

Developing Proteomics Techniques for Glycosylated Proteins

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This is to certify that I have examined this copy of an honors thesis by

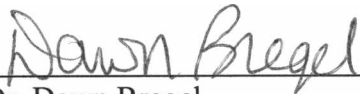
Adrienne Udelhoven

And have found that it is complete and satisfactory in all respects, and that any and all revisions required by the final examining committee have been made.


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Abstract

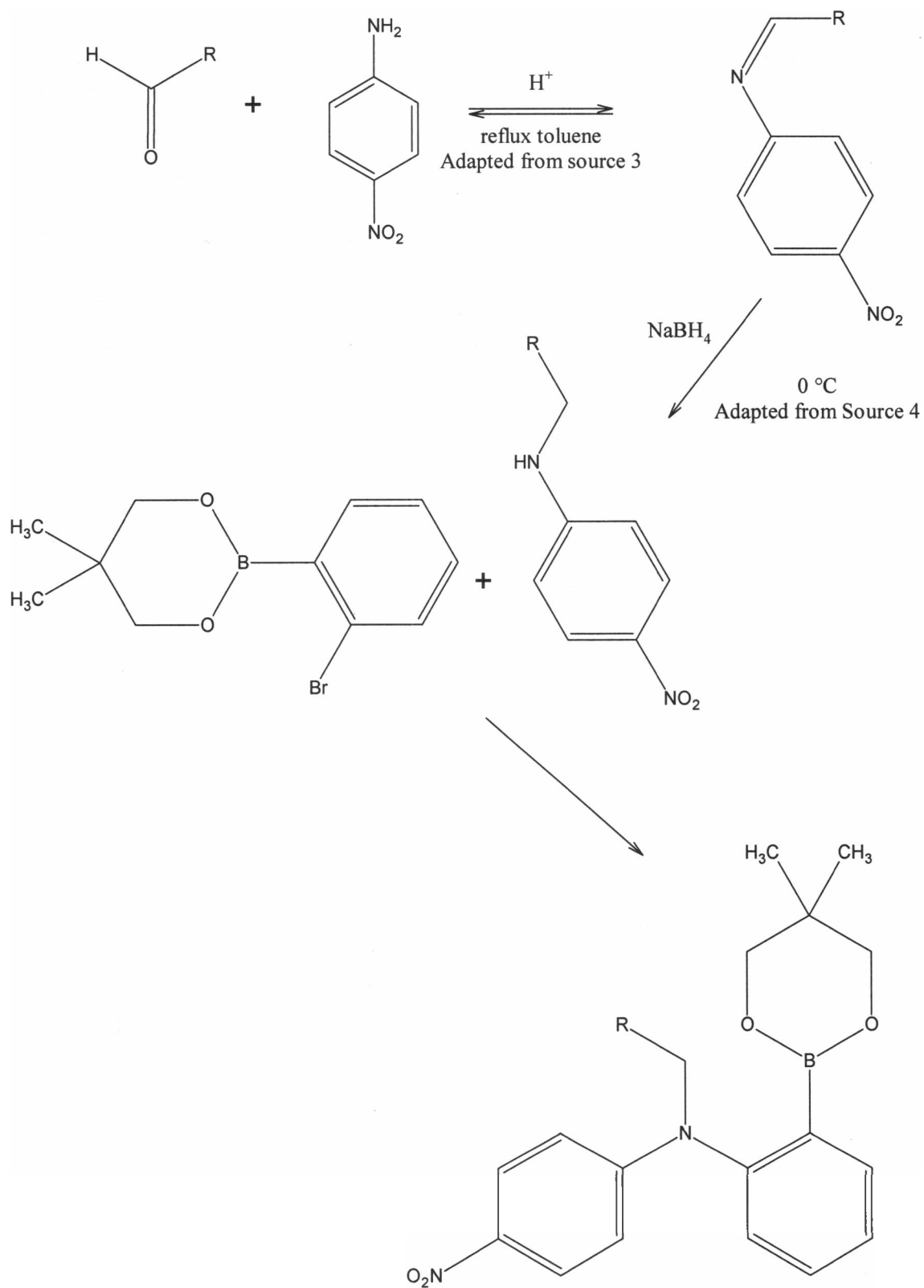
The ability to study proteins and their role in cell function is a central area of scientific study. To learn more about proteins and glycosylation, a form of post-translational modification, a means of detecting specifically for glycosylation is necessary. The modification of an existing dye to meet this need was the goal of the project. N-benzyl-4-nitroaniline and methyl ((4-(4-nitrophenyl)amino)methyl)benzoate, two intermediate compounds in the synthesis, were created and purified.

Introduction

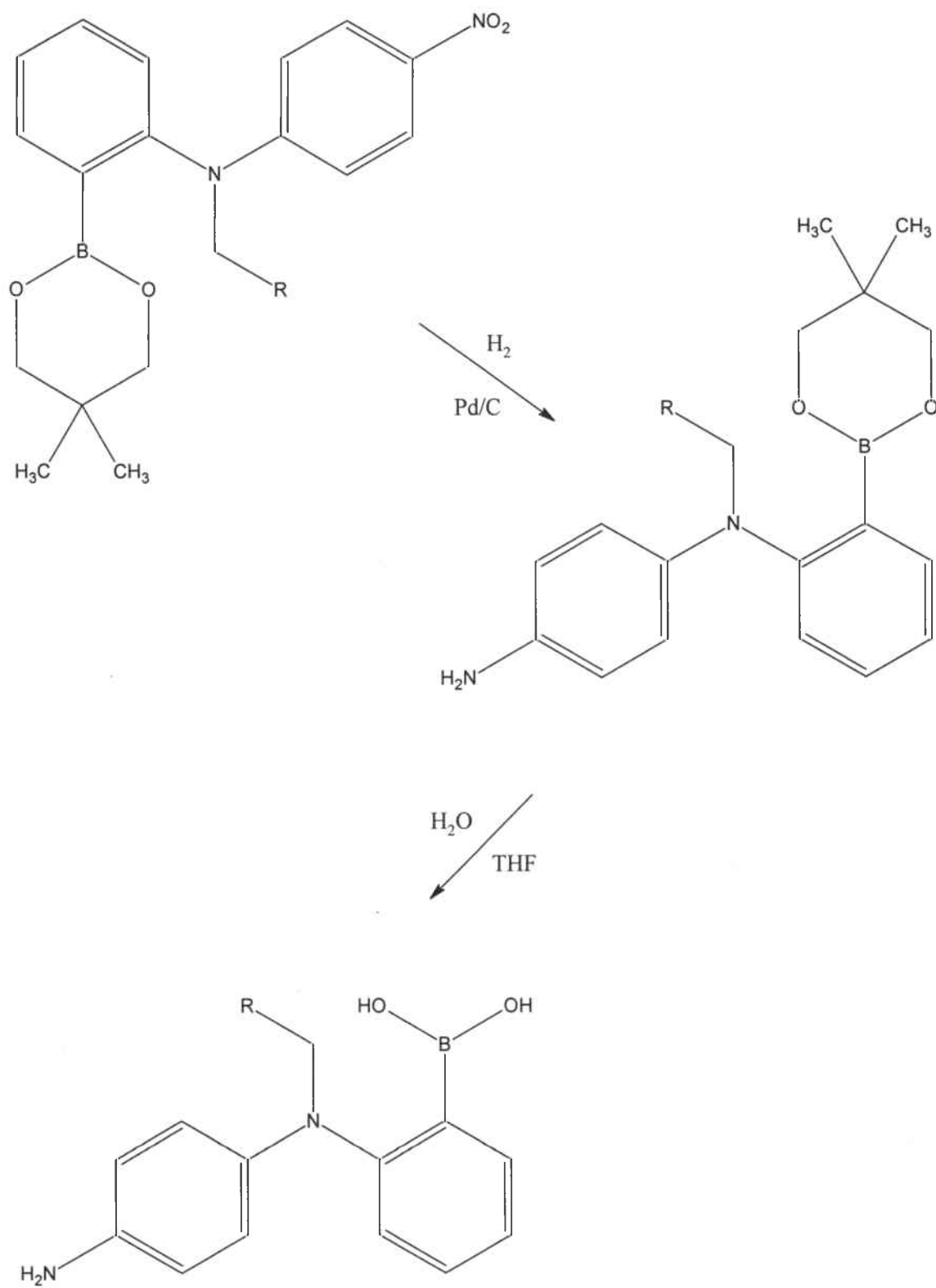
The ability to study proteins and their role in cell function is a central area of scientific study. The proteome is the protein complement of the genome, and includes genetic expression, ribosomal synthesis, and proteolytic degradation.¹ Essentially proteomics is studying the life cycle of a protein. It is known that proteins have a role in nearly all aspects of cell function with this involvement being highly regulated by post-translational modifications.¹ Therefore, understanding why these post-translational modifications occur is absolutely essential to the understanding of the roles proteins play within a cell.

Once an RNA sequence has been translated into a protein, it is susceptible to post-translational modifications such as phosphorylation and glycosylation. The goal of the research was to modify a dye created by the Dratz and Grieco groups at Montana State University-Bozeman so that this dye is specific for the detection of glycosylation. The following proposed reaction schemes 1 and 2, if successful, will result in the specific detection of glycosylation.

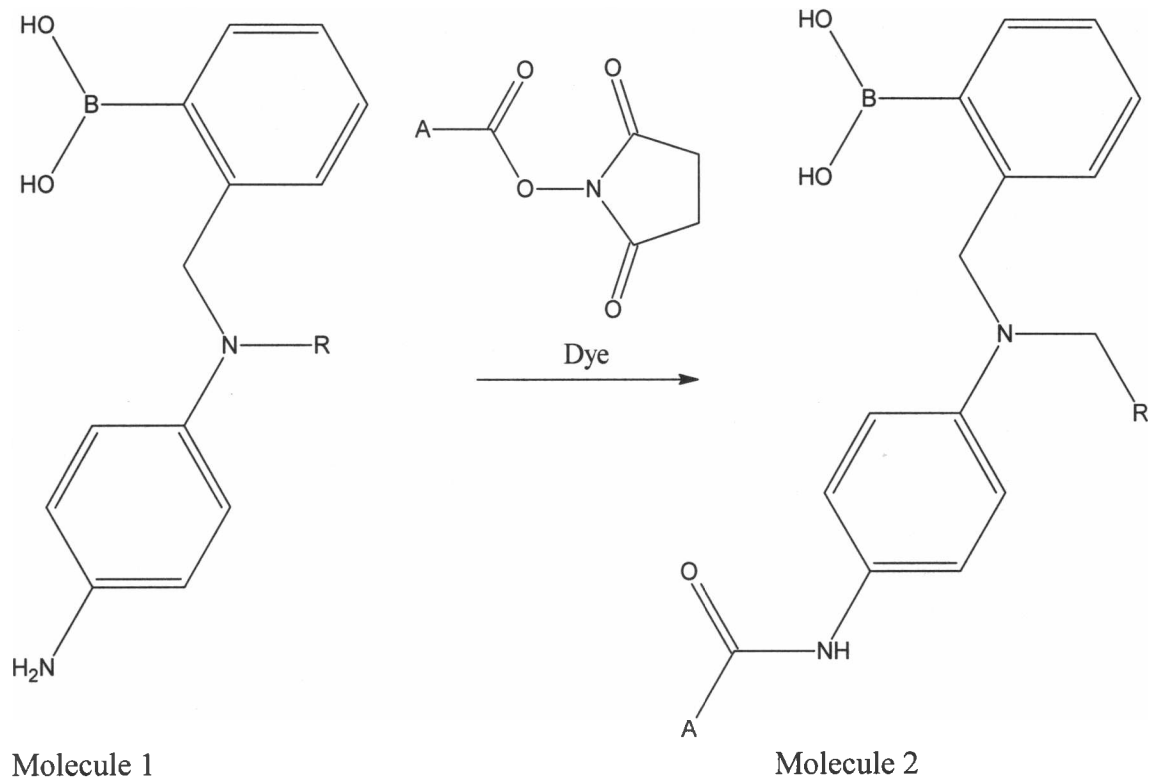
Scheme 1



Scheme 2

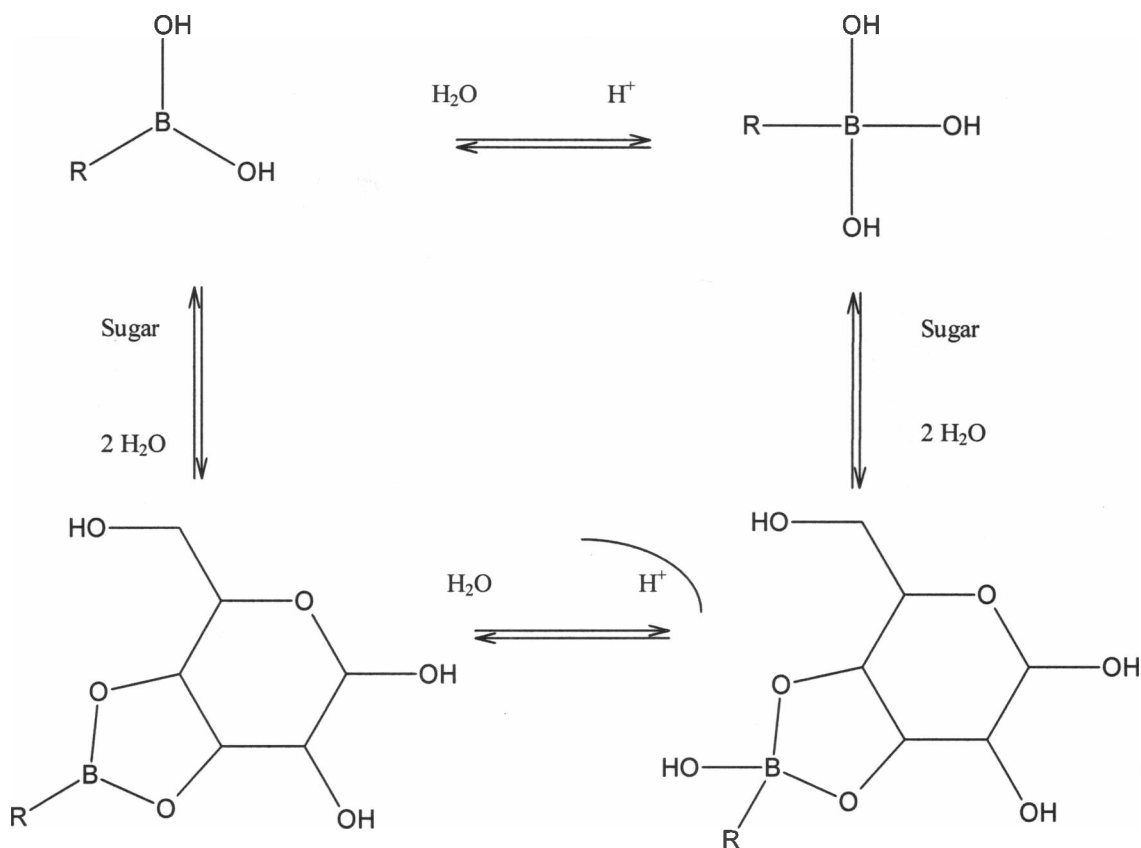


Scheme 3



The modified dye contains a boronic acid. Boronic acids react with geminal diols to form boronic esters.² As the only known biological geminal diol is a sugar, this modified dye is specific for detection of glycosylation.²

Scheme 4



R represents the three different aldehydes: benzaldehyde (3), 9-anthraldehyde (4), and methyl 4-formylbenzoate (5). Molecules 3, 4, and 4 were each reacted with 4-nitroaniline (6). The resultant imines were reduced with NaBH₄. Three different aldehydes were used during the course of my research so that I could gain experience with the reaction conditions and purification of the desired product before using an aldehyde that would give molecule 2 water solubility (a necessary physical property when working with biological molecules). Demonstrating that schemes 1, 2, and 3 could be performed successfully was the goal of my research.

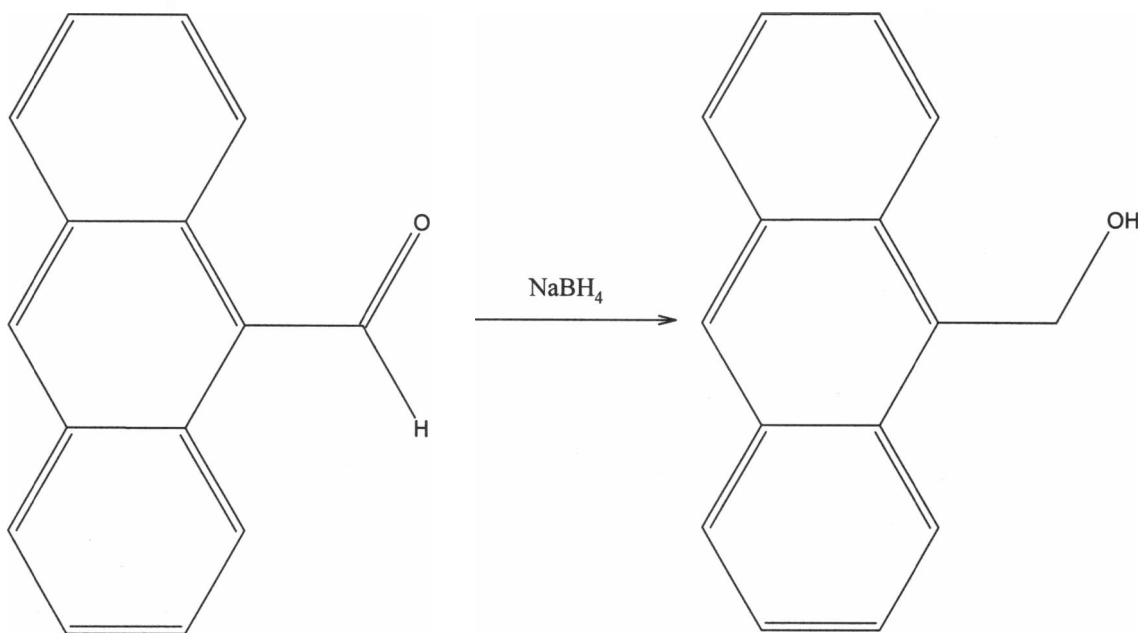
Results

The amine condensation reaction was performed successfully for all three aldehydes (3, 4, and 5) when refluxed with a Dean-Stark Trap sidearm. N-benzyl-4-nitroaniline (7), 9-(4-nitrophenyl)aminomethylanthracene (8), and methyl ((4-(4-nitrophenyl)amino)methyl)benzoate (9) were produced. However, final yields have yet to be determined. 7 and 9 were successfully purified through the use of column chromatography. However, purification of 8 through column chromatography or recrystallization was unsuccessful.

Discussion

The first amine condensation reaction was performed with 4 and 6 at room temperature. This reaction was base catalyzed with NaBH_4 present to avoid isolation of an imine. However, NMR analysis identified only 4-hydroxymethylanthracene as the product. Reduction of the aldehyde by NaBH_4 to produce an alcohol occurred too rapidly for the imine to form.

Scheme 5



The second means of synthesis for these compounds was to condense via a Dean-Stark Trap. This technique removes water so the equilibrium is forced toward the desired products. This method of imine formation was successful for both 7 and 8. 9 was formed when a smaller Dean-Stark Trap and acid catalyst were used. When formation of 9 was first attempted, no product was obtained. However, success was achieved by scaling up the reaction and decreasing the size of the sidearm on the Dean-Stark Trap. Unfortunately, this reaction proceeded only to 60 % within 48 hours of refluxing. Refluxing further might possibly have improved the final yield, but this project was being performed under a deadline and it was decided to proceed to the next step of the synthesis. The rate of this reaction suggests that the addition of substituent groups results in a slowing of product formation.

An unexpected method of purification for 9 was discovered. 9 was insoluble in a 7:3 hexanes/ethyl acetate mixture. While unwanted side products went into solution.

Rinsing with the hexanes/ethyl acetate mixture helped to purify the product and 9 was serendipitously left. This product was then dissolved in dichloromethane and run on a column of silica gel with a solvent system of 7:3 hexanes/ethyl acetate mixture. Fortunately, the product was brightly colored and could be followed in the column by watching the movement of the colored band eliminating the need for taking a large number of fractions. Only a portion of the three products have been run on a column so the yields have yet to be optimized. Pure anthracene amine could not be obtained by column chromatography or recrystallization using a wide range of solvent systems.

Experimental

To create one of the three imines, one of the three aldehydes and 6 were combined in a round bottom flask attached to a Dean-Stark Trap then refluxed in toluene. This reaction was run for a minimum of 24 hours, and the progress of the reaction was monitored by the use of $^1\text{H-NMR}$. As the environment of the aldehyde proton changes, the peak shifts downfield. With 3, the size of the glassware was not important, but with 5 glassware size was critical to the success of the reaction. Also, a catalytic amount of *p*-toluene sulfonic acid was added to force the reaction of 5 to proceed. This acid was not necessary with the reaction of 3.

Formation of benzaldehyde imine

3 (2.9 mL, 2.9×10^{-2} mol), VI (3.929 g, 2.845×10^{-2} mol), and toluene (114 mL) were combined in a 250 mL round bottom flask. This reaction was stirred under reflux for 28 hours.

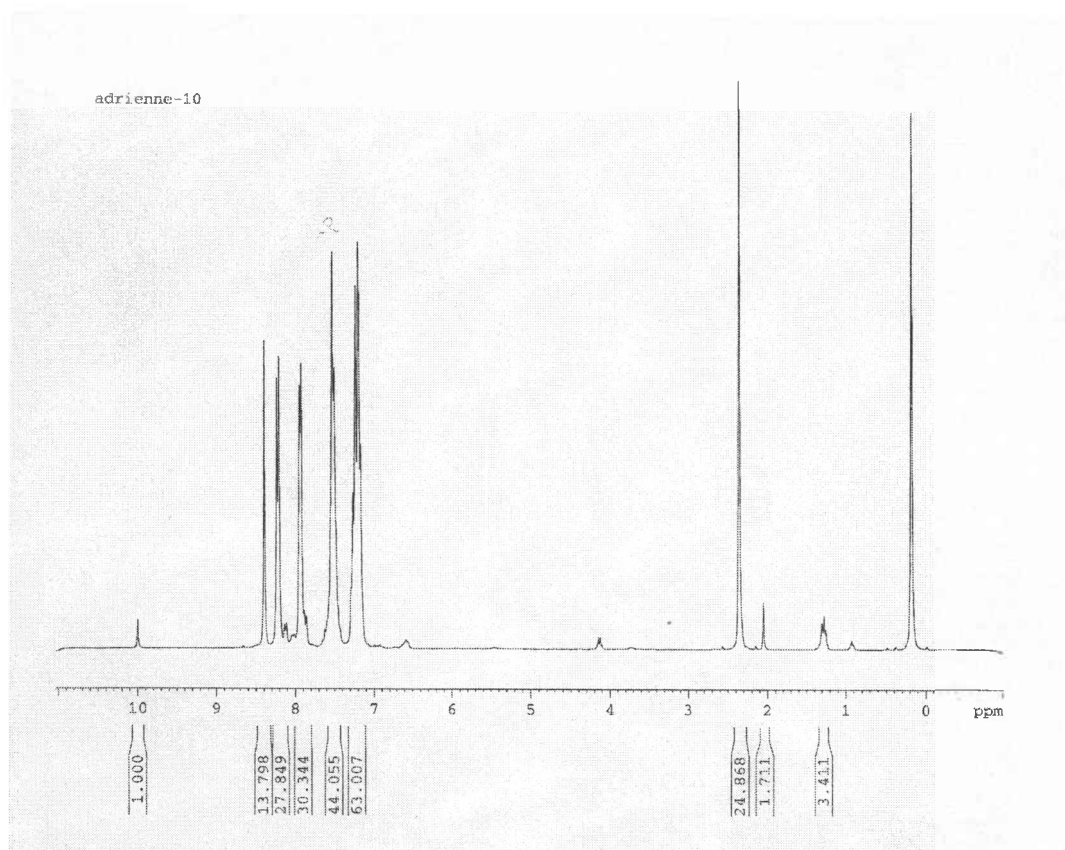


Figure 1 $^1\text{H-NMR}$ of benzaldehyde imine

N-benzyl-4-nitroaniline

Benzaldehyde imine (6.327 g, 2.797×10^{-2} mol), NaBH_4 (2.137 g, 5.648×10^{-2} mol, 2eq), and ethanol (112 mL) were combined in a 250 mL round bottom flask. After two hours at 0°C the reaction was quenched with a saturated solution of sodium bicarbonate. A characteristic color change was observed with the following purification. The reaction mixture was a deep brown. Once the water washes were performed the organic layer became a clear amber color. The organic layer changed to a vibrant yellow when dried with magnesium sulfate. This solution was poured into a 500 mL separatory funnel and the round bottom was rinsed with ethyl acetate. Due to the large scale of the reaction, approximately 1200 mL of ethyl acetate was added to create an organic layer. The

organic layer was washed with water, separated, and dried with magnesium sulfate. The organic layer was then separated into six portions, and the solvent volume was reduced under vacuum. Five of the portions were combined and set aside for purification at a later date. The sixth portion was purified by column chromatography on silica using a solvent mixture of 7:3 hexanes/ethyl acetate. Since only a fraction of the total product was purified final yields have not yet been determined. ¹H- NMR (CDCl₃, 300 MHz) δ 8.06 (d, J=9.1 Hz, 2H), δ 7.70 (d*,J=7.4 Hz,1H), δ 7.33 (m, 5H), δ 6.55 (d, J=9.1 Hz, 2H), δ 4.87 (s, 1H), δ 4.41 (s, 2H).

Formation of ester imine

6(0.856 g, 6.197x10⁻³ mol), 5 (1.007 g, 6.137x10⁻³mol), *p*-toluene sulfonic acid (0.0279 g), and 60 mL of toluene were added to a 250 mL round bottom flask and refluxed for approximately 48 hours. The Dean-Stark Trap for this reaction had a significantly smaller side arm than the Dean-Stark Trap for the reaction of 3. NMR analysis showed the reaction had proceeded approximately 60 percent toward the desired product. ¹H-NMR (DMSO, 300 MHz) δ 10.08 (s, 1 H), δ 8.74 (s, 2H), δ 8.27 (d, J=8.6 Hz, 2H), δ 8.11 (m, *), δ 8.01 (d, J=7.0 Hz, *), δ 7.91 (d, J=9.0 Hz, *) δ 7.46 (d, J=8.4 Hz, 3 H), δ 6.67 (s, *), δ 6.56 (d, J=9.0 Hz, *) *One or more peaks were integrated together so a true H count could not be obtained.

Methyl ((4-(4-nitrophenyl)amino)methyl)benzoate

Ester imine (1.836 g, 6.457x10⁻³mol), NaBH₄ (0.489g, 1.293x10⁻²mol, 2eq) and ethanol (66 mL) were combined for two hours at 0°C before quenching with a saturated solution of sodium bicarbonate. The ethanol was removed under vacuum. Then the product was dissolved in ethyl acetate. This reaction mixture was poured into a 250 mL separatory

funnel and the round bottom flask rinsed with ethyl acetate. Approximately 150 mL of ethyl acetate was added. A characteristic color change from dark brown to yellow was observed. This solution was washed with six 50 mL fractions of water and dried with magnesium sulfate. The solvent was removed under vacuum. The product was dissolved in ethyl acetate and then was rotational-evaporated to obtain an $^1\text{H-NMR}$ in CDCl_3 . $^1\text{H-NMR}$ (CDCl_3 , 300 MHz) δ 8.05 (m, 4H), δ 7.39 (d, $J=7.8$ Hz, 2H), δ 7.24 (s, 4H), δ 6.61 (d, $J=8.9$ Hz, *), δ 6.55 (d, $J=9.0$ Hz, *) δ 4.88 (s, 1H), δ 4.49 (s, 1H), δ 4.31 (s, *), δ 3.90 (s, 3H).

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