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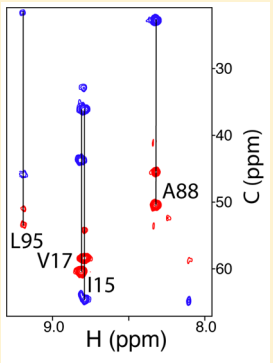
Heteronuclear Multidimensional Protein NMR in a Teaching Laboratory

Nathan T. Wright*

Department of Chemistry and Biochemistry, James Madison University, 901 Carrier Drive, Room 1174, Harrisonburg, Virginia 22807, United States

Supporting Information

ABSTRACT: Heteronuclear multidimensional NMR techniques are commonly used to study protein structure, function, and dynamics, yet they are rarely taught at the undergraduate level. Here, we describe a senior undergraduate laboratory where students collect, process, and analyze heteronuclear multidimensional NMR experiments using an unstudied Ig domain (Ig2 of human obscurin) in order to sequence-specifically assign this domain. In this way, this project-oriented laboratory tries to simulate an authentic biomolecular NMR research experience. Through the semester, students gain a more thorough understanding of both the specific technical aspects of multidimensional NMR analysis and the process by which raw data are converted into sequence-specific secondary structural information.



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INTRODUCTION

X-ray crystallography and NMR spectroscopy are two of the most common experimental techniques used to generate atomic-level protein structures. All undergraduate biochemistry students see the result of these techniques in pictures of protein models in their textbooks. Students thus recognize the ubiquity of three-dimensional protein structure and, through brief overviews in their biochemistry textbooks, have a general idea of how such structures are generated (for example, see ref 1). Outside of several notable exceptions, in-depth explorations of how raw data are converted into relevant structural information are usually not covered in undergraduate biochemistry curricula.[2−5]

This disconnect is not the result of a failure to teach the basic physical phenomena governing protein structure determination. Almost all organic chemistry students learn basic rules of how to interpret 1D NMR spectra.[6,7] Likewise, many students learn about Bragg's law and light diffraction in introductory physics.[7] Thus, most undergraduates who have taken these courses know the basic concepts underlying high-resolution protein structure techniques, but are not exposed to the intermediate steps that link these basic physical principles to protein structures. Crystallographers have taken significant steps to address this disconnect (for example, see refs 8−11). In contrast, there exists relatively few undergraduate protein NMR resources. One explanation for this is technical limitations; the expense of hardware for triple-resonance experiments, labeled samples, and a sufficiently high-field magnet are all reasons why undergraduates have limited exposure the instrumentation necessary for protein heteronuclear multidimensional NMR experimentation.[2] Therefore, the raw data described in this manuscript are available as Supporting Information on the *J. Chem. Educ.* Web site.

As structural techniques, both NMR and X-ray have advantages and drawbacks. Protein X-ray crystallography can be used to solve the high-resolution structure of anything that forms a well-diffracting single crystal. However, one first has to produce a crystal, and this can be difficult and time-consuming. Protein NMR structure determination generally takes longer, and the resulting structures have been traditionally limited to smaller sizes. However, the data from NMR provides a convenient platform for further protein binding or protein dynamics studies.

The process of converting NMR data into structurally useful data is multilayered. In its most robust form, experimental data such as distance restraint data (via Nuclear Overhauser effect data; NOEs), angle restraint data (via torsion angles and Residual Dipolar Coupling data; RDCs), chemical shift data, and dynamics data are all synthesized into a single protein model. To integrate these data, a user adds progressively more restraints to a computer minimization algorithm in an iterative fashion. The computer protocol then tries to fit all the experimental data into an ensemble of structures (this process is described more fully in refs 12 and 13). The structure is complete when all the data added agree, and the resulting models are highly reproducible. While in aggregate these data

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J. Chem. Educ. XXXX, XXX, XXX−XXX

give a full description of the protein of interest, it is often not necessary to collect and analyze this entire data set. For instance, many solution NMR structures use only NOE data, and there has been recent interest in solving structures using solely RDC measurements.[14,15] As an even simpler structural analysis, a residue-by-residue description of secondary structure is accomplished by mapping how far protein backbone chemical shifts deviate from random-coil values.[16]

A prerequisite for all of the above experiments is a fully assigned protein. To attain this, one must experimentally determine the chemical shifts of each atom in each residue in the protein. As an example, a given alanine residue will have shifts corresponding to its amide, alpha and beta hydrogen, its amide nitrogen, and its carbonyl, alpha and beta carbon. For a folded protein, each residue will occupy a unique chemical environment, and thus, each residue will have a unique combination of chemical shifts. These chemical shifts can then be compared to literature values of the same amino acid. Systematic deviations in chemical shifts from the literature values indicate that a section of residues adopt either a helical structure or an elongated secondary structure. Thus, if one knows the NMR assignments of a protein, one can quickly and easily determine the secondary structure either manually or through online programs such as TALOS+.[16]

We have developed a laboratory course (CHEM 368L) that is intended to give senior undergraduates further insight into the process of secondary structure determination by NMR (as described in Putney et al.[17]). Here, students sequence-specifically assign protein NMR data of an Ig-like domain through multidimensional NMR analysis. While this procedure alone does not produce a high-resolution three-dimensional structure, the data generated from these techniques are required for NOE-based or NOE/RDC based solution structure determination.[18] Chemical shift assignments are also a prerequisite for determining protein−target interactions via chemical shift mapping (for an example, see ref 19). This lab was designed to provide technical training in analyzing multidimensional NMR protein solution structure data and to develop the knowledge base necessary to critically evaluate the validity of protein NMR-based data. If students understand the utility and pitfalls of these experiments, they gain significant insight into the methodology underlying most solution protein NMR experimentation.

As a required part of JMU's new Biophysical Chemistry major, the second semester of a new two-semester biochemistry lab, with 4 h of laboratory per week, was designed around the theme of biomolecular NMR spectroscopy. Students had completed three semesters of biochemistry/biophysical chemistry class, one semester of biochemistry laboratory, two semesters of organic chemistry, and two semesters of physics. This laboratory, conducted in the spring of 2015, was completed in 11 weeks, with an additional 2 weeks for presentations and final papers. The course covered the specific learning objectives of preparing a suitable NMR sample, understanding the theory and technique of multidimensional NMR data collection, processing NMR data, expressing magnetization transfers between homo- and heteronuclear species, identifying and analyzing appropriate NMR experiments for data analysis, and recognizing strengths and pitfalls of individual NMR experiments. For each student, these goals were assessed on their ability to sequence-specifically assign all the peaks of a well-dispersed HSQC spectrum, determine the protein's secondary structure, and to write a publication-quality manuscript about their data.

NMR SAMPLE PREPARATION AND DATA ACQUISITION

All experiments described here were conducted on the second Ig domain from human obscurin A (Ig2) (GenBank accession number CAC44768.1; residues 105−211), overexpressed in pET24a in *Escherichia coli* and purified with a nickel affinity and size exclusion column (as previously described[20,21]). Most Ig-like domains are good candidates for student-led characterization: they are of small size (90−115 residues), soluble, monomeric to a concentration of at least 3 mM, and stable for several months at 20 °C.[22] The Ig2 domain had not yet been structurally characterized, but presented a well-dispersed HSQC spectrum (Figure 1) and was thus deemed an

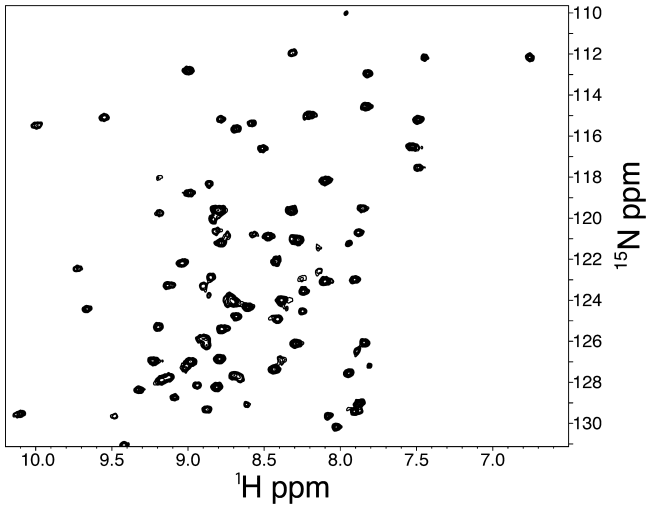

Figure 1. Unassigned HSQC of human obscurin Ig2 at 600 MHz.

appropriate model system for this laboratory. Due to cost, instructors purified one ^{13}C, ^{15}N-labeled protein sample that the students then used through the entire semester.

This sample was placed in a Shigemi tube containing 300 μL of 1.5 mM Ig2, 10% D_2O, 20 mM Tris pH 7.5, 50 mM NaCl, and 0.35 mM NaN_3. Students practiced purification and NMR sample preparation on unlabeled protein. All data were collected in-house on a 600 MHz Bruker Avance II spectrometer equipped with a TXI room temperature 5 mm probe with *z*-axis pulse field gradient coils. Triple resonance room temperature probes are sufficiently sensitive for these experiments, and for instructional purposes have the additional benefit of being physically robust compared to cryoprobes.[23] As a class, students collected one complete set of publication-quality NMR spectra for use among all the students, including multiple 2D HSQCs and one set of standard triple resonance (i.e., 3D) experiments including ^{15}N-edited HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, C(CO)NH, H(CCCO)NH, and an ^{15}N-edited TOtal Correlation SpectroscopY experiment (TOCSY). The spin lock time for the TOCSY was set to 70 ms. All experiments are included in the standard Bruker pulse program library on Topspin 2.1, PL6 (Billerica, MA), and are easily interfaced with automated Bruker pulse program calibration software such as "pulsecal". A full list of the pulse programs and references is included as Supporting Information. Most 3D experiments were collected with 128, 64, and 1024

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J. Chem. Educ. XXXX, XXX, XXX−XXX

points in the *T*1 (Carbon dimension, except in the H(CCCO)-NH and TOCSY), *T*2 (Nitrogen), and *T*3 (Hydrogen) dimensions, respectively. 2D ^{15}N HSQC experiments were taken every week to monitor sample stability. NMR data were processed with NMRPipe v. 8.2,[24] extended in the indirect dimension via linear prediction, and the resulting spectra were manually analyzed via Sparky v. 3.144.[25]

HAZARDS

D_2O is slightly hazardous if ingested, and sodium azide was used as a bactericidal agent and can be fatal if swallowed. While all chemicals pose some health risk, the remaining chemicals in the experiment are considered nonharmful. Extreme care should always be exercised around strong magnetic fields. Students with pacemakers, cochlear implants, or other electrical implants should not go within the 5 G line of such magnets. Additionally, students should be reminded to remove any metal not firmly attached to clothing, i.e., earrings and bobby pins. Credit cards near NMR magnets are erased. Students should be mindful of the liquid helium and nitrogen within the magnet, and the potential danger of asphyxiation if the magnet quenches. These points were discussed in depth on the first day of lab, so that students were aware of potential hazards.

RESULTS AND DISCUSSION

A small laboratory class (2 students) spent 11 weeks working together on NMR assignments, with the first 2 weeks were devoted largely to data management preparation. Students first downloaded a series of free online computer programs, consisting of XQuartz[26] (for Apple computers), NMRPipe and NMRDraw,[24] and Sparky.[25] Sparky is a NMR visualization program that assists with data management, peak labeling, and NMR assignments. NMRPipe and NMRDraw are used to process the NMR data, and to make publication-quality figures (as can be seen in Figures 1 and 2), and XQuartz is an X11 command terminal platform used for interfacing with the Sparky and NMRPipe programs. These programs were installed on the students' private laptops (all Apple machines), but could also be installed on most off-the-shelf Linux or Windows systems. Students found it more efficient to complete the NMR assignments in lab as opposed to on their own, primarily because this allowed them uninterrupted access to the instructor. Before this course, students had not worked with command prompts, and this aspect of training was initially time-consuming. To ensure competency in basic computer skills, students performed a tutorial of how to navigate through a C-shell prompt and perform basic tasks in the computer programs. Tutorials and templates used in this lab are available as Supporting Information for this manuscript. Students were given appropriate template processing scripts and were then instructed on how to adapt these scripts to process various 2D or 3D NMR spectra correctly. This was supplemented with oral instructions as needed.

NMR data collection was conducted on the same sample over the first 8 weeks of the semester. Most 3D experiments were collected for 6 days each. As a group, students learned how to shim the sample, set the 90° H pulse correctly, optimize water suppression, and set the number of points in each dimension. Students then set up an HSQC spectrum with minimal assistance, allowing them to explore which parameters are important for data acquisition. For instance, students calculated the acquisition time in each dimension after being given the number of time points and the sweepwidth, and compared their answers to the values calculated by the NMR software. Experimental setup also proved a suitable time to explain theoretical concepts such as through-bond magnetization transfer, INEPT pulses, and multidimensional data collection.

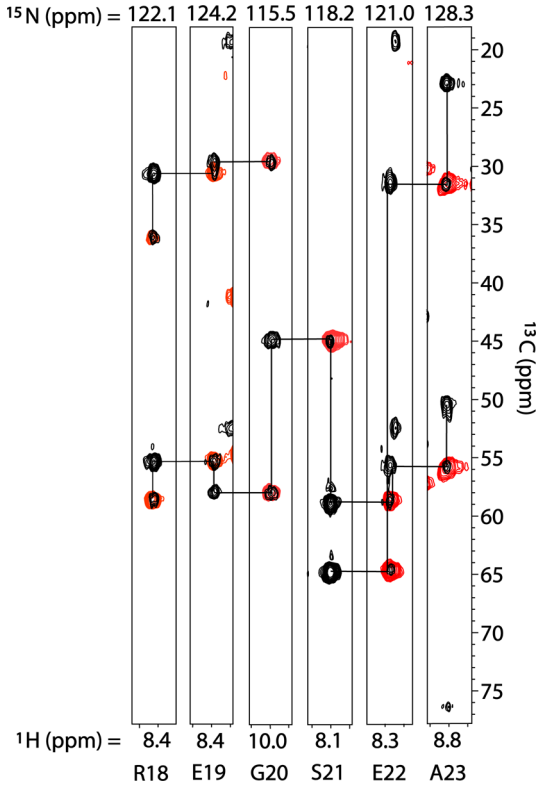

Figure 2. Example of student-generated "backbone walking" data, showing ^{1}H−^{13}C strips of nitrogen planes from the CBCA(CO)NH (in red) overlaid with strips from the HNCACB (in black). These data show the α and β carbon connectivities between E18 and A23.

All spectral data were visualized in Sparky.[25] This program allows the user to sync spectra to each other, thus greatly simplifying data analysis. Students labeled peaks in Sparky and also in an online spreadsheet. This setup allowed students to work independently on their own computers but also interface with other people in their group in real-time. Students first learned how to navigate Sparky by labeling each peak in the HSQC with an arbitrary identifier. These identifiers were then correlated to all subsequent experiments. For instance, a peak in the CBCA(CO)NH might be correlated to ^{13}C = 34 ppm, ^{15}N = 125 ppm, and ^{1}H = 9.2 ppm. The nitrogen and hydrogen shifts align with an HSQC peak already denoted "peak #20". The carbon shift of this CBCA(CO)NH peak would thus be labeled as 'the previous beta carbon of H−N peak 20', or '20CB-1'. Using this kind of nomenclature, students labeled the CBCA(CO)NH and the HNCACB, keeping track of all the shifts in the online spreadsheet. After this labeling exercise, the concept of "backbone walking" and peak assignment could be introduced.[27] Students quickly recognized the common NMR problem of chemical shift degeneracy, and they then labeled the HNCO and HN(CA)CO to resolve these issues.[28] When analyzing together, students were able to assign roughly 85% of the HSQC peaks. The C(CCO)NH experiment was necessary to differentiate the spin systems for the last ∼15% of peaks (Figure 3).

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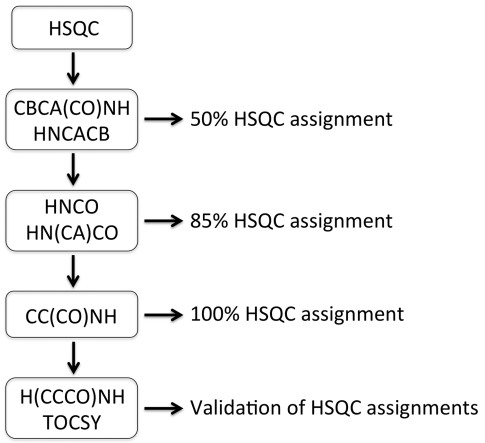

Figure 3. Outline of the multidimensional NMR experiments used for assigning Ig2. The arrows indicate the order that the spectra were analyzed and how data from various spectra contribute to the overall goal of complete HSQC assignment.

As should be expected, students at first needed significant assistance with this kind of data analysis. As an introduction to peak picking and backbone walking/assignments, the instructor assigned 3−4 peaks as a demonstration of the process. After this tutorial, the students assigned several peaks with the instructor's help, after which students quickly became mostly self-sufficient (for example, Figure 2). This basic teaching format was repeated as additional experiments were added to the data set. As students became more comfortable with the data interpretation and making assignments, they became more independent until by the middle of the semester students were working almost completely independently. The instructor then served primarily as a sounding-board for working through difficult assignments. Students were able to complete all assignments in the allotted laboratory time.

While moderately well dispersed, the HSQC spectrum of Ig2 contains several areas of overlapped resonances. As tends to happen in such spectral regions, students often mislabeled carbon peaks as belonging to the wrong N−HN resonance. As a result, the corresponding Excel tables contained incorrect labels on these amide resonances. To manage this, students were initially encouraged to avoid the regions of HSQC spectra that looked crowded. In this way, students could gain experience before they analyzed the more challenging data. This approach also decreased the total number of assignment possibilities, thereby simplifying their analysis.

Students studied the H(CCCO)NH and TOCSY data after all of the amide chemical shift assignments were completed. This not only allowed for sequence-specific assignment of the hydrogen atoms, but also functioned as an extra internal check that their amide assignments were correct.

After assignments were complete, the chemical shifts were uploaded into the online program TALOS+.[16] From this output, students were able to differentiate secondary structural elements on a residue-by-residue basis.

As an alternative approach to the assignment process, students could have used free automated assigning programs such as MARS.[29] We chose not to pursue this route. The major goal of this lab is for students to gain insight into the entire assignment process, and we worried that any automation would result in a less developed technical proficiency of the technique. Additionally, while these assignment programs work well, they rarely result in fully assigned data set. Therefore, students would likely need to complete the assignments manually, and if all the easy assignments were done automatically, students would have insufficient experience to properly analyze the more difficult spectral regions.

Since the Ig2 chemical shift data and secondary structure were unknown a priori, this lab was graded through written lab reports and oral presentations.[30,31] Students turned in iterative drafts of their lab report to demonstrate evolving technical and theoretical comprehension of the subject. Along with writing criteria, students were graded on their ability to generate figures that would be appropriate for a peer-reviewed publication (for example, Figure 2).

The final versions of these papers were in the style of the *Journal of Biomolecular NMR Assignments*, and an edited version of one student's paper has been accepted in this journal.[20] These papers, along with informal week-to-week conversations and a formal oral presentation, allowed sufficient assessment of whether students had sufficient mastery of this subject.

Pedagogically, one area of concern was whether teaching this NMR lab necessitated the omission of other biophysical topics. In this particular case, students had already been exposed to a several semesters of related coursework, and thus, this kind of special topics course was deemed appropriate. One alternative to this semester-long course is shorter 4−5 week module (see Supporting Information). In this scenario, 4−5 weeks can be spent on data analysis of a preselected group of peaks, culminating in the partial assignment of this Ig domain. While student understanding of the material may be correspondingly diminished, this would be balanced by the benefit of covering additional structural biology/biochemistry techniques.

CONCLUSIONS

This laboratory was offered as an advanced undergraduate lab for students interested in additional protein structure training. Here, students collected, processed, and fully analyzed through-bond experiments on the human obscurin Ig2 protein fragment. This allowed students to study the secondary structure of this domain in a residue-by-residue basis. This lab not only helps students better understand how protein NMR data is analyzed, but also alerts them to inherent strengths and weaknesses in such data. This is an important skill, since these data form the underpinning for solution protein structure determination, NMR protein-target binding analysis, and NMR protein dynamics work. There are three significant hurdles to conducting a lab such as this. First, one must obtain a high-quality and long-lasting NMR sample. To this end, we demonstrate that Ig-like domains work well for this kind of undergraduate laboratory. Alternatively, uniformly ^{13}C, ^{15}N-labeled ubiquitin is commercially available (Cambridge Isotope). The structure and assignments of ubiquitin are known, and thus, a modified version of this laboratory would be necessary using this sample. Second, students often do not have sufficient computer skills to independently process the data. To address these considerations, we have posted multiple tutorials of how to navigate each required computer program. These tutorials are written at an undergraduate level, and thus may be more accessible than other online tutorials whose target audience possesses a computer science background. Last and most significant, students must have access to a spectrometer equipped with a triple-resonance probe that can indirectly detect ^{15}N. Such a prerequisite is untenable in many institutions. To help alleviate this problem, we have posted

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J. Chem. Educ. XXXX, XXX, XXX−XXX

the raw NMR data from this paper as Supporting Information. Thus, anyone who has access to both a computer and to the online portion of this journal could conduct the data analysis portion of this lab. We are in the process of developing a comprehensive Web site with multiple structural biology raw data sets, tutorials, and instructions. This will be the focus of a future paper.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00331.

The lab and lecture schedule (PDF, DOCX)
One possibility of how to create a shorter biomolecular NMR-based laboratory (PDF, DOCX)
A full list of the pulse programs used for this class (PDF, DOCX)
Tutorial on how to analyze these data (PDF, DOCX)
Tutorial on how to navigate Sparky (PDF, DOCX)
Tutorial NMR-Draw, on how to process NMR data, how to edit the NMR macros (PDF, DOCX)
Tutorial scripts, on how to install the requisite programs (PDF, DOCX)
Entire Ig2 assignments (XLSX)
Raw NMR data: CBCACONH (ZIP)
Raw NMR data: CCCONH (ZIP)
Raw NMR data: HCCCONH (ZIP)
Raw NMR data: HNCACB (ZIP)
Raw NMR data: HNCACO (ZIP)
Raw NMR data: HNCO (ZIP)
Raw NMR data: HSQC (ZIP)
Raw NMR data: TOCSY (ZIP)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wrightnt@jmu.edu.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Voet, V. P.; Voet, J. G.; Pratt, C. W. *Fundamentals of Biochemistry*, 4th ed.; John Wiley and Sons: Hoboken, NJ, 2013; pp 142−145.
(2) Gonzalez, E.; Dolino, D.; Schwartzenburg, D.; Steiger, M. A. Dipeptide Structural Analysis Using Two-dimensional NMR for the Undergraduate Advanced Laboratory. *J. Chem. Educ.* **2015**, *92* (3), 557−560.
(3) Rehart, A.; Gerig, J. T. Proton NMR Studies of the Conformation of an Octapeptide. An NMR Exercise for Biophysical Chemistry. *J. Chem. Educ.* **2000**, *77* (7), 892−894.
(4) Yarger, J. L.; Nieman, R. A.; Bieber, A. L. NMR Titration Used to Observe Specific Proton Dissociation in Polyprotic Tripeptides: An Undergraduate Research Lab. *J. Chem. Educ.* **1997**, *74* (2), 243−246.
(5) Harris, R.; Driscoll, P. C. The Ubiquitin NMR Resource. In *Modern NMR spectroscopy in Education*; American Chemical Society: Washington, DC, 2007, 114−126.
(6) Soulsby, D. *NMR Spectroscopy in the Undergraduate Curriculum*; American Chemical Society: Washington, DC, 2013.
(7) What's on the MCAT Exam. https://students-residents.aamc.org/applying-medical-school/article/whats-mcat-exam/ (accessed Nov 30, 2015).
(8) Crundwell, G.; Phan, J.; Kantardjieff, K. A. The Incorporation of a Single-Crystal X-ray Diffraction Experiment into the Undergraduate Physical Chemistry Laboratory. *J. Chem. Educ.* **1999**, *76* (9), 1242−1245.
(9) Lonsdale, K. Crystal Structure Analysis for Undergraduates. *J. Chem. Educ.* **1964**, *41* (5), 240.
(10) Wilson, C. C.; Parkin, A.; Thomas, L. H. Frontiers in Crystallography: a Project-Based Research-Led Learning Experience. *J. Chem. Educ.* **2012**, *89* (1), 34−37.
(11) Noriega, G. O.; Vazquez, E. S. A Strategy for Introducing Undergraduate Students to Protein Structure. *Biochem. Mol. Biol. Educ.* **2000**, *28*, 200−202.
(12) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley and Sons: New York, 1986; Vol. *2*−*6*, pp 162−198.
(13) Teng, Q. *Structural Biology: Practical NMR Application*; Springer: New York, 2005; pp 211−229.
(14) Ruan, K.; Briggman, K. B.; Tolman, J. R. De novo Determination of Internuclear Vector Orientations for Residual Dipolar Couplings Measured in Three Independent Aligment Media. *J. Biomol. NMR* **2008**, *41* (2), 61−76.
(15) Mei, S.; Zhang, J.; Zhang, X.; Tu, X. Solution Structure of the Leptospiral LigA4 Big Domain. *Biochem. Biophys. Res. Commun.* **2015**, *467*, 288.
(16) Shen, Y.; Delaglio, F.; Cornilescu, G.; Bax, A. TALOS+: A Hybrid Method for Predicting Protien Backbone Torision Angles from NMR Chemical Shifts. *J. Biomol. NMR* **2009**, *44* (4), 213−223.
(17) Putney, D. R.; Todd, E. A.; Berndsen, C. E.; Wright, N. T. Chemical Shift Assignments for S. cerevisiae Ubc13. *Biomol. NMR Assignments* **2015**, *9* (2), 407−10.
(18) Wright, N. T.; Cannon, B. R.; Wilder, P. T.; Morgan, M. T.; Varney, K. M.; Zimmer, D. B.; Weber, D. J. Solution Structure of S100A1 Bound to the CapZ peptide (TRTK12). *J. Mol. Biol.* **2009**, *386* (5), 1265−77.
(19) Cavalier, M. C.; Pierce, A. D.; Wilder, P. T.; Alasady, M. J.; Hartman, K. G.; Neau, D. B.; Foley, T. L.; Jadhav, A.; Maloney, D. J.; Simeonov, A.; et al. Covalent Small Molecule Inhibitors of Ca(2+)-bound S100B. *Biochemistry* **2014**, *53* (42), 6628−40.
(20) Temple, J. E.; Oehler, M. C.; Wright, N. T. Chemical Shift Assignments for the Ig2 Domain of Human Obscurin A. *Biomol. NMR Assignments* **2015**, DOI: 10.1007/s12104-015-9638-7.
(21) Rudloff, M. W.; Woosley, A. N.; Wright, N. T. Biophysical Characterization of Naturally Occurring Titin M10 Mutations. *Protein Sci.* **2015**, *24* (6), 946−55.
(22) Meyer, L. C.; Wright, N. T. Structure of Giant Muscle Proteins. *Front. Physiol.* **2013**, *4*, 368.
(23) Kovacs, H.; Moskau, D.; Spraul, M. Cryogenically Cooled Probes- a Leap in NMR Technology. *Prog. Nucl. Magn. Reson. Spectrosc.* **2005**, *46*, 131−155.
(24) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **1995**, *6* (3), 277−93.
(25) Goddard, T. D.; Kneller, D. G. SPARKY. https://www.cgl.ucsf.edu/home/sparky/ (accessed Sept 15, 2015).
(26) Apple, Inc. XQuartz. http://xquartz.macosforge.org/landing/ (accessed Sept 15, 2015).
(27) Jacobsen, N. *NMR Spectroscopy Explained: Simplified Theory, Applications and Examples for Organic Chemistry and Structural Biology*; John Wiley and Sons: Hoboken, NJ, 2007.
(28) Rule, G. S.; Hitchens, T. K. *Fundamentals of Protein NMR Spectroscopy*; Springer: New York, NY, 2006; pp 25−27.
(29) Jung, Y. S.; Zweckstetter, M. Mars − Robust Automatic Backbone Assignment of Proteins. *J. Biomol. NMR* **2004**, *30* (1), 11−23.
(30) MacDonald, G. Teaching Protein Purification and Characterization Techniques. *J. Chem. Educ.* **2008**, *85* (9), 1250−1252.
(31) Amenta, D. S.; Mosbo, J. A. Attracting the New Generation of Chemistry Majors to Synthetic Chemistry without Using Pheromones: A Research-Based, Group Approach to Multistep Syntheses at the College Sophomore Level. *J. Chem. Educ.* **1994**, *71* (8), 661.

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J. Chem. Educ. XXXX, XXX, XXX−XXX