that is not available from scalar coupling pathways but only from dipolar interactions (e.g., interglycosidic trNOEs). Except for such special cases, STD TOCSY spectra are the method of choice to identify binding activity even in the presence of severe signal overlap.

Library C was remarkable because it contained only compounds that had identical molecular weights, identical functional groups, and therefore almost identical biophysical properties resulting in severe resonance overlap. Sequential screening

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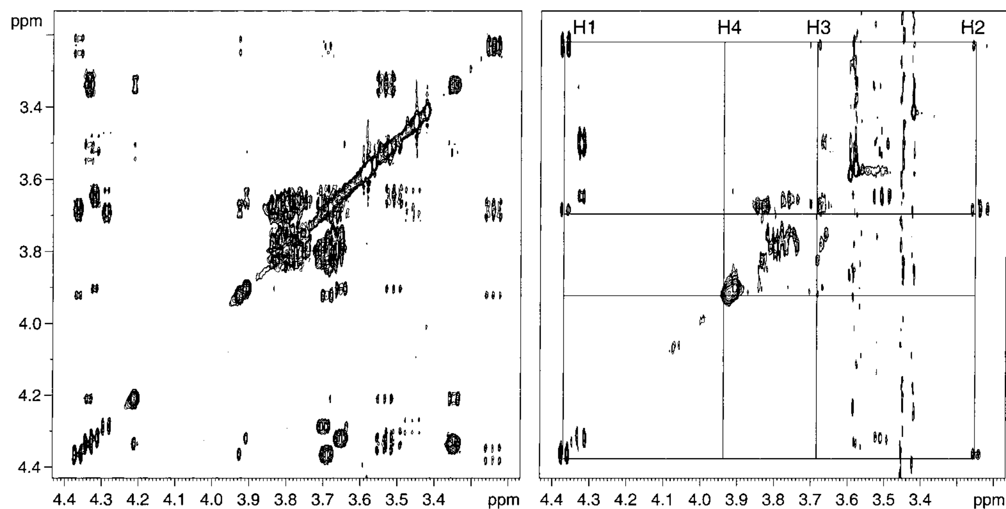

Figure 4. Comparison of a TOCSY (left) and a STD-TOCSY (right) spectrum of library A in the presence of SNA. The spin systems of the bound compounds **2** and **5** are well resolved with the spin system of compound **2** indicated in the STD TOCSY. The mixing time was 60 ms.

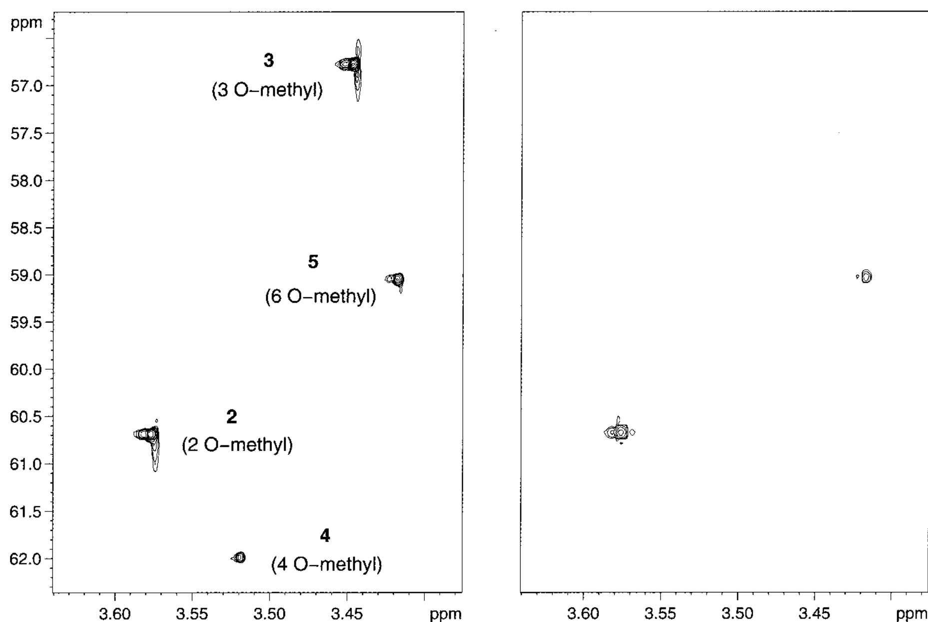

Figure 5. HMQC (left) and STD HMQC (right) spectra of library B in the presence of SNA. The region of *O*-methyl ^{13}C resonance signals is shown. Subtraction of on- and off-resonance HMQC spectra to generate the STD HMQC spectrum was achieved by shifting the receiver phase by 180. The presaturation time was 2 s. It is immediately obvious that only compounds **2*** and **5*** bind to SNA.

protocols such as ELISA would also detect binding activity for library C, but to identify the individual compounds that are responsible for this activity the compound mixture would have to be separated into its components. It is obvious that a clean separation of library C in its individual components is not straightforward. This clearly highlights the advantages of parallel NMR screening protocols. It should be mentioned that identification of monosaccharides **2** and **5** as the bioactive components was guided by the preknowledge about libraries A and B, and by the fact that the composition of library C was known. When dealing with synthetic libraries such preknowledge usually is present. In other cases where, e.g., natural compound mixtures are targeted unambiguous identification of binding components would probably require additional experimental effort. One option is the application of 3D NMR experiments to deliver unambiguous assignments.[17]

STD NMR allows the combination with almost any type of NMR experiment.[9] It is well established that the use of heteronuclear NMR experiments leads to much better dispersion of signals in multidimensional heteronuclear NMR experiments. This strategy is further enhanced by introducing ^{13}C labels. To combine STD NMR with heteronuclear pulse sequences ^{13}C labeling is not absolutely necessary, but the acquisition of spectra would require significantly more time without ^{13}C enrichment. The random methylation approach followed in the present study allowed straightforward and cheap incorporation of ^{13}C labels into carbohydrates. Library B that was composed of randomly ^{13}C-methylated galactose derivatives had been used to demonstrate the feasibility of this approach. By acquiring STD HMQC spectra the methyl groups of the components with binding activity were readily assigned. It is obvious that the good dispersion in the ^{13}C dimension would allow much more complex mixtures to be handled. The trNOE-based screening protocol could also be extended to the use of ^{13}C-labeled compounds. In that case one would apply ^{13}C-filtered trNOE sequences. This approach has the disadvantage that the methyl proton signals are much less well dispersed than the corresponding carbon signals, and was therefore not further evaluated

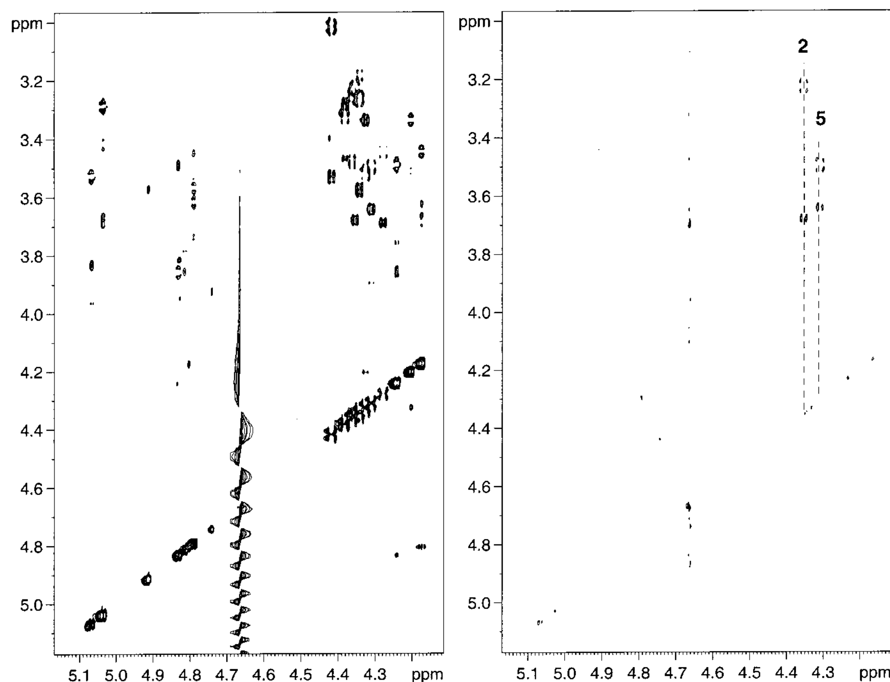

Figure 6. TOCSY (left) and STD TOCSY (right) spectrum of library C in the presence of SNA (conditions as in Figure 4). Compounds **2** and **5** with binding activity for SNA are easily identified in the STD TOCSY spectrum.

here. Our experiments suggest that the random modification of key hydroxyl groups by simple ^{13}C methylation provides a very useful tool for the analysis of binding activities of carbohydrates.

Certainly, carbohydrates represent an important class of biological macromolecules.[18] Therefore, their conformational features and their interaction with protein receptors is in the focus of current research.[19] To identify key-hydroxyl groups in saccharides transformation into several functional groups other than *O*-methyl groups is possible. For instance, introduction of fluoro, deoxy, or amino groups also generates compounds that may be tested for binding activity against protein targets.[20] In principle, compound libraries containing such derivatives can be synthesized, and also subjected to the NMR screening protocols described above. The approach clearly is not limited to carbohydrate derivatives. Limitations may arise when ligand signals of compound libraries are spread over the whole spectral range. It could prove difficult then to selectively saturate the protein without also affecting some of the ligand resonances in STD experiments. Control experiments where the frequency for on-resonance irradiation is shifted, and application of other classes of NMR experiments such as NOE-pumping,[6d] or trNOESY[7a] will help to eliminate artifacts in such cases. Fortunately, such a situation will only rarely arise because at least in synthetic libraries all compounds are derived from common ancestors sharing common structural elements that lead to rather similar NMR spectra. Other artifacts may arise from unspecific binding. In principle, such effects are easily eliminated by performing reference experiments with other proteins, or by testing the ability of bioactive ligands to compete with known inhibitors or substrates of the target protein. In cases where several binding components are present at the same time, relative affinities of ligands may be evaluated.

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Conclusion

Our study shows that STD NMR in combination with a simple ^{13}C isotope labeling protocol to produce a ^{13}C labeled compound library is a very powerful method for identifying binding activity directly from mixtures. In the present case it was demonstrated how a well-established methylation procedure can be used to synthesize a carbohydrate library with selectively blocked hydroxy groups. This approach may be easily extended to larger saccharides and also to other classes of compounds. The study also demonstrates the potential of the lately developed STD NMR technique for the investigation of small compound libraries. It was shown that a library (library C) consisting of 20 almost identical diasteromeric saccharides is easily analyzed for binding activity. We are currently extending this protocol to larger saccharides and to other classes of compounds.

Experimental Section

Preparation of SNA. SNA (Vector) was dialyzed three times against 500 mL of 20 mM NaCl, 50 mM sodium phosphate at pH 7.6 to remove lactose (as added by the manufacturer). Protein content was determined by UV absorption at 280 nm. The dialysate was lyophilized twice using D_2O as solvent, and was finally dissolved in D_2O (99.98%, Sigma-Aldrich). Insoluble precipitates were removed by filtration. To prevent bacterial growth, small amounts of sodium azide were added.

Preparation of Randomly Methylated Monosaccharide Libraries. The monosaccharide libraries were prepared following a standard protocol.[15] One gram of the respective methylglycoside and 0.5 mL of iodomethane were dissolved in dry DMF and cooled to 0 °C. Two grams of silver oxide (freshly prepared following ref 21) were added in small portions and the mixture was brought to room temperature. The reaction was stopped when the fraction of monomethylated derivatives (TLC, $CHCl_3$/MeOH 9:1) reached a maximum (2−3 h) by filtering off the silver oxide and evaporating to dryness. The products were subjected to silica gel chromatography ($CHCl_3$/MeOH 9:1) to yield 40−50% of monomethylated methylglycosides.

NMR Spectroscopy. NMR samples were prepared in D_2O with d_4-TSP (trimethylsilylpropionic acid, Sigma-Aldrich) as internal reference.

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Protein/ligand ratios of ca. 1:15 to 1:50 were employed, with typical ligand concentrations in the millimolar range. NMR spectra were measured on Bruker DRX500 and DRX600 spectrometers at 500 and 600 MHz proton resonance frequency, respectively, and 310 K measuring temperature. Homonuclear 2D spectra were acquired phase sensitive in F1 using TPPI. Heteronuclear correlation spectra were acquired with gradient selection. In some cases protein signals were suppressed with a T_2 spinlock filter.[22] Water suppression was achieved by presaturation except for the STD spectra where the WATERGATE sequence was used. NOESY spectra were recorded with 100−400 ms mixing time. In STD spectra[9] saturation was achieved by a series of equally spaced 50 ms Gaussian shaped pulses, with a peak amplitude of 10 Hz, 10 ms delay between the pulses, and a total saturation time of ca. 2 s. The frequency of the protein irradiation was set on the maximum of the broad protein hump either in the aromatic or in the aliphatic region, the frequency of the offset irradiation to −5.0 or +20 ppm; however, the result turned out to be not dependent on the irradiation frequencies. FIDs with protein saturation were recorded alternating with reference FIDs (irradiation in a peak-free region) and directly subtracted. A minimum of 32 dummy scans was employed to reduce subtraction artifacts. For the STD HMQC sequence, subtraction of on- and off-resonance spectra was achieved by alternating the receiver phase every second scan. For this the standard HMQC pulse sequence (Bruker) was modified. Typical measurement times were less than 1 h.

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