Tracking Enzyme Hydrolysis of an Amide Bond Using Highly Simplified 4-nitroanilide Colorimetric Substrate

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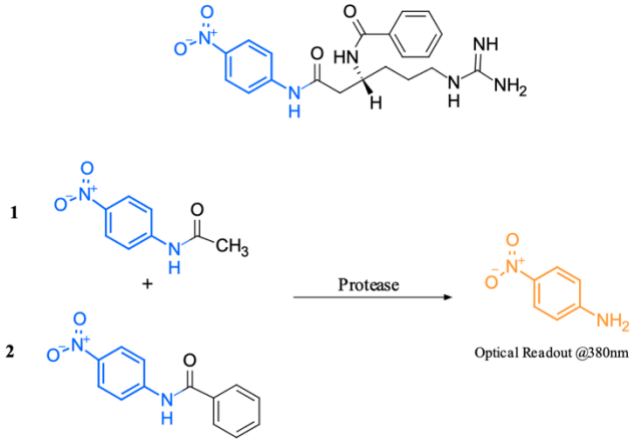
ABSTRACT: Proteases, enzymes that hydrolyze peptide bonds, have tremendous implications in the life sciences, ranging from the treatment of various diseases to enabling chemoselective bond cleavage in chemical synthesis in chemical synthesis. The kinetics of proteases can be monitored spectroscopically using nitroanilide substrates, many of which are structurally complex and often require long, multi-step syntheses. This study sought to determine the rate of hydrolysis by various serine proteases—nattokinase, trypsin, and pepsin—on two highly simplified 4-nitroanilide colorimetric substrates—4-nitroacetanilide and 4-nitrobenzanilide—via UV-visible (UV-vis) spectroscopy. Each substrate was chemically synthesized in one step via acylation of 4-nitroaniline. Solutions of both substrates were prepared, incubated with buffered enzyme solutions, and enzymatic cleavage of the amide bond was spectroscopically monitored on time-course. After experimentation, it was determined that nattokinase and trypsin were able to hydrolyze the amide bond of 4-nitroacetanilide at 25e-10 moles per hour and 4e-10 moles per hour respectively, while the results for pepsin were inconclusive as 4-nitroacetanilide is unstable in acidic conditions. None of the proteases were able to hydrolyze the amide bond in 4-nitrobenzanilide. Computational studies suggest that this is a result of differences in amide reactivity between the two substrates rather than differences in binding affinity to each protease.

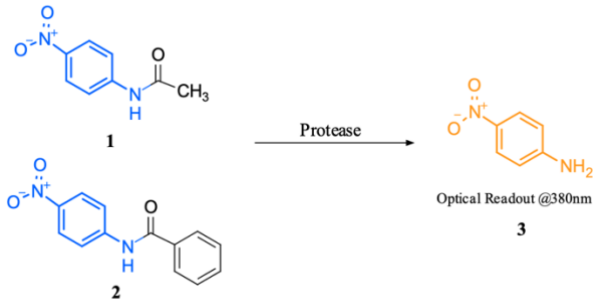
INTRODUCTION

Proteases are an important class of enzymes that hydrolyze peptide bonds; serine proteases have a catalytic triad consisting of a serine nucleophile, histidine base, and aspartate acid.1 Nattokinase is a biologically active serine protease currently being investigated for the treatment of cardiovascular diseases by hydrolyzing fibrin. Fibrin is a protein responsible for the formation of fibrous meshes found on atherosclerotic plates. These fibrous meshes are further calcified, becoming an advanced plaque that covers a necrotic core, which is vulnerable to cap rupture—forming blood clots and resulting in thrombosis.2 By hydrolyzing fibrin, nattokinase has been shown to delay thrombus formation with near-full inhibition at 75mg/kg 2. In this way, nattokinase is a natural blood thinner and can be used to treat cardiovascular disease. Pepsin is a serine protease that breaks down proteins into smaller peptides found in the stomach, where it helps digest the proteins in food and has applications in laryngeal diseases.3 Trypsin is also a serine protease produced in the pancreas responsible for the metabolism of peptide bonds in the small intestine.4 Given the biological significance of trypsin, it is associated in the treatment of many diseases, such as immunoreactive serum trypsin in pancreatic diseases 3. The ability to quantify the rate of hydrolysis by these enzymes is essential to understanding their catalytic ability.

4-nitroanilide substrates containing peptide chains such as L-Alanine 4-nitroanilide hydrochloride and N-α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (shown in Figure 1A), are commercially available from Sigma-Aldrich and other suppliers, where the peptide chain functions as the enzymatic recognition site. Enzymatic cleavage of the 4-nitroaniline amide bond releases 4-nitroaniline, which can be tracked spectroscopically at 380 nanometers (nm).5 However, many of these compounds require multi-step syntheses, which can prove to be time-consuming and uneconomical to produce.6, 7 By simplifying the synthesis of these colorimetric substrates, the function of the compound still remains the same, but both time and resources are conserved in their preparation. Herein, we report the hydrolysis kinetics of three serine proteases using two highly simplified 4-nitroanilide colorimetric substrates. A general experimental overview is shown in Figure 1B. A picture of the experimental cuvettes is shown in Figure 2. Additionally, subsequent docking experiments revealed that difference in enzymatic hydrolysis of the amide bond in our substrates is most likely a function of differences in native reactivity rather than differences in binding to the enzymatic active site.8



1A



1B

Figure 1A (top): Sigma-Aldrich sells N-α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (substrate for trypsin) at $911/g. It is structurally complicated and requires a multi-step synthesis. The 4-nitroanilide core scaffold is highlighted in blue.

Figure 1B (bottom): A general experimental overview. Two simple 4-nitroanilide substrates—4-nitroacetanilide 1 and 4-nitrobenzanilide 2—that are accessible via one-step synthesis from 4-nitroaniline ($4/g from Sigma-Aldrich). The 4-nitroanilide core scaffold is highlighted in blue to emphasize the structural similarities between these two compounds and Figure 1A. Although these compounds are much simpler, they have the same function as Figure 1A, where the cleavage of the amide bond releases 4-nitroaniline 3, which can be tracked spectroscopically at 380 nm.

Figure 2: Picture of cuvettes, left to right: N0 — Blank, N1 — 4-nitroacetanilide and Nattokinase, N2 — 4-nitroacetanilide control, N3 — Nattokinase control.

MATERIALS AND METHODS

Chemical Synthesis

4-nitroacetanilide was synthesized via acetylation of 4-nitroaniline. 4-nitroaniline (1g, 1 eq., 7.2 mmol) was added to a vacuum-dried round-bottom flask equipped with a stir bar, along with 15 eq. acetic anhydride and 10 mL acetonitrile. The round-bottom flask was septum-sealed and stirred for 24 hours and monitored to completion via thin-layer chromatography (TLC). Unreacted acetic anhydride was quenched with methanol and the crude material was concentrated in a rotary evaporator. The product was extracted 5 times with ethyl acetate over saturated sodium carbonate. The resulting organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo to yield yellow crystals of 4-nitroacetanilide in 89% yield. The reaction schematic is shown in Figure 3.

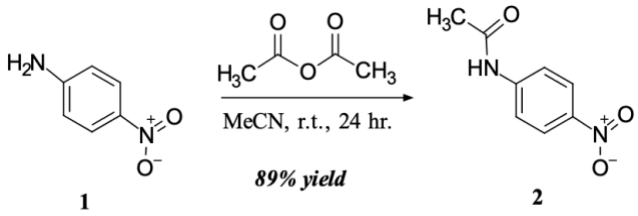


Figure 3: 4-nitroaniline 1 was acetylated to yield 4-nitroacetanilide 2 in 89% yield.

4-nitrobenzanilide was synthesized via reaction between benzoyl chloride and 4-nitroaniline. 4-nitroaniline (1.0g, 1 eq., 7.2 mmol) was dissolved in methylene chloride and added to a vacuum-dried round-bottom flask equipped with a stir bar, along with 1 eq. triethylamine. The round-bottom flask was septum-sealed and stirred until 4-nitroaniline was fully dissolved. Then, 1.2 eq. benzoyl chloride was injected through the septum and into the reaction mixture. A precipitant was observed after 3 minutes and the reaction was monitored to completion via TLC. Unreacted benzoyl chloride was quenched with water and the crude product was concentrated in vacuo. The product was extracted 5 times in ethyl acetate over saturated sodium carbonate. The resulting organic layers were dried over anhydrous magnesium sulfate and concentrated in vacuo to yield yellow crystals of 4-nitrobenzanilide in 99% yield. The reaction schematic is shown in Figure 4.

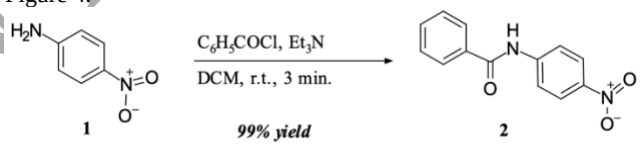


Figure 4: 4-nitrobenzanilide 2 was synthesized via reaction between 4-nitroaniline 1 and benzoyl chloride in the presence of triethylamine.

Both compounds were characterized by UV-visible spectroscopy (BioRad SmartSpec 3000 spectrophotometer) and FTIR (Thermo Scientific iS5 Nicolet FTIR spectrometer, iD5 ATR assembly). Full characterization of 4-nitroacetanilide and 4-nitrobenzanilide can be found in the online supplemental information Figure 1.

UV-Visible Spectroscopy

1.0 mM solutions of both 4-nitroacetanilide and 4-nitrobenzanilide were prepared in 95% deionized water and 5% DMSO. Enzyme buffers were prepared with 15 mM TRIS and 100 mM sodium chloride in deionized water. For each enzyme, the solution was adjusted to 0.5 mg per mL of buffer and adjusted to the optimal pH. No further pH adjustments were needed for nattokinase or trypsin as the buffer was already at a pH of 8; however, for pepsin, the buffer was adjusted to a pH of 1.5 with 1 M HCl since pepsin functions best in acidic environments.

The maximum absorbances for 4-nitroaniline, 4-nitroacetanilide, and 4-nitrobenzanilide were determined to be at 380, 320, and 325 nm respectively. Solutions at various concentrations—0.1 mM, 0.2 mM, 0.5 mM, and 1 mM—were prepared to create Beer's Law graphs for 4-nitroaniline at 320, 325, and 380 nanometers, 4-

nitroacetanilide at 320 and 380 nanometers, and 4-nitrobenzanilide at 325 and 380 nanometers. Beer's Laws graphs were used to derive extinction coefficients, which were necessary to calculate the rate of enzymatic hydrolysis. Full UV-vis spectra and extinction coefficients for each compound at 320, 325, and 380 nm were collected and can be found in the online supplemental information Figures 2, 3, 4, and 5.

Enzyme Kinetics

A BioRad SmartSpec 3000 spectrophotometer was used to collect all UV-Vis data. All samples were incubated at 40°C unless otherwise specified.

After the substrate and enzyme solutions were prepared, one mL of the substrate solution, and one mL of the buffered enzyme solution were added to a cuvette. Separate substrate and enzyme solutions were also prepared as controls to account for enzyme and substrate degradation over time. Cuvettes were placed into the spectrophotometer and absorbances were collected at 320, 325, and 380 nanometers. Spectra was collected before incubation and after various lengths of incubation. A full UV-vis spectrum of nattokinase incubated with 4-nitroacetanilide at various time points is shown in Figure 5.

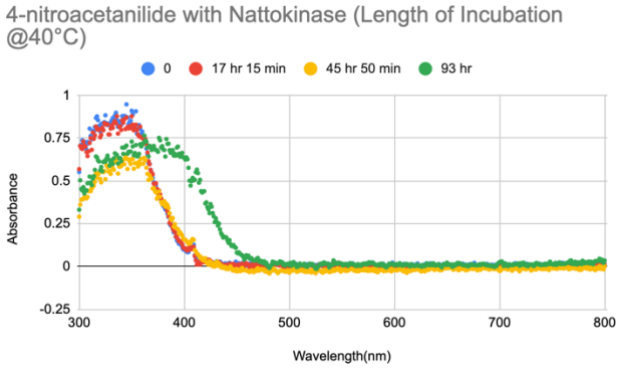


Figure 5: After 93 hours of incubation with nattokinase, the UV-vis spectrum of 4-nitroacetanilide begins to look like the UV-vis spectrum of 4-nitroaniline, showing nattokinase was able to successfully hydrolyze the amide bond. To ensure that the change in absorbance was enzymatic hydrolysis, separate substrate and enzyme solutions were prepared as controls to account for degradation. We found that there was no substrate degradation and only slight enzyme degradation, which was subtracted from the final spectrum shown above.

In-Silico Approaches

Molecular docking was done in order to identify potential differences in binding affinity—giving predictive binding affinities of various substrates with respective enzymes in silico. The binding pocket interactions between 4-nitroacetanilide and trypsin are shown in Figure 6. Table 1 gives the highest binding affinities of each docking experiment. Other binding pocket interactions can be found in the online supplemental information Figures 6, 7, 8, 9, and 10.

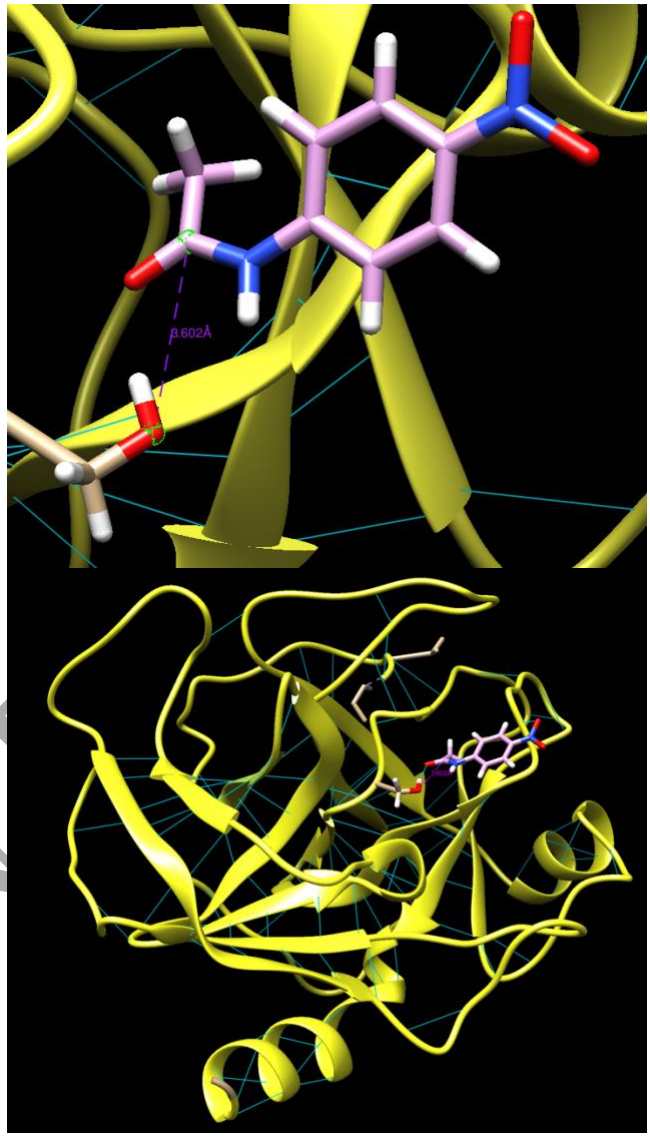


Figure 6A (top): 4-nitroacetanilide (purple) bound to trypsin (yellow) [PDB code: 4J2Y], hydrogen bond interactions shown in blue and distance between oxygen of serine 196 (SER 196) and carbonyl carbon of 4-nitroacetanilide shown in dark purple.

Figure 6B (bottom): Close-up of SER 196 nucleophile and 4-nitroacetanilide.

Table 1: Binding affinities and distances of various docking experiments.

Protease	Substrate	Lowest ΔG — Best Binding Affinity (Corresponding Distance)	Closest Distance — Oxygen of Serine Nucleophile to Carbonyl Carbon of Substrate

			(Corresponding ΔG)
Nattokinase	4-nitroacetanilide	-6.53 kcal/mol (12.32 Å)	3.353 Å (-5.39 kcal/mol)
Nattokinase	4-nitrobenzanilide	-6.91 kcal/mol (11.26 Å)	5.539 Å (-5.99 kcal/mol)
Pepsin	4-nitroacetanilide	-6.48 kcal/mol (8.47 Å)	3.484 Å (-5.82 kcal/mol)
Pepsin	4-nitrobenzanilide	-7.02 kcal/mol (13.95 Å)	3.542 Å (-5.60 kcal/mol)
Trypsin	4-nitroacetanilide	-6.555 kcal/mol (3.602 Å)	3.216 Å (-5.91 kcal/mol)
Trypsin	4-nitrobenzanilide	-7.01 kcal/mol (3.22 Å)	3.017 Å (-6.65 kcal/mol)

RESULTS AND SIGNIFICANCE

All UV-visible data was analyzed using the extinction coefficients derived from the Beer's Law graphs of 4-nitroaniline, 4-nitroacetanilide, and 4-nitrobenzanilide. These extinction coefficients were used to determine the concentrations of substrate vs. 4-nitroaniline over time. Nattokinase and trypsin demonstrated successful proteolysis of 4-nitroacetanilide at 25e-10 moles per hour and 4e-10 moles per hour respectively, while the results for pepsin with 4-nitroacetanilide were inconclusive due to the instability of 4-nitroacetanilide in acidic conditions, shown in Figure 6. None of the proteases were able to hydrolyze the amide bond of 4-nitrobenzanilide, most likely due to the increase in energy that is required to break the conjugation of the benzoate. An arrow-pushing mechanism is shown in Figure 7. After calculating the concentrations of substrate and 4-nitroaniline at each time point, the data was graphed with trend lines and shown in Figure 8. The rates of hydrolysis by these three serine proteases on 4-nitroacetanilide are shown in Figure 9.

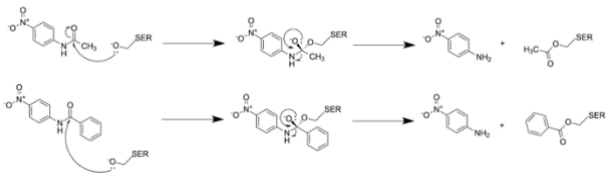
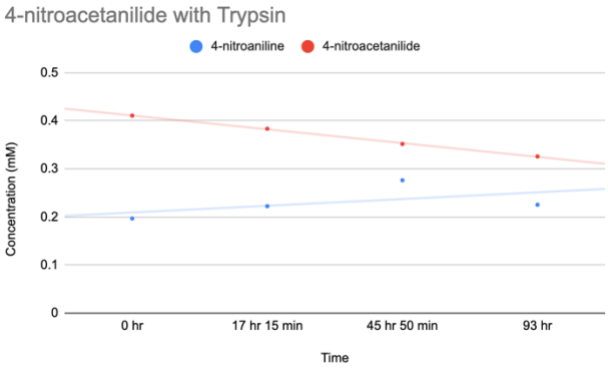
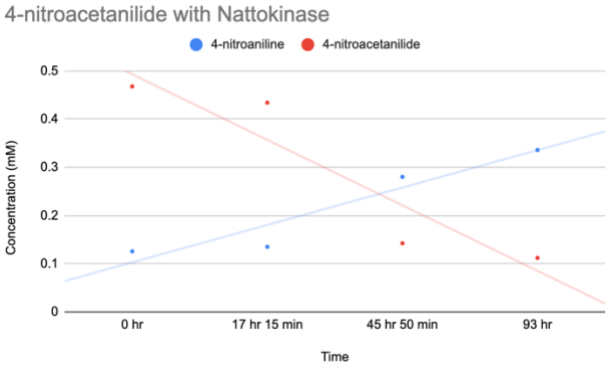
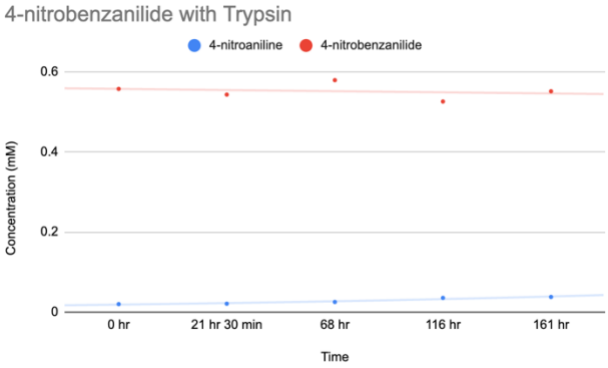
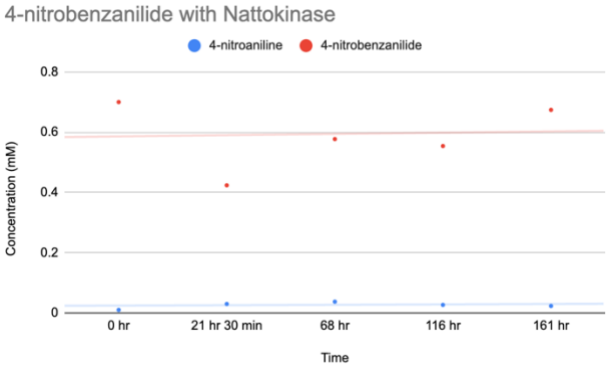


Figure 7: An arrow-pushing mechanism showing the reactivity between the serine nucleophile and our two 4-nitroanilide substrates.









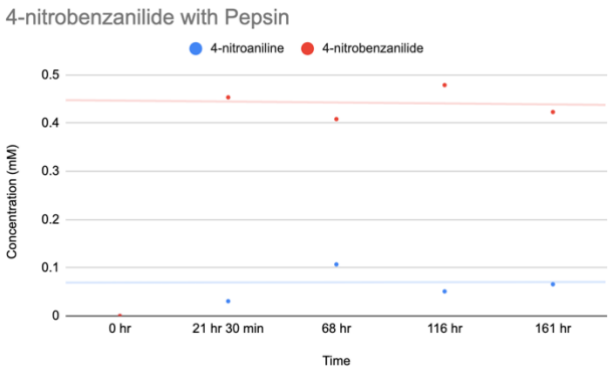


Figure 8A (top left): When 4-nitroacetanilide is incubated with nattokinase, the concentration of 4-nitroaniline increases as the concentration of 4-nitroacetanilide decreases over time. The rate of hydrolysis by nattokinase was determined to be 25e-10 moles per hour.

Figure 8B (top right): When 4-nitroacetanilide is incubated with trypsin, the concentration of 4-nitroaniline increases as the concentration of 4-nitroacetanilide decreases over time. The rate of hydrolysis by trypsin was determined to be 4e-10 moles per hour, 6.25 times slower than nattokinase.

Note: The rate of hydrolysis of 4-nitroacetanilide by pepsin was inconclusive because 4-nitroacetanilide is unstable in acidic conditions.

Figure 8C (middle left): When 4-nitrobenzanilide is incubated with nattokinase, the concentrations of 4-nitroaniline and 4-nitrobenzanilide remain the same.

Figure 8D (middle right): When 4-nitrobenzanilide is incubated with trypsin, the concentrations of 4-nitroaniline and 4-nitrobenzanilide remain the same.

Figure 8E (bottom left): When 4-nitrobenzanilide is incubated with pepsin, the concentrations of 4-nitroaniline and 4-nitrobenzanilide remain the same.

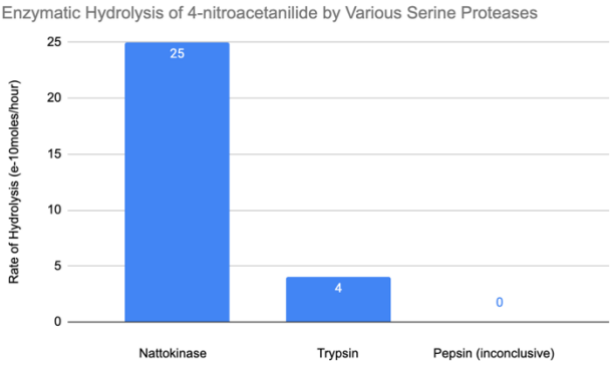


Figure 9: The rates of hydrolysis by various serine proteases on 4-nitroacetanilide.

CONCLUSION

In conclusion, nattokinase demonstrated the highest rate of hydrolysis on 4-nitroacetanilide. Trypsin was also able to successfully hydrolyze the amide bond in 4-nitroacetanilide, but at a slower rate compared to nattokinase. Results for pepsin are inconclusive due to the instability of 4-nitroacetanilide in acidic conditions. However, none of the enzymes were able to hydrolyze the amide bond in 4-nitrobenzanilide because more energy is required to break the conjugation of the benzoate. Based on the results of our enzyme hydrolysis data, we plan on extending our work to quantifying the effectiveness of protease inhibitors. We also plan to design and synthesize other 4-nitroanilide colorimetric substrates to determine the hydrolysis kinetics and substrate specificities of various enzymes, in an effort to better understand their biological mechanisms. Lastly, this study demonstrated the feasibility of using biological catalysts to deacylate anilines, and in the future, this could be employed for the chemoselective deacylation of amide containing compounds.

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