2015

The Synthesis of Arginine-based Heterocyclic Amines

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J. N. Andrews Honors Program
Andrews University

HONS 497
Honors Thesis

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March 30, 2015

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ABSTRACT

Heterocyclic Amines (HCAs) are a group of mutagenic and carcinogenic chemicals found in muscle meat after grilling, frying, or broiling. Research studies show that very low amounts of HCAs are created from creatin(in)e reacting with another amino acid at temperatures greater than 200 °C. Other studies show that plant-based arginine can substitute for creatin(in)e forming a new class of mutagenic HCAs, but also in low yields. We attempted to develop a direct, higher yielding method of producing these arginine-HCAs based on a procedure previously used to synthesize creatine-HCAs. With greater amounts of arginine-HCA, chemical characterization and toxicity assessment could be improved.

INTRODUCTION

Heterocyclic Amines (HCAs) are a group of mutagenic and carcinogenic chemicals that have been found in muscle meat such as beef, poultry, and pork when cooked at elevated temperatures during grilling, frying, or broiling.[9] Laboratory experiments have shown that substantial exposures to HCAs in animal models have led to tumors of the colon, liver, skin, lung, and prostate.[7] Despite the public health concern in most scientific communities, the carcinogenicity and mutagenicity of HCAs are not well understood in the human model, but it is well accepted as a risk factor in human cancer.[1] Research studies link the formation of HCA to creatine reacting with another amino acid at temperatures near 200°C.[8] Very few peer-review literature sources show any connection to non-creatine based HCAs formation. However, two 1994 papers[3] show that arginine, prevalent in plants, is a potential source of a new class of HCAs due to its structural similarity to creatine.[2] Dr. Hayes' research group has identified one lead arginine-based HCAs, and several other potential candidates, but they are produced in such minute amounts that it makes molecular structural analysis challenging. Our research group has identified these new chemical species using high-pressure liquid chromatography (HPLC), UV-vis absorbance spectroscopy, and fluorescence spectroscopy. To complete the chemical characterization of this new arginine-HCA, nuclear magnetic resonance (NMR) and mass spectroscopy will need to be performed but NMR requires milligrams of sample, preferably ten milligrams or more. The current production amounts of purified arginine-HCA are around a couple of micrograms.

With this research project, we sought to increase the production of this arginine based HCA following an analogous literature procedure that has been used to make creatine-based HCA. This portion of the project is significant because it provides the research group, and other researchers outside of Andrews University, with an ample supply of arginine-HCA

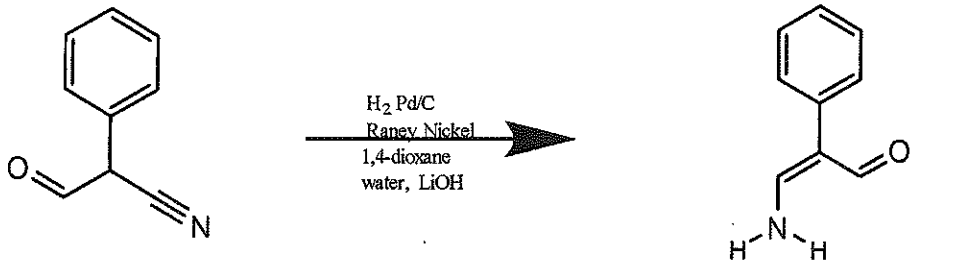
to be used as analytical standards, and for more materials to be used in toxicity and exposure tests, which will help improve the understanding of the health and safety issues of arginine-based HCA.

In step 1 of this project, we attempted to synthesize and analyze 2-Amino-1-Methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) using the techniques from previous established projects. In step 2 of this research, we hoped to replace creatine with arginine to see if an alternative synthesis route could be establish for the arginine-based HCA by integrating the procedure from step 1 of our research. In future stages of this project (step 3), the newly synthesized product will be analyzed, purified and identified by Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and High Performance Liquid Chromatography (HPLC). This project aimed to develop a route for the production of a new class of potential carcinogens that will provide a chemical foundation to improve our knowledge of health risks in the over-cooking of proteinaceous plant products.

METHODS

This project was organized in various stages that were crucial to achieving our goal of a high yield production of the arginine-based HCA. The key step in the synthesis, Figure 2, of our control molecule, PhIP, is the reaction of 3-amino-2-phenylpropenal with creatinine. The goal would be to simply replace creatinine with arginine and re-attempt the synthesis and then compare the products via HPLC to the arginine-HCA already produced by our group. In our attempt to synthesize PhIP, it was noticed that 3-amino-2-phenylpropenal was not commercially available from any vendor. Therefore, in order to synthesize PhIP, we had to first manufacture the important ingredient, 3-Amino-2-phenylpropenal. The procedure reported below, focuses on Stage 1, the synthesis of 3-

amino-2-phenylpropenal from 3-hydroxy-2-phenyacrylonitrile (Figure 1). The literature reference at a proposed hydrogen reduction from a commercially available material in 55% yield. While important procedural details were missing from this protocol, we anticipated the process was quite repeatable and easily accomplished. However, the difficulty of stage 1 reaction meant that numerous procedures were attempted to synthesize 3-amino-2-phenylpropenal. The final stages of this project are listed in the future work section below.



3-hydroxy-2-phenyacrylonitrile

3-amino-2-phenylpropenal

Figure 1. Stage 1 reaction of 3-hydroxy-2-phenylacrylonitrile to create the product, 3-amino-2-phenylpropenal.

STAGE 1. THE PREPARATION OF 3-AMINO-2-PHENYLPROPENAL.

Nitrile Reduction by Catalytic Hydrogenation over Palladium-Activated Raney-Nickel.[4] The reaction was performed using the hydrogenation of 3-hydroxy-2-phenylacrylonitrile in the presence of a Raney nickel catalyst and 10% palladium on carbon at 55 psi (Figure 1). The product was purified through filtration of the catalyst and rinsing of the mixture of 1,4-dioxane and water. Rotary evaporation was utilized to remove all excess solvents from the reaction, thus leaving behind the final product. The mixture obtained was clear. Thin Layer Chromatography (TLC) and ninhydrin spray test were employed as product verification techniques. Benzonitrile was used as the control product.

Reduction of Nitrile using $NABH_4$ and $CoCl_2$. Solid $CoCl_2$-$6H_2O$ (0.689 mmol) and substrate (benzonitrile or 3-hydroxy-2-phenylacrylonitrile, 6.89 mmol) were added to 42 mL THF/H_2O (2:1 v:v) while $NABH_4$ (13.74 mmol) was slowly added cooled solution. TLC analysis was completed 50 minutes into the experiment to test for any trace amounts of the starting material. When the presence of starting material was noticed, more $NaBH_4$ was slowly added. After a total time of 2 hours, 28% NH_4OH was added and the mixture was centrifuged. The combined supernatant was removed using roto-evaporation and extracted with dichloromethane (DCM). The combined DCM layers were dried with $MgSO_4$. TLC analysis (pH 3 buffer/Acetonitrile, 2:1) and ninhydrin spray was used for analytical testing.

Reduction of Nitrile using $LiAlH_4$ and $CoCl_2$. The same procedure above was duplicated with a much stronger reducing agent, $LiAlH_4$. Solid $CoCl_2$-$6H_2O$ (0.689 mmol) and substrate (benzonitrile or 3-hydroxy-2-phenylacrylonitrile, 6.89 mmol) were added in 42 mL THF/H_2O (2:1 v:v) while $LiAlH_4$ (13.74 mmol) was slowly added cooled solution. TLC analysis was completed 50 minutes into the experiment to test for any trace amounts of the starting material. When the presence of starting material was noticed, more $LiAlH_4$ was slowly added. After a total time of 2 hours, 28% NH_4OH (1 mL) was added and the mixture was centrifuged. The combined supernatant was removed using roto-evaporation and extracted with dichloromethane (DCM). The combined DCM layers were dried with $MgSO_4$. TLC analysis (pH 3 buffer/Acetonitrile, 2:1) and ninhydrin spray was used for analytical testing.

Reduction of Nitriles using I_2 in a Dry System. $NaBH_4$ (9.2 mmol) and the control substrate, 4-Acetylbenzonitrile (4 mmol), were placed in flask along with I_2 (4 mmol) in dry THF at

0°C for 2.5 hours. After which, the reaction was refluxed at 70°C for 3 hours and then cooled to 0°C. The contents were then refluxed for 30 minutes upon the addition of 6 M HCl (3 mL). The mixture was once again cooled to 0°C and neutralized with NaOH (3 g). The organic layer was extracted with ether and wash with brine. The combined organic layer was dried with $MgSO_4$ and concentrated. Analysis was completed by TLC (hexane/ethyl acetate, 60:40) and ninhydrin spray test.

Catalytic Hydrogenation with Nickelous Acetate under Argon. Appropriate amounts of nickelous acetate (0.433 mmol), ethanol (10 mL), and sodium borohydride (0.433 mmol) were added together and mixed vigorously under argon. Our control product of choice, 4-Acetylbenzonitrile (3.44 mmol), was later added while keeping argon on the system. When the system was completely flushed with argon, the reaction was capped and allowed to run overnight. Analysis was completed by ninhydrin spray test and TLC (ethanol/hexane, 60:40).

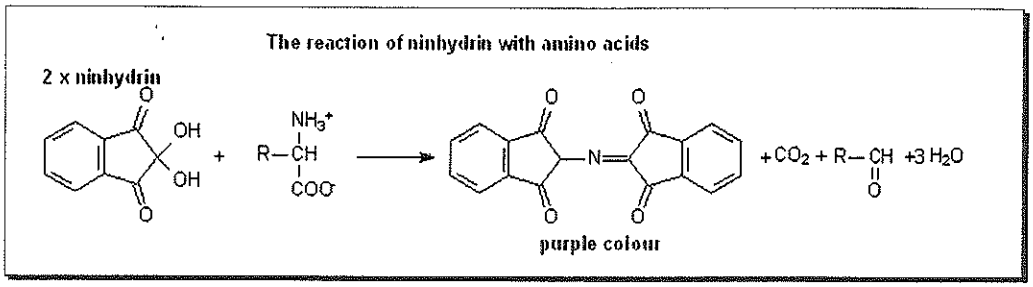
ANALYSIS

Ninhydrin Spray Test

Ninhydrin is an organic compound that is used to visualize the presence of amino groups. The ninhydrin solution utilized in this experiment was developed in lab as a 1 wt% solution in isopropanol and stored in spray bottles.

When a reaction was completed, one drop of solution was pipetted onto a silica gel TLC plate and air dried for minute. The 1 wt% ninhydrin solution was sprayed onto the TLC plate. After heating the spot on a TLC plate for approximately 15 seconds with a heat gun, a successful ninhydrin test yielded a striking purple pigment known as Ruhemann's

Purple. This test was utilized to test for the presence of a primary amine after the hydrogenation reactions (Figure 1).



NOTE: Thin Layer Chromatography techniques utilized silica gel plates.

Future Stages

Stage 2. **The synthesis of 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP).** PhIP will be synthesized by the reaction of creatine and 3-Amino-2-phenylpropenal under dry nitrogen. The product of this reaction will be recovered by extraction with 1-butanol and water. Further purification of the product will be accomplished by recrystallization and TLC, yielding PhIP. Mass Spectrometry and NMR will serve to verify the identity of this product. Our production of PhIP will be compared with the control PhIP purchased from Toronto Research Chemicals. HPLC will be a quick, direct, and informative instrument to use for this purity analysis.

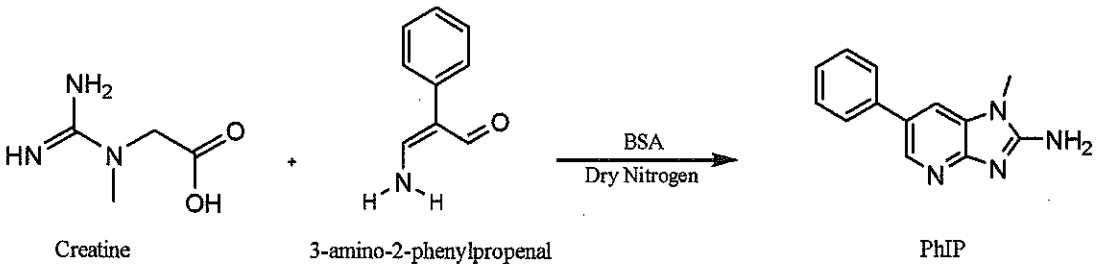


Figure 2. Reaction mechanism of Stage 2.

Stage 3. **Production of the Arginine HCA.** With a successful production of PhIP, we will continue our research by substituting the amino acid arginine for creatinine in the procedure utilized in Stage 2. Arginine is chosen because of its structural similarity to creatine and its vast presence in plant products.

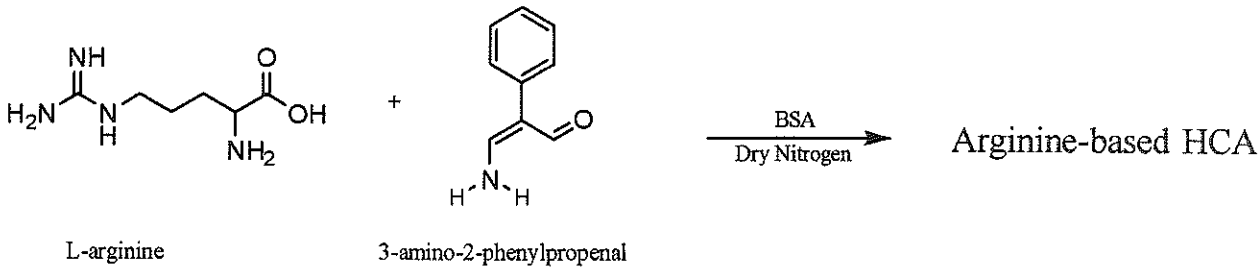


Figure 3. Reaction Mechanism of Stage 3. L-arginine has been substituted into the reaction for creatine.

Stage 4. **Use analysis and purification techniques to identify the product.** NMR, Mass Spectrometry, and HPLC will be used to complete analysis of all products. The research group has created samples of products created by burning arginine with various amino acids. We will compare the final product from Stage 4 with the products already created by the research group. We hope to find a match through the use of the instruments listed above.

RESULTS & DISCUSSION

Hydrogenation of 3-hydroxy-2-phenylacrylonitrile with the various reduction methods listed above displayed moderate results with the control of benzonitrile or 4-acetobenzonitrile. Utilizing the ninhydrin spray test on a thin layer chromatography plate, a positive result was obtained for the reduction of a nitrile to an amine for all model systems

with the exception of the reduction using I_2 and nickelous acetate when under the control system (Table 1).

Model Systems	Control Product with Ninhydrin Spray test	Experimental Product with Ninhydrin Spray test
Nitrile Reduction by Catalytic Hydrogenation over Palladium-Activated Raney-Nickel	Benzonitrile **(Positive)**	3-hydroxy-2-phenylacetonitrile **(Negative)**
Reduction of Nitrile using $LiAlH_4$ and $CoCl_2$	Benzonitrile **(Positive)**	2-Formyl-2-Phenylacetonirtile **(Negative)**
Reduction of Nitrile using $NABH_4$ and $CoCl_2$	Benzonitrile **(Positive)**	2-Formyl-2-Phenylacetonirtile **(Negative)**
Reduction of Nitriles using I_2 in a Dry System.	4-Acetyl Benzonitrile **(Negative)**	2-Formyl-2-Phenylacetonirtile **(Negative)**
Catalytic Hydrogenation with Nickelous Acetate.	4-Acetyl Benzonitrile **(Negative)**	2-Formyl-2-Phenylacetonirtile **(Negative)**

Table 1. Amines Test. In stage 1 of the procedure, we attempted to transform a nitrile to an amine. In order to test the presence of this amine, a ninhydrin spray, test was utilized. A positive test would indicate a dark purple color, while a negative test would not cause a change in color.

A deep purple color, Ruhemann's purple, was easily identifiable for the presence of an amine compared to the lack of an amine as shown in Figure 4. We were unable to observe a positive ninhydrin test with our experimental starting product, 3-hydroxy-2-phenylacrylonitrile as shown in Table 1 above.

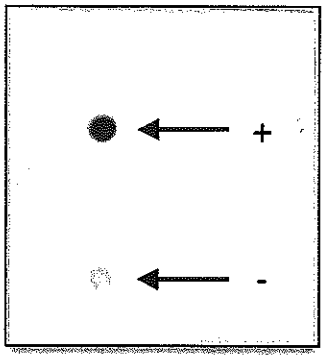


Figure 4. A successful (positive) ninhydrin test is shown by the positive notion at the top, while an unsuccessful test is established by a negative symbol.

Different options were employed to gain success on the experimental starting product. Most procedures were allowed to run over night to ensure that all reactions had sufficient time to react. We also applied moderate heating to various model systems when the procedure allowed for such manipulations. On numerous occasions, various model systems were run with and

without heat in order to seek the optimal results. We employed various reaction conditions such as temperature, pressure, and concentration to ensure success but all attempts failed to produce the intended primary amine.

CONCLUSIONS

Unlike the Friedländer synthesis[5] that demonstrated success with the synthesis of 3-amino-2-phenylpropenal, we report difficulty in finding success with synthesizing this product. We believe that our lack of success in this area of the project is due to the influence of the aldehyde group on the starting material, 3-hydroxy-2-phenylacrylonitrile. The carbonyl group is known to be an electron-withdrawing group, because of the electronegative oxygen, and that could have changed the mechanism of the reaction. Thus, the starting material was being consumed and leading to the formation of metal chelates under the different model systems we had utilized. It became apparent to us that important chemical information was omitted from the literature method that would have been very instrumental for proper success in this hydrogenation process.

At the current moment, we are looking at a possible route where the carbonyl group is protected by glycols and acid work up is used to create the enamine that we desire. While this thought is at the infancy stage, we believe it is the next best step forward in gaining success on stage 1 of this project. We are still attempting to develop a direct, higher yielding method of producing these arginine-HCAs based on new research. The importance of greater amounts of arginine-HCA is vital to chemical characterization and toxicity assessment. On going research will correct the stage 1 hydrogenation process and continue to the final stages of this project.

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