JOURNAL OF CHEMICAL EDUCATION

LABORATORY EXPERIMENT

pubs.acs.org/jchemeduc

Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding

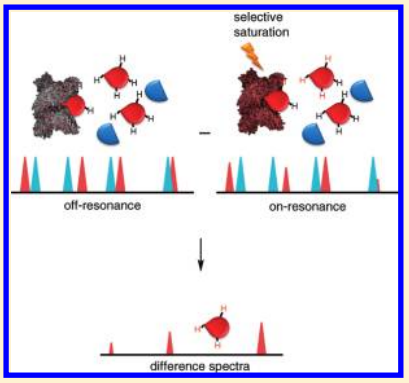
Aldino Viegas, João Manso, Franklin L. Nobrega, and Eurico J. Cabrita*

REQUIMTE, CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

Supporting Information

ABSTRACT: Saturation transfer difference (STD) NMR has emerged as one of the most popular ligand-based NMR techniques for the study of protein−ligand interactions. The success of this technique is a consequence of its robustness and the fact that it is focused on the signals of the ligand, without any need of processing NMR information about the receptor and only using small quantities of nonlabeled macromolecule. Moreover, the attractiveness of this experiment is also extendable to the classroom. In the context of a practical NMR class, this experiment is ideal to illustrate some fundamental NMR concepts, such as the nuclear Overhauser effect and relaxation in a multidisciplinary context, bridging chemistry and biochemistry with a taste of medicinal chemistry.

We use the readily available human serum albumin (HSA), 6-D,L-methyl-tryptophan (6-CH_3-Trp), and 7- D,L-methyl-tryptophan (7-CH_3-Trp) to introduce the STD-NMR experiment and to illustrate its applicability for ligand screening, mapping of binding moieties, and determination of the dissociation constant, in a context that can be explored or adapted to the student's course level and topic (chemistry or biochemistry). We also cover the most important theoretical aspects of the STD experiment, calling attention to some of its limitations and drawbacks without a complex theoretical approach.



KEYWORDS: Graduate Education/Research, Upper-Division Undergraduate, Biochemistry, Interdisciplinary/Multidisciplinary, Laboratory Instruction, Hands-On Learning/Manipulatives, Bioanalytical Chemistry, Drugs/Pharmaceuticals, NMR Spectroscopy, Proteins/Peptides

Nuclear magnetic resonance (NMR) spectroscopy is a unique tool to study molecular interactions in solution, and it became an essential technique to characterize events of molecular recognition and to obtain information about the interactions of small ligands with biologically relevant macromolecules (proteins or nucleic acids).[1] Ligand-based NMR screening and the NMR determination of the bound conformation of a ligand are important tools in the rational drug-discovery process. In this context, the saturation transfer difference NMR (STD-NMR) experiment has been used for some years to characterize ligand−receptor complexes. The STD-NMR experiment is based on the nuclear Overhauser effect and in the observation of the ligand resonance signals. It can be used as a screening technique, for identification of lead structures or as a tool useful for identifying ligand moieties important for binding. The term *binding epitope* is frequently used in the STD-NMR literature to characterize the hydrogens of the ligand that are closer to the protein upon binding.[2]

The STD-NMR experiment relies on the fact that, for a weak-binding ligand (dissociation constant, K_D, ranging from 10^{-8} mol L^{-1} to 10^{-3} mol L^{-1}), there is exchange between the bound and the free ligand state. Basically, an STD experiment involves subtracting a spectrum in which the protein was selectively saturated (*on-resonance* spectrum obtained by irradiating at a region of the spectrum that contains only resonances of the receptor/protein such as 0 ppm to −1 ppm) with signal intensities I_{SAT}, from one recorded without protein saturation (*off-resonance* spectrum), with signal intensities I_0. In the difference spectrum ($I_{STD} = I_0 - I_{SAT}$) only the signals of the ligand(s) that received saturation transfer from the protein (via spin diffusion, through the nuclear Overhauser effect; see the Supporting Information, section 2.1) will remain. Other compounds that may be present but do not bind to the receptor will not receive any saturation transfer; their signals will be of equal intensity on the *on-resonance* and the *off-resonance* spectra and, as a consequence, after subtraction no signals will appear in the difference spectrum from the nonbinding small molecule(s) (Figure 1). The difference in intensity due to saturation transfer can be quantified ($I_{STD} = I_0 - I_{SAT}$) and constitutes an indication of binding. For a molecule that binds to the receptor, only the signals of the hydrogens that are in close contact to the protein (≤5 Å) and receive magnetization transfer will appear in the difference spectrum and from those, the ones that are closer to the protein will have more intense signals, owing to a more efficient saturation transfer.

The experiment using human serum albumin (HSA) as a receptor and 6-D,L-methyl-tryptophan (6-CH_3-Trp) and 7-D,L-methyl-tryptophan (7-CH_3-Trp) as the ligands under investigation is straightforward to implement in modern NMR spectrometers and is suitable for any chemistry, biochemistry, or biological chemistry course as an example of the use of NMR

Published: April 18, 2011

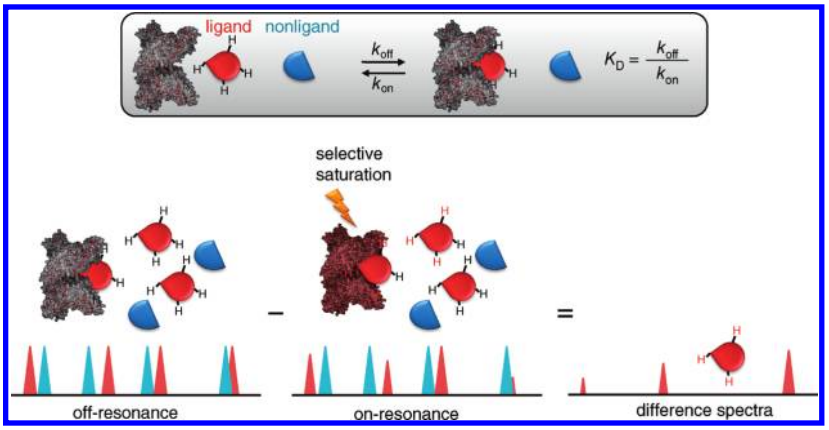


Figure 1. Scheme of the STD-NMR experiment. The exchange between free and bound ligand allows intermolecular transfer of magnetization from the receptor to the bound small molecule.

to characterize molecular interactions in a biological context. The STD-NMR experiment and data analysis are currently part of the NMR practice in structural analysis and structural biochemistry courses for organic chemistry and biochemistry master degree students, respectively.

The experiments were adapted from Wang and co-workers.[3] As reported in the literature, 6-CH_3-Trp is used as an example of weak ligand (K_D = 37 μM) and 7-CH_3-Trp is used as an example of a nonbinding compound.[3−5] Using this system as a model, we will demonstrate the application of STD-NMR to

- A ligand-based NMR screening experiment to determine in a qualitative manner which compound binds to the protein in the context of drug discovery.[2,6,7]
- Ligand mapping: a more advanced example of the use of NMR for a direct characterization of protein−ligand interactions at the molecular level through the identification of important ligand moieties.[2,8,9]
- The determination of the dissociation constant (K_D) between the protein and the ligand.

One of the many advantages of this experiment is that it does not require the use of high-field spectrometers (400 MHz was used in this work) and therefore should be possible to implement in most colleges and universities with access to a NMR facility.

The explanation of the STD-NMR experiment was kept very simple to be accessible to different student levels. More details about the outcome and limitations of the STD-NMR experiment can be found in the Supporting Information, section 2.2.

EXPERIMENTAL PROCEDURE

Preparation of the Samples

HSA was purchased from Fluka; 6-methyl-D,L-tryptophan and 7-methyl-D,L-tryptophan were purchased from Sigma. A 50 μM HSA stock solution was prepared in a phosphate buffer in D_2O (75 mM potassium phosphate, 150 mM sodium chloride at pH 7.5). For both ligands, 5 mM stock solutions in DMSO-d_6 were prepared. Samples for NMR analysis were prepared from the stock solutions as described in the Supporting Information, section 3.1

NMR Experimental Details and General Setup of the STD-NMR Experiment

NMR spectra were acquired in a Bruker Avance III spectrometer operating at a proton frequency of 400 MHz with a conventional inverse 5 mm probehead with *z*-gradients at 25 °C using standard Bruker pulse programs. Spectral acquisition and processing parameters are included in the Supporting Information, sections 3.2 and 3.3.

The STD-NMR Ligand Based-Screening Experiment

The general setup of the STD-NMR experiment was followed using sample B (HSA + 20-fold excess of 6-CH_3-Trp and 7-CH_3-Trp). Details are in the Supporting Information, section 3.3.2

The STD Build-Up Experiment and Ligand Mapping

The general setup of the STD-NMR experiment was followed and a set of 10 STD-NMR experiments were performed using sample C (200 μL of 6-CH_3-D,L-tryptophan was added to 200 μL of HSA stock solution and 100 μL of buffer solution). The saturation times were 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 4.00, and 5.00 s. Details are in the Supporting Information, section 3.3.3

Determination of K_D

The protein was titrated with 6-CH_3-Trp and the general setup for the STD-NMR experiment was followed with samples D1 to D7 (10−200 μL of 6-CH_3-D,L-tryptophan was added to 200−400 μL HSA and 290−0 μL buffer solution), corresponding to a ligand excess from 5- to 100-fold. Details are in the Supporting Information, section 3.3.4

HAZARDS

There are no significant hazards in running this laboratory. Local safety rules in the NMR lab should be followed.

RESULTS AND DISCUSSION

The STD-NMR Ligand Based-Screening Experiment

The STD-NMR spectrum obtained for the mixture of HSA, 6-CH_3-Trp, and 7-CH_3-Trp and the reference spectrum for the mixture under study are shown in Figure 2. The interpretation of this experiment is straightforward; in the STD-NMR spectrum, strong STD signals from 6-CH_3-Trp are readily observable, which indicate that this is an active ligand, whereas the absence of STD signals from 7-CH_3-Trp is in accordance with the fact that this compound does not interact with the protein. This example shows how easily the detection of low-affinity compounds can be achieved with STD-NMR in a screening context, analogous to the one used to screen compound libraries for ligands in the drug-discovery process.

dx.doi.org/10.1021/ed101169t |*J. Chem. Educ.* 2011, 88, 990–994

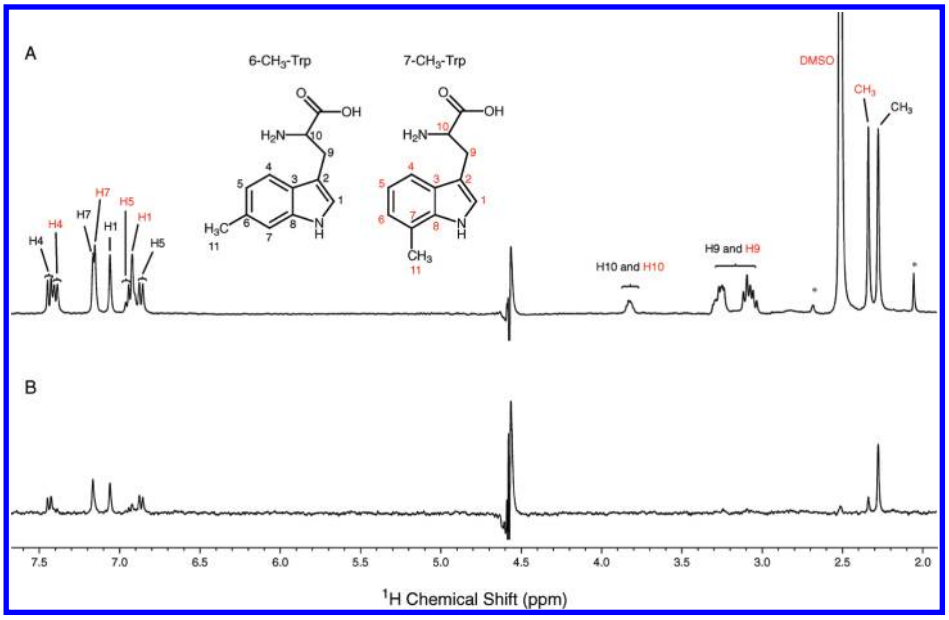


Figure 2. The STD-NMR ligand based-screening experiment: (A) reference spectrum for the mixture of HAS, 6-CH_3-Trp, and 7-CH_3-Trp and (B) corresponding STD-NMR spectrum. The hydrogens in black are from 6-CH_3-Trp and the hydrogens in red are from 7-CH_3-Trp. The asterisk indicates impurities present in the sample. Note that the signals of the impurities are also not present in the STD-NMR spectrum.

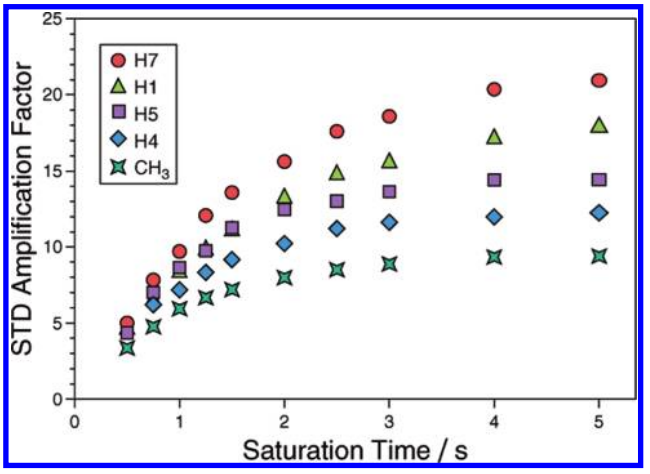


Figure 3. STD amplification factor as a function of saturation time.

The STD Build-Up Experiment and Ligand Mapping

The STD effect can be best analyzed if the amplification factor (A_{STD}) is used.[2] The STD amplification factor is obtained by multiplying the relative STD effect of a given hydrogen (I_{STD}/I_0) at a given ligand concentration ($[L]_T$) with the molar ratio of ligand in excess relative to the protein ($[L]_T/[P]$):

$$A_{STD} = \frac{I_0 - I_{SAT}}{I_0} \times \frac{[L]_T}{[P]} = \frac{I_{STD}}{I_0} \times \frac{[L]_T}{[P]} \qquad (1)$$

For a determined saturation time, the A_{STD} can also be depicted as the average number of ligand molecules saturated per molecule of receptor. In principle, the longer the saturation time and the more ligand used, the stronger the STD and the higher the A_{STD} due to ligand turnover at the binding site (see the Supporting Information, sections 2.2.1 and 2.2.2 for a more detailed explanation). In Figure 3, the A_{STD} was plotted against the saturation time for a 100-fold ligand excess. A clear build-up of the STD effect with increasing saturation time can be observed, reflecting the amplification of the STD. It is also clear from Figure 3 that the A_{STD} is not the same for all hydrogens.

The differences in A_{STD} for different hydrogens can be quantitatively expressed by analyzing the relative STD effects at a given saturation time, mapping of moieties of the ligand important for interaction. Provided that all the ligand hydrogens have similar relaxation rates, then the differences in the relative STD response (I_{STD}/I_0 or A_{STD}) for each ligand hydrogen should reflect the relative proximity of that hydrogen to the receptor-binding site. The procedure is simple, for a given saturation time, the relative STD (or A_{STD}) with the highest intensity is set to 100%, and all other STD signals are calculated accordingly. This was done for the data obtained with 1.50 s saturation time, and the relative STD was mapped onto the structure of 6-CH_3-Trp as depicted in Figure 4.

Accordingly, hydrogens H1 and H7, should be the ones more directly involved in binding and closer to the receptor, followed by hydrogens H5 and H4. The methyl group has a lower relative STD value indicating that it should be more distant. Hydrogens H9 and H10 give almost no STD response indicating that they should be the ones more distant from the protein.

Determination of K_D

There are several STD based methodologies for the determination of the K_D.[10] A comprehensive paper about the direct determination of association constants from STD data was recently published by Angulo et al.[11] Here we have chosen to illustrate a simple method based in the analysis of STD data obtained for increasing ligand concentration that uses an analogy to enzyme kinetics and to the well-known Michaelis−Menten equation:[1]

$$v_0 = \frac{V_{MAX}[L]}{K_M + [L]} \qquad (2)$$

dx.doi.org/10.1021/ed101169t |J. Chem. Educ. 2011, 88, 990–994

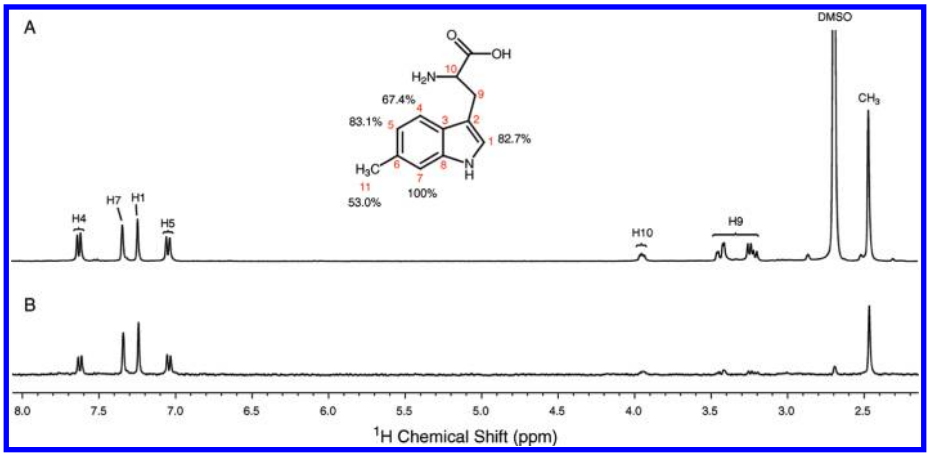


Figure 4. STD-NMR of 6-CH_3-Trp with HSA: (A) reference ^{1}H spectrum of 6-CH_3-Trp with HSA and (B) STD spectra of the solution of 6-CH_3-Trp (2.0 mM) with HSA (20 μM) (1.50 s of saturation time). The relative degree of saturation of the individual hydrogens are mapped into the structure and normalized to that of hydrogen H7.

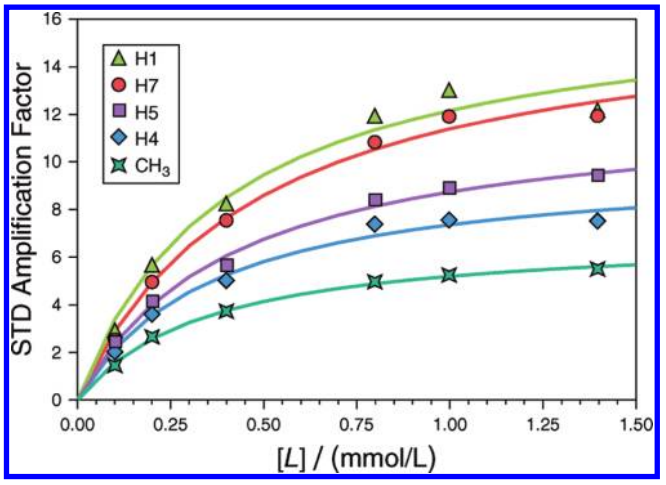


Figure 5. STD amplification factor as a function of ligand concentration. Curves represent the best fit according to eq3.

An analogous equation can be written for the amplification factor, A_{STD}:[1]

$$A_{STD} = \frac{\alpha_{STD}[L]}{K_D + [L]} \qquad (3)$$

where α_{STD} is the maximum amplification factor and $[L]$ is the ligand concentration. This expression is valid if $[L] = [L]_T$, which is usually the case in STD experiments since $[L]_T \gg [P]_T$. A_{STD} will increase with increasing $[L]_T$, in a manner similar to v_0, until a maximum amplification (α_{STD}) is reached, when $[L]_T \gg K_D$ and the receptor binding site is saturated.

In Figure 5, A_{STD} was plotted against the concentration of ligand. The typical hyperbolic behavior, as expected from eq 3, is readily observed as well as the expected decrease in A_{STD} for higher ligand concentrations due to saturation of the binding site (see Supporting Information, sections 2.2.1 and 2.2.2 for further details).

The estimation of K_D and α_{STD} can be done by linearization of this equation in a manner similar to a Lineweaver−Burk plot[10] or by fitting directly the experimental data with eq 3, using appropriate software. We have chosen to follow this last approach using the Solver utility from Microsoft Excel.[12] The curves representing the best fit are depicted in Figure 5. The average K_D determined was 400 ± 73 μmol L^{-1}. This value is about 10 times higher than the value reported in literature for this system (K_D = 37 μmol L^{-1})[3−5] and illustrates one of the limitations of the STD experiment for the direct calculation of dissociation constants, the fact that the STD-NMR experiment does not discriminate between specific and nonspecific ligand binding.[13]

CONCLUSION

We have presented a NMR laboratory activity, extendable to a classroom activity, based in the STD-NMR experiment, one of the most widespread NMR methods for the study of the interactions between small ligands and macromolecular receptors. Its applicability for ligand screening, identification of ligand moieties important for binding, or association constant determination was illustrated and a detailed protocol for the acquisition, processing, and data interpretation was given covering the most important theoretical aspects and drawing attention for some of its limitations and drawbacks.

ASSOCIATED CONTENT

Supporting Information

Notes for the instructor; basic theory of NOE and STD-NMR; instructions for the students including a detailed protocol for the setup of the NMR experiments; NMR spectra and data. This material is available via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ejc@dq.fct.unl.pt.

ACKNOWLEDGMENT

We would like to thank one reviewer for valuable comments and suggestions that significantly improved this article. We are grateful to the Fundação para a Ciência e Tecnologia −FCT (projects PTDC/QUI-QUI/65187/2006 and PTDC/QUI-QUI/098892/2008 and grant SFRH/BD/35992/2007). The NMR spectrometers are part of the National NMR Network and were

purchased in the framework of the National Programme for Scientific Re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and FCT.

REFERENCES

(1) Lepre, C. A.; Moore, J. M.; Peng, J. W. Theory and Applications of NMR-Based Screening in Pharmaceutical Research. *Chem. Rev.* **2004**, *104* (8), 3641–3675.

(2) Meyer, B.; Peters, T. NMR Spectroscopy Techniques for Screening and Identifying Ligand Binding to Protein Receptors. *Angew. Chem., Int. Ed.* **2003**, *42* (8), 864–890.

(3) Wang, Y. S.; Liu, D. J.; Wyss, D. F. Competition STD NMR for the Detection of High-Affinity Ligands and NMR-Based Screening. *Magn. Reson. Chem.* **2004**, *42* (6), 485–489.

(4) Dalvit, C.; Fasolini, M.; Flocco, M.; Knapp, S.; Pevarello, P.; Veronesi, M. NMR-Based Screening with Competition Water-Ligand Observed via Gradient Spectroscopy Experiments: Detection of High-Affinity Ligands. *J. Med. Chem.* **2002**, *45* (12), 2610–2614.

(5) McMenamy, R. H.; Oncley, J. L. The Specific Binding of l-Tryptophan to Serum Albumin. *J. Biol. Chem.* **1958**, *233* (6), 1439–1435.

(6) Klages, J.; Coles, M.; Kessler, H. NMR-Based Screening: A Powerful Tool in Fragment-Based Drug Discovery. *Mol. BioSyst.* **2006**, *2* (6−7), 319–331.

(7) Streiff, J. H.; Juranic, N. O.; Macura, S. I.; Warner, D. O.; Jones, K. A.; Perkins, W. J. Saturation Transfer Difference Nuclear Magnetic Resonance Spectroscopy As a Method for Screening Proteins for Anesthetic Binding. *Mol. Pharmacol.* **2004**, *66* (4), 929–935.

(8) Viegas, A.; Bras, N. F.; Cerqueira, N. M. F. S. A.; Fernandes, P. A.; Prates, J. A. M.; Fontes, C. M. G. A.; Bruix, M.; Romao, M. J.; Carvalho, A. L.; Ramos, M. J.; Macedo, A. L.; Cabrita, E. J. Molecular Determinants of Ligand Specificity in Family 11 Carbohydrate Binding Modules—An NMR, X-ray Crystallography and Computational Chemistry Approach. *FEBS J.* **2008**, *275* (10), 2524–2535.

(9) Viegas, A.; Macedo, A. L., Cabrita, E. J. Ligand-Macromolecular Interactions in Drug Discovery: Methods and Protocols. *Methods in Molecular Biology*; Roque, A. C., Ed.; Humana Press: United Kingdom: 2010; Vol. 572, pp 81−100.

(10) Fielding., L. NMR Methods for the Determination of Protein−Ligand Dissociation Constants. *Prog. Nucl. Magn. Reson. Spectrosc.* **2007**, *51*, 219–242.

(11) Angulo, J.; Diaz, I.; Enríquez-Navas, P. M.; Nieto, P. M. Saturation Ligand−Receptor Binding Affinities from Saturation Transfer Difference (STD) NMR Spectroscopy: The Binding Isotherm of STD Initial Growth Rates. *Chem.—Eur. J.* **2010**, *16*, 7803–7812.

(12) Harris, D. C. Nonlinear Least-Squares Curve Fitting with Microsoft Excel Solver. *J. Chem. Educ.* **1998**, *75*, 119–121.

(13) Ji, Z.; Yao, Z.; Liu, M. Saturation Transfer Difference Nuclear Magnetic Resonance Study on the Specific Binding of Ligand to Protein. *Anal. Biochem.* **2009**, *385* (2), 380–382.

NOTE ADDED AFTER ASAP PUBLICATION

The version of this paper published on April 18, 2011, had an incorrect grant number in the Acknowledgment paragraph. The grant numbers that appear as of April 21, 2011, are correct.