Improving the Synthesis of an Unnatural Fluorescent Amino Acid

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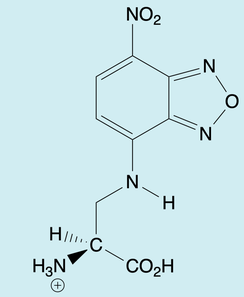
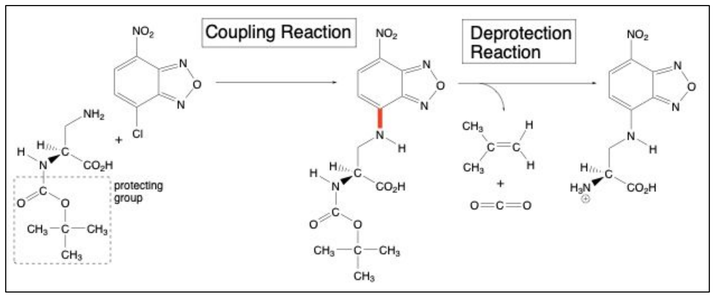


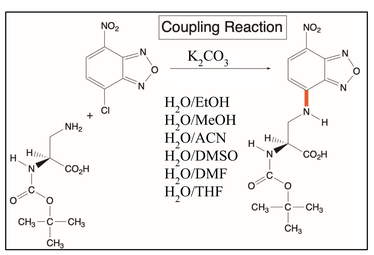
Abstract

The long-term goal of this project is to more efficiently synthesize an unnatural fluorescent amino acid, 3-[7-nitro-2,1,3-benzoxadiazol-4-yl]-L-alanine; diazole for short. This fluorescent amino acid can be incorporated into a transparent protein, converting it to a "glow-in-the-dark" protein. Two parallel pathways are being explored to synthesize this diazole amino acid: traditional organic synthesis and biocatalysis. ^{1}H-NMR and LC-MS are being used to analyze the organic route by monitoring the reaction kinetics of the key coupling reaction. The goal of these kinetics experiments is to identify optimal reaction conditions (solvent, temperature, etc.). For the biocatalytic route, the enzyme glutathione S-transferase from the cyanobacterium *T. elongatus* is being used to catalyze the key coupling reaction. Enzyme kinetics are monitored using LC-MS.



Background

Amino acids can be utilized in tagging to better observe living cells. There are 20 amino acids that occur in nature, none of which naturally glow in the dark. By creating an unnatural amino acid that fluoresces, the amino acid can be used for tagging, turning a once clear and colorless cell into a glowing cell. By improving the synthesis of the diazole amino acid, this unnatural fluorescent amino acid can be more readily used in protein analysis. The figure below illustrates the reaction conditions being studied to optimize the organic synthesis.



As an alternative synthetic route, the biocatalytic route makes use of biological catalysts known as enzymes. Enzymes serve as a means to improve the rate at which a reaction occurs. The figure below depicts the target biochemical reaction which is by Glutathione S-transferase (GST). GST is capable of coupling glutathione to NBD-Cl. Then, the product can undergo further synthesis to achieve the desired diazole amino acid.

Methods

Organic Synthesis

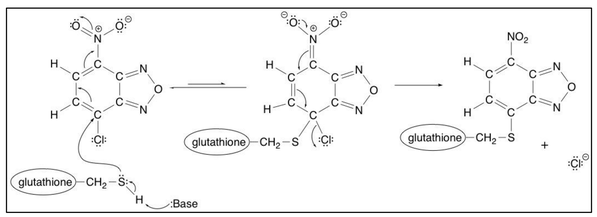
^{1}H NMR kinetics: NBD-Cl was reacted with Boc-Dap-OH at final concentrations of 0.060 M each in the deuterated mixed solvents listed in the table below. Reactions were monitored over 12 hours, taking ^{1}H-NMR spectra every 30 minutes.

LC-MS kinetics: Same reaction conditions as described above, except the solvent mixtures were undeuterated. A 5 µL sample was removed each hour, and diluted with 3 mL of the mixed solvent. The sample was stored at 4 ℃. The next day, reaction progress was monitored using LC-MS.

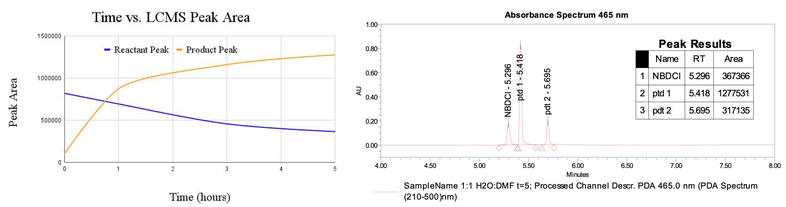
1:1 H_2O:EtOH	1:1 H_2O:MeOH	1:1 H_2O:ACN	1:1 H_2O:DMSO	1:1 H_2O:DMF	1:1 H_2O:THF

Biocatalysis

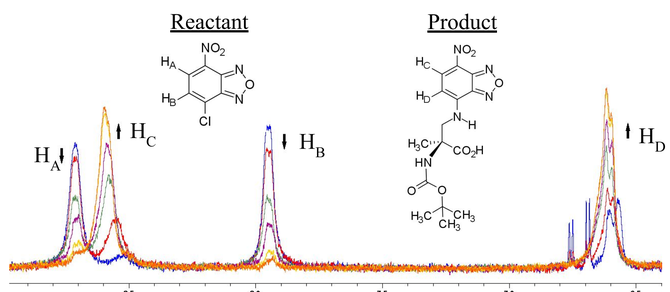
Glutathione S-transferase from the cyanobacterium *T. elongatus* was expressed in *E. coli*. The purified enzyme catalyzed the coupling reaction between NBD-Cl and glutathione.



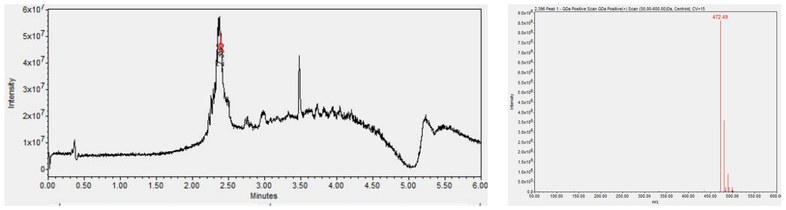
Results



LCMS Results from organic reaction in DMF solvent. The graph shows the change in peak areas of the reactant and product peaks overtime. The spectrum on the right shows the reaction after 5 hours.



An overlay of H-NMR results from the organic reaction in ethanol solvent. The blue spectrum represents time 0 minutes and the orange spectrum represents time 600 minutes.



LC-MS monitoring of the enzyme-catalyzed reaction. The figure on the left is the total ion current (positive mode) for the reaction mixture after 120 minutes. The figure on the right is the mass spectrum extracted at a retention time of 2.3 minutes.

Discussion

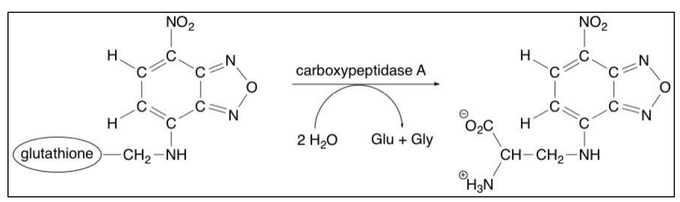
With data from LC-MS kinetics, we concluded that we are converting our reactant to a product, as the area of the product peak is increasing over time and the area of the reactant peak is decreasing overtime. We also were able to confirm a side product forming, which elutes at 5.7 minutes. Overall, LCMS looks promising as a form of monitoring the reaction over time.

As shown on the NMR spectrum, the peaks representing the protons on the reactant are showing a decrease in size from 0 minutes to 600 minutes. The peaks representing the protons on the product, therefore, show an increase in size. This confirms that the reactant is being converted into the desired product. An internal standard of TMS is being studied for more quantitative analysis of NMR data.

The LC-MS was used to confirm the identity of the coupling product formed during the enzyme catalyzed reaction. From this, we concluded that the product formed was the protonated form of the desired product.

Future Work

- Finish kinetic reactions with all solvents and with TMS standard.
- Confirm identities of products of organic reaction using LC-MS.
- Monitor enzyme catalyzed reaction in different solvents using LC-MS.
- Replace the glutathione thiol with an amine.
- Hydrolyze glutamic acid and glycine to yield the diazole product.



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