The Development of an Alternative Synthesis of Trichloroacetyl Isocyanate

Katherine J. Mackenzie
Biological Sciences and Chemistry

Author

Katherine Mackenzie took a summer class from Professor Shaka and, several months later, asked if she could work in his lab. She took over a project from a graduating undergraduate researcher, considering the prior work while pursuing a new avenue of inquiry. In her research, Katherine enjoyed being able to apply the techniques and theories she learned in her classes. By experiencing the real-life situations to which her education could be applied she found it exciting to realize that the answers to puzzling research problems could be found in abstract theories. Katherine will graduate from UCI in Spring 2009, and hopes to go on to graduate school to pursue a Ph.D. in Chemistry.

Key Terms

- Carbohydrate
- Nuclear Magnetic Resonance (NMR)
- Trichloroacetyl Isocyanate (TAI)

Abstract

The ^{1}H-NMR spectra of carbohydrates are difficult to analyze because they exhibit spectral crowding. The compound trichloroacetyl isocyanate (TAI) replaces the hydroxyl groups of carbohydrates with protein-like structures containing NH groups. The NH groups appear downfield in the ^{1}H-NMR spectra, allowing them to be counted. The proximal ring protons of TAI-derivatized carbohydrates become dispersed and can be identified as primary or secondary. TAI doubly enriched with ^{13}C and ^{15}N can be reacted with carbohydrates, producing a derivatized product with NMR-active isotopes. Multidimensional NMR analyses can then be carried out on the derivatized carbohydrates. This project was the development of a new synthesis procedure for TAI that would allow for isotopic enrichment. A novel procedure previously developed had low yield and was difficult to perform; this new procedure is more efficient and has a higher yield. ^{1}H-NMR spectra of carbohydrates derivatized with unenriched TAI obtained from this procedure show the product is comparable to commercially obtained TAI and support the conclusion that this synthesis is a reasonable alternative to the one previously developed.

Faculty Mentor

Glycans are on the cutting edge of research in chemical signaling in biological systems, playing roles in immune response, cancer, and cell-cell signaling. Normally these sugars do not crystallize and therefore cannot be characterized by X-ray diffraction. In addition, the NMR spectrum of the unmodified glycan is usually intractable. However, by introducing a chemical group enriched with ^{15}N and/or ^{13}C (an "isotag"), much better NMR spectra can be obtained. The streamlined synthesis of trichloroacetylisocyanate that Ms. Mackenzie implemented gives us access to new options for characterization of this important class of molecules. Her work has been instrumental in making high-sensitivity multidimensional NMR of glycans a reality rather than a dream.

Athan J. Shaka
School of Physical Sciences

Introduction

Nuclear magnetic resonance (NMR) is a powerful analytical tool used to determine the three dimensional structures of molecules in solution. Atoms with a spin-½, such as ^{1}H, ^{15}N, and ^{13}C, give narrow resonance lines in an NMR graph and therefore molecules containing these atoms can be analyzed. The natural abundance of ^{13}C is only 1.1%, which means that of all carbon atoms, 1.1% are the ^{13}C isotope. The majority of carbon atoms are ^{12}C, which has spin zero and is NMR inactive. Similarly, ^{15}N is only 0.38% abundant. The majority of nitrogen atoms are ^{14}N, which is spin-1 and gives wider lines with less resolution. To improve ^{13}C-NMR and ^{15}N-NMR resolution and sensitivity, ^{13}C and ^{15}N isotopic enrichment is often used. One method of enrichment is to synthesize the molecule of interest using ^{13}C and ^{15}N enriched reagents. This results in a product with a higher percentage of isotopes than natural abundance. The synthesis of trichloroacetyl isocyanate (TAI) developed in this project allows for ^{13}C and ^{15}N enrichment by using isotopically enriched reagents. Doubly-enriched TAI is not available commercially.

Determining the structure of a carbohydrate is necessary to understand its function. Carbohydrates are essential in cell signaling, biological structural units, and are present in many pharmaceutical drugs. Due to their size and complexity, their structures are difficult to ascertain without a way to visualize where each atom is in space relative to the others. These molecules also have many chiral centers and therefore many isomers. To understand the reactions and roles of carbohydrates, the exact structure of the correct isomer of the molecule in question must be determined. Carbohydrates are mainly composed of hydrogen and carbon, so ^{1}H-NMR analysis is an obvious choice to determine their structure. Unfortunately, ^{1}H-NMR spectra of large, complex molecules such as carbohydrates are difficult to analyze because they exhibit spectral crowding—peaks overlap, disguising splitting patterns and concealing coupled peaks (Figure 1). The spatial relationships between atoms are difficult to determine.

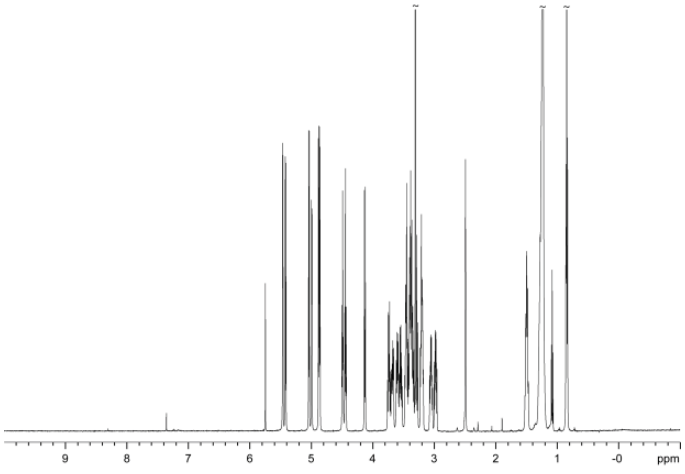

Figure 1

^{1}H-NMR of underivatized dodecyl-β-D-maltoside. There is spectral crowding between 2.8 and 3.8 ppm. The ring proton peaks are overlapped and splitting patterns are not discernible.

One method of obtaining information on carbohydrate structure is to actively substitute the free hydroxyls of the sugar. This affects the shift of the atoms at the substitution site, often causing a downfield shift in the proton signals. This increases the range over which ring proton signals are observed, decreasing the spectral crowding in that region and making data easier to analyze (Duus et al., 2000). The use of TAI in structural determination of carbohydrates takes advantage of the effect that hydroxyl substitution with an electron-withdrawing substituent has on ring protons. Goodlett (1965) reacted TAI with glycols and alcohols to esterify the hydroxyl groups, producing a downfield shift of the protons alpha to these groups. Shifts of 0.5 to 0.9 ppm were observed for protons alpha to primary hydroxyl groups and shifts of 1.0 to 1.5 ppm were observed for protons alpha to secondary hyroxy groups. In addition, the signal from the hydroxyl proton is replaced by a signal from the N-H proton of the new carbamate group, which appears far downfield of the ring protons, in the range of 10 ppm (Figure 2). These NH peaks can be counted and correspond to the number of hyroxyl groups on the compound of interest (Goodlett, 1965). By counting the number of NH peaks, the number of TAI groups bonded to the carbohydrate can be determined.

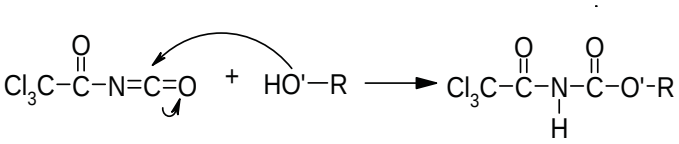

Figure 2

Substitution of a hydroxyl group using TAI. The proton is replaced by a carbamide group.

Furthermore, TAI is so reactive that it completely derivatizes all primary, secondary, and tertiary hydroxyl groups in a matter of minutes, provided the TAI and its partner are soluble in the same solvent. TAI does not have any hydrogen atoms and therefore no ^{1}H-NMR spectra itself. This means that the derivatization procedure can be carried out in the NMR tube without ^{1}H-NMR signals from TAI obscuring the NMR peaks of the derivatized product. No

by-products are produced and purification is unnecessary (Goodlett, 1965). Goodlett's findings have been applied to small sugars, and the effect of TAI on the spectra of sugars is consistent with his observations involving alcohols and glycerols (Dermenci, 2005). While the effects of TAI on the spectra of small carbohydrates is documented, TAI is not commonly used to characterize the structures of complex sugars of unknown structure.

TAI reacts with the hydroxyl group of carbohydrates, substituting all the hydroxyl groups with carbamide groups. The electron withdrawing properties of TAI cause the signals of the ring protons to be shifted downfield, and the size of the shift can be used to determine if the signal is from a primary or secondary ring proton. This spreads out the ring proton signals, making the spectra much easier to resolve and therefore analyze. The number of NH peaks that appear far downfield corresponds to the number of hydroxyl groups present on the underivatized carbohydrate, allowing the hydroxyl groups to be easily counted (Figure 3).

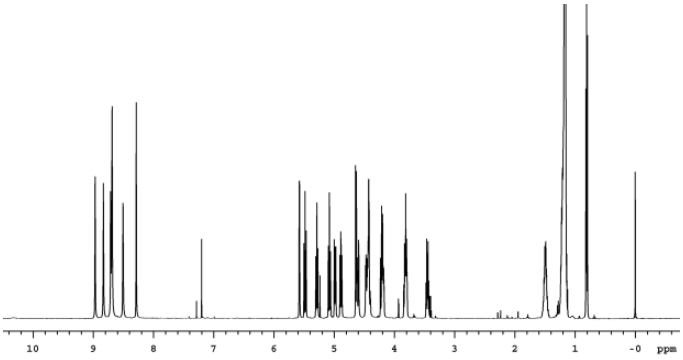

Figure 3

The ^{1}H-NMR of dodecyl-β-D-maltoside derivatized with TAI exhibits dispersed ring proton peaks from 3.4 to 5.6 ppm and NH peaks between 8 and 9 ppm.

Aside from 1.1% ^{13}C and 0.38% ^{15}N, there are no NMR-active nuclei in TAI. By enriching TAI to 100% ^{13}C and ^{15}N in the isocyanate group using chemical synthesis, the derivatives are suitable for high-sensitivity, high resolution 3-D and 4-D NMR spectra. By introducing NMR-active isotopes into the molecule, multidimensional NMR analyses can be carried out on derivatized sugars. These experiments allow the relationships between atoms through space to be determined, rather than simply through bonds. ^{13}C and ^{15}N isotopes are relatively common in NMR studies and a wide range of reagents are commercially available with these isotopes, but ^{13}C,^{15}N-enriched TAI is not commercially available. ^{13}C,^{15}N-enriched TAI allows for 3-D NMR experiments on derivatized sugars, which provide a much clearer picture of the structure than 1-D ^{1}H-NMR experiments (Figure 4). Thus ^{13}C,^{15}N-enriched TAI must be synthesized because it cannot be simply purchased, and the procedure used must allow for double labeling. We developed a working synthesis for ^{13}C-enriched TAI (Figure 5). While the procedure was successful, gas chromatography was necessary for purification. This method is difficult and time consuming for an air-sensitive product like TAI, and resulted in a very low yield. Furthermore, to introduce both ^{13}C and ^{15}N, doubly-labeled K^{13}C^{15}N was required. This reagent is especially costly to obtain because it is enriched with two different isotopes. Oxidation of KOCN to OCN^- was problematic and OCN^- could not be separated from Cl^- in the synthesis of TAI. OCN^- did not displace Cl^- to the extent desired in the last step (Dermenci, 2005).

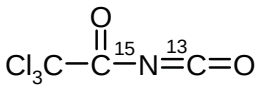

Figure 4

^{13}C,^{15}N-enriched trichloroacetyl isocyanate.

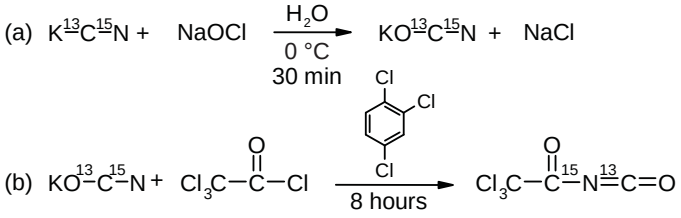

Figure 5

Previous synthesis of TAI.

The purpose of this project was to develop an alternative synthesis of TAI that could be used to produce ^{13}C,^{15}N-enriched TAI (Figure 6). The goal was a synthesis with a higher yield than the procedure developed by Dermenci. By introducing each isotope one at a time, a higher yield would be obtained, increasing the efficiency of the synthesis. The product would be used to derivatize carbohydrates, allowing the use of multidimensional NMR experiments to elucidate the structures of these carbohydrates. The procedure is use-

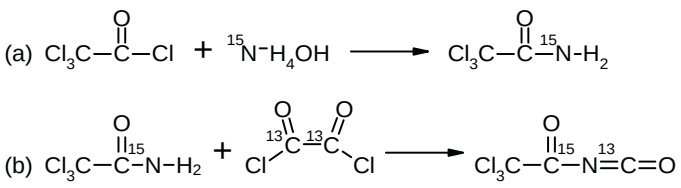

Figure 6

Alternative synthesis of trichloroacetyl isocyante, in which ^{13}C,^{15}N-labelling is possible. Trichloroacetyl chloride is reacted with ammonium hydroxide to produce trichloroacetamide. This compound is then reacted with oxalyl chloride to produce TAI.

ful because doubly-labeled TAI cannot be purchased. The synthesis was adapted from a procedure to produce acyl isocyantes (Weikert et al.) The adapted two-part synthesis allows for the efficient introduction of ^{15}N and ^{13}C to produce ^{13}C,^{15}N-enriched TAI.

Experimental Methods

Synthesis of Trichloroacetamide

In a 500 mL round bottom flask equipped with an addition funnel, 25.0 mL of 14.8 N ammonium hydroxide (370 mmol Fisher Scientific) was added to 250.0 mL ethyl acetate. The solution was stirred in an ice bath for 5 minutes. 8.8 mL (79 mmol) of trichloroacetyl chloride (Aldrich) was dissolved in 50.0 mL ethyl acetate in the addition funnel. The system was put under nitrogen by connecting it to a Schlenk manifold. The trichloroacetyl chloride solution was added to the round bottom flask dropwise via the addition funnel until the addition was complete. The solution in the round bottom flask was stirred in an ice bath under nitrogen until all white solids dissolved, yielding a clear, colorless solution.

The organic layer of the solution was extracted and washed twice with 100.0 mL deionized water. The organic layer was then washed twice with 75.0 mL of a saturated brine solution. The organic layer was dried with magnesium sulfate for 10 minutes and filtered using a Buchner filter. The ethyl acetate was removed using a rotary evaporator at room temperature, yielding a white, crystalline solid. The solid was triturated with hexanes, which were removed using a rotary evaporator, yielding the white, crystalline solid trichloroacetamide (yield: 78%). The purity of the solid was checked with thin layer chromatography using a 96/3.9/0.1% CH_2Cl_2/MeOH/NH_4OH solution (one spot, Rf=0.43). The product was placed in a 60 ºC oven for 20 minutes then stored in a desiccator.

Synthesis of Trichloroacetyl Isocyanate (TAI)

All glassware was dried in an oven overnight (125 ºC). The reaction took place under nitrogen, connected to a Schlenk manifold, which contains a drying filter. A 10 mL round bottom flask equipped with a cold finger condenser and a magnetic stir bar was placed under nitrogen. 1.498 g (9.224 mmol) of trichloroacetamide was placed in the flask, then 3.0 mL of 1,2-dichloroethane (Dri-Solve, EMD Biosciences) was added. 1.2 mL (14 mmol) oxalyl chloride (Acros Organics) was added quickly to the flask. The flask was heated to reflux using a silicone oil bath. The solution reacted at reflux for 5 hours and was then returned to room temperature.

The cold finger apparatus was replaced by a distillation apparatus with a collection flask that had four graduated receiving tubes. The round bottom flask was immersed in a silicone oil bath with any exposed area wrapped in aluminum foil, and the condenser was wrapped in glass tape. The solution was distilled until the flask was almost dry. Four fractions were collected. The first two fractions were collected between 64 ºC and 80 ºC; the third fraction was collected between 80 ºC and 110 ºC; the fourth fraction was collected from 110 ºC to 134 ºC. After the system cooled to room temperature, the collection flask was immediately removed and quickly capped, being sure to minimize contact with air. The purity of the product was determined by using it to derivatize a carbohydrate and comparing the ^1H-NMR spectrum with that of the same carbohydrate derivatized with commercially obtained TAI.

The flask was moved into a glove box in which an inert nitrogen atmosphere was maintained, and the fourth fraction collected during distillation was transferred to a glass vial for storage.

Preparation of Derivatized Carbohydrate NMR Samples

The derivatization of each carbohydrate was performed in a glove box under an inert nitrogen atmosphere. Approximately 8 mg (0.016 mmol) of dodecyl-β-D-maltoside was added to a glass vial. 0.75 mL of deuterated chloroform was added (D, 99.96% Cambridge Isotope Laboratories). TAI collected in the third fraction of the distillation was added to the vial in excess, 13 drops, and the solution was mixed. The carbohydrate began to dissolve after about 5 minutes of mixing and the solution was completely clear and colorless after 10 minutes, producing a 21 mM sample. The sample was then transferred to a Wilmad J. Young 535-grade NMR tube and removed from the glove box. This procedure was repeated with 10 mg (0.067 mmol) of D-ribose to produce an 89 mM sample and 6 mg (0.015 mmol) of sucralose to produce a 20 mM sample.

Preparation of Underivatized Carbohydrate NMR Samples

Samples of underivatized D-ribose and sucralose were prepared for comparison. 1.6 mg (0.011 mmol) of D-ribose was dissolved in 1.0 mL D_2O (D, 99.96% Cambridge Isotope Laboratories) to yield an 11 mM solution. The sample was transferred to a Wilmad 535-grade NMR tube. This procedure was repeated with 4.1 mg (0.010 mmol) of sucralose to yield a 10 mM sample.

Results

The purpose of this project was to develop a better synthesis of TAI that would allow for isotopic enrichment. The purity of the product was determined by using it to derivatize dodecyl-β-D-maltoside and then comparing the ^{1}H-NMR spectrum of that sample to a spectrum of the sugar derivatized with commercially obtained TAI (Aldrich). Figure 7 shows the ^{1}H-NMR spectrum of the same carbohydrate derivatized with TAI synthesized by the procedure outlined above. Figure 8 is the ^{1}H-NMR spectrum of dodecyl-β-D-maltoside derivatized with TAI purchased commercially.

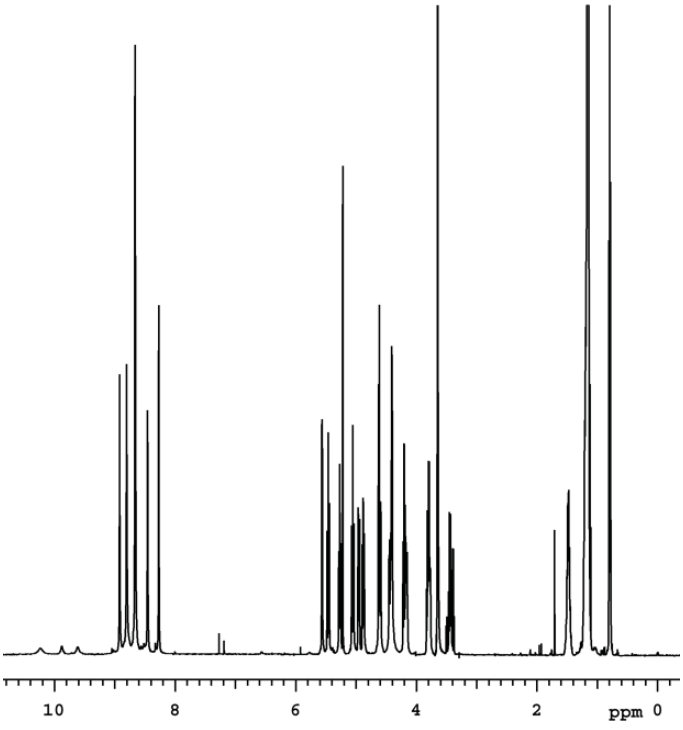

Figure 7

^{1}H-NMR spectrum of dodecyl-β-D-maltoside derivatized with synthesized TAI. The peak at 3.6 ppm is a signal from 1,2-dichloroethane.

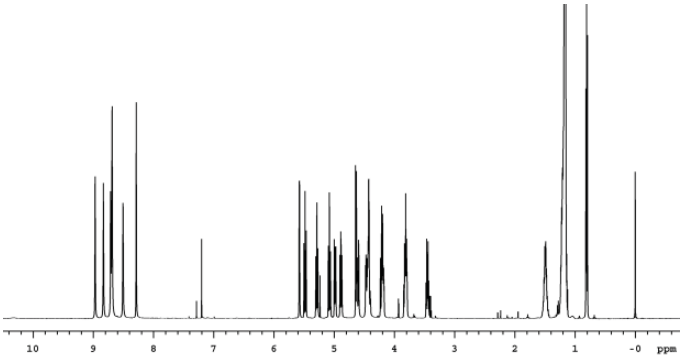

Figure 8

^{1}H-NMR spectrum of dodecyl-β-D-maltoside derivatized with commercially obtained TAI. The spectrum is nearly identical to that of the carbohydrate and synthesized TAI.

The spectra are nearly identical, with peaks at the same shifts and with the same splitting patterns. There are a few significant differences between the two spectra, but the synthesis is still an effective method to attain TAI. The larger peak at 7.27 ppm and the peak at 0 ppm in the spectrum with commercial TAI are due to slight differences in the specific deuterated chloroform used as a solvent. The other differences between the two spectra are due to impurities in the distilled product. The two wide "horns" visible at 10.2 ppm and 9.6 ppm in Figure 7 are due to the product of TAI and water, introduced through exposure to air. Residual 1,2-dichloroethane in the product produces the large peak at 3.6 ppm in Figure 7. While these two sets of peaks do result in a cluttered spectrum and impure sample, they do not impede data analysis. Once recognized and marked, the peaks can be neglected when determining carbohydrate stereochemistry. What is significant is that these slight impurities do not affect the ability of TAI to derivatize carbohydrates, as evidenced by the similarity between the two spectra.

The spectrum of TAI produced through this synthesis has the desired properties of dispersing the ring proton signals and replacing the hydroxyl proton signals with carbamide proton signals. In the spectrum of underivatized dodecyl-β-D-maltoside, the ring proton signals overlap between 2.8 and 3.8 ppm (Figure 9). The splitting patterns cannot be easily deduced and peaks overlap, making it difficult to determine

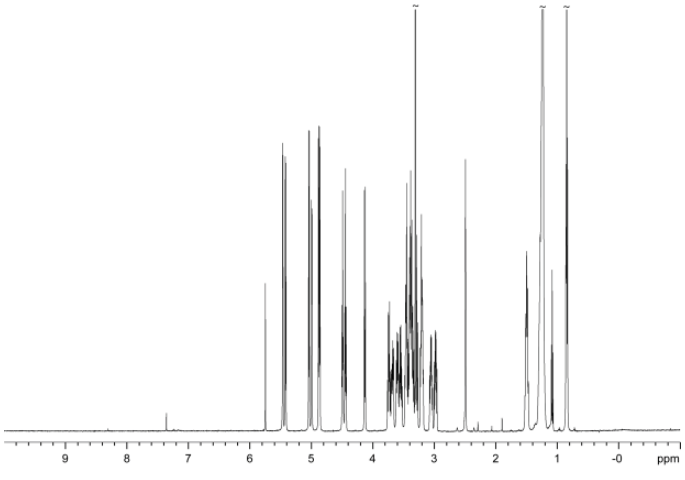

Figure 9

^{1}H-NMR of underivatized dodecyl-β-D-maltoside. There is spectral crowding between 2.8 and 3.8 ppm. The ring proton peaks are overlapped and splitting patterns are not discernible.

the strength of the peaks. The spectrum of dodecyl-β-D-maltoside derivatized with synthesized TAI shows that the ring proton peaks have been dispersed between 3.2 and 5.6 ppm (Figure 7). A closer look at this region shows that the splitting patterns of each signal are now clearly visible as a result of this dispersion (Figure 10). Because the splitting patterns and peak strength of each ring proton are observable, the spectrum of derivatized maltoside provides more insight into its structure. The hydroxyl proton peaks have been replaced by carbamate proton signals, which are seen between 8.2 and 9 ppm. All of these effects agree with the characteristics of TAI-reacted carbohydrates discussed in the literature, supporting the conclusion that the synthesis was successful.

Similar results are observed when comparing the spectra of underivatized D-ribose (Figure 11) with derivatized D-ribose (Figure 12), and the spectra of underivatized sucralose (Figure 13) with derivatized sucralose (Figure 14).

For both compounds, the spectra of the underivatized carbohydrates show overlapping ring proton peaks. In the

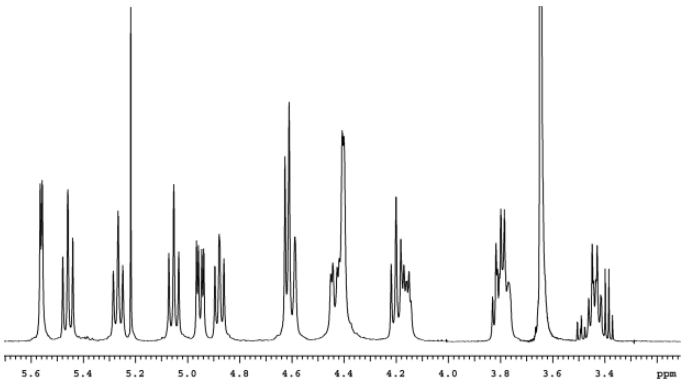

Figure 10

Expanded view of the dispersed ring proton peaks of dodecyl-β-D-maltoside derivatized with synthesized TAI. The splitting patterns of each signal are clearly discernible, providing a great deal of information about the stereochemistry of the compound.

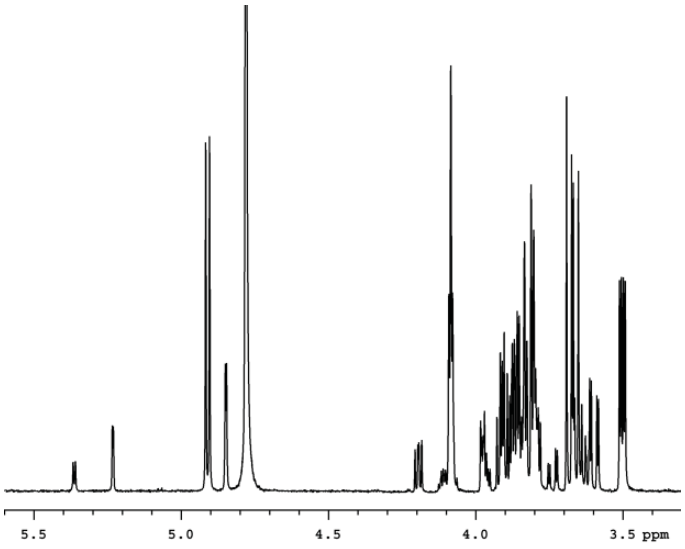

Figure 11

^{1}H-NMR spectrum of underivatized D-ribose. Spectral crowding is observed between 2.8 and 4.0 ppm. The peak at 4.78 ppm is H_2O.

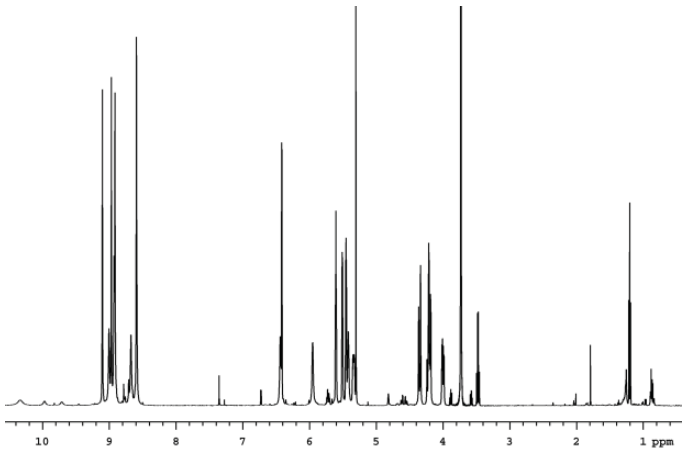

Figure 12

^{1}H-NMR spectrum of D-ribose derivatized with synthesized TAI. Ring proton peaks are dispersed and carbamide proton peaks are observed between 8.5 and 9.2 ppm.

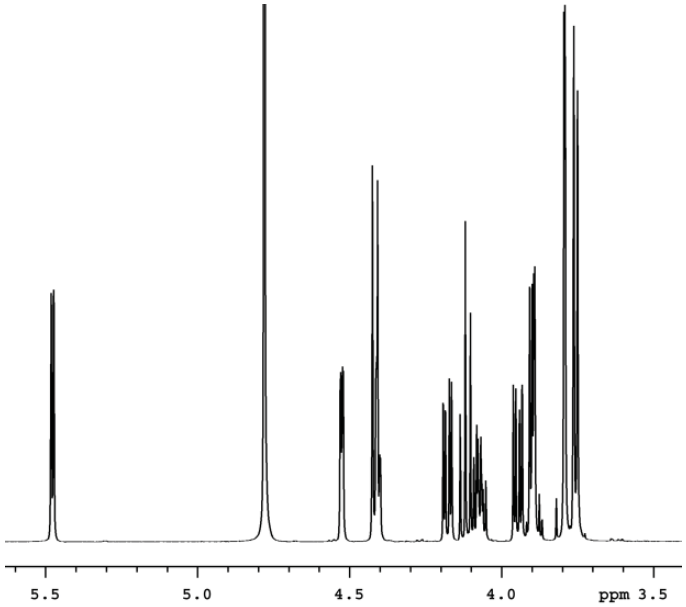

Figure 13

^{1}H-NMR spectrum of underivatized sucralose. Spectral crowding is observed between 3.7 and 4.2 ppm. The peak at 4.78 ppm is H_2O.

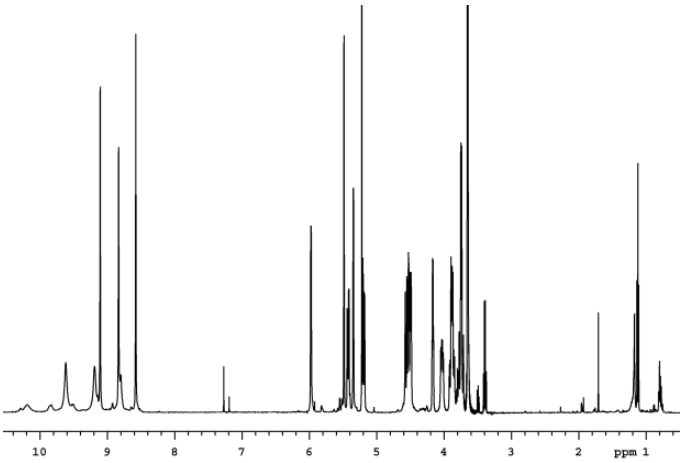

Figure 14

^{1}H-NMR spectrum of sucralose derivatized with synthesized TAI. Ring proton peaks are dispersed between 3.2 and 6 ppm and carbamide proton peaks are observed between 8.5 and 9.2 ppm.

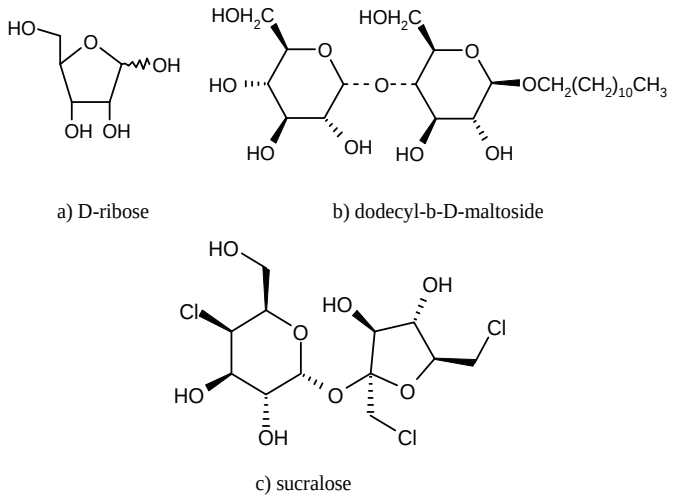

Figure 15

Carbohydrates successfully derivatized with TAI synthesized through the described procedure.

spectra of the derivatized carbohydrates, however, these signals have become dispersed as a result of the electron-withdrawing properties of TAI. This dispersion allows the signals from individual ring protons to be discernible, making analysis of these signals easier.

Discussion

The synthesis was successfully adapted to produce TAI with a higher yield in an easier to purify procedure than the one developed previously. Carbohydrates reacted with this TAI exhibit the expected dispersed ring proton peaks and NH peaks. The successful derivatization of D-ribose, dodecyl-β-D-maltoside, and sucralose show that the synthesized TAI is a viable derivatizing agent for a variety of carbohydrates (Figure 15). Currently, TAI is not used to characterize the structures of large and very complex carbohydrates. For TAI to be an effective tool in determining the structure of unknown carbohydrates, it must be able to derivatize a wide array of sugars. The sugars used in this experiment were chosen partly to show the potential range of carbohydrates with which TAI can be used. The derivatization of D-ribose, a very simple one-ring sugar, illustrates the basic ability of TAI to react with simple sugars and affect their ^{1}H-NMR spectra. Dodecyl-β-D-maltoside is a more complex two-ring carbohydrate. The successful derivatization of this compound shows that TAI is not limited to simple carbohydrate monomers, but could be used with larger, more complex multi-ring carbohydrates. Sucralose is also a two ring carbohydrate, but has the added complexity of three hydroxyl groups substituted with chlorine atoms. This carbohydrate was also successfully derivatized by the synthesized TAI, demonstrating the range of sugars TAI will fully derivatize. This range is important if TAI is to be used with complex carbohydrates with unknown structures.

The previous procedure of TAI synthesis required gas chromatography, which is a difficult and time-consuming purification technique when used with air-sensitive TAI. As a result of this, the yield was extremely low (Dermenci, 2005). A low yield is never desirable, but is especially costly when dealing with isotopically enriched reagents. The TAI synthesis developed here does not require gas chromatography for purification and has a reasonable yield (approximately 39.2% overall). The residual 1,2-dichloroethane present in the product does not affect derivatization and can easily be taken into account when analyzing NMR spectra for structure determination. This synthesis also provides a method by which the isocyanate group of TAI can be enriched with ^{13}C and ^{15}N beyond natural abundance (Figure 16).

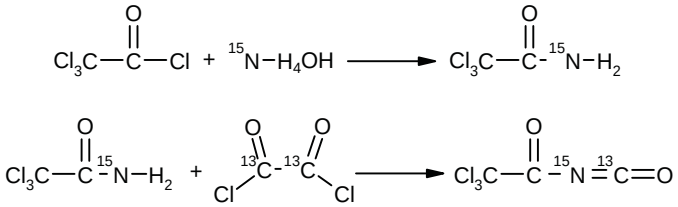

Figure 16

Potential synthesis of ^{13}C,^{15}N-enriched TAI. $^{15}NH_4OH$ is used to produce ^{15}N-trichloroacetamide. This is combined with ^{13}C-oxalyl chloride to achieve the doubly-enriched product.

Derivatizing carbohydrates with ^{13}C,^{15}N-enriched TAI allows for multi-dimensional NMR experiments to be carried out. A carbohydrate derivatized with ^{13}C,^{15}N-enriched TAI

would have its free hydroxyl groups replaced by enriched carbamate groups. By combining ^{1}H-NMR, ^{13}C-NMR, and ^{15}N-NMR data, the correlations between specific ^{1}H, ^{13}C, and ^{15}N signals could be determined. Doubly-enriched TAI is a potentially powerful tool in the determination of complex carbohydrate structure. The procedure developed here for doubly-enriched TAI is easier, more efficient, and more practical than the previously devised synthesis.

Acknowledgements

I would especially like to acknowledge the support of my faculty mentor Dr. A.J. Shaka. This project was also made possible with help from Dr. James Nowick, Omid Khakshoor, Alpay Dermenci, and Dr. Patrick Farmer. I would like to thank UROP for their financial support of this project in the form of UROP and SURP grants, as well as the NIH grant GM-66763.

Works Cited

Dermenci, A. "Introducing 'Protein-like' Functional Groups into Carbohydrate Molecules: A New Way to Obtain Well-Resolved Nuclear Magnetic Resonance Spectra." UCI Undergraduate Research Journal 8 (2005): 35-46.

Duus, Jens, Gotfredsen, Charlotte, Bock, Klaus. "Carbohydrate Structural Determination by NMR Spectroscopy: NMR Methods and Limitations." Chemical Reviews 100 (2000): 4589-4614.

Goodlett, V.W. "Use of In Situ Reactions for Characterization of Alcohols and Glycols by Nuclear Magnetic Resonance." Analytical Chemistry 37.3 (1965): 431-432.

Weikert, R.J., Bingham, S., Emanuel M.A., Fraser-Smith, E.B., Loughhead, D.G., Nelson, P.H., Poulton, A.L. "Synthesis and Anthelmintic Acivity of 3'-Benzoylurea Derivatives of 6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole." Journal of Medicinal Chemistry 34.5 (1991): 1630-1633.