Isotope Labelling Strategies to Assess Metabolic Pathways of Riboside Derivatives

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ABSTRACT

Experimentation involving isotopic ribose provides a means to assess the metabolic pathways of riboside derivatives. Such derivatives that pertain to this study consist of dihydronicotinamide ribose (NRH) and nicotinamide riboside (NR). From this, identification and purification of such compounds is necessary, which is achieved through thin layer chromatography (TLC) and nuclear magnetic resonance (NMR). After drying and viewing under UV at 254 nm, or charring, the progression of the reaction was observed. From NMR, proton spectra were produced, detailing factors such as spin multiplicity and proton quantification to identify samples. Samples of EtONaR and isotope D-ribose (99%), as compared to non-isotope ribose, were used in both protocols. Results indicated the success of each reaction and provided insight into the effect of polarity altering-environments on each substance. NMR data indicated identification of compounds that appropriately corresponds with predictions. From this research, new light can be shed upon the all-encompassing NAD+ enzyme, with associations to cancer therapy and neurodegenerative disorders. This study served as a first step in further research relating metabolic pathway discovery and its implications, to which compound identification must be completed first.

INTRODUCTION

The implications surrounding NAD+ regulation are all-encompassing, ranging from neurodegenerative disorders to mitochondrial damage. A key principle that has been narrowly researched is NR+ as a precursor to NAD+ synthesis. As an enzyme, NAD+ is particularly relevant in the aging process as aging associated disorders pose a demand for NAD+ synthesis that exceeds accessibility. Because of this, nicotinic acid and nicotinamide have been manufactured as Vitamin B3 supplements to restore NAD+ levels. Most recently, the phosphorylated form of NR+, NMN, has been manufactured as an oral supplement to induce NAD+ production (Makarov and Migaud, 2019). To the same effect, growing evidence has supported the notion that NAD+ regulates various biological processes, such as lifespan (Sauve, 2008).

Stemming from implications of NAD+ in geriatrics, pharmacological agents have been studied to maximize the beneficial capabilities that NAD+ possesses. Specifically, NRH has been found as a potent NAD+ enhancer in vivo and in vitro; in which NRH provides 2-10 fold increase of NAD+ concentration after 1 hour of administration (Yang et al, 2019). Similarly, NMN serves as an NAD+ precursor as well, impacts age related impairment of endothelial angiogenic processes (Kiss, 2019).

In respect to this study, assays were evaluated through thin layer chromatography. Thin layer chromatography (TLC) and high performance liquid chromatography (HTLC) has long been used as a reliable, simple, and cost efficient means to identify compounds through purification, while observing the progression of a reaction in real time. Within a TLC system, a solvent system can be optimized to best suit a reaction given the polarity of the compounds and separation needed, all while sacrificing only nanograms of the solution. Additionally, once properly dried, TLC plates may be viewed under UV light to observe (Santiago and Strobel, 2013). Similarly, HPLC is a useful technique to implemented in stationary or reverse phase polarity if a pure chemical standard is available (Aniszewski, 2015).

Another means to analyze samples that was implemented within this study is NMR spectroscopy. NMR provides the basis for the parameters of chemical shift, spin-spin splitting, linewidths, nuclear Overhauser effect, and chemical exchange. The generation of proton NMR

spectrum is achieved through inducing a magnetic field that exerts a resonance conditions on all protons within the sample (James, 1998). Techniques of TLC and NMR, taken together are able to efficiently identify samples, alongside any chemical parameters needed to be evaluated.

In this particular study, both aforementioned methods were utilized to identify and purify samples of NRH, NR, EtONaR, and isotopic ribose. ^{17}C isotopes used within riboside derivative, such as isotopic ribose, provide insight into metabolic pathways of such compounds, which provides a basis to which further research on the implications surrounding NAD+, a riboside derivative, can be found.

METHODS

Column priming:

Using a 250 mL column with a top spherical socket joint, 250 mL of CH_3OH was poured through and completely filtered out. Then, 100 mL of 50% CH_3OH and 50% H_2O was filtered through. Following this, 100 mL of 25% CH_3OH and H_2O was poured into the column and allowed to filter through. Finally, 100 mL H_2O was poured through. This was to ensure that the column is free of contaminants.

Methanolic sulfuric acid preparation:

5 mL of 18.4 M H_2SO_4 in 200 mL of methanol was used as the acid bath to which TLC plates were coated before being charred.

NR and NRH purification:

1 g of NRH was dissolved completely in 2 mL H_2O and added to the column, followed by the slow addition of 10 mL H_2O. The solution was allowed to flow through into collection tubes. After 13 mL of the solution were collected into the tube, a new tube was introduced until the column was filtered to completion. Collections tubes were labelled numerically.

TLC plates were run on every odd numbered sample using 100% C_3H_6O in the chromatography column (from #3-#25 as the first sample was significantly diluted and did not visibly indicate a high NRH concentration). TLC plates were then charred by dipping in the

methanolic sulfuric acid bath and drying, then placed on a hot plate at 200ºC, waiting for black spots to emerge.

Ethyl 1-(2R, 3R, 4S, 5R) - 3, 4 - dihydroxy - 5 - (tetrahydrofuran-2-yl) - 1H - pyrrole -3-carboxylate (EtONaR):

Various ratios of methanol to water content in the product were tested before the reaction was successful. First, 50 mg EtONaR was dissolved in 1 mL CH_3OH in a scintillation vial. In another vial, 1pellet of NaOH, weighing 94.7 mg, was dissolved in 0.6 µL H_2O. In a vial designated for the final reaction, 10 µL of the NaOH mixture was dissolved in 500 µL of the EtONaR mixture.

In order to simultaneously experiment with the polarity of the substance in regard to its spotting on a TLC plate, another reaction of the same compound was prepared. The same starting material (50 mg EtONaR in 1 mL CH_3OH), was used however 1 pellet of NaOH was instead dissolved in a 1:9 ratio of CH_3OH to H_2O. SCR TLC plates were run: the "S" (starting material) was spotted with the original EtONaR compound, the "R" (product) was spotted with the reaction of 500 µL EtONaR compound + 10 µL of the NaOH mixture, the "C" (combined) was co-spotted with both the starting material and product.

Similarly, the same EtONaR reaction was ran with 20% final water content in overall solution. The starting material was 50 mg EtONaR in 200 µL H_2O and 800 µL CH_3OH. The same ratio was maintained while dissolving 1 pellet of NaOH.

The previous reaction was repeated, however the final product consisted of 20 µL of NaOH in water. TLC plates were run in a chromatography column of 3:1 C_3H_6O to CH_2Cl_2.

Isotopic ribose synthesis and neutralization process:

In a 50 mL round bottom flask, 100 mg isotopic ribose was dissolved completely into 2 mL CH_3OH using a magnetic stirrer. To this, 375 µL methanolic sulfuric acid was added (0.6868 g H_2SO_4 dissolved in 10.5113 g CH_3OH). Once dissolved completely, this reaction was kept on ice throughout the day and in -20ºC conditions overnight.

Every hour, SCR TLC plates were run. The starting material, "S", was 50 mg 99% D-ribose in 1 mL CH_3OH. This solution ensured a similar solute:solvent ratio to the product

("R"), which was 100 mg isotope ribose in 2 mL CH_3OH. The "C" was a co-spot of both the S and R. The chromatography column conditions for this experiment was 3:1 C_3H_6O to CH_2Cl_2.

Once the reaction went to completion, the final product was neutralized. To do so, a strip of glass wool was inserted into a glass pipette, added with excess Na_2CO_3. Then, the final compound was slowly added to the plugged pipette and allowed to flow through into a scintillation vial. CH_3OH is used to wash the round bottom flask with the compound to remove any excess solution. pH was taken of the compound before and after the neutralization process, and SCR TLC plates were run, following the same conditions and procedure as outlined for TLC of the same compound before neutralization-- with the "R" as now the neutralized product.

Determination of chromatography chamber conditions:

A 7.6 cm by 2.5 cm chromatography TLC developing chamber has reverse phase polarity. Therefore, more polar substances move higher on the plate compared to less polar substances. Because of this, several TLC tests were prepared with the analyte in varying chamber conditions. For example, ribose was tested in 3 conditions: 100% CH_2Cl_2, 1:1 C_3H_6O to CH_2Cl_2, and 3:1 CH_2Cl_2 to C_3H_6O. Additionally, when determining the conditions for the EtONaR reaction, variations of C_3H_6O and CH_3OHwere tested, including 3:1, 1:1, 1:3, 100% C_3H_6O, and 100% CH_3OH.

The polarity of a compound indicated its position on a reverse phase TLC plate; depending on the area in which spots need to emerge, a proper ratio between solutions was prepared into the chromatography chamber.

Data analysis (NMR procedure):

Before running NMR, all compounds were evaporated in a rotary evaporator and placed in NMR tubes with 750 µL deuterium oxide. NMR was run on both starting material and the final product for comparison. Once a spectra was produced, peaks were integrated and any impurities were zeroed.

RESULTS

TLC plates (NRH, EtONaR, Ribose):

TLC plates were analyzed by evaluating the progression and movement of spots after 7 hours of the reaction. Figure 1 demonstrates 12 fractions of NRH, in comparison to NR, that were collected. Beside it are the corresponding UV images at 254 nm for each plate. Higher numbered fractions, categorized by a greater NRH/NR concentration rose higher on each plate.

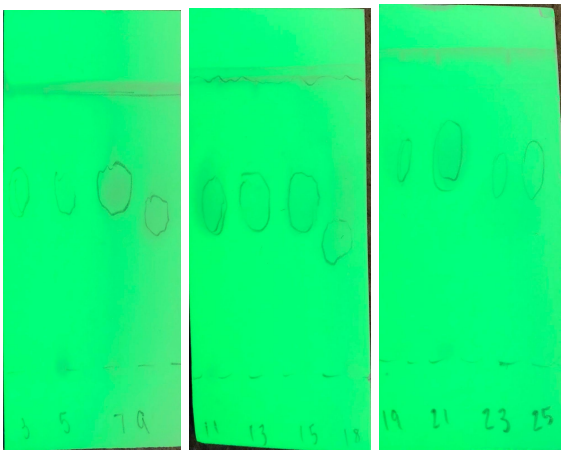

Figure 1: Odd fractions collected from sample #3-#25 of NRH and NR, viewed under UV light at 235 nm.

In Figure 2, TLC plates taken every hour from the EtONaR reaction are shown. Throughout the day, the R spot moved higher on the plate while S spot stays constant. This is due to the fact that the starting material was being converted to the product. Figure 3 are TLC plates from the same reaction with slightly altered conditions of water content. There is no visible difference between the TLC plates with NaOH in 100% H_2O and NaOH dissolved in 1:9 ratio of CH_3OH to H_2O. This suggests that the added CH_3OH did not result in a polarity change

significant enough to alter the position of the spots on the plate. The adjustment of 20% final water content is seen in Figure 2. The adjustment of the additional NaOH in the final reaction is seen in Figure 3.

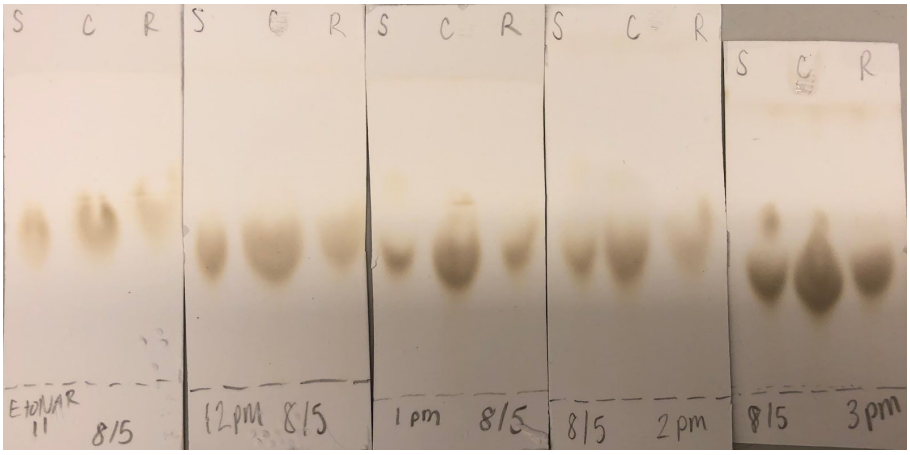

Figure 2: EtoNaR, trial 1: 20% water content in solvent

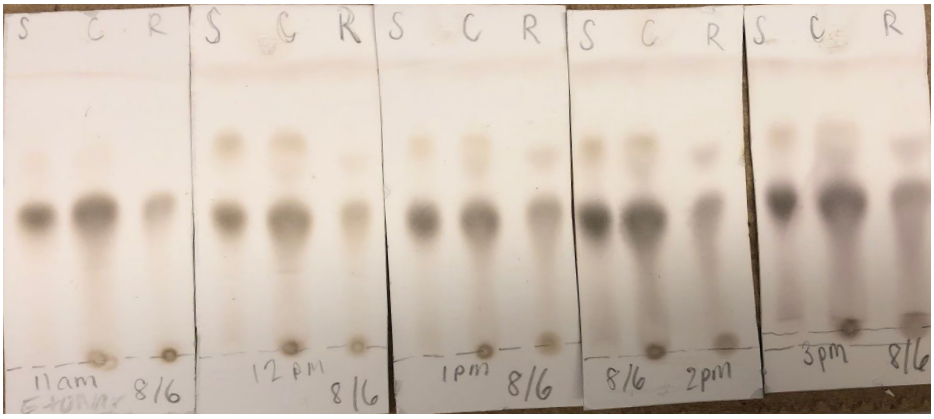

Figure 3: EtONaR, trial 2: NaOH in 100% H_2O

Figure 4 are TLC plates of D-ribose against isotopic ribose taken hourly. This reaction visibly went to completion and was successful as the R spot progressively rose while S spot stayed constant, suggesting that starting material was converted to product. Within the same experiment, after neutralization a TLC plate was ran again, seen in Figure 5.

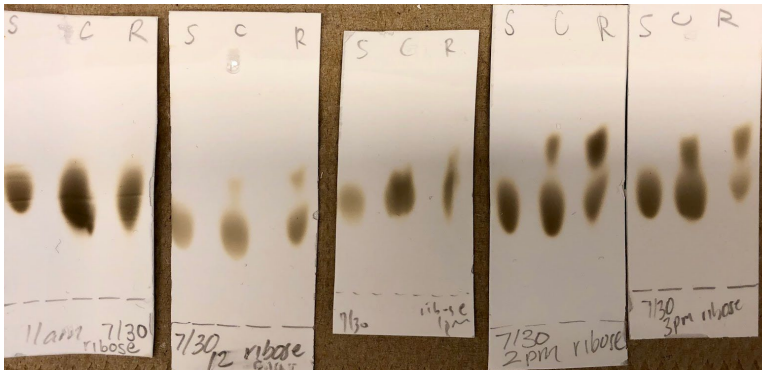

Figure 4: Progression of isotope ribose throughout 4 hours

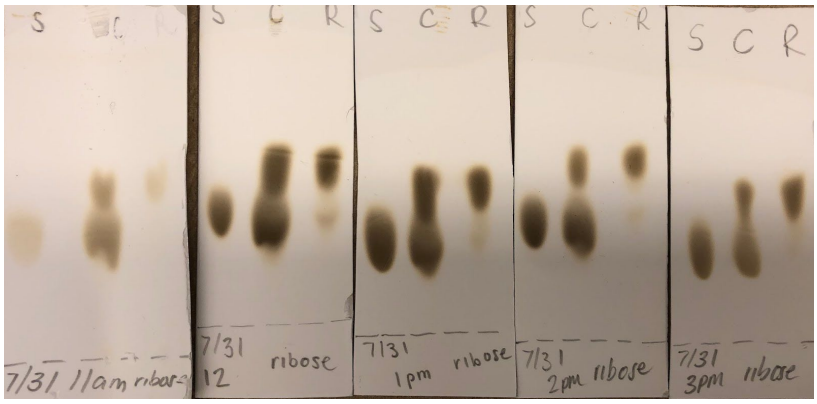

Figure 5: Progression of isotope ribose after neutralization

NMR spectra:

Proton emission spectra were run and spin multiplicity and coupling constants were evaluated.A side by side comparison of pure NRH and the collected fraction #10 is provided in Figure 6. The peaks within the circled area represent impurities. In Figure 7, the proton emission spectra for isotopic ribose is shown. For comparison, non-isotope ribose emission spectra is adjacent.

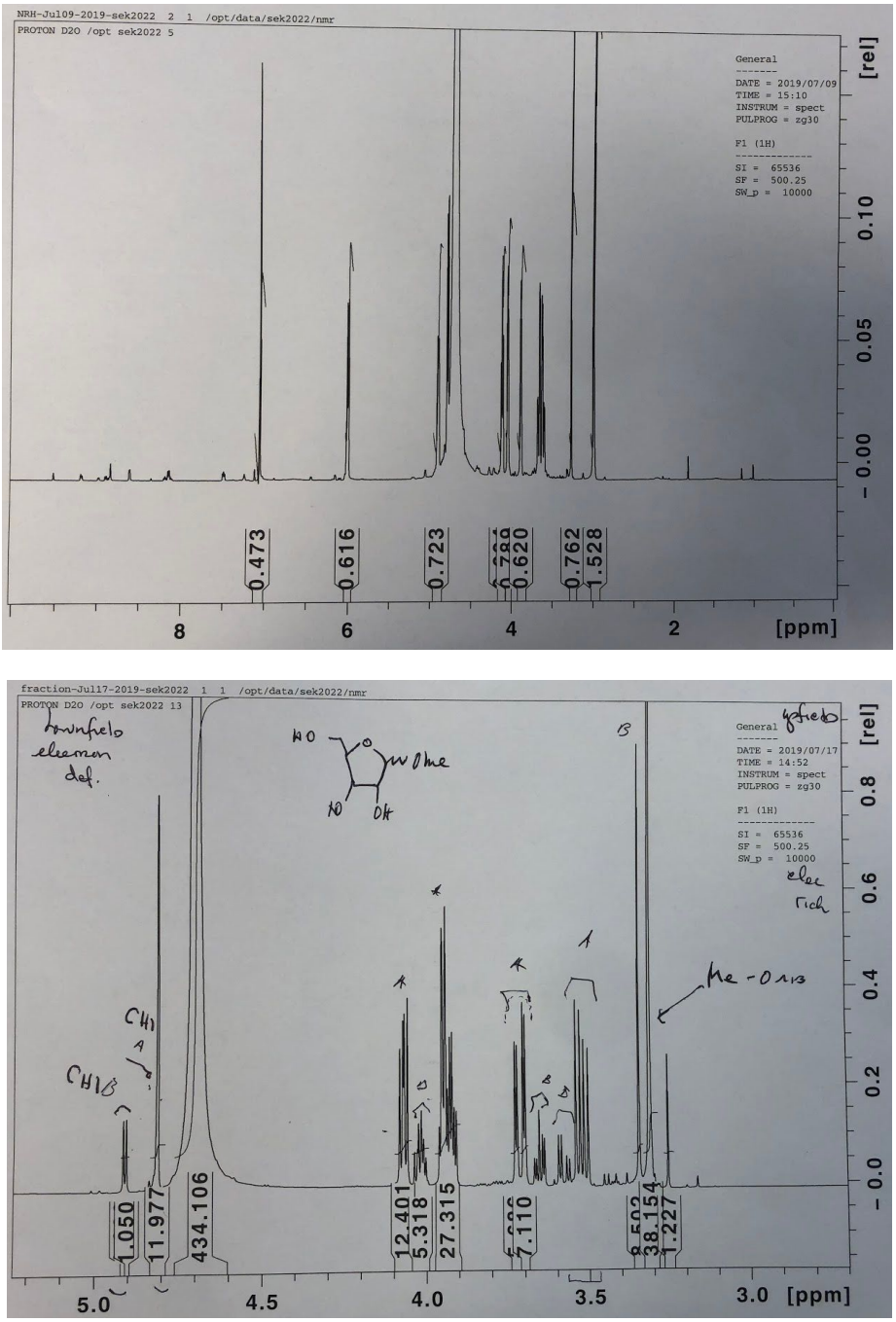

Figure 6: NRH and collected fraction proton emission spectra with integrated peaks.

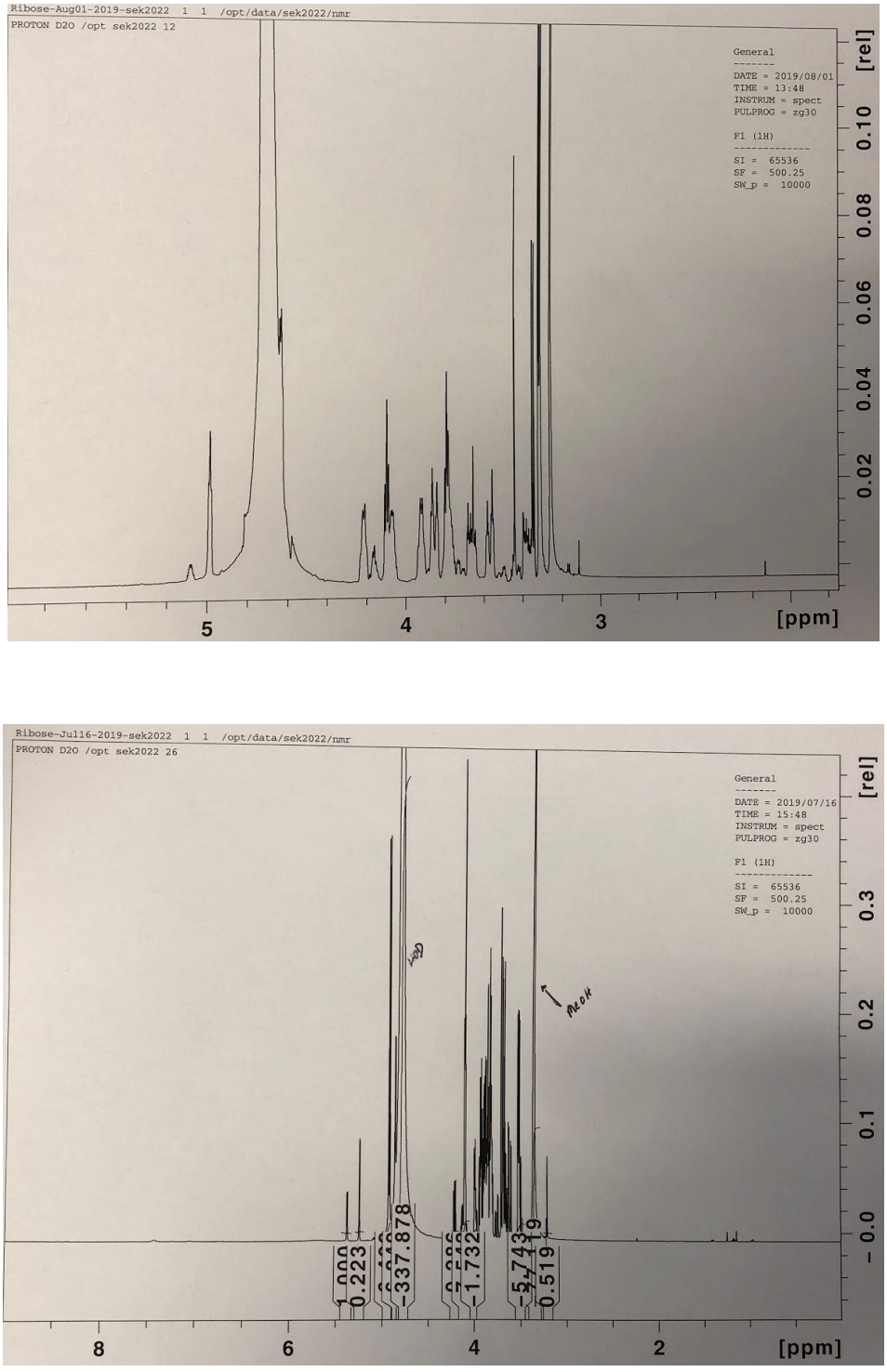

Figure 7: D-Ribose (99%) and isotope ribose, peaks integrated.

DISCUSSION AND CONCLUSIONS

At the start of this study, the aim was to purify and identify samples of NRH, NR, EtONar, and isotope ribose. Previous literature has suggested the importance of such research in order to later understand implications of the derivatives of such compounds, such as NAD+. The results from this study are intended to serve as a stepping stone to which further research can be founded.

In regard to sources of error within experimentation, some discrepancy within data may be attributed to temperature differences, as some compounds were not kept on ice during the day, yet kept in -20ºC overnight.

Both TLC plates and NMR data indicate successful product preparation. However, oftentimes the peaks of the spectra were so close that integration of singular peaks was not possible. Additionally, while evaporating every product to be placed in an NMR tube, it is possible that residual solvent was incorporated as well. To counteract this, the solvent peak was used as a reference point.

Recent research has found that NAD+ most notably has implications in cancer therapy, as NAD+ signaling impacts DNA repair, cell proliferation, and oxidative stress. Regulating metabolic pathways within NAD+ can provide insight into tumor development and progression (Zhu, 2019). Additionally, the relationship between NAD+ regulation and lifespan has been largely studied, particularly in mice. One study has suggested that a precursor, NAM (nicotinamide) improves healthspan, though not lifespan. Healthspan was categorized by reduced hepatic steatosis and improvement in glucose homeostasis (Mitchell et al, 2018). Another study has supplemented these findings by suggesting that metabolism derivatives of NAD+ affect both the random patterns of aging and mechanistic patterns as well (Xu and Sauve, 2010). Because of this, moving forward, NAD+ can be researched through its properties as a non-redox electrophile and its implications in the aging process.

The level of complexity presented by this project allows for more intrinsic and specific research to be compounded upon. In terms of the procedure efficacy, it would have been beneficial to prepare experiments chronologically, as opposed to starting one trial of one experiment then moving on to another focus before completing trials of the first experiment. The

differing dimensions in the TLC plate may play a minute role in accurately interpreting the plates. This would have resulted in a more cohesive experimentation process.

In all, this experiment marks an investigation into riboside derivatives, NRH and NR, and shed insight into polarity altering conditions.

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