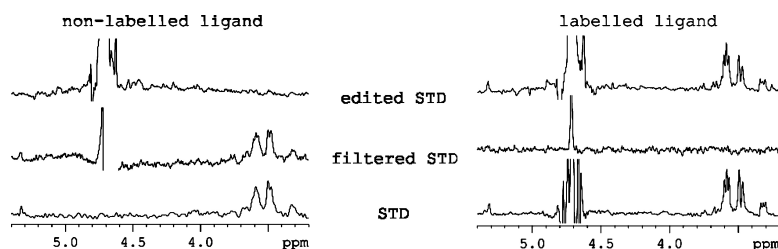## Article

Competition Saturation Transfer Difference Experiments Improved with Isotope Editing and Filtering Schemes in NMR-Based Screening

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Competition Saturation Transfer Difference Experiments Improved with Isotope Editing and Filtering Schemes in NMR-Based Screening

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Abstract: Competition binding experiments are used in NMR-based screenings to match up to the binding
site with that of a known ligand and to determine the strength of the interaction, thus providing a ranking
of hits according to receptor affinity. These competition titration experiments must use a reference ligand
for which the binding site on the receptor and the affinity of the interaction is known. These experiments
rely on the observation of separate signals of the reference and hit compounds, which is very often hampered
by signal overlap. Here, we present a combination of isotope editing and filtering schemes with saturation
transfer difference (STD) experiments that allows the separation of the STD signals of the labeled reference
ligand from that of the natural abundance hit compound even in the case of severe signal overlap. Thus,
the measurement of well-defined titration curves in competition STD titration experiments is feasible and
allows the quantitative determination of binding constants. Note that the method requires the availability of
the reference ligand in an NMR-active, stable-isotope-labeled form.

Introduction

The recognition of ligands by their receptors regulates essential processes for life. Identification of lead compounds or bioactive fragments that bind specifically to the target receptor in screens is an essential step in the design of new compounds of pharmaceutical significance. Ligand-observed NMR methods,[1] are frequently used to search for ligands, both in academic and industrial laboratories. A particular strength of NMR-based screening is that, in addition to finding hits, further information is provided, such as the characterization of the binding epitope, comparison of the binding site of the hit with that of a reference ligand as well as binding affinity toward a target receptor.

Among ligand-observed NMR methods, the saturation difference (STD) experiment, in particular,[2] features high sensitivity and robustness and requires only minute amounts of unlabeled macromolecules. Moreover, it is applicable for high-molecular weight complexes and, last but not least, is highly amenable to automation. The STD experiment can be flexibly modified with coupling to other NMR pulse sequence building blocks to give homo- or heteronuclear multidimensional spectra, such as STD-TOCSY,[2a,5] STD-HSQC/HMQC,[5a] which efficiently facilitate the characterization of the binding epitope.

The primary objective of the screen is the identification of compounds (so-called "hits", "binders") binding to the receptor. The discovery of hits is carried out either "*consecutively*", by testing each compound in separate measurements, or "*simultaneously*", by using mixtures of compounds, which is followed by identifying the hits. While the second approach might significantly reduce the total number of experiments, the first strategy is more reliable and robust. Subsequent to finding a hit, the binding epitopes can be characterized by the assignment of the ligand signals affected in binding.

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(1) (a) Middleton, D. A. *Annu. Rev. NMR Spectrosc.* **2007**, *60*, 39–75. (b) Jahnke, W. *J. Biomolecular NMR* **2007**, *39*, 87–90. (c) Peng, J. W.; Moore, J.; Abdul-Manan, N. *Prog. Nucl. Magn. Reson. Spectrosc.* **2004**, *44*, 225–256. (d) Stockman, B. J.; Dalvit, C. *Prog. Nucl. Magn. Reson. Spectrosc.* **2004**, *44*, 187–231. (e) Zartler, E. R. *Curr. Top. Med. Chem.* **2003**, *3*, 25–37.

(2) (a) Mayer, M.; Mayer, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 1784–1788. (b) Mayer, M.; Mayer, B. *J. Am. Chem. Soc.* **2001**, *123*, 6108–6117. (c) Meyer, B.; Peters, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 864–890.

(3) (a) Fielding, L. *Curr. Top. Med. Chem.* **2003**, *3*, 39–53. (b) Fielding, L. *Prog. Nucl. Magn. Reson. Spectrosc.* **2007**, *51*, 219–242.

Further information on the binding is delivered by competition experiments if a reference ligand exists. First, in a competition experiment, one can test whether the hit binds to the same binding pocket of the receptor as the reference ligand. In such an experiment, the signals of the hit compound are reduced upon addition of the reference ligand if the binding is competitive. This can be carried out using both mixtures and samples containing single compounds. Subsequently, in a competition titration experiment, the dissociation constants (K_d) of the binders can be determined, and thus, the hits can be ranked according to their binding affinities.[3] Importantly, the competition titration experiments can only be carried out in a sample of a *single* hit compound (together with the macromolecule) to which the reference ligand is added as the titration proceeds. During a competition titration followed with STD,[2b] the STD signals of the ligand with unknown affinity and those of the reference ligand are monitored simultaneously. These competition experiments, however, rely on the observation of separate signals of the reference ligand and that of the hit compound. A similar chemical structure for both reference ligand and hit, however, inherently results in similar proton chemical shifts yielding fully or partial overlapping STD signals. In such cases, only the overall change of the STD effect can be detected, preventing accurate assessment of the ligand K_d.

Overlap of STD signals is frequently found when constitutional or configurational isomers of a known ligand (or lead compound) are tested. A typical case is carbohydrate recognition.[4] A given receptor can have strikingly different affinities for sugars that differ in the configuration of only one carbon atom within the ring (i.e., glucose versus galactose) or are joined by different interglycosidic linkages. As the range of the ^{1}H NMR resonances and also the STD signals is compressed within a narrow spectral region, ~3−4.5 ppm for ring protons and ~4.5−6 ppm for the anomeric protons, having a carbohydrate reference ligand in the screen of carbohydrate compounds is likely to lead to overlapping signals.

Extension of the STD experiment to a second dimension, such as STD-TOCSY[2a,5] and STD-HSQC/HMQC,[5a] was suggested to overcome the overlap problem. Although these 2D NMR experiments lead to much better dispersion of signals, their acquisition requires significantly more time. Competition STD titration for K_d measurement requires at least 6−8 titration steps for all potential hit compounds and thus renders the application of 2D STD experiments in competition titration, due to time requirements, impractical. In contrast, the 1D STD competition titration is highly sensitive and robust and requires only minute amounts of macromolecules.

As opposed to high-throughput biological screens, an NMR-based screen is able to test only a few hundred compounds a day.[6] It is therefore desirable that the NMR screen is carried out in a fast, reliable and efficient way[7] while retaining the advantages of providing additional information about the binding in competition experiments, such as affinity and identity of the

binding site in comparison with a reference ligand. Signal overlap prevents, however, partly or completely the quantitative evaluation of the competition titration experiment. Here we propose the use of a stable isotope-labeled reference ligand in conjunction with isotope edited/filtered STD experiments to overcome the problems arising from signal overlap.

Results and Discussion

In a small-scale screening process aimed at identifying and characterizing further potential ligands for malectin, D-maltose was used as a reference ligand in STD-based competition titrations to determine the K_d of the potential ligands found in the screen. STD spectra of malectin with various disaccharides[8] are shown in Figure 1. The binding affinity for the reference ligand, D-maltose, was determined using isothermal titration calorimetry (ITC). For ligands featuring at least some nonoverlapping signals with D-maltose, the affinity could easily and quickly be obtained by STD competition titrations. In our hands, 0.5 mL of a 20 μM solution of the receptor was employed for STD versus 2 mL of 800 μM solution for ITC. However, the STD NMR signals of the various sugar ligands appeared in a narrow spectral region, as shown in Figure 1. For instance, in the case of the α- and β-*p*-nitrophenyl-maltosides as well as for methyl-α-glucoside, all the STD signals overlap. The STD experiments also showed that the aromatic aglycon of the sugars does not take part in the binding, since no or only very small STD signals in the aromatic region of the spectrum were observed. Thus, this region could not be utilized for the affinity determination, and the conventional STD approach could not be used to determine the affinities of these particular sugar derivatives.

We propose the use of ^{13}C-isotope edited and filtered STD experiments in order to separate the STD effects on two different ligands having overlapping STD signals. If a reference ligand with known affinity is available in a stable isotope labeled form, then the editing-filtering schemes can be used to separate their particular STD signals and to obtain separate titration curves for the decreasing and the increasing STD responses. The pulse schemes of the corresponding experiments are depicted in Figure 2. The original STD experiment is modified by the insertion of known editing/filtering schemes[9] both of which consist of a combination of the spin−echo sequence with a pulsed field gradient purging element. During the spin−echo tuned to the one-bond heteronuclear coupling constant, the ^{1}H magnetization of the ^{13}C-labeled ligand evolves into antiphase magnetization, while that of the nonlabeled ligand is unaffected (disregarding relaxation losses). In the filtering scheme, this latter [1]H(^{12}C) magnetization is flipped to the *z* axis by the subsequent 90° [1]H pulse, leaving the antiphase [1]H(^{13}C) magnetization in the transverse plane, where it can be eliminated by the purging gradient. In the editing scheme, the phase of the 90° [1]H pulse is shifted by 90° to convert the antiphase ^{1}H magnetization of the ^{13}C-labeled ligand into two-spin order (*zz*-magnetization) for preservation, while the transverse [1]H(^{12}C) magnetization of the nonlabeled ligand is eliminated with the purging gradient.

(4) (a) Angulo, J.; Rademacher, C.; Biet, T.; Benie, A. J.; Blume, A.; Peters, H.; Palcic, M.; Parra, F.; Peters, T. *Methods Enzymol.* **2006**, *416*, 12−30. (b) Johnson, M. A.; Pinto, B. M. *Carbohydr. Res.* **2004**, *339*, 907−928.

(5) (a) Vogther, M.; Peters, T. *J. Am. Chem. Soc.* **2000**, *122*, 6093−6099. (b) Johnson, M. A.; Pinto, B. M. *J. Am. Chem. Soc.* **2002**, *124*, 15368−15374.

(6) (a) Schwardt, O.; Kolb, H.; Ernst, B. *Curr. Top. Med. Chem.* **2003**, *3*, 1−9. (b) Fejzo, J.; Lepre, C.; Xie, X. *Curr. Top. Med. Chem.* **2003**, *3*, 81−97.

(7) Ross, A.; Senn, H. *Curr. Top. Med. Chem.* **2003**, *3*, 55−67.

(8) Schallus, T.; Jaeckh, C.; Feher, K.; de Palma, A.; Liu, Y.; Simpson, J.; McKeen, M.; Stier, G.; Gibson, T. J.; Feizi, T.; Pieler, T.; Muhle-Goll, C. *Mol. Biol. Cell* **2008**, *19*, 3404−3414. Malectin: A Novel Carbohydrate-Binding Protein of the Endoplasmic Reticulum and a Candidate Player in the Early Steps of Protein N-Glycosylation.

(9) (a) Breeze, A. *Prog. Nucl. Magn. Reson. Spectrosc.* **2000**, *36*, 323−372. (b) Otting, G.; Wüthrich, K. *Q. Rev. Biophys.* **1990**, *23*, 39−96. (c) Griffey, R. H.; Redfield, A. G. *Q. Rev. Biophys.* **1987**, *19*, 51−82.
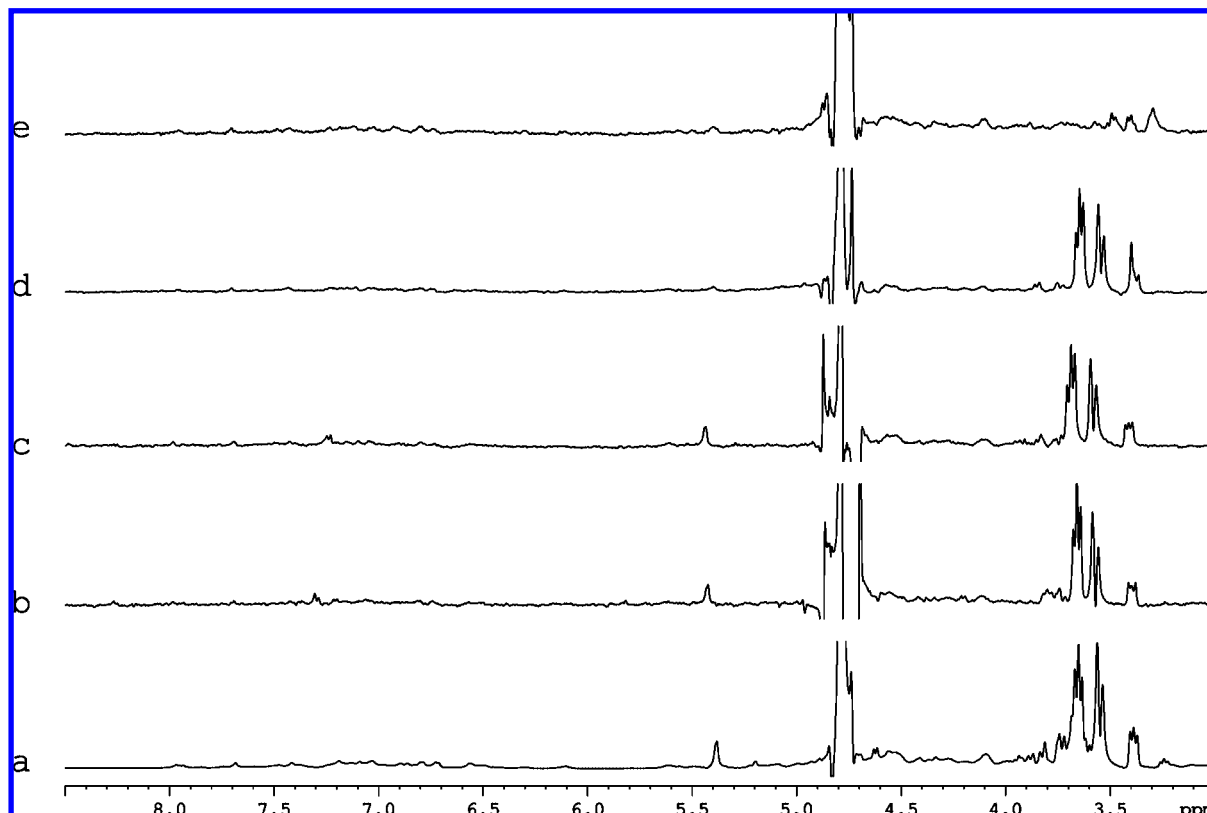
Figure 1. STD spectra of related disaccharides in the presence of lectin-type protein malectin: (a) D-(+)-maltose, (b) *p*-nitrophenyl-α-D-(+)-maltoside, (c) *p*-nitrophenyl-β-D-(+)- maltoside, (d) Me-α-D-glucoside, (e) D-(+)-cellobiose.
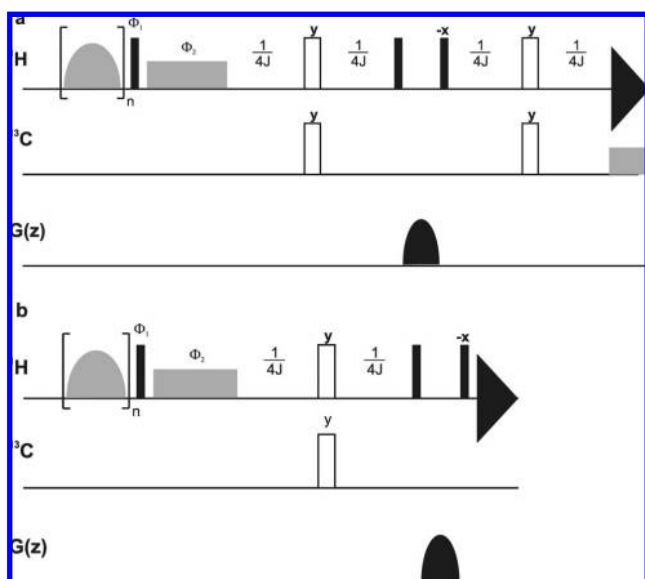

Figure 2. Pulse sequence of the (a) edited and (b) filtered STD experiments. Filled and open bars represent 90° and 180° pulses, respectively with phases of *x*, unless stated otherwise. Φ_1 is *x*, *x*, −*x*, −*x*, while Φ_2 is *y*, *y*, −*y*, −*y* and the receiver phase is *x*, −*x*, *x*, −*x*.

In both experiments, after purging, the magnetization stored along the *z*-axis is flipped back into the transverse plane. Note that in the filtering experiment, the $^1H(^{12}C)$ magnetization of the nonlabeled ligand is ready for detection, while in the edited scheme, one additional spin−echo is needed to refocus the $^1H-^{13}C$ antiphase magnetization prior to decoupling during the acquisition period. Regarding sample conditions (ligand excess, protein concentration) and NMR parameters for the setup of

the STD part (effectiveness of spin diffusion for different sizes of macromolecules, length of saturation, selectivity of pulses, etc.) the proposed experiment behaves identically to the original STD experiment.[2] A 40:1−50:1 excess of the ligand is a typical starting point, but this excess may vary, depending on the particular system.[2b] As the STD experiments take advantage of the large correlation time of the receptor molecule to ensure efficient saturation transfer, the receptor concentration could be reduced for larger biomolecules. Moreover, using high field magnets with sensitive cryogenic probes may permit further decrease the required amount of ligand and receptor.

To demonstrate the performance and the quality of isotope-selection of the proposed experiments, we have used samples containing either unlabeled or ^{13}C-labeled D-maltose in the presence of malectin. As expected, the ^{13}C-filtered STD spectrum displays signals for the unlabeled ligand, while in the edited experiment no signals are detected (Figure 3). The naturally occurring 1% ^{13}C isotope content could, in principle, produce STD signals in the ^{13}C-edited spectrum. These signals were, however, under the detection limit. When using a labeled ligand, ^{13}C-labeled maltose, only the ^{13}C-edited experiment produces STD signals, which are efficiently suppressed with the ^{13}C-filtering scheme. Note that, due to the necessity of the additional refocusing period in the ^{13}C-edited experiment, this becomes longer, thus leading to some unavoidable relaxation losses compared with the filtered STD experiment.

In case of mismatch of the spin−echo time, due to variations in the one-bond heteronuclear couplings, part of the magnetization of the ^{13}C-labeled ligand will not develop into antiphase magnetization and will remain aligned with that of the nonlabeled ligand. Consequently, the ^{13}C-filtered experiment will show a mixture of the STD signals of the labeled and the
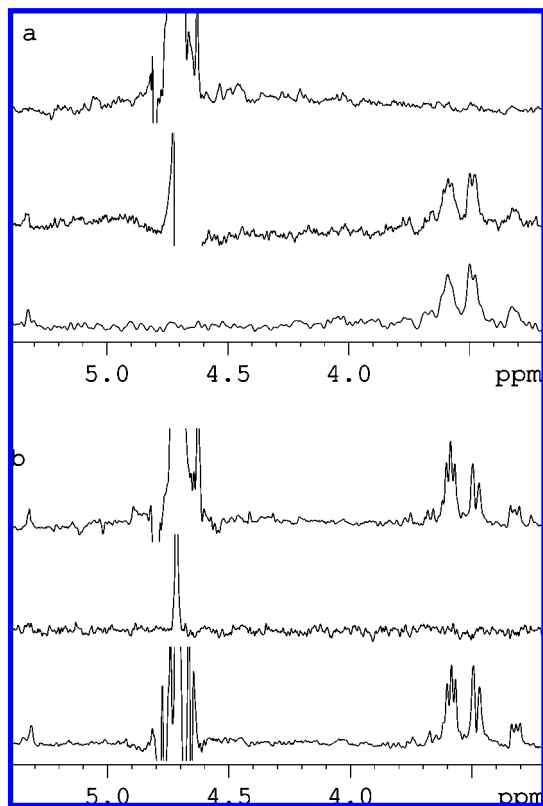
Figure 3. Results of the filtered (upper rows), edited (middle rows) and original (lower rows) STD experiments with samples containing (a) ^{13}C-labeled and (b) unlabeled maltose ligand. The STD spectrum of ^{13}C labeled maltose (the lower row in panel (a)) was recorded with ^{13}C decoupling during acquisition.

Figure 4. Competition titration of ^{13}C-labeled maltose with *p*-nitrophenyl-*β*-maltoside (a) STD experiment with ^{13}C-decoupling during acquisition, (b) edited STD and (c) filtered STD. Concentration of *p*-nitrophenyl-*β*-maltoside is shown on the left side.

Figure 5. Competition titration curves of the edited and filtered STD schemes for the *p*-nitrophenyl-*β*-maltoside. ▲: filtered STD (triangles) and ■ edited STD (squares). Data points corresponding to higher ligand concentrations were measured, but are not shown.

nonlabeled ligand when severe mismatch occurs. The ^{13}C-edited experiment, however, will deliver only the STD responses of the labeled ligand without any "crosstalk" from the unlabeled ligand, but with slightly reduced intensity due to the mismatch. Thus, regarding robustness and selectivity, the edited experiment is clearly superior to the filtered experiment. However, due to the almost uniform values of the one-bond heteronuclear [1]H—[13]C couplings in carbohydrates (not including anomeric carbons) crosstalk is not a real concern even in the filtered experiments. The ^{13}C-filtered STD signal of the anomeric proton, however, likely contains a contribution from the labeled reference ligand in addition to the STD response of the unlabeled ligand.

The current implementation of isotope filtering/editing is based on the principle of purging the undesired coherences. An experiment in which the edited and filtered STD signals would be simultaneously obtained was also considered; however, due to the described crosstalk no perfect separation of the STD effects could be expected.

As a proof of principle, the STD competition titration was carried out in the following way: the initial sample contained unlabeled protein together with an isotope-labeled reference ligand with known affinity (^{13}C-labeled D-maltose) in 50-fold excess. This sample was titrated with an unlabeled potential ligand (*p*-nitrophenyl-*β*-maltoside), for which the K_d is to be determined. The resulting spectra recorded by the original STD experiment and by the modified edited/filtered STD schemes are shown in Figure 4. The spectra in Figure 4a show the overlapping STD signals of both molecules. The sum of the increasing and the decreasing components shows an overall change, which is strongly attenuated compared to the change
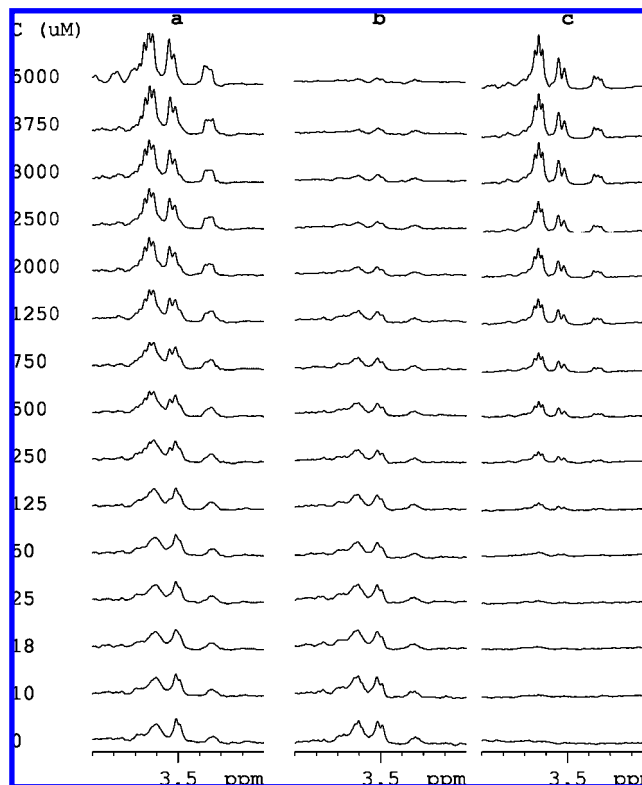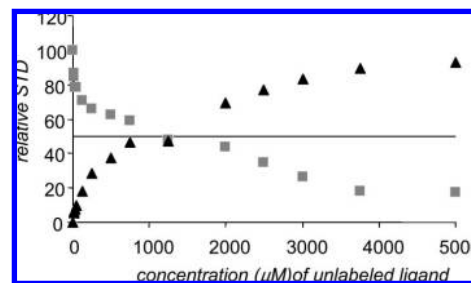
observed for nonoverlapping signals. This overall change is the result of the different intensity of the final STD signals of the two ligands and the slight differences in their ^{1}H spectra. The final STD intensity of a particular ligand is dependent on the affinity, kinetics of the binding interaction and the ligand excess. In the case of similar final STD intensities for the two ligands, no change would be observed during the entire titration. In contrast, upon addition of aliquots of the unlabeled molecule the ^{13}C-edited STD spectra show the monotonically decreasing STD signal of the labeled reference ligand from the full STD down to zero signal, while the ^{13}C-filtered STD spectra showed an increasing STD signal of the natural abundance ligand starting from no signal to the final STD response.

The titration curves are shown in Figure 5. Both isotope separated STD curves show a complete intensity change from no signal to full STD signal or vice versa. The edited STD curve displays larger deviation from a smooth saturation curve because

Scheme 1. Structures of (a) D-(+)-maltose, (b) *p*-nitrophenyl-α-D-(+)-maltoside, (c) *p*-nitrophenyl-β-D-(+)-maltoside, (d) Me-α-D-glucoside, (e) D-(+)-cellobiose
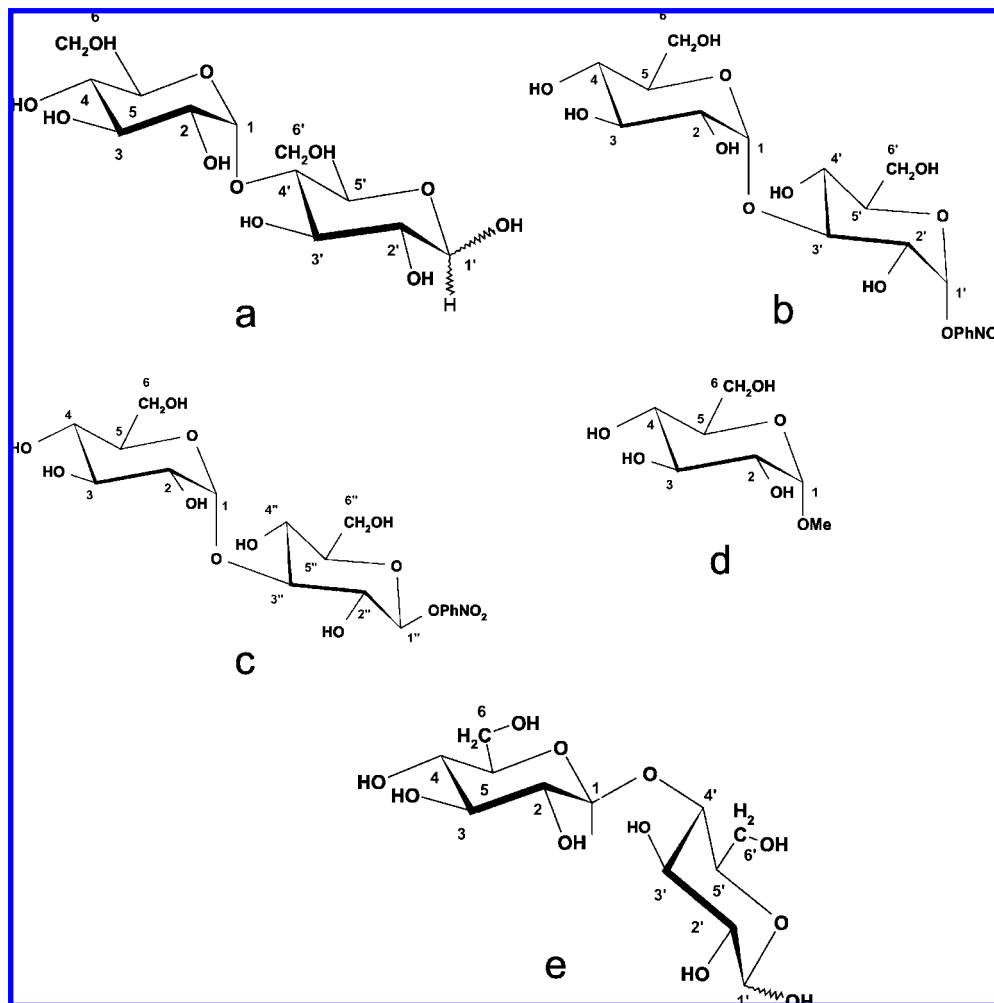
of its lower sensitivity due to relaxation losses. Both curves reach their 50% value at approximately the same concentration, 1100 μM and 1050 μM for the filtered and the edited experiments, respectively.

The titration curves were quantitatively evaluated to obtain the affinity constant of the aromatic maltoside (Figure 5). IC$_{50}$ values were determined by curve fitting, and then the affinity of the natural abundance ligand was calculated according to the following equation:[10]

$$K_{dref} = \frac{[L_{ref}]K_d}{IC_{50} - K_d}$$

where K_{dref} refers to the dissociation constant of the reference ligand, L_{ref} is the concentration of the reference ligand, K_d is the dissociation constant of hit compound and IC$_{50}$ is the concentration of hit compound at half-signal intensity.

Using the binding constant of the reference ligand of 50 μM (obtained by ITC), the affinity of the *p*-nitrophenyl-β-maltoside was found to be 58 μM, which is in satisfactory agreement with the affinity value of 51 μM as measured by ITC. Thus, in this particular case the lectin-type protein, malectin, has an affinity toward the *p*-nitrophenyl β-isomer approximately the same as that for the reference ligand, D-maltose.

Note that the proposed isotope editing/filtering approach requires having the reference ligand in ^{13}C-labeled form. However, there is no need of labeling in all positions if the labeled compound will only be used for screening purposes in the ^{13}C-edited experiment. For example, a ^{13}C-labeled acetate or methyl ether/glycoside can be introduced via chemical modification into a position that gives rise to an STD effect. A straightforward and inexpensive incorporation of such ^{13}C labels into whole libraries of carbohydrates was demonstrated by Vogther et al.[5a] More importantly, the application of the proposed edited/filtered STD schemes requires labeling of only a single reference compound.

Conclusion

Competition experiments in NMR screening deliver valuable additional information about the binding event and thereby provide advantages over traditional high-throughput screens. Overlap of the reference ligand and the hit signals hampers obtaining reliable information on the binding site and/or ranking the hits according to their receptor affinity. Failure of the competition experiments due to signal overlap reduces the overall effectiveness and efficiency of the NMR screen. The combination of an isotope-labeled reference ligand and the edited/filtered STD scheme makes it possible to separate the STD signals of the natural

(10) Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

abundance hits from that of the labeled reference ligand; thus, the corresponding titration curves can be individually recorded and evaluated.

In fact, there is no need for the acquisition of both the edited and filtered STD spectra. For quantitative assessment of K_d the edited experiment is more suitable, because it is not affected by crosstalk due to J mismatch. Therefore, in the case of one binding site an optimal setup of the NMR-based screening may be the following: first the hit compounds are identified in a "consecutive" NMR screen, and after that only the previously identified hits are titrated one by one with the isotope-labeled reference ligand. The titration curves of the edited STD experiment start from zero intensity and end at the full STD signal of the labeled reference compound. These curves are comparable and allow a relative ranking of the hits without the need for precise quantitative evaluation. In the case of multiple binding sites, however, competition for the same binding site must be confirmed by observing the disappearance of the hit signal in the STD filtered spectrum recorded in the final stage of the titration.

Experimental Section

The NMR measurements were carried out on Bruker DRX 500- and 600-MHz spectrometers each equipped with a 5 mm inverse HCN cryogenic probe at 295 K. The resonances of the carbohydrate ligands were assigned using a combination of TOCSY, HSQC, HSQC-TOCSY and HMBC experiments on natural abundance samples in deuterated water. For the STD NMR measurements, unlabeled protein samples of 20 μM were used with a 50-fold ligand excess, containing 20 mM potassium phosphate buffer at pH 6.8 and 150 mM KCl. The STD spectra were measured by using a pulse sequence in which the difference between the on- and off-resonance experiments was created by phase cycling. Saturation of the protein NMR signals was performed using a train of selective Gaussian pulses of 49 ms duration each (maximum field strength of 75 Hz) separated by short delays of 1 ms. The on-resonance frequency used for saturation was set to 0.8 ppm, while the off-resonance irradiation was applied at −40 ppm, where no NMR resonances were present. For the suppression of the background protein signals, a $T_{1\rho}$ relaxation filter of 30 ms was used, with field strength of 5 kHz. The STD spectra were processed with Bruker Topspin 1.3. All spectra were multiplied by an exponential window function with 5 Hz line broadening prior to integration. The curve fitting was carried out using MATLAB.

Supporting Information Available: ITC titration curves for (a) D-maltose and (b) *p*-nitrophenyl-β-maltoside. This material is available free of charge via the Internet at http://pubs.acs.org.

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