M. Tuberculosis IGP Synthase: Preparation, Purification, and Characterization of the Substrate CdRP.

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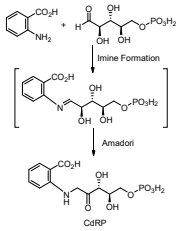
Introduction

Indole-3-glycerol Phosphate Synthase (IGPS) is required for the survival of mycobacterium tuberculosis, which makes this enzyme a potential target for anti-tuberculosis treatment. IGPS catalyzes the ring closure of 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP) to form indole-3-glycerol phosphate (IGP) during tryptophan biosynthesis. Analysis of the crystal structure of IGPS from Mycobacterium tuberculosis (MtIGPS) suggested nine amino acid residues that could be important for drug binding. Experiments designed to examine the role of these amino acids require CdRP substrate of high purity.



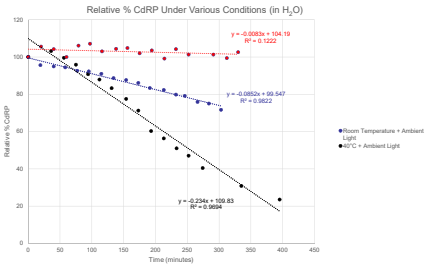
Materials and Methods

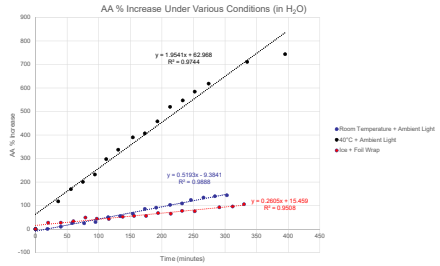
The preparation of the substrate CdRP[1] started by weighing out 274 mg (2 mmol) of recrystallized anthranilic acid and placing it in a glass tube. 2 mL of ethanol (EtOH) was then added, dissolving the anthranilic acid. 310 mg (1 mmol) of ribose-5-phoshate (R5P) from Sigma-Aldrich was also weighed out and placed in another glass tube with 1 mL distilled water, allowing the ribose-5-phosphate to dissolve. 2 mL of EtOH was pipetted into a small glass vial, along with a small stir bar, to be placed on a magnetic stirrer. The ribose-5-phosphate solution was then slowly added into the vial containing EtOH, followed by the anthranilic acid solution. The vial was then covered with foil and left to stir in a dark room for 4 hours. After stirring, an oily bottom layer was formed.

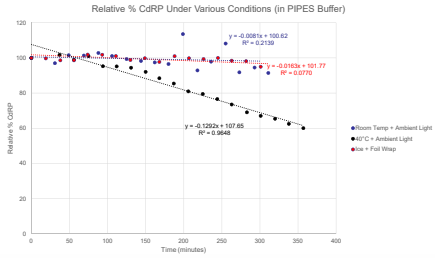
To start the purification, the vial was then placed in the fridge to allow the oil to firm up. After refrigeration, the top aqueous layer was carefully pipetted out, leaving just the oil in the vial. The oil was then washed with ethyl acetate multiple times. After the final wash, ethanol was added, and the oil was triturated with a metal spatula, forming a fine, yellow powder. After trituration, the solution was placed in a 15 mL falcon tube and centrifuged for 7 minutes, ultimately forming a pellet of the fine yellow powder. The EtOH solution was carefully poured out, and fresh EtOH was added. The pellet was also disturbed to allow the EtOH to wash the fine powder. The solution was then centrifuged again, and this process was repeated twice more. After the final centrifuging, the aqueous solution was poured out, wrapped falcon tube, and placed inside a vacuum to allow the powder to fully dry.

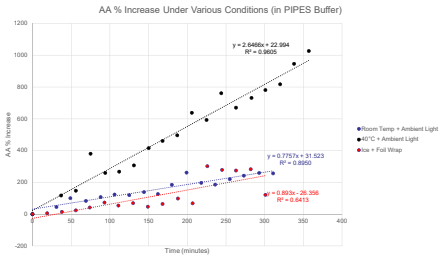
In order to check the purity of the synthesized CdRP, analysis of the sample via HPLC-MS and NMR (in D_2O) was carried out– both providing quality results that indicated the presence of the desired substrate as well as anthranilic acid and other unidentified minor impurities. Once the purity level of the sample was verified, it was necessary to observe CdRP degradation (and ultimately anthranilic acid formation) over time under various conditions. Such conditions included leaving the sample in room temperature and ambient light, in 40°C and ambient light, and in ice, wrapped in foil. 1.00 mg/mL samples of CdRP first in water, then in PIPES buffer were prepared for HPLC-MS analysis, and the results of the observed substrate degradation are shown in the figures above.

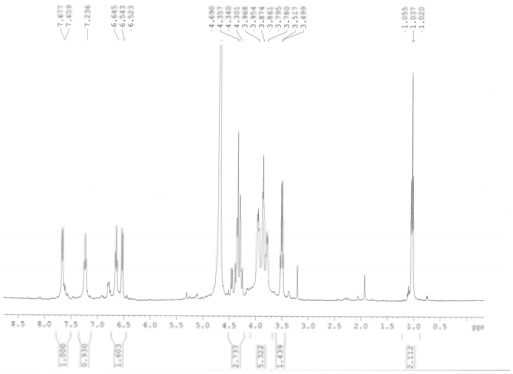
Results from HPLC-MS and NMR











Proposed DEAE Purification

In order to achieve higher purity within the synthesized CdRP samples, diethylaminoethyl (DEAE) purification is currently being tested. This method is anion-exchange chromatography, which will be used to purify the water-soluble compound based on differences in charge. With this, DEAE-sepharose is our resin of choice.

First, several solutions with increasing concentrations of ammonium bicarbonate buffer are prepared. The actual process is carried out in a cold room to prevent sample degradation. The resin is first equilibrated with the lowest concentration buffer until the flow-through reaches a pH of ~8. Then, the CdRP sample solution is placed in the chromatography column, flowing through with the lower concentration buffers. Over time, higher salt concentration buffer solutions are added into the column to bind competitively with the sample in order to elute it from the column. Finally, sample fractions are collected and analyzed via HPLC-MS.

Reference

1. Doy, C.H., *Chemical Synthesis of the Tryptophan Path Intermediate 1-(o-Carboxyphenylamino)-1-deoxy-D-ribulose 5-Phosphate,* Nature, 1965, pp 736-737.

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