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Laboratory Experiment

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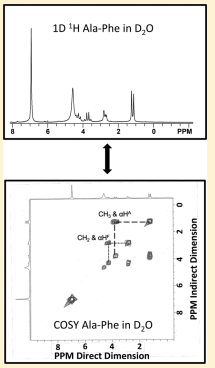
Dipeptide Structural Analysis Using Two-Dimensional NMR for the Undergraduate Advanced Laboratory

Elizabeth Gonzalez, Drew Dolino, Danielle Schwartzenburg, and Michelle A. Steiger*

Department of Chemistry and Physics, University of St. Thomas, Houston, Texas 77006, United States

Supporting Information

ABSTRACT: A laboratory experiment was developed to introduce students in either an organic chemistry or biochemistry lab course to two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy using simple biomolecules. The goal of this experiment is for students to understand and interpret the information provided by a 2D NMR spectrum. Students are provided three unknown samples: a dipeptide and each of the two amino acids that make up the dipeptide. A Fourier transform-NMR (60 MHz) instrument is used to record standard proton (^{1}H) NMR spectra for each of the unknown samples. By interpreting the ^{1}H NMR spectra for the two single amino acid unknown samples, students identify the amino acids in the dipeptide. For the dipeptide molecule, students record the ^{1}H and correlation spectroscopy (COSY or ^{1}H−^{1}H) NMR spectra. The students use the COSY spectrum information to assign all of the proton peaks in the more complicated 1D ^{1}H spectrum of the dipeptide. A comparative analysis of the NMR spectra for dipeptides with the same amino acid constituents reveals that the order in which the amino acids are connected in the dipeptide influences the chemical shift of at least the α-protons.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Organic Chemistry, Hands-On Learning/Manipulatives, Amino Acids, NMR Spectroscopy, Laboratory Instruction*

INTRODUCTION

NMR analysis can be incorporated into a wide number of undergraduate chemistry courses that span the curriculum.[1−3] NMR spectroscopy is widely used in biochemistry laboratories to analyze the structure and chemistry of small proteins and peptides.[4−8] Multidimensional NMR spectroscopy is necessary for the structural analysis of many biomolecules. The advantage of two-dimensional (2D) NMR spectroscopy is that students can obtain structural information about larger molecules where the NMR signals are often coincident (or overlapping). 2D NMR analysis often requires the use of a high-field NMR spectrometer.[9−11] Many smaller universities are limited to use of a 60 or 90 MHz NMR spectrometer given the cost and maintenance constraints of a more powerful NMR instrument. Previous reports have used only single amino acids with low-field NMR instruments.[12−14] By using biological molecules, an advanced NMR exercise that involves 2D NMR analysis with a low-field NMR instrument (60 MHz) was incorporated into an upper-division undergraduate biochemistry lab course.

The experiment is included as part of a larger amino acid lab experiment. Both single amino acids and dipeptide molecules are analyzed using a 60 MHz NMR instrument. Students were exposed to simple proton NMR spectroscopy in a prerequisite organic chemistry course; therefore, they are able to interpret a one-dimensional (1D) proton NMR spectrum. Here, students use the 2D NMR spectrum of a dipeptide molecule to interpret the 1D NMR spectrum of the dipeptide.

The design of the lab experiment is outlined in Figure 1. Students work in groups of two and are first provided with samples of two different amino acids for the 1D proton (^{1}H) NMR analysis. The students identify the amino acids by interpreting the simple ^{1}H NMR spectrum of each single amino acid.

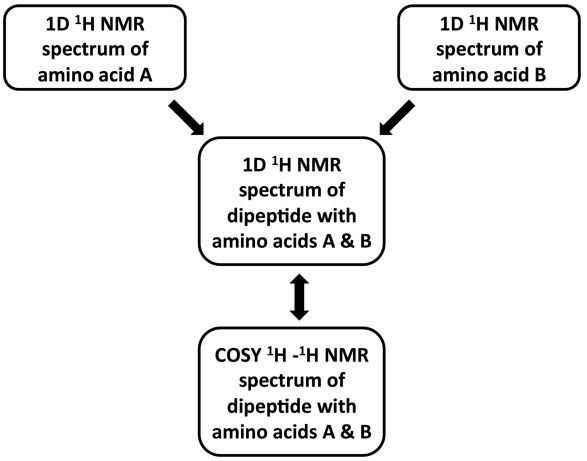


Figure 1. Outline of the NMR analysis of single amino acids and the corresponding dipeptide. The arrows indicate the relationship between the spectra and how the various spectra help in the interpretation process of the 1D NMR spectrum of the dipeptide.

Next, each group is provided a sample dipeptide that contains the two amino acids. Students record a 1D ^{1}H NMR spectrum as well a correlation spectroscopy (COSY) spectrum for this dipeptide. By comparing the 1D ^{1}H single amino acid

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and dipeptide spectra, the students immediately realize that two amino acids in a peptide molecule result in a more complex NMR spectrum. Often, students cannot assign every peak in the ^{1}H NMR spectrum for the dipeptide molecule since some of the proton signals may overlap and give rise to a complex signal. Thus, they record the COSY spectrum of the dipeptide and use it to interpret each proton peak in the 1D NMR spectrum of the dipeptide. By building on their previous knowledge of NMR, the students are able to see directly the information added by the 2D spectrum and how it is useful for structural analysis.

EXPERIMENTAL OVERVIEW

The students work in groups of two, and each group is given three samples: a 155−250 mg dipeptide sample labeled with a numerical identifier and two different 55 mg single amino acid samples with corresponding numerical identifiers. All of the amino acid and dipeptide samples are provided as unknowns to the students, but the students are told that the single amino acid samples correspond to the two amino acids in the dipeptide. The students use heavy water (D_2O) (0.6 mL) to dissolve each sample and add DCl or KOD dropwise as needed to dissolve the samples. More information about the sample preparation is given in the Supporting Information. Once in solution, the students obtain the various spectra (Figure 1).

This experiment requires close to 40 min of NMR run-time per student group to record all four spectra. Prior to the running of any sample, the tetramethylsilane (TMS) reference peak is set to zero ppm. One biochemistry lab section has, at most, eight groups; as a result, the students complete the experiment outside of an assigned 3 h lab time. The acquisition of a COSY spectrum takes at least 20 min per sample on a 60 MHz NMR. The 20 min COSY run-time is used for a short lecture that covers the theory of multidimensional NMR, what specific type of information is obtained from a COSY spectrum, and how students should interpret a COSY spectrum.

HAZARDS

All chemicals pose some risk; none of the lab chemicals used should be ingested or directly inhaled. All of the chemicals for this experiment should be handled by students in a vent hood to avoid inhalation. Students should wear gloves, lab coats, and safety glasses throughout the experiment. The amino acid samples are premeasured to limit a student's exposure to the amino acids; any amino acid contact with skin is thoroughly washed with soap and water. DCl and KOD are corrosive and toxic. Contact with the skin or eyes can lead to severe burning; any contact requires prompt and thorough rinsing with water and may require medical attention. All of the amino acid solutions are collected, neutralized, and properly disposed after the lab experiment.

RESULTS

The NMR experiment has been performed for three years in a biochemistry lab by 60 students. The amino acid and peptide samples were dissolved in D_2O, which resulted in the proton exchange of solvent-exchangeable protons; these exchangeable protons included amine, amide, carboxylic acid, alcohol, or sulfhydryl functional group protons that were present on the molecule. Thus, in all of the spectra, there were no clearly resolved amine, amide, carboxylic acid, alcohol, or sulfhydryl functional group protons because these hydrogens were replaced by deuterium atoms from the solvent. Additionally, an HOD solvent peak between 4.5 and 6 ppm was observed as a result of the hydrogen−deuterium exchange.[15] This simplified the spectra but also limited the amino acids that work best in this lab experiment; amino acid side chains with these functional groups were generally avoided. Amino acid combinations of methionine, valine, isoleucine, phenylalanine, alanine, glycine, proline, tyrosine, and leucine worked best and were routinely used in the lab experiment. In cases where tyrosine was given as one of the amino acid unknowns, students were told that there was one proton group associated with the amino acid side chain that was not observed; this was given as additional information to help the students identify their amino acid correctly. To avoid proton exchange with the solvent, dimethyl sulfoxide (DMSO) was tried as a solvent, but the amino acids and dipeptide samples were not dissolved in a high enough concentration for a 60 MHz NMR instrument to record clear 1D and 2D NMR spectra.

All of the student groups generally had at least one amino acid in common with another lab group. In the next lab meeting, the students compared their NMR spectra to find groups with at least one or two amino acids in common. The student groups were instructed on which specific dipeptide samples were to be compared; this prevented students from comparing samples that were not related. The students compared the dipeptide 1D and 2D spectra and, through a series of postlab questions given in the Supporting Information, explained what differences they observed and how they accounted for these differences. Figure 2 is a dual display version of the 1D NMR spectra for alanine-phenylalanine (Ala-Phe) and phenylalanine-alanine (Phe-Ala), which were two of the dipeptides routinely used in this lab exercise, that clearly showed the sequence effects observed in the dipeptides.

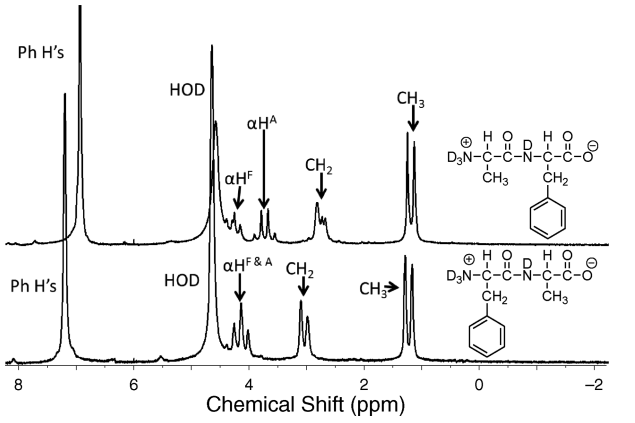

Figure 2. Student-generated ^{1}H NMR spectra of the Ala-Phe (top) and Phe-Ala (bottom) dipeptides shown as a dual display NMR spectrum. All of the proton peaks are assigned; αH^A refers to the α-proton on the alanine amino acids, and αH^F refers to the α-proton on the phenylalanine amino acids.

The 1D ^{1}H NMR spectrum for Phe-Ala only distinguished four different types of protons in addition to the HOD proton peak. The α-proton peaks of Phe and Ala appear to overlap in this peptide spectrum; however, in the Ala-Phe dipeptide 1D ^{1}H NMR spectrum, all five different types of protons were resolved. This demonstrates that sequence differences cause different chemical shifts in the peaks for the α-protons. This

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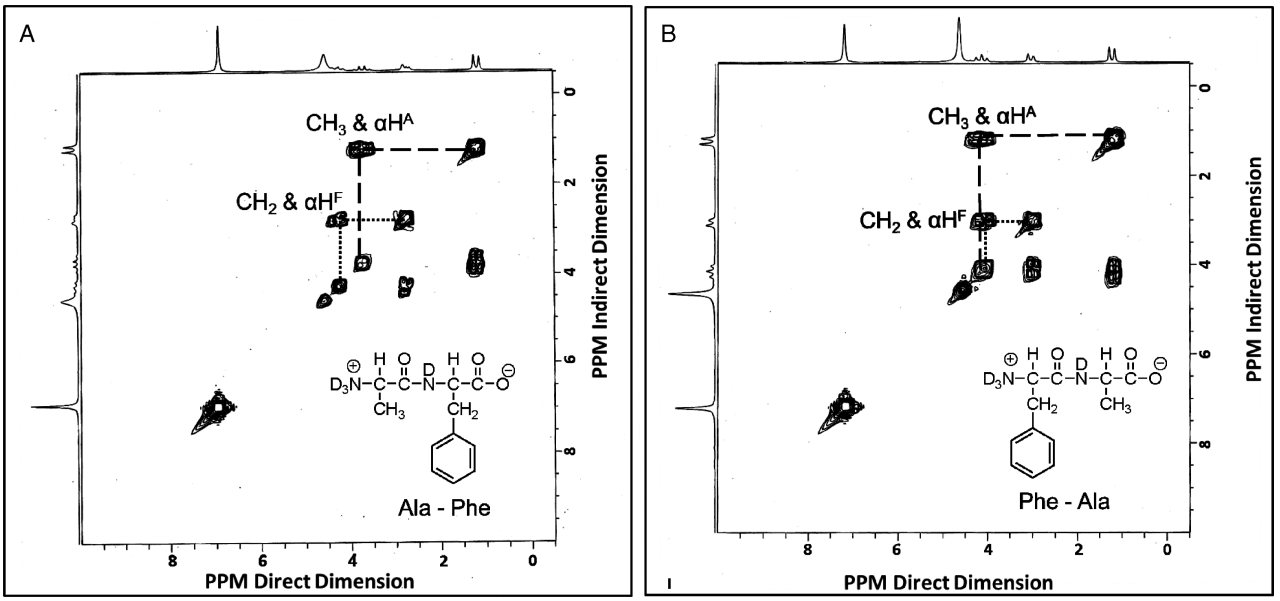

Figure 3. Student-generated COSY spectra of Ala-Phe (A) and Phe-Ala (B). In each panel, the off-diagonal peaks for the α-protons are labeled with dotted lines that show the α-H on the Phe (F) amino acid that gives an off-diagonal peak through its interaction with the CH_2 group on the F side chain. The dashed line shows the α-H on the Ala (A) amino acid that gives an off-diagonal peak through its interaction with the CH_3 group on the A side chain.

allows the students to see how detailed and valuable NMR data is for the chemical analysis of proteins.

Since the NMR spectra are more complex and have apparently overlapping proton peaks, further analysis is done. The further analysis is provided by the COSY spectrum. The COSY spectrum for each dipeptide was used to assign the α-proton peaks. The information obtained from the COSY spectrum was in the form of the off-diagonal cross peaks that served as evidence that these protons were 2 or 3 bonds apart. These off-diagonal peaks are likely the result of spin coupling between groups of protons.

The α-proton at ~3.7 ppm was the alanine α-proton, and the proton at ~4.2 ppm was the phenylalanine α-proton (Figure 2). These proton identities were confirmed by both COSY (Figure 3) and heteronuclear correlation (HETCOR) analyses (Supporting Information). Similar differences of the α-protons were noted for valine-methionine (Val-Met) and methionine-valine (Met-Val) and for proline-leucine (Pro-Leu) and leucine-proline (Leu-Pro) (Supporting Information).

The students were given prelab questions and postlab questions (Supporting Information) that were submitted, along with fully interpreted NMR spectra, for grading.

■ DISCUSSION

All of the students were able to interpret the ^{1}H NMR spectrum of the single amino acid sample using their NMR knowledge from the organic chemistry prerequisite course. The interpretation of the single amino acid NMR spectra revealed the identities of the amino acids in the dipeptide. The students initiated the analysis of the ^{1}H NMR spectrum of the dipeptide by drawing out the dipeptide and comparing the single amino acid spectra to the dipeptide spectrum. Over the three years in which this experiment was done, 75% of the students showed a solid grasp on the meaning of the COSY spectrum and were able to assign the individual protons that yield the off-diagonal peaks. Furthermore, 75% of the students were able to interpret the 1D ^{1}H NMR spectrum of the dipeptide.

Overall, the goal of introducing 2D NMR spectroscopy to students was met based on the student surveys. Prior to this lab exercise, no student had an understanding of multidimensional or 2D NMR spectroscopy. After this experiment, 70% of the students indicated that they gained a much better understanding of the information obtained from a COSY NMR spectrum and how this information directly aided in the structural analysis of larger biomolecules. In the course evaluations, students found that this exercise flowed well with the lecture material on protein structure and helped to demonstrate the use of NMR spectroscopy beyond the simple standard use that they had encountered in other courses.

■ ASSOCIATED CONTENT

Supporting Information

Instructor notes; student hand-out; and additional ^{1}H NMR, COSY, and sample HETCOR spectra for the routinely used dipeptides in this lab exercise are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

* E-mail: steigerm@stthom.edu.

Notes

The authors declare no competing financial interest.

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