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Laboratory Experiment

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^{1}H NMR Spectroscopy-Based Configurational Analysis of Mono- and Disaccharides and Detection of β-Glucosidase Activity: An Undergraduate Biochemistry Laboratory

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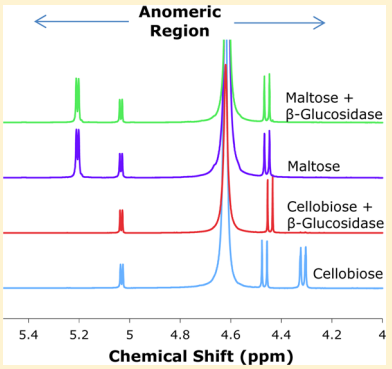
Supporting Information

ABSTRACT: A ^{1}H NMR spectroscopy-based laboratory experiment explores mono- and disaccharide structural chemistry, and the enzyme-substrate specificity of glycosidic bond cleavage by β-glucosidase towards cellobiose (β-linked gluco-disaccharide) and maltose (α-linked gluco-disaccharide). Structural differences between cellobiose, maltose, and the glycolytic product glucose are established in terms of chemical shift changes and J-coupling constants of the anomeric proton peaks. The distinct anomeric proton signals observed in the 4.0−5.5 ppm range of glucose, cellobiose, and maltose spectra serve as a signature NMR feature to monitor the substrate specificity of β-glucosidase towards cellobiose as a substrate. This experiment consolidates carbohydrate structural chemistry and NMR spectroscopy as applied to investigate enzyme specificity in the context of a biochemistry laboratory experiment with hands-on NMR experience for upper-division undergraduates.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Hands-On Learning/Manipulatives, Carbohydrates, NMR Spectroscopy*

INTRODUCTION

Carbohydrate structural concepts and related enzyme catalyzed reactions are important topics that are covered in biochemistry lecture courses, but often not adequately represented in biochemistry laboratory courses. Similarly, despite the fact that nuclear magnetic resonance (NMR) spectroscopy use is ubiquitous in modern biochemical research, NMR spectroscopy is not often introduced into the modern biochemical classroom. Several published studies have suggested wet chemistry tests to identify unknown carbohydrates,[1,2] but there have been few undergraduate exercises that use NMR spectroscopy for this purpose, despite the fact that NMR spectroscopy, in addition to identification, gives direct insight into carbohydrate structure. Anomerization in glucose has been well-studied by optical rotation[3] as well as by NMR spectroscopy.[4−6] A few publications in this *Journal* and other journals have used NMR spectroscopy to follow the kinetics or analyze the products of carbohydrate reactions.[7−12] One nonlaboratory paper discussed the anomeric specificity of enzymes acting on sugars, pointing out widespread errors in biochemistry textbooks in reporting selectivity.[13] There have been a few laboratory exercises designed to demonstrate enzyme selectivity on substrates other than carbohydrates.[14,15] This experiment focuses on the application of NMR spectroscopy to demonstrate substrate specificity of an enzyme-catalyzed reaction toward disaccharides in terms of carbohydrate structural concepts, while lending itself to a discussion of anomerization in glucose and disaccharides.

This experiment was introduced into our biochemistry laboratory course, required for and taken primarily by upper-division students who are pursuing a chemistry degree with a biochemistry concentration. Students enrolled in this course have generally completed two semesters of organic chemistry (lecture and lab), as well as one course in a three-semester biochemistry lecture sequence. They have been introduced to NMR spectroscopy in their organic lecture courses and have also used NMR spectroscopy for product identification and structural analysis in the two-semester organic laboratory sequence. Although most organic chemistry textbooks include a section on carbohydrate structure, time constraints often prevent coverage of carbohydrates in organic classes. Students are, therefore, first introduced to carbohydrate structure and chemistry in the first-semester biochemistry lecture class. If students were given the necessary background, this experiment may also be appropriate for a second-semester or advanced organic chemistry lab course to emphasize NMR spectroscopy and structural aspects.

NMR spectroscopy is one of the most powerful tools available to chemists. Application of ^{1}H NMR spectroscopy for the characterization of organic compounds is well-known, and recent developments in advanced NMR instrumentation and data processing capabilities have broadened the use of NMR spectroscopy in the fields of medicine, biology, and physical sciences. Introductory organic chemistry courses introduce NMR spectroscopy; however, large class sizes often limit instructors' ability to provide hands-on experience for the students to get sufficient training. Furthermore, despite the

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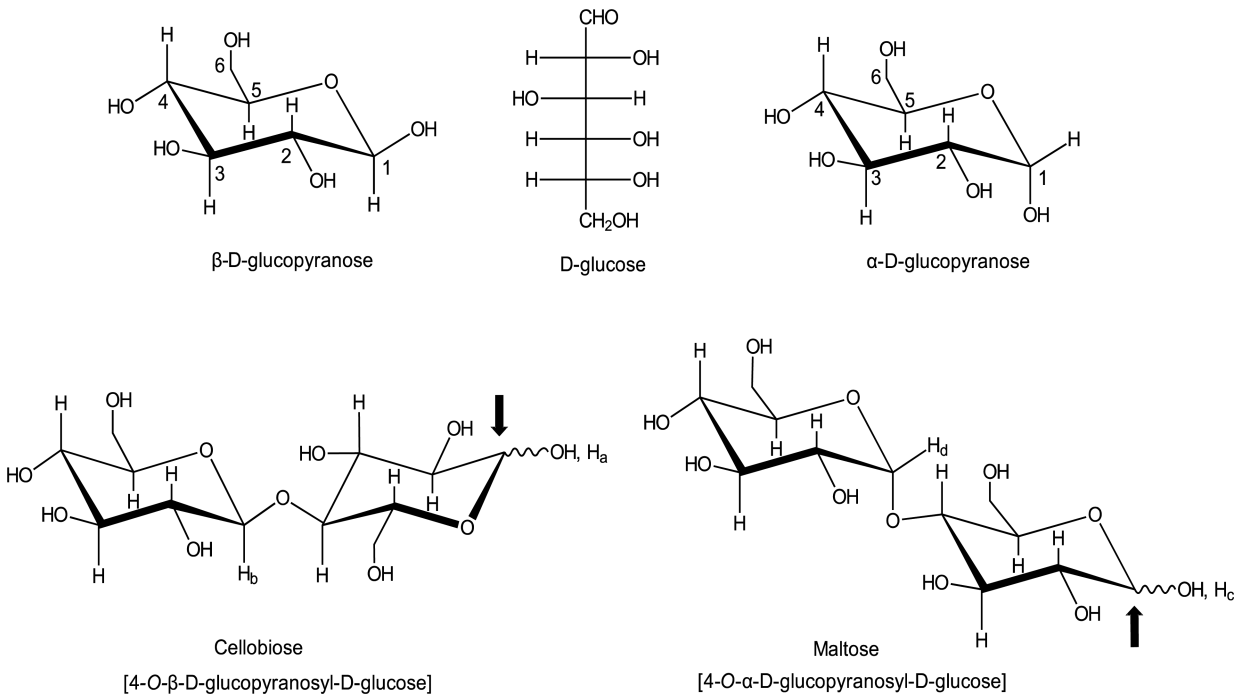

Figure 1. Chemical structures of glucose, cellobiose, and maltose showing anomeric variations among the structures. Arrows in cellobiose and maltose structures show the reducing end of the disaccharides. Formation of cyclic forms of D-glucose produces α- and β-anomeric protons at C-1. The anomeric protons of cellobiose are labeled H_a and H_b and those maltose are labeled H_c and H_d.

usefulness of NMR spectroscopy in modern biochemistry, this topic is not addressed in many biochemistry courses.

Carbohydrate chemistry is one of the major topics of an undergraduate biochemistry curriculum. A thorough understanding of this topic is necessary for students to comprehend a number of advanced topics related to cell biology, medicinal chemistry, pharmaceutical chemistry, and plant biomass chemistry. This experiment gives students opportunities to review carbohydrate structure, to observe how the structure is revealed by NMR spectroscopy, and to study the substrate specificity of an enzyme-catalyzed reaction where carbohydrate structure is the key.

Selective and efficient catalytic reactions carried out by different enzymes are essential for life. β-Glucosidases (BGLs) are ubiquitous among all domains of life and carry out many important biological functions by removing glycosyl residues from the nonreducing ends of saccharides and glucose derivatives.[16] β-Glucosidases play roles in many biological processes, such as nutrient acquisition, defense, biomass degradation, and plant-microbe interaction. Different BGLs can be found in biological systems carrying out a wide range of catalytic reactions, yet all BGLs target only those substrates with β-linked glycosidic bonds, a unique enzyme property that is demonstrated in this experiment using cellobiose and maltose as examples.

Cellulose, the most abundant polymer in nature and the major component of plant biomass, is a promising source for renewable energy and material production. Its structural unit is cellobiose, a disaccharide of glucose linked via a $\beta(1\rightarrow4)$ glycosidic linkage (Figure 1). Depolymerization of cellulose into glucose requires the cleavage of the β-glycosidic linkage between glucose residues; such a cleavage can be mediated by acid/base or enzymatic hydrolysis. Enzyme-mediated hydrolysis of the β-glycosidic linkage in cellobiose requires BGLs, whereas maltose, the disaccharide unit of the biologically important polysaccharide starch, requires α-glucosidases as the glucose residues are linked by $\alpha(1\rightarrow4)$ glycosidic bonds. Here, the enzyme prefixes α and β denote the substrate specificity of glucosidase enzymes. β-Glucosidase activity is commonly monitored by a coupled-enzyme assay[17] that detects the glucose released by the BGL reaction or by using 4-nitrophenyl-β-D-glucopyranoside (p-NPG) as the BGL substrate with visible spectroscopy.[18,19] This experiment, which uses NMR spectroscopy to detect BGL activity, provides a simple, direct, low-cost alternative experimental method with the additional benefit of reinforcing students' understanding of carbohydrate structure and NMR structural analysis.

LEARNING OUTCOMES

This experiment and the associated pre-lab assignment and post-lab quiz are designed to integrate concepts that have been introduced in different areas of the undergraduate chemistry curriculum (carbohydrate chemistry, spectroscopy, and enzymology) and to introduce the use of NMR spectroscopy to follow biochemical reactions, specifically those involving mono- and disaccharides. An opportunity to revisit some of the important organic and biochemical concepts at a more advanced level in an independent hands-on setting provides an enhanced learning opportunity for students and serves as an opportunity for instructors to assess retention from previous organic and biochemistry courses. Specific learning outcomes expected from this laboratory exercise fall, therefore, into three different subject areas: carbohydrate structural chemistry, ^{1}H NMR spectroscopy (as applied to address a biochemical problem), and enzyme specificity.

Carbohydrate structure has many subtleties that students struggle to learn. Therefore, a review can help students solidify structural concepts such as anomerization and glycosidic bonding. In addition, although conformation and configuration of cyclic compounds are introduced in earlier organic chemistry

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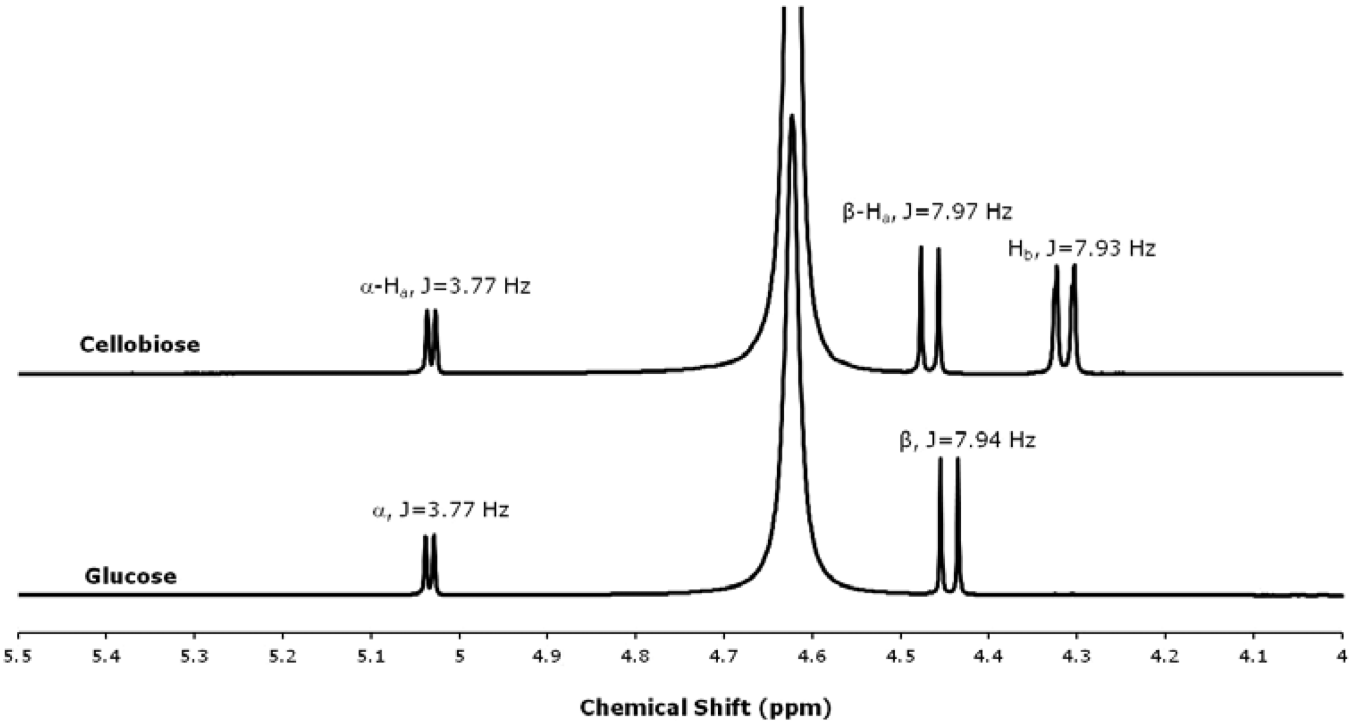

Figure 2. Anomeric region of a sample of student-generated ^{1}H NMR spectra of glucose and cellobiose. Assignments of all anomeric protons along with the measured coupling constants are given above the peaks according to the notation given in Figure 1. Students are expected to make these assignments.

courses, students tend to use these terms interchangeably. This experiment presents a timely opportunity for review of these concepts in cyclic mono- and disaccharide structures.

NMR topics are extensively covered in organic chemistry courses, but are not generally seen again by many chemistry undergraduates who focus on biochemistry, despite the importance of NMR spectroscopy in biochemical research. A review of NMR principles is, therefore, especially beneficial for these students. The use of chemical shifts and coupling constants to assign the spectra of specific mono- and disaccharides affords an excellent review. Anomerization, the conversion process of one anomer to the other, can also be explained with the help of NMR data.[4] In addition, this experiment also introduces students to the implications of NMR samples prepared with aqueous solvents, where an intense peak stemming from water protons can dwarf the lower-intensity peaks of interest. Most, if not all, of the organic samples analyzed using NMR spectroscopy in introductory organic chemistry laboratory courses involve nonaqueous solvents; thus, students have not had previous opportunities to observe this phenomenon. In this experiment, students learn about problems associated with a large solvent signal and about how to mitigate those problems.

Substrate specificity, the ability of an enzyme to act on a specific substrate and catalyze a reaction to form product(s), is characteristic of enzymes. This experiment offers an opportunity to revisit the theoretical knowledge of substrate specificity gained in a biochemistry lecture course by observing an example in a laboratory setting. In particular, the specificity of β-glucosidase toward a specific carbohydrate bond configuration is observed in this experiment.

EXPERIMENTAL OUTLINE

Overview

A total of 12 chemistry majors have conducted this experiment over two different semesters, four in one semester and eight in the other semester. The lab was completed over two, 3 h lab periods that included the actual experimental work, pre-lab and post-lab discussions, and related worksheet exercises. Specific activities and approximate time allotments are given in the Supporting Information. In the pre-lab assignment, students are guided through the details of carbohydrate structure and the implications for NMR spectra. This assignment was designed to help students analyze NMR spectra and to understand the enzyme reaction observed in terms of substrate and product structures.

For the experimental work, the class was divided into groups of two so that each student would have experience with all aspects of the experiment, including collection of NMR data. With the larger class, additional time outside of class was required to collect all the NMR spectra. The amount of time needed will vary with the number of students and availability of an NMR spectrometer.

Solution Preparation and Enzyme Reactions

All solutions are prepared in sodium acetate buffer, pH 5. The enzyme reactions are conducted at 37 °C for 3 h in solutions containing one of the disaccharides and BGL. In addition, two control reactions are set up with glucose and BGL, as well as all substrates without BGL. The progress of glucosidase activity is monitored by thin-layer chromatography (TLC).

NMR Sample Preparation and Spectral Recording

At the end of the 3-h reaction time, the reaction mixture is dehydrated, dissolved in D_2O, and spiked with DMSO-d_6 (99.9 atom % D) to use the DMSO-d_5 present in the solvent as an

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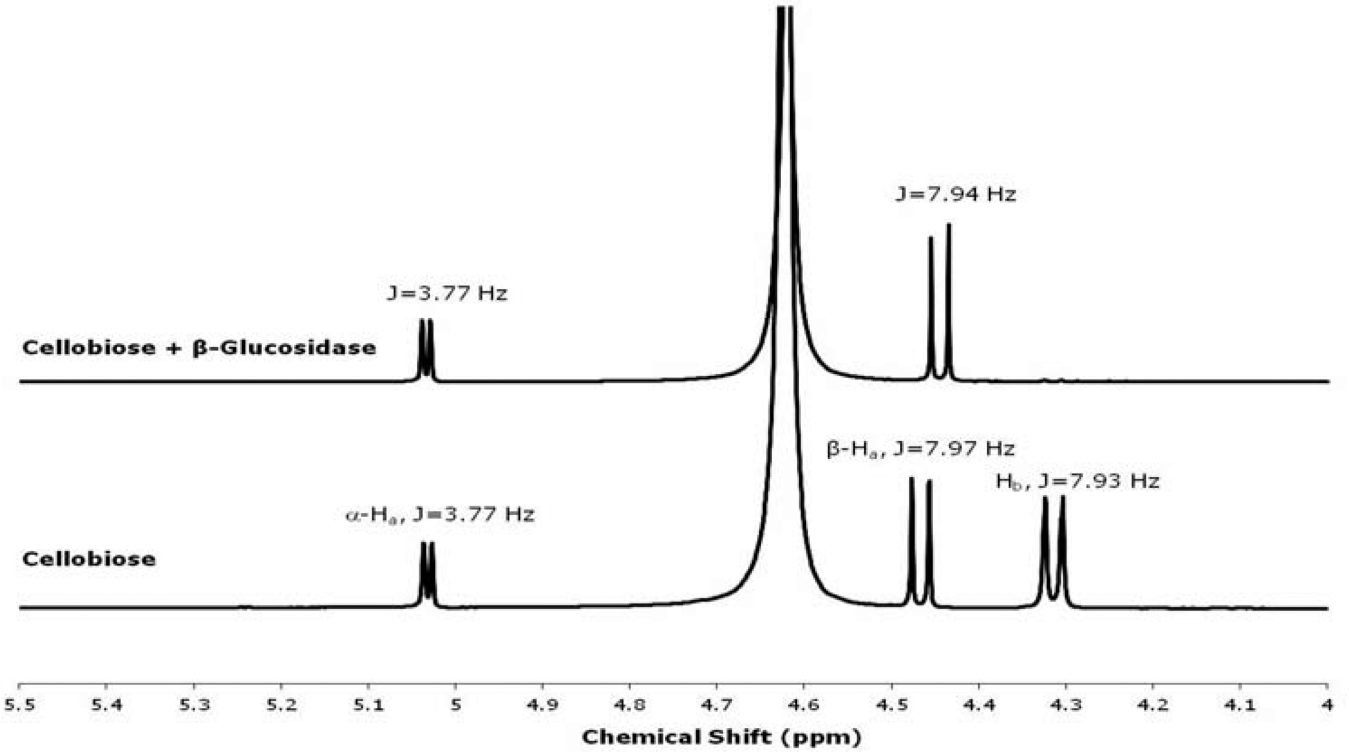

Figure 3. Anomeric region of the student-generated ^{1}H NMR spectra of cellobiose before (bottom) and after (top) the β-glucosidase activity.

NMR frequency reference. All proton NMR spectra are recorded on a 400 MHz spectrometer operating at 25 °C. The spectral parameters and other experimental details are given in the Supporting Information. After processing the spectra, students measure chemical shifts, coupling constants, and peak integrals, and use these values to assign spectra for their written reports.

HAZARDS

The TLC solvent mixture contains methanol, dichrolomethane, and ethyl acetate. These solvents are highly flammable, eye irritants, toxic, and may cause drowsiness; therefore, the TLC plate developing must be conducted inside a fume hood. TLC monitoring involves spraying of orcinol in 70% H_2SO_4. Both orcinol and sulfuric acid are eye and skin irritants and may cause severe respiratory irritation; therefore, TLC plates should be sprayed inside a fume hood. DMSO-d_6 is a combustible liquid that should be kept away from any source of ignition; inhalation of vapor or mist should be avoided. D_2O is hazardous if ingested. Appropriate safety equipment (laboratory coat, safety goggles, and gloves) should be used by students at all times. People with electronic or metallic implants should not get within the field line of the NMR magnet.

RESULTS AND DISCUSSION

All spectra shown in this article represent data collected by students. Spectral analysis focused on the anomeric region from 4.0 to 5.5 ppm where the anomeric carbon-attached proton signals occur (Figures 2−4).[4,20]

The intramolecular reaction between the aldehyde (i.e., aldose) and the alcohol functional groups in the linear open chain of a glucose molecule leads to the formation of cyclic hemiacetal. This reaction is reversible with the six-membered (pyranose) ring being the dominant species (>99%) (Figure 1). The hemiacetal carbon, also known as the anomeric carbon (C-1 in Figure 1), assumes two different major configurations in the cyclic form resulting in α (36.4%) and β (63.6%) anomers of glucose in aqueous solutions with minute amounts of the linear and other cyclic forms.[21] The proton attached to the anomeric carbon is relatively deshielded compared to other protons and, thus, appears within a 4.0−5.5 ppm range. This serves as a distinct ^{1}H NMR signature for carbohydrates in a region that is separated from other protons and usually produces well-resolved signals. The interconversion of α and β anomers via ring opening and closing, and the associated change in the specific optical rotation (i.e., mutarotation), as well as the concepts of reducing and nonreducing sugars, can also be discussed using the data and information from existing literature.[4,20,22]

To differentiate the signals originating from the protons attached to the anomeric carbon in cellobiose and glucose, standard ^{1}H NMR spectra of glucose and cellobiose were recorded. The anomeric region (4.0−5.5 ppm) of these spectra is shown in Figure 2. The presence of α and β anomers of glucose due to mutarotation is evident from the two sets of peaks observed for anomeric protons between 4.4 and 5.1 ppm in the glucose spectrum (Figure 2). The ratio of the α and β anomers (α:β) was measured as 38:62 under these experimental conditions. In cellobiose, mutarotation at the reducing end (shown by arrow in Figure 1) produces two anomeric protons (α-H_a and β-H_a) that, along with another anomeric proton H_b, results in three magnetically non-equivalent protons, thus three distinct chemical shifts: H_b at 4.31 ppm, β-H_a at 4.46, and α-H_a at 5.03 ppm in the cellobiose spectrum (Figure 2).[4]

The J-coupling constants provide information about the connectivity between atoms leading to important structural information. The magnitude of the ^3J-coupling constant is

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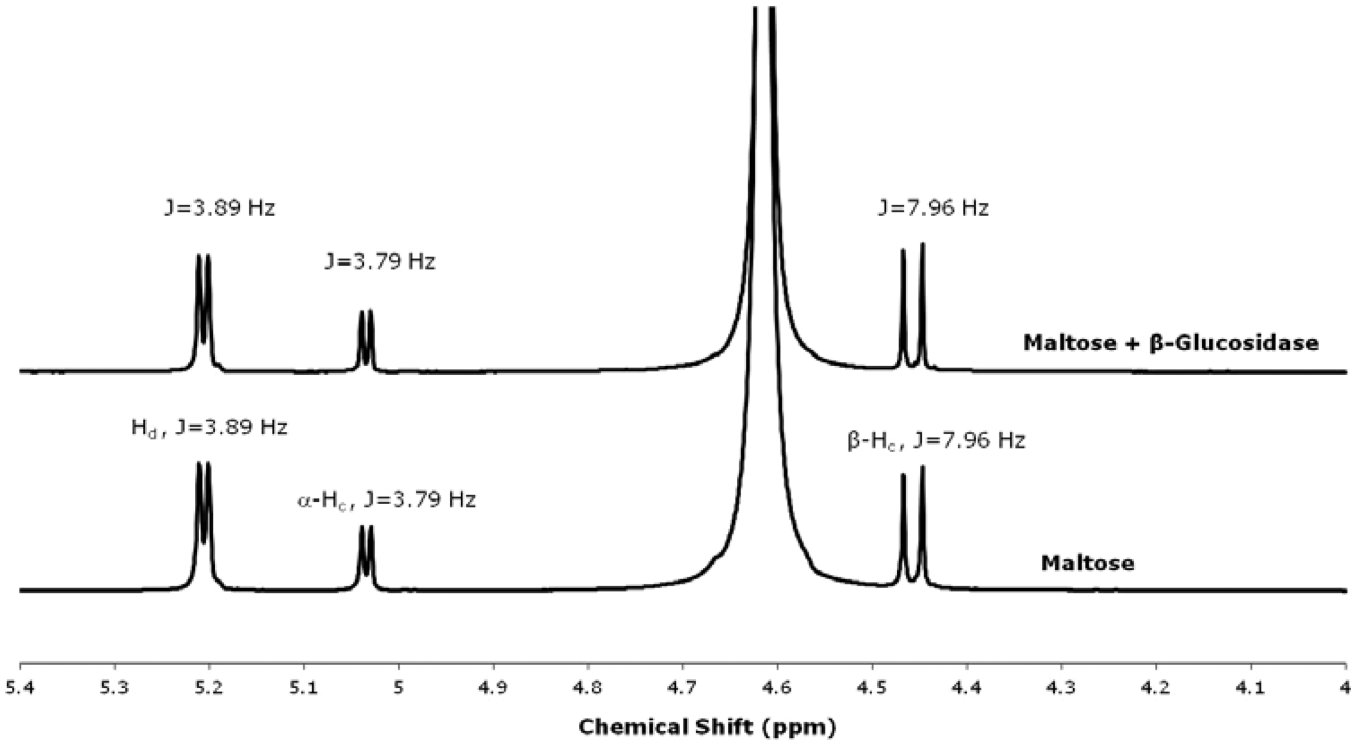

Figure 4. Anomeric region of student-generated ^{1}H NMR spectra of maltose before (bottom) and after (top) the treatment with β-glucosidase.

dependent on the dihedral angle between two interacting protons and, thus, serves along with the chemical shift to identify specific structural features. In glucose, the proton attached to the anomeric carbon is spin-coupled with the proton attached to C-2 resulting in a doublet. The coupling constant observed for the α-anomer (3.77 Hz) is smaller than that of the β-anomer (7.94 Hz) as the dihedral angle is smaller in the α-anomer than in the β-anomer.[20,23]

^{1}H NMR spectra of cellobiose and maltose (Figures 3 and 4) clearly distinguish the structural differences between these two disaccharides. Even though the connectivity between the anomeric carbon and other atoms in both cellobiose and maltose are the same, the difference in the glycosidic linkages, $\beta(1\rightarrow4)$ in cellobiose and $\alpha(1\rightarrow4)$ in maltose, makes the anomeric protons at these linkages in each of these disaccharides magnetically distinct as evidenced by the chemical shift difference between H_b and H_d (cf. Figures 3 and 4).[23] This distinct spectral feature observed for cellobiose and maltose makes ^{1}H NMR spectroscopy an ideal technique for the identification of these two disaccharides, as well as for monitoring the glycoside hydrolase activity on these disaccharides.

To minimize the water peak in NMR spectra, the samples were dried overnight in an oven at 37 °C, redissolved in D_2O and immediately transferred into NMR tubes for data collection. This procedure successfully reduced the signal from water so that well-resolved carbohydrate signals were observed. However, due to the hygroscopic nature of D_2O and the presence of multiple exchangeable protons in the OH groups of glucose or cellobiose, an intense water (HOD) peak was still observed. An alternative way to reduce the intensity or completely remove the water peak would be to use water suppression techniques, such as WATERGATE pulse sequence, during the collection of NMR spectra.[24]

The difference in the glycosidic bonding in cellobiose and maltose makes them substrates for different glucosidases: β-glucosidase and α-glucosidase, respectively. To demonstrate the substrate specificity of β-glucosidase toward cellobiose, cellobiose and maltose were treated with β-glucosidase and ^{1}H NMR spectra were collected after the completion of the reaction (Figures 3 and 4). The complete breakdown of cellobiose into glucose by β-glucosidase within the reaction time allowed is evident from the spectra (Figure 3), whereas maltose remains intact after the same period of reaction. This result demonstrates the inability of β-glucosidase to catalyze the breakdown of the α-glycosidic bond in maltose. This experiment could be extended by observing the reactivity of α-glucosidase toward cellobiose and maltose substrates to demonstrate the ability of α-glucosidase to break the α-glycosidic bond in a maltose substrate.

Over the two semesters where this experiment was included in the biochemistry laboratory, students demonstrated competency in the areas of carbohydrate structural chemistry, NMR spectroscopy, and enzyme specificity upon completion of this experiment. Comparison of student performance on these topics in a pre-lab assignment with that of post-lab quiz answers and a laboratory writeup clearly showed students' achievement of the defined learning outcomes. Hands-on NMR experience was appreciated by students who encouraged the inclusion of this laboratory experiment in future offerings of the course.

CONCLUSION

Enzymes and carbohydrates are major topics in the biochemistry curriculum and an integrated, hands-on teaching method enhances student learning and understanding of important concepts in these topics. NMR spectroscopy is a valuable analytical technique in physical and biological studies, and plays a crucial role in academic and industrial laboratories. This easy-to-implement laboratory experiment provides an

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opportunity to obtain significant hands-on experience in NMR spectral analysis and to reinforce the basic structural chemistry of mono- and disaccharides, as well as to demonstrate the substrate specificity of enzymes in a single experiment.

■ ASSOCIATED CONTENT

Supporting Information

Student handouts consisting of complete student laboratory procedure, pre-lab assignment and post-lab quiz, answer keys, and notes for instructor are provided. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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